Biofilm formation and EPS analysis of the thermoacidophilic Archaeon *Sulfolobus acidocaldarius*

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ABSTRACT

In their natural environment up to 99% of all microorganisms are assumed to live in microbial aggregates, so called biofilms. Bacterial as well as eukaryotic biofilms have been extensively studied for decades, while the formation of biofilms in the third domain of life, Archaea, only recently became of research interest. In the present study the biofilm formation and synthesis of extracellular polymeric substances (EPS) of the thermoacidophilic Archaeon *Sulfolobus acidocaldarius* was investigated. The cultivation of *S. acidocaldarius* in biofilms represented a major challenge, since the organism grows at temperatures of 75°C to 80°C and a pH of 2 - 3. Several cultivation methods frequently applied for bacterial biofilm formation, e.g. growth on glass slides or on solidified growth medium, were applied to establish a method, which yields sufficient biofilm mass required for subsequent analysis. Of the tested methods, growth as unsaturated biofilm on polycarbonate membrane filters or on gellan gum-solidified Brock medium plates, were successfully adapted to the conditions required for *S. acidocaldarius* biofilm growth. Microscopy studies of the biofilm formed on polycarbonate membrane filters revealed the occurrence of densely packed cells in a 110 µm thick biofilm, which were stainable with the N-acetylglucosamine specific lectin wheat germ agglutinin. Unsaturated biofilms grown on Brock medium plates were used to evaluate different EPS isolation methods. Five methods frequently applied for the isolation of EPS from bacterial biofilms and activated sludge, namely shaking, shaking in presence of a cation exchange resin (CER) as well as treatment with either NaOH, EDTA and crown ether were tested. The suitability of the five methods for the isolation of EPS from *S. acidocaldarius* biofilms was determined by comparing the concentration of isolated carbohydrates, proteins and eDNA. Moreover, interferences of the isolation methods with subsequent analytical methods and impact on cell integrity were determined. Applying these criteria, the CER method was determined the best suited EPS isolation procedure resulting in EPS carbohydrate, protein and eDNA concentrations of 4.7 ± 0.1 fg cell^{-1}, 4.2 ± 1.1 fg cell^{-1} and 0.91 ± 0.48 fg cell^{-1}, respectively. Visualization of the extracellular proteome of CER isolated EPS revealed approximately 1,000 protein spots mainly with a molecular mass of 25 kDa to 116 kDa and a pI of 5 to 8. Functional analysis of the EPS proteins using fluorogenic substrates as well as zymography demonstrated the presence of diverse groups of hydrolytic enzymes within the EPS of *S. acidocaldarius*, capable of degrading a variety of

macromolecules. In-gel activity staining for proteases and esterases suggests expression of at least one extracellular protease, the so called thermopsin, and one extracellular esterase. Presence and the diversity of extracellular enzymes indicate the importance of EPS as an external digestive system for *S. acidocaldarius* biofilms. First analysis concerning the composition of the EPS polysaccharides within the biofilm matrix using acid hydrolyzation of the exopolysaccharides and thin layer chromatography revealed D-glucose as the main component. Additionally, a yet unidentified sugar was detected. The involvement of glycosyltransferases (GTs) of a gene cluster comprising 12 GTs, in the formation of the exopolysaccharides was proven by quantitative analysis of the carbohydrate concentration of GT deletion mutants. EPS composition of certain GT gene deletion mutants revealed significant changes of EPS protein and carbohydrate concentrations and ratios compared to the reference strain.

In conclusion, this study revealed first insights into the general composition and function of EPS from the thermoacidophilic Archaeon *S. acidocaldarius*. For the first time, a method to isolate EPS from Archaea was successfully applied. This study identified polysaccharides as the main component of *S. acidocaldarius* EPS, followed by proteins and eDNA and revealed the individual importance of these polymers for the integrity of the biofilm. The EPS isolation method established in this study was used in a collaboration with the MPI Marburg revealing the involvement of an Lrs14 regulator in the *S. acidocaldarius* submersed biofilm formation (Orell *et al.* 2013b) as well as the involvement of an α-mannosidase in the EPS composition of *S. solfataricus* unsaturated biofilms (Koerdt *et al.* 2012). Furthermore, several GTs were identified to play crucial roles in the synthesis of exopolysaccharides. The expression and characterization of GTs with respect to substrate specificity will reveal their role in the formation of exopolysaccharides of *S. acidocaldarius*.

1. INTRODUCTION

1.1 Archaea – The third domain of life

The third domain of life, Archaea, was classified based on comparison of DNA sequences of the small subunit rRNA, which is a universal marker found in all organisms on earth (Woese *et al.* 1990). Initially, Archaea have mostly been studied due to their adaptation to extreme environments, being able to cope with e.g. extreme temperatures, increased salt concentrations, desiccation and even radioactive radiation. However, molecular studies revealed that Archaea do not only inhabit extreme environments. Instead, they were shown to be ubiquitously present, even colonizing the human gut, and are of great importance in the biogeochemical cycling of carbon, nitrogen, sulfur and iron (Gill *et al.* 2006; Weidler *et al.* 2008; Justice *et al.* 2012). Nowadays, this domain is divided into four approved kingdoms, namely Euryarchaeota, Crenarchaeota, Thaumarchaeota and the so far uncultivated Korarchaeota as well as two additionally proposed kingdoms, the Nanoarchaeota and Aigarchaeota, which are still under discussion (Woese *et al.* 1990; Auchtung *et al.* 2006; Elkins *et al.* 2008; Pester *et al.* 2011). Most (hyper)-thermophiles belong to the Crenarchaeota or the Euryarchaeota, the latter also comprising several methanogens and extreme halophiles (Woese *et al.* 1990). The phylum of Nanoarchaeota consists of the recently isolated Nanoarchaeota Nst1 (Podar *et al.* 2013) and *Nanoarchaeum equitans,* which is only culturable in co-cultures with the Crenarchaeum *Ignicoccus hospitalis* (Huber *et al.* 2002). The Thaumarchaeota are represented by several mesophilic species (Brochier-Armanet *et al.* 2008; Pester *et al.* 2011). Finally, the latest proposed phylum, the Aigarchaeota is represented by the species *Caldiarchaeum subterraneum* (Nunoura *et al.* 2011).

Additionally to several unique features, Archaea show a mosaic of bacterial and eukaryal characteristics. For instance, archaeal transcription and translation machineries as well as DNA repair mechanisms resemble eukaryotic systems while bacteria-like features include the lack of a nuclear membrane as well as organelles and the organization of genes in operon structures (Kelman and White 2005). Also the paracrystalline surface layer (S-layer) surrounding most Archaea in combination with the absence of a cell wall composed of murein or pseudomurein, is a common archaeal feature (Sleytr and Sára 1997). Another

unique archaeal feature is their lipid membrane composed of polyisoprenyl groups, which are linked to the polar head group of glycerol via an ether group in contrast to the ester-link found in bacteria or eukaryotes (Kandler and König 1978; Albers and Meyer 2011). Moreover, Archaea possess modified metabolic pathways, compared to bacterial or eukaryal pathways, comprising previously unknown and unusual enzymes (Siebers and Schönheit 2005; Van der Oost and Siebers 2006; Sato and Atomi 2011). These so called extremozymes, are of special research interest due to their adaptation to the harsh environmental conditions, which makes them well suitable for diverse biotechnological applications (Piller *et al.* 1996; Eichler 2001; Kim *et al.* 2004; Egorova and Antranikian 2005; De Miguel Bouzas *et al.* 2006).

1.1.1 The Crenarchaeon *Sulfolobus acidocaldarius*

The thermoacidophilic Crenarchaeon *Sulfolobus acidocaldarius* was first isolated by (Brock *et al.* 1972) at Yellowstone National Park (USA), prior to the pioneering work of Woese and his co-workers identifying Archaea as a new domain of life (Woese *et al.* 1990). Thus, *S. acidocaldarius* was initially classified as a bacterial species. *S. acidocaldarius* was the first *Sulfolobus* strain identified and also the first thermoacidophilic Archaeon reported. It was isolated from hot acidic mud ponds and grows best at temperatures of 75°C to 80°C and a pH of 2 to 3. It is an obligate aerobic and heterotrophic lobe-shaped organism using complex organic substrates as carbon sources, including yeast extract, tryptone and a limited number of amino acids and sugars (Grogan 1989; Chen *et al.* 2005). The genome of *S. acidocaldarius* was sequenced in 2005 (Chen *et al.* 2005). So far, *S. acidocaldarius* is one of the few Archaea, in which an efficient genetic system allowing for generation of mutants as well as recombinant over-expressions via plasmids was developed. Hence, *S. acidocaldarius* is one of the model organisms frequently used within the domain of Archaea (Chen *et al.* 2005; Wagner *et al.* 2012).

1.2 Bacterial and archaeal biofilms

Biofilms are microbial communities embedded in a self-produced matrix that can be found at interfaces like air-water and water-solid surfaces in almost any environment (Flemming and Wingender 2010). Under natural conditions 99% of all bacteria are assumed to live in the biofilm mode but also eukaryotic microorganisms were shown to grow in biofilms (Donlan and Costerton 2002). The biofilm matrix consists of extracellular polymeric substances (EPS), including polysaccharides, proteins, lipids, nucleic acids, glycolipids and also humic substances as well as water. Interactions of the polymers provide a high mechanical stability of biofilms, which is further enhanced by crosslinking of polymers via multivalent cations such as Ca^{2+} or Mg^{2+} (Figure 1.1)(Karatan and Watnick 2009). Additionally to EPS, cell surface structures like flagella, pili and fimbriae were also shown to stabilize the matrix (Flemming and Wingender 2010).

The nature of the EPS as well as surface structures strongly affect biofilm formation. The stages of biofilm formation include initial reversible attachment, irreversible attachment, formation of microcolonies, formation of a mature biofilm and dispersion of parts of the biofilm (Stoodley *et al.* 2002). The attachment of cells to a substratum is mediated by the occurrence of fimbriae, flagella and pili, the presence, quantity and composition of the EPS as well as cell surface molecules (e.g. proteins, lipopolysaccharides) (O'Toole *et al.* 2000; Flemming and Wingender 2001; Donlan 2002). The physico-chemical properties of the substratum also determine the attachment rate. It has been demonstrated that increased surface roughness promotes attachment, most likely due to reduced shear forces under flow conditions induced by the topography of the substratum (Fletcher and Loeb 1979; Percival *et al.* 1999).

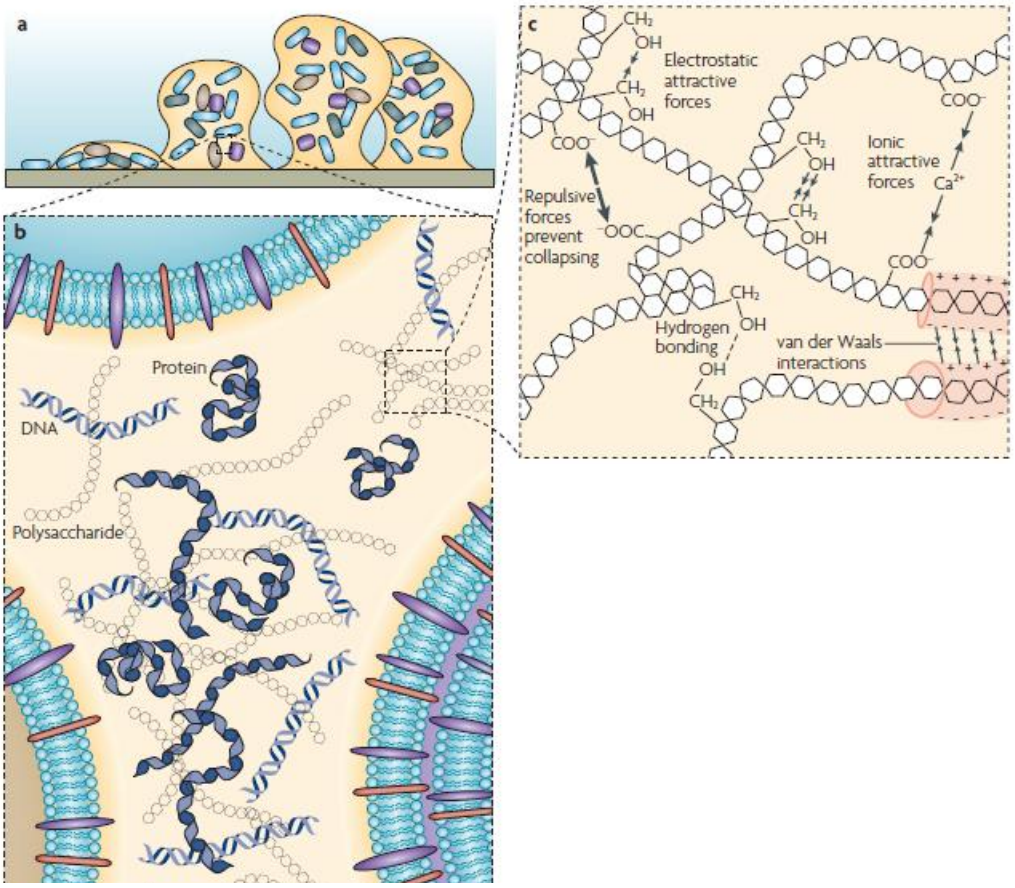

Figure 1.1: Schematic of a biofilm. Scheme showing (a) phases of biofilm formation leading to mushroom like structures (b) the major EPS components, eDNA, polysaccharides and proteins, scattered between the cells and (c) physico-chemical interactions between the different EPS components (Flemming and Wingender 2010).

Living in the biofilm mode offers several advantages over planktonic growth, for instance higher tolerance against antimicrobial agents and desiccation, enhanced gene exchange and better nutrient availability (Stewart and Costerton 2001; Donlan 2002; Molin and Tolker-Nielsen 2003). The transition from the planktonic to the biofilm state in bacteria can be a response to altered environmental conditions including stresses such as unfavorable temperatures, desiccation, starvation or chemical agents (Gilbert *et al.* 1990; Hall-Stoodley *et al.* 2004). In bacteria, biofilm formation has been well studied for decades, while the formation of such communities in Archaea only recently became a major research interest (for an overview see Table 1.1 and Fröls (2013)). Apart from the single-species biofilms so far studied, Archaea were also found in environmental mixed population biofilms like methane-rich marine sediments or in waste water treatment sludge and also in acid mine drainage (Bond *et al.* 2000; Boetius *et al.* 2000; Fernández *et al.* 2008).

Like in bacteria, the formation of biofilms in Archaea can be triggered by environmental stresses e.g. pH, temperature, salt concentrations or UV radiation like it was shown for

biofilms of *Archaeoglobus fulgidus* (LaPaglia and Hartzell 1997). Application of such stress factors on *A. fulgidus* resulted in increased biofilm formation as well as an enhanced formation of EPS 2 to 12 hours after stress exposure. Additionally, *Sulfolobus* strains were shown to react to UV radiation by the formation of cell aggregates and by the expression of UV-induced pili (Ups pili), which are involved in cell aggregation and DNA exchange (Fröls *et al.* 2008; Koerdt *et al.* 2010; Ajon *et al.* 2011; Henche *et al.* 2012b; Henche *et al.* 2012a). Aggregation of cells by Ups pili was also detected after treatment with e.g. the antibiotics bleomycin or mitomycin, which cause breaks in double stranded DNA (Fröls *et al.* 2008; Ajon *et al.* 2011).

Comparison of the biofilm formation in microtiter plates of three *Sulfolobus* strains, *S. acidocaldarius*, *S. solfataricus* and *S. islandicus* under different growth conditions with respect to pH, temperature and iron concentrations revealed that *S. acidocaldarius* produced the highest amount of biofilm (Koerdt *et al.* 2010). Biofilm formation for this strain was especially increased at low temperatures (60°C) and a near neutral pH. The comparison of proteomic and transcriptomic data of biofilms and planktonic cells of *S. acidocaldarius*, *S. solfataricus* and *S. islandicus* showed that similar to bacteria (Sauer *et al.* 2002; Karatan and Watnick 2009) an altered gene expression between planktonic and biofilm state was evident with 15% of the genome differently expressed in *S. acidocaldarius*. However, only 9 genes showing significant differential expressions between both stages were shared between the three closely related strains (Koerdt *et al.* 2011; Orell *et al.* 2013a). One of the three up-regulated genes, a putative Lrs14-like transcriptional regulator was further investigated in *S. acidocaldarius* and shown to be involved in biofilm formation and cell motility. Moreover, a DNA binding protein as well as a heat-shock protein were up-regulated in the biofilm state.

Besides *Sulfolobus* one of the best studied archaeal biofilms are those of haloarchaea. Biofilm formation was observed in different species of *Halobacterium*, *Haloferax* and *Halorubrum* (Fröls *et al.* 2012) (see Table 1.1).

1.2.1 Role of archaeal surface structures in attachment

Surface structures play a crucial role in the attachment of cells to surfaces and were also detected in several Archaea, such as different *Sulfolobus* species (Zolghadr *et al.* 2010) and also in haloarchaea like *Halobacterium salinarum* (Fröls *et al.* 2012). Recent studies revealed that *S. acidocaldarius* possesses three major surface structures namely the archaellum (archaeal flagellum), Ups pili (UV induced pili) and Aap pili (archaeal adhesive pili), which are involved in cell adhesion to surfaces as well as in cell to cell contact (Henche *et al.* 2012b; Henche *et al.* 2012a; Meyer and Albers 2013). Similar to the bacterial type IV pili, archaeal pili as well as the archaellum consist of an ATPase, a transmembrane protein and a prepilin peptidase, which cleaves the pilin precursor (Albers and Pohlschröder 2009; Pohlschroder *et al.* 2011). However, in contrast to their bacterial counterparts, archaeal pili were shown to possess an N-terminal prepilin peptidase-processing motif, suggesting the possibility of different combinations of pilin subunits. Hence, several slightly different type IV pili with different roles in surface attachment might be produced in Archaea, explaining the diverse functions of the identified type IV pili structures in surface adhesion of for instance, *S. acidocaldarius* and *S. solfataricus* (Fröls *et al.* 2008; Zolghadr *et al.* 2010; Pohlschroder *et al.* 2011). In *S. acidocaldarius* deletion of the genes required for expression of the archaellum or the Aap pili led to reduced adhesion (Henche *et al.* 2012b). Furthermore, it was demonstrated that the Ups pili are required for the initial attachment of planktonic *S. acidocaldarius* cells to a substratum, since deletion mutants, lacking the Ups pili, were no longer able to attach to abiotic surfaces or to form multilayered biofilms as observed for the parental reference strain (Zolghadr *et al.* 2010).

1.3 Extracellular polymeric substances

1.3.1 Isolation of EPS

In order to analyze EPS compounds with respect to quantity, identity and function an important step is the isolation of the EPS fraction from the biofilms. This step is most crucial for all following analyses, since too mild conditions during EPS isolation might lead to low EPS yield while too harsh conditions result in contamination of the EPS with intracellular material due to cell lysis. Hence, a compromise between EPS yield and cell lysis has to be met. Prior to this study, no method to isolate EPS from biofilms of thermoacidophilic Archaea, or Archaea in general, was reported.

For EPS isolation from bacterial and mixed species biofilms as well as from activated sludges, several chemical and physical methods have been applied and compared. Common chemical isolation methods are the application of NaOH, EDTA, crown ether or formaldehyde (Brown and Lester 1980; Wuertz *et al.* 2001; Aguilera *et al.* 2008; Tapia *et al.* 2009) while physical methods include shaking, stirring, ultrasound, filtration and centrifugation (Brown and Lester 1980; Liu and Fang 2002). Also combinations of physical and chemical methods have been applied in particular the use of cation exchange resins (Jahn and Nielsen 1995; Frølund *et al.* 1996). Comparative studies about EPS isolation from bacterial or mixed species biofilms and sludges demonstrated the difficulty to establish a suitable method for EPS isolation for a particular sample (e.g. Park and Novak 2007; Tapia *et al.* 2009). Certain environmental factors like challenging pH values or temperatures, as is the case for biofilms of *Sulfolobus*, further complicate the choice of a suitable EPS isolation method. Aguilera *et al.* (2008) for instance compared five common EPS isolation methods for benthic eukaryotic biofilms in an acidic river. Of the three chemical methods (treatment of biofilms with MilliQ water, NaCl or EDTA) and two physico-chemical methods (cation exchange resin, crown ether cation exchange resin) all led to different efficiencies with respect to total EPS yield. NaCl, which led to the highest total EPS yield also revealed the highest levels of glucose-6-phosphate-dehydrogenase activity, a strictly intracellular enzyme, used as a marker to indicate cell lysis. Like several other studies, this demonstrates the difficult balance between high EPS yield and low cell damage and the need for determination of cell lysis (Jahn and Nielsen 1995). Aside from the overall EPS yield also the chemical nature of the isolated EPS depends on the chosen method. Park and Novak (2007) for instance showed that the composition of the

isolated EPS varied with the isolation method applied and that depending on the objective of the isolation several methods have to be applied in order to determine all fractions of the EPS matrix. To further evaluate the suitability of a certain EPS isolation method, the extent of cell lysis and consequential contamination of the EPS with intracellular material needs to be considered. For this, several methods have been applied in the past including determination of the activity of strictly intracellular enzymes (e.g. glucose-6-phosphate-dehydrogenase) in the EPS (e.g. Frølund *et al.* 1996; Aguilera *et al.* 2008), determination of culturability (e.g. Jahn and Nielsen 1995) or the determination of the membrane integrity of cells (e.g. Wu and Xi 2009) via e.g. live/dead staining. Moreover, in several studies the ratio of proteins to carbohydrates was used as an indicator for cell lysis as well as the release of extracellular DNA (e.g. Sheng *et al.* 2005; D'Abzac *et al.* 2010). For a detailed summary of EPS isolation methods applied to bacterial and mixed species biofilms see Michalowski (2012).

1.3.2 Function of EPS

The EPS represent the major constituents of microbial biofilms. Aside from the immobilization of cells, EPS possess various functions, vital for the survival of the biofilm organisms. One major function is for example the protection of biofilm residents against environmental stress e.g. desiccation, starvation, antimicrobial agents or radiation. Furthermore, nutrients provided by lysed cells but also their DNA can be retained within the EPS matrix thus enhancing horizontal gene transfer (Decho 2000; Flemming 2002). Also, the EPS compounds themselves can be used as a nutrient source.

The nature of the individual EPS components, in particular polysaccharides, proteins and eDNA, determines the physico-chemical characteristics of a biofilm. Neutral or charged polysaccharides are often the major components of the EPS. They are involved in adhesion of cells to abiotic and biotic surfaces and are important for the cohesion of biofilms (Sutherland 2001; Vu *et al.* 2009). They form a three-dimensional polymer network, contributing to a large extent to the architecture and the mechanical stability of biofilms, often in conjunction with multivalent bridging cations such as Ca^{2+}. Furthermore, they are involved in the retention and stabilization of enzymes, like for instance lipase in *Pseudomonas aeruginosa* biofilms, which is stabilized by alginate (Tielen *et al.* 2013).

Like polysaccharides, proteins are involved in initial attachment and aggregation of cells to the surface and can also possess a structural role. Structural proteins are e.g. lectins, which are carbohydrate-binding proteins involved in initial biofilm formation and binding to exo-polysaccharides and glycoconjugates. Due to their attachment to the cell they can serve as an anchor binding the EPS matrix to the cell (Higgins and Novak 1997; Tielker *et al.* 2005). Another major function of proteins is their enzymatic activity enabling digestion of exogenous macromolecules, which can be taken up by the cells and used as nutrients. Common enzyme classes found in bacterial biofilms are for instance proteases, lipases, esterases and phosphatases (Wingender and Jaeger 2003; McDougald *et al.* 2012).

Several studies indicate that DNA is released into the biofilm matrix and can be taken up by cells in a higher rate compared to planktonic cells (Molin and Tolker-Nielsen 2003; Madsen *et al.* 2012). The release and prolonged retention of DNA in the biofilm matrix facilitate horizontal gene transfer and hence, the adaptation of organisms to altered environmental conditions (Whitchurch *et al.* 2002; Allesen-Holm *et al.* 2006). In *P. aeruginosa* large amounts of eDNA were detectable during biosynthesis of alginate and were identified as requirement for cell attachment and initial stages of biofilm formation (Whitchurch *et al.* 2002; Steinberger *et al.* 2002). Moreover, eDNA was shown to be a structural component in a bacterial strain isolated from river snow of the South Saskatchewan River in Canada, which showed the formation of a filamentous network made of eDNA (Böckelmann *et al.* 2006).

Depending on the microorganism and environmental conditions such as pH, temperature, oxygen concentration, nitrogen concentration, shear forces and the availability of nutrients, EPS composition and structure can vary significantly. This leads to variable properties of the biofilm with respect to e.g. density, porosity, charge, sorption, ion exchange properties, hydrophobicity and mechanical stability (Mayer *et al.* 1999).

In Archaea little is known about the composition and function of the EPS. Previously some Archaea including species of *Methanosarcina*, *Haloferax* and *Haloarcula* but also *Sulfolobus* were shown to secrete extracellular polysaccharides (Sowers and Gunsalus 1988; Antón *et al.* 1988; Nicolaus *et al.* 1993). Koerdt *et al.* (2010) compared the biofilm formation of *S. acidocaldarius*, *S. solfataricus* and *S. islandicus* in microtiter plates and concluded from lectin staining that all three strains produced exopolysaccharides containing glucose, galactose, N-acetylglucosamine and mannose residues. The same sugar residues were

detected in biofilms of *Thermococcus litoralis* DSM 5473 and haloarchaea (Rinker and Kelly 1996; Fröls *et al.* 2012). In *T. litoralis* DSM 5473 the production of extracellular mannose and an increased biofilm formation was detected upon supplementation of the growth medium with maltose (Rinker and Kelly 1996). Moreover, a correlation between presence of glucose in the growth medium and production of exopolysaccharides was detected for e.g. *Haloferax volcanii* DSM 3757 (Fröls *et al.* 2012). Nevertheless, the structural role of exopolysaccharides in the EPS matrix of archaeal biofilms remains unknown.

The first enzyme involved in archaeal EPS formation, an α-mannosidase, was reported recently for *Sulfolobus solfataricus* PBL2025. Overexpression of the α-mannosidase as well as gene deletion studies in this strain demonstrated the involvement in the biofilm formation (Koerdt *et al.* 2012). Other archaeal enzymes have been found to be secreted actively into the growth medium like amylopullulanases (Guan *et al.* 2013) and α-amylases (Worthington *et al.* 2003; Moshfegh *et al.* 2013) (see section 1.3.3). Their function in biofilms, however, was not evaluated.

Even though eDNA was detected in *Sulfolobus* biofilms as well as in several haloarchaeal biofilms, a structural role could not be assigned so far (Koerdt *et al.* 2010; Fröls *et al.* 2012).

1.3.3 Secretion of EPS

In Archaea two main protein secretion systems are known; the general secretion (Sec) pathway and the twin-arginine translocation (Tat) pathway. Both of these systems are also known in bacteria and function analogously. The Sec pathway is a universal secretion pathway translocating unfolded proteins through small pores within the cytoplasmic membrane. Conclusively, folding as well as post-translational modifications have to occur subsequent to secretion. Proteins targeted for secretion by the Sec pathway reveal conserved features like an amino-terminal signal peptide showing a tripartite structure, which is composed of a charged amino terminus, a hydrophobic stretch and a signal peptidase recognition motif (Bardy *et al.* 2003). In contrast to the Sec pathway, the Tat secretion is only found in prokaryotes, chloroplasts and a small number of protists (Weiner *et al.* 1998; Bogsch 1998). The pore generated for this secretion is much larger since proteins are translocated in a folded state including post-translational modifications (Pohlschröder *et al.* 2005). Proteins, targeted by the Tat pathway, carry a signal peptide similar to the signal

peptide required for Sec pathway, however, with a less hydrophobic stretch and a highly conserved twin-arginine motif (Bendtsen *et al.* 2005b; Bendtsen *et al.* 2005a). Secreted proteins can remain directly associated with the cell by interaction with the outer part of the membrane, attachment to the outer cell wall, or indirectly, by association with other membrane-anchored proteins (Szabo and Pohlschroder 2012). Proteins secreted by Archaea can take part in different functions like defense mechanisms via peptides or proteins with antimicrobial activity (archaeocins), enzymatic degradation of polymeric substances, binding and uptake of nutrients as well as the formation of cell surface structures such as pili or archaella (Szabo and Pohlschroder 2012). In *S. solfataricus* as well as in the halophilic Archaeon *Halorubrum xinjiangense*, extracellular α-amylases were detected (Worthington *et al.* 2003; Moshfegh *et al.* 2013). Recently, an extracellular amylopullulanase of the hyperthermophilic anaerobic *Thermococcus kodakarensis* KOD1, with a possible industrial application, was characterized (Guan *et al.* 2013). Moreover, in *Haloferax mediterranei* a cyclodextrin glycosyltransferase was found to be secreted into the growth medium via the Tat pathway (Bautista *et al.* 2012). In the hyperthermophilic *Acidianus hospitalis* filamentous particles, so called ZLPs (zipper-like proteins), were demonstrated to be a secreted form of tetrathionate hydrolases, which are involved in the sulfur metabolism (Krupovic *et al.* 2012).

Secretion of exopolysaccharides has been documented in archaeal species belonging to *Sulfolobus*, *Thermococcus* and several haloarchaea (Antón *et al.* 1988; Nicolaus *et al.* 1993; Rinker and Kelly 1996; Paramonov *et al.* 1998; Nicolaus *et al.* 1999; Parolis *et al.* 1999; Rinker and Kelly 2000; for a detailed review see Poli *et al.* 2011). *Haloferax mediterranei* planktonic cells secrete a hetero-exopolysaccharide made of glucose, galactose as well as unidentified sugars into the growth medium (Antón *et al.* 1988). In *Sulfolobus solfataricus* DSM 5833 the same monosaccharides as well as mannose and glucosamine were detected in a sulfated hetero-exopolysaccharide isolated from the growth medium of a planktonic culture (Nicolaus *et al.* 1993). Biofilms of *Thermococcus litoralis* produce a homo-exopolysaccharide made of mannose, which is involved in biofilm formation (Rinker and Kelly 1996).

Additionally to the Sec and Tat secretion pathways, several archaeal species were shown to produce membrane vesicles as an alternative process of secreting e.g. proteins and DNA, similar to some gram-negative bacteria, which are released by budding from the cell membrane (Schooling and Beveridge 2006). It was demonstrated that *Sulfolobus* species

grown in liquid cultures produce membrane vesicles consisting of tetraether lipids coated with S-layer proteins which could be involved in the secretion of sulfolobicins (Prangishvili *et al.* 2000; Ellen *et al.* 2009; Ellen *et al.* 2011). Membrane vesicles formed by *Thermococcales* were shown to contain DNA, which is more resistant to degradation compared to free DNA suggesting a possible involvement in horizontal gene transfer (Soler *et al.* 2008). In *Thermococcus kodakaraensis* membrane vesicles were indeed demonstrated to be capable of shuttling DNA from one cell to another (Marguet *et al.* 2013; Gaudin *et al.* 2013). However, it is still unknown if membrane vesicles are also constituents of archaeal biofilms.

1.3.4 Role of glycosyltransferases in synthesis of exopolysaccharides

Glycosyltransferases (GTs) are enzymes, which catalyze glycosidic bond formation during biosynthesis of oligosaccharides, polysaccharides and glycoconjugates (e.g. glycoproteins, glycolipids) by utilization of an activated sugar donor. Hence, they are involved in the biosynthesis of exopolysaccharides, which represent a main component of the EPS matrix. Several studies indicate the involvement of GTs in the formation of biofilms in Bacteria (Table 1.2). Mutations of genes encoding certain GTs led to altered biofilm formation confirming the importance of polysaccharides in the establishment and maintenance of bacterial biofilms (Teng *et al.* 2009; Zhou *et al.* 2010). In *S. acidocaldarius* a gene cluster comprising 12 GTs was identified in the genome, which is suspected to have a major function in exopolysaccharide synthesis (Orell *et al.* 2013b).

As stated before, the biofilm matrix of *S. acidocaldarius* was shown to contain a large number of sugar residues detected via lectin staining. Thus, it can be assumed that they play a crucial role in the formation and maintenance of *S. acidocaldarius* biofilms. Using BLASTp analyses and the CAZy database (Carbohydrate Active Enzymes database; Cantarel *et al.*, 2009) 12 GTs of GT families 2 and 4 as well as several putative membrane proteins and two methyltransferases were predicted in a gene cluster comprising 24 genes (Saci_1904 – Saci_1927)(Figure 1.2). Two of the genes within the cluster (Saci_1905 and Saci_1912) show homologies to wzx flippases, which in bacteria are involved in the translocation of lipopolysaccharides and sugar polymer precursors across the membrane (Islam *et al.* 2010; Islam and Lam 2013). In recent studies one of the genes encoding a putative GT, i.e. Saci_1909, was deleted and the deletion mutant *S. acidocaldarius* Δ1909 showed an overproduction of unevenly distributed exopolysaccharides in submersed biofilms cultivated

at the bottom of µ-dishes compared to the reference strain MW001 (Orell *et al.* 2013b). This strongly suggests a role of this GT in the exopolysaccharide formation in *S. acidocaldarius*. Moreover, involvement in the EPS synthesis or EPS secretion was demonstrated for Saci_1908, a membrane protein with 15 predicted transmembrane helices. The so far uncharacterized membrane protein Saci_1908 was shown to be regulated by an Lrs14 regulator (Saci_0446). A Lrs14 regulator had previously been reported from *S. solfataricus* where it was shown to be negatively auto-regulated in the late growth phase (Napoli *et al.* 1999). So far, however, its target genes remain unknown. In *S. acidocaldarius* the Lrs14 regulator was suggested to be involved in the regulation of biofilm formation (Orell *et al.* 2013b).

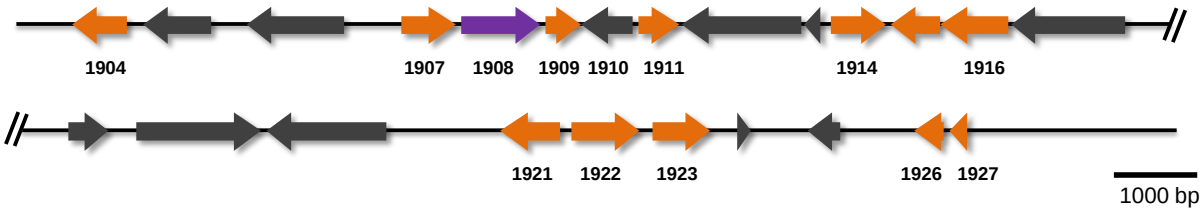

Figure 1.2: Schematic overview of the glycosyltransferase gene cluster in *S. acidocaldarius*. Numbers correspond to the respective *S. acidocaldarius* ORF ID. Orange: GTs; purple: gene under control of Lrs14 transcriptional regulator (Saci_0446); black: further genes (for annotation see Table 4.1).

Genome analysis of more than 500 bacterial, eukaryotic and archaeal genomes revealed that 1-2% of the number of gene products of any organism are GTs (Lairson *et al.* 2008). Magidovich and Eichler (2009) used the CAZy database to examine 56 archaeal genomes in order to identify putative genes coding for GTs and oligosaccharyltransferases. The study revealed that hyperthermophilic Archaea encode fewer GTs than non-hyperthermophilic Archaea. Furthermore, GTs found in hyperthermophiles are usually scattered within the genome while in non-hyperthermophiles predicted GTs are commonly located in the vicinity of an oligosaccharyl-transferase encoding sequence. Up to date GTs have been classified into 94 families by amino acid sequence similarities, which are collected in the CAZy database. For *S. acidocaldarius* CAZy predicts 28 GTs of 6 families, and 3 unclassified GTs. 21 of the 28 GTs belong to GT families 2 and 4. In addition, members of the GT35 and GT66 family were identified in the genome, which are present in most Archaea. It was shown that GT2 and GT4 families are predominantly found in Archaea (Lairson *et al.* 2008).

However, even though GTs are of great interest for biotechnological applications (Egorova and Antranikian 2005) so far only very few GTs have been characterized from *S. acidocaldarius* and Archaea in general (Kobashi *et al.* 1996; Meyer *et al.* 2013). Therefore, their enzymatic properties as well as physiological function still need to be elucidated.

Table 1.1: Overview of studies of archaeal biofilms and/or EPS.

Organism	Growth conditions and support material	Applied methods for biofilm and EPS analysis	Major results	Reference
Archaeoglobus fulgidus	• Growth under standard conditions with induction of biofilm formation on wall of culture vessel by application of extreme temperatures, addition of antimicrobials (e.g. antibiotics and metals) and increased pH and oxygen	• EPS extraction with phenol and ethanol • SDS-PAGE • Inductively coupled plasma for metal analysis	• Biofilm matrix is composed of polysaccharides, proteins and metals • Stress (application of extreme temperatures, antimicrobials (e.g. antibiotics and metals, increased pH and oxygen) led to biofilm formation, hence, biofilm formation is proposed to be a stress response in *A. fulgidus* • Biofilm structure varies depending on intensity of exposure to stress	LaPaglia and Hartzell 1997
Ferroplasma acidarmanus Fer1	• Growth on pyrite in either batch or air lift continuous cultures	• Scanning confocal microscopy analysis of biofilm development	• 10 up-regulated proteins identified via 2 DE including enzymes associated with anaerobic growth	Baker-Austin *et al.* 2010
20 different haloarchaea, (e.g. species of *Halobacterium, Haloferax, Halorubrum*)	• Adhesion to plastic surfaces and glass slides	• Examination of cell layers on glass surfaces via differential interference contrast, fluorescence and confocal microscopy	• Several types of cellular appendages detected for *H. salinarum* DSM 3754 • Two types of biofilm structures: (1) Carpet-like, multi-layered biofilms containing micro- and macrocolonies and (2) large aggregates of cells adhering to glass • presence of extracellular polymers (eDNA and glycoconjugates)	Fröls *et al.* 2013
Haloferax mediterranei	• Growth in a fermenter and as batch culture in medium with varying composition of sugars, NH_4Cl and KH_2PO_4	• EPS precipitation of the supernatant with cold ethanol • Infrared spectrum obtained with KBr technique • DEAE-Sepharose chromatography • TEM • TLC	• Thick pellicle on top of liquid unshaken cultures (TEM) • Composition of EPS independent of tested composition of growth medium • EPS consists of hexoses, hexoamines, uronic acids and proteins • Mannose is a major component (TLC) • Anionic groups present (DEAE- Sepharose chromatography)	Antón *et al.* 1988

Organism	Growth conditions and support material	Applied methods for biofilm and EPS analysis	Major results	Reference
Methanosarcina thermophila	• Growth in low saline medium with subsequent transfer to marine medium	• Phase contrast and thin-section electron microscopy	• Cell surface associated heteropolysaccharide layer only formed in low saline medium • Cell aggregation only in low saline medium	Sowers and Gunsalus 1987
Methanosarcina mazei	• Growth in serum tubes at standard growth conditions	• SEM • TEM • Acid-silver methenamine staining • Fluorescein lectin labeling • Gold lectin labeling • Isolation of matrix material • TLC • Gas-liquid chromatography • Amino acid analysis	• Cell aggregates (up to 100 µm), which accumulate at the bottom of the tube reaching a layer of up to 5 mm (SEM) • After prolonged growth time colonies began to disaggregate • TEM showed cells closely packed together surrounded by an EPS layer of 30-60 nm • Positive lectin stain using peanut agglutinin, soybean agglutinin and *R. communis* agglutinin • Negative lectin stain using concanavalin A, *D. bifloris* agglutinin, *U. europaeus* agglutinin and wheat germ agglutinin • Main monosaccharides obtained with TLC of EPS matrix: galactosamine, galactouronic acid and glucuronic acid	Robinson *et al.* 1985
Sulfolobus acidocaldarius *S. solfataricus* *S. islandicus*	• Growth of three *Sulfolobus* strains in polystyrol 96-well tissue culture plates under varying conditions (pH 2-7, temperature 60°C-85°C, iron 0.015 g/L - 0.065 g/L) • Cultivation of strains in uncoated plastic dishes	• Screening for biofilm formation efficiency using 96-well plate attachment assay • Staining with DAPI and different lectins and detection of signals using CLSM • SEM of biofilms cultivated in plastic dishes	• *S. solfataricus* forms less biofilm in 96-well plates under the tested conditions compared to *S. acidocaldarius* and *S. tokodaii* • SEM revealed different structures of biofilms of static biofilms formed by the three tested strains • CLSM imaging using different lectins revealed occurrence of glucose, galactose, mannose and N-acetylglucosamine residues in the EPS	Koerdt *et al.* 2010
S. acidocaldarius	• Shaking planktonic cultures with glass slide • Biofilm formation in static cultures in µ-dishes	• Attachment assays on glass slides and enumeration of attached cells • Staining of different EPS components and visualization via CLSM of biofilms of	• Deletion of adhesive pili led to increase in cell density • Deletion of Ups pili led to increased cluster formation	Henche *et al.* 2011

Organism	Growth conditions and support material	Applied methods for biofilm and EPS analysis	Major results	Reference
		mutants lacking genes for expression of certain cell surface structures		
S. solfataricus	• Surface: Glass, mica or carbon coated gold grids in shaking planktonic cultures	• Application of different stains, including lectins, to visualize certain components in the EPS	• Deletion of flagella or pili led to an inability to attach to the tested surfaces • Lectin stains indicated presence of glucose, α-D-mannose, α-D-galactose, and N-acetyl-D-glucosamine	Zolghadr *et al.* 2010
Thermococcus litoralis	• Biofilms on polycarbonate membrane filters and glass slides in standard growth medium • Varying additives to growth medium (maltose, yeast extract)	• Fluorescence microscopy (acridine orange) • Congo red (EPS) and carbol fuchsin (cells) for light microscopic analysis of biofilms on glass slides • Scanning electron microscopy • Polysaccharide characterization (HPLC)	• Biofilm formation on hydrophilic surfaces is increased by addition of maltose and/or yeast extract to the growth medium; highest biofilm formation when both were added • Light microscopy showed presence of polymeric material covering the cells • Mannose was detected with HPLC in biofilms and isolated EPS as the only monomer	Rinker and Kelly 1996
Unknown euryarchaeal and bacterial species	• Formation of "string of pearls" in sulfurous marsh water of the Sippenauer Moor (Regensburg, Germany)	• FISH	• Outer part of the pearls composed of bacteria, predominately filamentous bacteria • Within the pearls archaeal cocci are predominant • Archaea appeared to be embedded in a polymer	Rudolph *et al.* 2001

Abbreviations: 2 DE, two-dimensional gel electrophoresis**; CLSM,** Confocal laser scanning microscope; **DAPI,** 4',6-diamidino-2-phenylindole; **HPAE-PAD,** High performance anion exchange chromatography with pulsed amperometric detection; **HPLC**, High performance liquid chromatography; **SDS-PAGE**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **SEM,** Scanning electron microscope; **TEM,** Transmission electron microscope; **TLC,** Thin layer chromatography.

Table 1.2: Glycosyltransferase encoding genes[(a)] and polysaccharide synthesis clusters demonstrated to be involved in bacterial biofilm formation

Organism	Genes/Gene cluster	Function/Effect/Annotation	Reference
Bordetella bronchiseptica *B. pertussis* *B. parapertussis*	*bps*ABC[(a)]D	• BpsC is a member of the GT2 family. Disruption of *bps*C led to altered 3-dimensional structure of biofilms	Parise *et al.* 2007
Enterococcus faecalis	*epa*A[(a)] *epa*B[(a)] *epa*N[(a)]	• Disruption of *epa*A (GT4), *epa*B (GT2) and *epa*N (GT2) resulted in alteration in the Epa polysaccharide content and decreased biofilm formation	Teng *et al.* 2009
Enterococcus faecalis	*bgs*A[(a)]	• Inactivation of *bgs*A led to an almost complete arrest of biofilm formation on plastic surfaces • Overexpression of *bgsA* resulted in increased biofilm production	Theilacker *et al.* 2009
Escherichia coli	*pga*ABC[(a)]D	• PgaC is predicted to be a GT of family 2 • Each gene of the cluster is required for optimal biofilm formation	Wang *et al.* 2004
Pseudomonas aeruginosa	*psl*A[(a)]BC[(a)]DEF[(a)]GH[(a)]I[(a)]JK[(a)]LMNO	• In-frame deletion and complementation studies of the *psl* genes revealed that except for *psl*BMNO each gene is required for Psl exopolysaccharide production and surface attachment	Byrd *et al.* 2009 Matsukawa and Greenberg 2004
Pseudomonas aeruginosa	*pelABCDE*[(a)]*F*[(a)]*G*	• Cluster is required for pellicle formation and biofilm formation on glass and plastic • PelF is a cytosolic glycosyltransferase	Friedman and Kolter 2003 Ghafoor *et al.* 2013
Streptococcus parasanguinis	*gtf*1[(a)] *gtf*2[(a)] *gtf*3[(a)]	• Deletion mutants showed decreased biofilm formation	Zhou *et al.* 2010

[(a)] : Glycosyltransferase encoding genes

1.4 Aims of this study

Even though Archaea have recently gained research interest especially due to adaptation to extreme environments little is known about their potential to form biofilms. In several archaeal species, biofilm formation has been demonstrated. Nevertheless, the composition of the biofilm matrix with respect to extracellular polymeric substances (EPS) as well as genes involved in the formation of the biofilm matrix have not been a major research focus.

Therefore, in this study, the major aim was to shed light on the biofilm formation, the composition and function of extracellular polymeric substances (EPS) and the synthesis of exopolysaccharides of the hyperthermophilic crenarchaeal model strain *Sulfolobus acidocaldarius*.

The initial focus was on the establishment of a cultivation method, yielding sufficient amounts of *S. acidocaldarius* biofilm mass required for the isolation and subsequent analysis of the EPS components. Despite the fact that Archaea have previously been demonstrated to secrete EPS, prior to this study, no technique for the isolation of these substances had been established. Hence, a comparison of different isolation methods, previously applied for the isolation of EPS from bacterial biofilms and activated sludge, was pursued in order to evaluate their suitability to isolate EPS from *S. acidocaldarius* biofilms. The EPS isolation methods were compared with respect to EPS isolation yield, quality of isolated EPS and impact on cell integrity. Additionally, the interferences with subsequent analyses, e.g. the visualization of the extracellular proteome via 2 D gel electrophoresis were evaluated. In the further course of the study, a quantitative, qualitative and functional analysis of the individual isolated EPS components, more precisely exopolysaccharides, proteins and eDNA, was aspired. The function of extracellular proteins was evaluated with emphasis on extracellular enzymes with a focus on proteases and esterases. To obtain first insights into the composition of the exopolysaccharides of *S. acidocaldarius* biofilms, thin layer chromatography of hydrolyzed exopolysaccharides was performed.

Identification of genes involved in the synthesis of exopolysaccharides was determined by construction of gene deletion mutants (in collaboration with Dr. S.-V. Albers and B. Meyer of the MPI Marburg), each lacking a gene of a glycosyltransferase (GT) cluster found within the genome of *S. acidocaldarius*. The impact of the deletion on the EPS composition with respect

to carbohydrate and protein quantity was determined for biofilms of each deletion mutant. In order to characterize the function of the respective GTs, cloning and expression strategies were applied to purify the GTs for further characterization with respect to substrate specificity.

2. MATERIALS & METHODS

2.1 Chemicals and plasmids

Chemicals and enzymes were purchased from Amersham Pharmacia Biotech Europe GmbH (Uppsala, Sweden), Bio-Rad Laboratories GmbH (Munich, GER), Biometra (Göttingen, GER), Difco Laboratories (Augsburg, GER), MEB Fermentas Life Science (St. Leon Rot, GER), Gerbu Biotechnik GmbH (Wieblingen, GER), Life Technologies, (Karlsruhe, GER), Merck, QIAGEN (Hilden, GER), Roche Diagnostics GmbH (Mannheim, GER), Roth GmbH (Karlsruhe, GER), SERVA Electrophoresis GmbH (Heidelberg, GER), Sigma-Aldrich (Taufkirchen, GER) and VWR International (Langenfeld, GER) in analytical grade. For heterologous expression, the pET vector system (pET11c, pET15b, pET28b (Merck (Novagen) Darmstadt, GER). For the construction of gene deletion mutants, the auxotrophic *S. acidocaldarius* strain MW001 was kindly provided by Dr. S.-V. Albers (Wagner *et al.* 2012).

2.2 Commercial kits

Table 2.1: Commercial kits

Name	Manufacturer	Art. No.
CandyCane™ Glycoprotein Molecular Weight Standard	Invitrogen/Molecular Probes	C21852
DNeasy Blood and Tissue Kit	Qiagen	69504
GeneJET Plasmid Miniprep Kit	Thermo Scientific	K0502
Live/Dead® BacLight Bacterial Viability Kit	Invitrogen/Molecular Probes	L7012
Protino® Ni-TED	Machery-Nagel	745100.50
Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit	Invitrogen/Molecular Probes	P21857
QIAfilter Plasmid Midi Kit	Qiagen	12243
QIAquick Nucleotide Removal Kit	Qiagen	28304
Quant-iT™ PicoGreen® dsDNA Reagent Kit	Invitrogen/Molecular Probes	P7589
Wizard® SV Gel and PCR Clean-Up System	Promega	A9281

2.3 Instruments

Table 2.2: Equipment

Instrument	Specification	Manufacturer
2D electrophoresis chamber	Protean II xi cell	Bio-Rad
Agarose gel electrophoresis system and power supply	B1A EasyCast™ Consort E835	Owl Separation Systems Power supply: MS Laborgeräte
Analytical scales	EW 4200-2NM TE124S TE601	Kern & Sohn GmbH Sartorius Sartorius
Autoclaves	H+P Varioklav, 25T H+P Varioklav, 75S	Federgari Autoklav, Integra Bioscience (IBS)
Calibrated Imaging Densitometer	GS710	Bio-Rad
Centrifuges	5415D Sorvall Centrifuge RC26 Rotor: Sorvall SS-34	Eppendorf Kendro Kendro
Confocal laser scanning microscope	Axiovert 100M	Zeiss
Cooling centrifuge	Biofuge Fresco	Heraeus instruments
Fluorescence microscope	Leitz Laborlux	Leitz Wetzlar Germany
Fluorometer	SFM25 Bio-TEK	Kontron Instruments
Freeze dryer	Alpha 1-2	Christ
Gel electrophorese system	Model No. Mini-PROTEAN® 3 Cell	Bio-Rad
Heater/Stirring device	POWERTHERM VARIOMAG®	H+P Labortechnik AG
IEF cell	Protean IEF cell	Bio-Rad
Incubator (37°C)	Heraus B6	Kendro
Incubator (shaking) (20/30/37°C)	MULTITRON	Infors
Incubator (78°C)	Heraus T20	Kendro
Incubator (shaking) (78°C)	THERMOTRON	Infors
Laser scanner	Molecular imager FX pro plus	Bio-Rad
Manifold vacuum stainless steel filtration module		Millipore
Microwave	HF1612	Siemens AG
Molecular Imager Gel Doc	Universal Hood II	Bio-Rad

Instrument	Specification	Manufacturer
pH meter	WTW Series inoLab pH 720; pH-Electrode: SenTIX 81 pH0-14/0-100°C/3mol/KCl	WTW GmbH
Phase contrast microscope	Leica DM LS	Leica Microsystems
Plate Reader	Infinite Pro M200	Tecan
Spectrophotometer	Cary 50 Bio	Varian
Thermocycler	Mastercycler personal Thermocycler C1000	Eppendorf Bio-Rad
Thermoblock	Tsc ThermoShaker	Biometra GmbH
Thoma counting chamber		Optik Labor
Sonicator	UP 200s	Hielscher Ultrasonics GmbH
Vacuum centrifuge	RVC 2-25	Christ
VersaDoc imaging model	VersaDoc Model 4000 System	Bio-Rad
Water bath	GFL 1013	Fa. Gesellschaft für Labor-Technik GmbH
Wet tank blotting system	Mini Trans-Blot®	Bio-Rad

2.4 Software and databases

Table 2.3: Software and databases

Program	Version or link	Manufacturer
AxioVision	3.1	Zeiss
BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	National Library of Medicine
Cary Win UV Simple Reads Application	02.00(25)	Varian
CAZy	http://www.cazy.org/	AFMB - CNRS - Université d'Aix-Marseille
Compute pI/Mw tool	http://web.expasy.org/compute_pi/	ExPASy SIB bioinformatics resource portal
HHpred	http://toolkit.tuebingen.mpg.de/hhpred	Dept. of Protein Evolution at the Max Planck Institute for Developmental Biology
i-control	1.8.50.0	Tecan
KEGG	http://www.genome.jp/kegg/	Kanehisa Laboratories
LSM Image Browser	4.0.0.157	Zeiss
NCBI protein database	http://www.ncbi.nlm.nih.gov/protein	NCBI
PSORTb	http://www.psort.org/	Yu *et al.* 2010
SMART	http://smart.embl-heidelberg.de/	EMBL Heidelberg
TMHMM	http://www.cbs.dtu.dk/services/TMHMM/	Center for Biological Sequence Analysis
Quantity One	4.6.3	Bio-Rad
UniProtKB protein knowledgebase	http://www.uniprot.org/	UniProt

2.5 Strains and precultivation

2.5.1 *Escherichia coli*

E. coli K-12 DH5α (DSM 6897) was used for cloning of *S. acidocaldarius* genes. *E. coli* Rosetta(DE3) (Agilent Technologies/Stratagene) was used for heterologous expression of recombinant proteins. Pre-cultures of all strains were grown aerobically at 37°C at 180 rpm in Luria Bertani Broth (LB) medium.

Solid medium plates were prepared by adding 1.5% (w/v) agar-agar to the LB medium. Antibiotics were added to liquid and solid media according to the resistance encoded by the respective plasmids and strain specific resistance genes. Cultures carrying plasmids pET15b or pET28b were supplemented with 100 mg L^{-1} ampicillin and 50 mg L^{-1} kanamycin, respectively. *E. coli* Rosetta(DE3) cultures were additionally supplemented with 34 mg L^{-1} chloramphenicol. Growth was monitored photometrically at 600 nm (BioPhotometer).

2.5.2 *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*

S. acidocaldarius DSM 639 (wild type) was cultivated in Brock medium (Table 2.4) supplemented with 0.1% (w/v) N-Z-amine (EZMix™ N-Z-Amine® a casein enzymatic hydrolysate, from bovine milk, C4464, Sigma). The pH value was adjusted to 3.5 with 50% (v/v) sulfuric acid. Depending on the experiment dextrin (0.2% (w/v); from potato starch, 31400, Sigma) was added. The uracil auxotrophic *pyr*EF-deletion strain *S. acidocaldarius* MW001 (Wagner *et al.* 2012) as well as single gene deletion mutants derived from this strain additionally required 10 µg mL^{-1} uracil. For selection of gene insertion mutants, medium without addition of uracil was used. Liquid cultures were grown aerobically for approximately 18 h (exponential phase, OD_{600} of approximately 0.7) at 78°C (180 rpm). For cultivation on solid media, Brock medium was supplemented with 3 mM $CaCl_2$ and 10 mM $MgCl_2$ and solidified with 6 g L^{-1} gellan gum (Gelzan™, Sigma).

In the following *S. acidocaldarius* refers to strain DSM 639 and Brock medium refers to the Brock minimal medium supplemented with 0.1% (w/v) N-Z-amine and 0.2% (w/v) dextrin.

Sulfolobus solfataricus P2, PBL2025, PBL2025 pSVA9 (encoding lacS), PBL2025 pSVA31 (encoding ABCE1, a cytoplasmic protein involved in ribosome recycling used as control) and PBL2025 pSVAα-man (encoding the α-mannosidase) (Koerdt *et al.* 2012) cultures were grown aerobically in Brock media supplemented with 0.1% (w/v) N-Z-amine, 0.2% (w/v) D-glucose and 0.2% (w/v) D-arabinose (2 d, 78°C).

Table 2.4: Composition of modified Brock minimal medium according to Brock *et al.* (1972).

Constituents		Amount per L stock solution	Amount per L final Brock medium
Brock I			
	$CaCl_2$ x $2H_2O$	70 g	0.07 g
Brock II			
	$(NH_4)_2SO_4$	130 g	1.3 g
	$MgSO_4$ x 7 H_2O	25 g	0.25 g
	H_2SO_4 (50% (v/v))	1.5 mL	
Brock III			
	KH_2PO_4	56 g	0.028 g
	H_2SO_4 (50% (v/v))	1.5 mL	
	Trace elements		
	$MnCl_2$ x 4 H_2O	36 mg	1.8 mg
	Na_2MoO_4 x 2 H_2O	44 mg	0.22 mg
	$ZnSO_4$ x 7 H_2O	10 mg	0.05 mg
	$CuCl_2$ x 2 H_2O	6 mg	0.03 mg
	$NaMoO_4$ x 2 H_2O	2 mg	0.01 mg
	$VOSO_4$ x 2 H_2O	0.9 mg	4.5 mg
	$CoSO_4$ x 7 H_2O	6 mg	0.03 mg
1 L final Brock			
	Brock I		1 mL
	Brock II		10 mL
	Brock III		5 mL
	$FeCl_3$ x 6 H_2O (20% (w/v))		1 mL
	H_2SO_4 (50% (v/v))		Adjust to pH 3.5

2.6 General characterization of *S. acidocaldarius* biofilms

2.6.1 Optimization of growth conditions

To induce biofilm formation in *S. acidocaldarius* methods commonly applied for bacterial biofilm formation were adapted to the growth conditions required for *S. acidocaldarius*. Biofilms were cultivated on the surface of different substrata.

Polycarbonate membrane filter

1 mL of different dilutions (1:10, 1:20, 1:100, 1:200, diluted in Brock medium) of an exponentially growing liquid culture (approx. 10^9 cells mL^{-1}) in Brock medium was filtered onto black polycarbonate membrane filters (pore size 0.2, 30 mm diameter, Millipore). The membrane filters were placed on top of liquid Brock medium in presence or absence of 0.2% (w/v) dextrin in a wide neck Erlenmeyer flask. The flask with the floating filter was covered with aluminum foil and placed into an incubator without agitation (6-14 d, 78°C).

Mixed cellulose ester membranes

1 mL of different dilutions (1:10, 1:100, diluted in Brock medium) of an exponentially growing liquid culture (approx. 10^9 cells mL^{-1}) was filtered onto mixed cellulose ester membranes (pore size 0.45 µm, diameter 4.7 cm, Pall) which were placed on top of Brock medium plates. Stacks of up to 12 plates were sealed in plastic bags and incubated for 4 days at 78°C.

Gellan gum-solidified Brock medium plates

For the cultivation of S*. acidocaldarius* on Brock medium plates, two different methods were tested. For the spread plate method, 100 µL of different dilutions (10^{-1}-10^{-8}, diluted in Brock medium) of an exponentially growing liquid culture (approx. 10^9 cells mL^{-1}) were spread on Brock medium plates with a spatula. For the streaking method, an inoculation loop was used to spread an exponentially growing liquid culture (approx. 10^9 cells mL^{-1}) in single lines onto Brock medium plates. Stacks of up to 12 plates were sealed in plastic bags and incubated for 4 days at 78°C.

Plastic, elastomeric and glass surfaces

Coupons (72 mm x 26 mm x 2 mm) made of polyvinylchloride (PVC, provided by IWW Mülheim/Ruhr), high density polyethylene (PEHD300, provided by IWW Mülheim/Ruhr) and ethylene-propylene-diene monomer (EPDM 65 Shore, Schmitztechnik GmbH) as well as glass slides (76 x 26 mm x 1 mm; microscope slides made of soda-lime glass, Roth) were placed into 100 mL wide neck Erlenmeyer flasks filled with 50 mL Brock medium. The medium was inoculated with a *S. acidocaldarius* liquid culture (exponential phase, OD_{600} of approximately 0.6) to a final OD_{600} of 0.05. Flasks were closed with alumina foil and incubated at 78°C at 180 rpm for 2-4 days. 50% of the culture were replaced with fresh medium after 2 days of incubation. After 4 days coupons and glass slides were removed and analyzed microscopically.

2.6.2 Determination of biofilm mass

Determination of wet weight, dry mass as well as water content of *S. acidocaldarius* DSM 639 biofilms was determined according to the standard DIN EN 12880. After 4 days of incubation biofilm was scraped off from Brock medium plates and transferred to a crucible, which had previously been heated in a muffle furnace at 150°C for at least 30 min and cooled down to room temperature in a desiccator. The crucible was heated again at 105°C overnight and cooled down to room temperature in a desiccator prior to weighing. The crucible was heated at 105°C again for 1 h, cooled down and weighed. This was repeated until a constant weight of the crucible was obtained (< 0.5% (m/m) difference from the previous determination).

Determination of loss and residue on combustion of the dry mass of the biofilm was determined according to DIN EN 12879 by combusting the dried biofilm mass in a muffle furnace at 550°C. After 2 h the crucible was transferred to a desiccator and cooled down to room temperature before being weighed. The crucible was reheated to 550°C for further 30 min, cooled down to room temperature and weighed. This step was repeated until a constant weight was achieved (< 0.5% (m/m) difference from the previous determination).

2.6.3 Determination of total cell counts

4 mL cell suspension or decimal dilutions in deionized water were mixed with 1 mL 4',6-diamidino-2-phenylindole (DAPI; dihydrochloride, 25 µg ml^{-1} in 2% (v/v) formaldehyde; D9542 Sigma) for 20 min. Stained cells were then filtered onto black polycarbonate membrane filters (pore size 0.2 µm, 30 mm diameter, Millipore) and counted using an epi-fluorescence microscope. For statistical validity, 20 counting grids with 20-200 cells per grid were included in the count.

2.6.4 Determination of colony counts

Colony forming units of *S. acidocaldarius* biofilm suspensions and cell suspensions after EPS isolation were performed testing three different approaches on gellan gum-solidified Brock medium plates:

1. spreading of 100 µL cell suspension using a spatula,
2. spreading of 100 µL using glass beads and
3. spreading 1 mL cell suspension by tilting of the plate until the plate was completely and equally covered.

Plates were then dried and incubated at 78°C for 4 days in a sealed plastic bag and plates showing 20-200 colonies were counted.

2.6.5 Microscopy of biofilms and EPS components

Biofilms grown on polycarbonate membrane filters were stained with either DAPI (25 µg mL^{-1} in 2% (v/v) formaldehyde), SYTO9 (5 µM final concentration in DMSO; S-34854 Life Technologies) or wheat germ agglutinin (WGA, 5 µM in DMSO; L4895 Sigma) for 25 minutes. The stain was removed by washing three times with H_2O. Afterwards the sample was placed on a glass slide on top of a drop of mounting medium. Another drop of mounting medium was put on the filter and covered with a cover slide. Images were acquired using a confocal laser scanning microscope (CLSM) or an epi-fluorescence microscope. For the detection of SYTO9, an excitation at 488 nm using an argon laser was applied. Emission was detected using a band-pass with a range of 505 – 530 nm. DAPI staining was visualized using an epi-fluorescence microscope (filter: PHACO 3).

2.6.6 Determination of cell surface hydrophobicity

The cell surface hydrophobicity of *S. acidocaldarius* was determined according to the microbial adhesion to hydrocarbons (MATH) method described by Rosenberg (1984 and 2006). To determine variations of the results due to the pH value a variation of medium in combination with pH value was performed. Additionally to the originally applied buffer used in this assay (phosphate buffer saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7), also Brock medium without supplements (pH 3.5) was applied. 2 x 2 mL of an exponentially growing *S. acidocaldarius* liquid culture were centrifuged (6,000 x g, 5 min, 4 °C) and the pellet was washed once with either PBS (pH 7) or Brock medium (pH 3.5). After centrifugation the pellet was suspended in the respective buffer/medium and the OD_{600} adjusted to 0.4 – 0.9 (OD_{Start}). Of each sample, 1.2 mL were transferred to Eppendorf reaction tubes, covered with 200 µL n-hexadecane (≥ 99%; Roth) and mixed for 2 min. After 15 min 0.8 mL of the lower phase were transferred into a cuvette and the OD_{600} was determined (OD_{End}). The hydrophobicity index (HPBI) was calculated according the following equation:

$$\boldsymbol{HBPI} = \frac{OD(Start) - OD(End)}{OD(Start)} * 100\%$$

The classification of strains in terms of their surface hydrophobicity was adapted from Martinez-Martinez *et al.* (1991):

HPBI < 20%: hydrophilic
HPBI > 20% < 40%: intermediate
HPBI > 40%: hydrophobic

Since no archaeal control strains are known for this assay, the hydrophilic bacterial strain *Klebsiella oxytoca* RW15 and the hydrophobic bacterial strain *Acinetobacter junii* RW3 (kindly provided by the group of Prof. H.-C. Flemming) were used as controls. Cultivation of the bacterial strains was performed in tryptic soy broth (30 g L^{-1}, Roth) at 25°C overnight (180 rpm).

2.7 EPS isolation

Five methods were tested for their suitability to isolate EPS from biofilm of *S. acidocaldarius*. *S. acidocaldarius* shaking cultures were grown aerobically in Brock medium. Exponentially growing liquid cultures (approx. 10^9 cells mL^{-1}) were streaked on gellan gum-solidified Brock medium using an inoculation loop as described in section 2.6.1. After 4 days the biomass was scraped from the surface of the solid medium, using a spatula, weighed and suspended in 6 mM phosphate buffer (0.1 g of wet biomass per 10 mL; 2 mM Na_3PO_4 x 12 H_2O, 4 mM NaH_2PO_4 x 1 H_2O, 9 mM NaCl, 1mM KCl, pH 7).

Shaking

Biofilms suspended in phosphate buffer were transferred into 50 mL centrifuge tubes in 10 mL aliquots and shaken at highest capacity for 20 min on a shaker (Vortex Genie®2, Scientific Industries).

Cation exchange resin (CER)

Biofilms suspended in phosphate buffer were transferred into 50 mL centrifuge tubes in 10 mL aliquots. The CER (Dowex® Marathon® C sodium form, Sigma) was washed twice with phosphate buffer (15 min; 1 g Dowex 10 mL^{-1} buffer). To each tube, 2 g of the hydrated CER were added. The samples were shaken at highest capacity for 20 minutes on a shaker (Vortex Genie®2, Scientific Industries).

NaOH

30 mL of the biofilms suspension in phosphate buffer were mixed with 12 mL NaOH (1 M in H_2O) and stirred on a magnetic stirrer for 3 h at 4°C.

EDTA

30 mL EDTA (disodium salt, 2% (w/v) in H_2O) were added to 30 mL biofilm suspended in phosphate buffer and the mixture was stirred on a magnetic stirrer for 3 h at 4°C.

Crown ether

30 mL biofilm suspended in phosphate buffer were centrifuged and the pellet resuspended in 30 mL crown ether in Tris buffer (50 mM Tris, pH 8, 30 mM dicyclohexyl-18-crown-6 ether; 158402 Sigma) and stirred on a magnetic stirrer for 3 h at 4°C.

After each EPS isolation samples were centrifuged (20 min, 20,000 x g, 4°C) and the supernatants (EPS and low molecular weight substances) were filter-sterilized (pore size 0.22 µm, Rotilabo®, Roth) and dialyzed against deionized water using a dialysis membrane of 3500 Da molecular weight cut-off (MWCO; Spectrum Laboratories). The dialyzed supernatant corresponded to the EPS solution. The cell pellet after EPS isolation, suspended in phosphate buffer, is referred to as "cells after EPS isolation".

2.8 Quantitative biochemical analysis

2.8.1 Multi-element analysis of biofilms and EPS

Multi-element analyses of biofilm and cell suspensions as well as EPS solutions were performed by the IWW Water Centre (Mülheim/Ruhr) using inductively coupled plasma optical emission spectrometry (ICP-OES) according to ISO 11885 (2007). Biofilm and cell suspensions were treated with a mixture of HNO_3 and H_2O_2 in combination with microwave treatment before the determination.

2.8.2 Quantification of carbohydrates

Carbohydrate concentrations were determined using the phenol/sulfuric acid method described by Dubois *et al.* (1956). 2.5 mL sulfuric acid (95% - 97%) and 0.5 mL phenol (0.5% (w/v)) were added to 500 µL sample, blank (water) and standards and mixed immediately. Samples were incubated at room temperature for 10 min and subsequently at 30°C for 30 min in a water bath. After additional 5 min at room temperature absorbance at 490 nm was measured in a macro cuvette against deionized water. A three-point calibration was performed using D-glucose standards in a concentration range of 0 µg mL^{-1} to 75 µg mL^{-1}. Each determination was performed in triplicates.

2.8.3 Quantification of proteins

Lowry assay

For the determination of protein concentrations in biofilm suspensions, cells (cell pellet in phosphate buffer after EPS isolation) and supernatant after EPS isolation as well as in the EPS solution a modified Lowry assay (Peterson 1977) was applied using the following commercially available reagents prepared according to the manufactures' instructions:

- Lowry reagent (Sigma L3540; 45.03% sodium dodecyl sulfate, 38.49% (w/v) sodium carbonate, 15.13% (w/v) lithiumhydroxide-monohydrate, 1.35% (w/v) copper-tartrate-complex; 2 g) was dissolved in 40 mL deionized water.
- Folin-Ciocalteu's phenol reagent (Sigma F9252; 61.2% (v/v) water, 12.2% (w/v) lithium sulfate, 2% (w/v) sodium tungstate dehydrate, 9.5% (v/v) hydrochloric acid (≥ 25%), 6.9% (v/v) phosphoric acid solution in water, 2% (w/v) sodium molybdate-dihydrate) was diluted with water in a ratio of 1:5.
- Contents of one vial bovine serum albumin (BSA, Sigma P5619; 2 mg) were dissolved in 5 mL deionized water to a final concentration of 400 µg mL^{-1}.

For protein concentration determination 0.5 mL of the Lowry reagent were added to 0.5 mL sample, blank (water) and standards and mixed immediately. After 20 min incubation at room temperature, 0.25 mL Folin-Ciocalteu's phenol reagent (2 N) were added and the samples were incubated for 30 min at room temperature. Absorbance was measured at 750 nm in a semi-micro cuvette against deionized water. A 3-point calibration was performed using BSA standards in a concentration range of 0 µg mL^{-1} to 60 µg mL^{-1}. Each determination was performed in triplicates.

Bradford assay

For the determination of protein concentrations of samples from heterologous expression in *E. coli* (section 2.14.16) the Bio-Rad protein assay based on the method of Bradford (Bradford, 1976), was applied following the manufactures' instructions. A standard calibration was performed using BSA in a concentration range of 1-25 µg mL^{-1}. Samples were diluted in water to a volume of 0.6 mL and 0.4 mL of the Bradford reagent were added. After 5 min incubation at room temperature in the dark absorbance at 595 nm was determined (BioPhotometer).

2.8.4 Quantification of DNA

PicoGreen assay

DNA concentration in EPS solutions were determined using the fluorescent dye PicoGreen (Quant-iT^{TM} PicoGreen® dsDNA Reagenz, Invitrogen). Two calibrations in 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) were performed using λ-DNA as standard.

Low range: 0, 0.05, 0.5, 5 and 50 ng mL^{-1} λ-DNA
High range: 0, 2, 20, 200 and 2000 ng mL^{-1} λ-DNA

1 mL sample or standard was mixed with 1 mL 1 x PicoGreen reagent and the mixture was incubated for 2 min in the dark. The relative fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Photometric quantification

DNA concentrations for molecular biology methods were measured spectrometrically at 260 nm (BioPhotometer). For this 2 µL sample were mixed with 78 µL RNase/DNase-free water and filled into a UV-cuvette. Purity of DNA was evaluated by determining the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}).

2.9 Identification of monosaccharides

2.9.1 Hydrolysis of polysaccharides

Hydrolysis of polysaccharides was performed as described by Rode (2004). 2 mL EPS solution were dried using a vacuum centrifuge. The dried EPS were suspended in 1 mL 0.1 M HCl and incubated at 100°C for 48 h. Neutralization was performed using 1 M NaOH. The hydrolyzed EPS was then concentrated to 100 µL using a vacuum centrifuge. 100 µL acetone were mixed with each sample. After 2 h at 4°C samples were centrifuged (16,000 x g, 45 min) and the supernatants used for thin layer chromatography.

2.9.2 Thin layer chromatography (TLC)

Identification of monosaccharides was performed using TLC modified after Batisse *et al.* (1992). As solvent a mixture of acetonitrile:1-pentanol:water (60:20:20, v/v/v) was used. The solvent was filled into the chromatography chamber at least 1 h prior to the run together

with a Whatman paper for saturation of the gaseous phase. For each run 6 µL of the respective monosaccharide standards (D-galactose, D-mannose, D-glucose, D-xylose, L-rhamnose; 30 mM) and 8 µL of the sample were slowly applied onto silica gel coated TLC plates (20 x 20 cm or 10 x 20 cm silicagel G60 plates, Merck) to produce small spots. After drying the plate was placed into the chromatography chamber and developed for 3 x 45 min with drying steps of at least 15 min between each run. Afterwards plates were dried and sprayed with the derivatization reagent N-(-1-naphthyl) ethylenediamine-dihydrochloride (3 g L^{-1} in 5% (v/v) methanol). The plate was then transferred to an oven and heated at 100°C for at least 10 min until spots appeared. Images were taken using a digital camera.

2.10 Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.10.1 1 D gel electrophoresis

Proteins were separated and visualized by SDS-PAGE according to Laemmli (1970). The concentration of polyacrylamide in the separating gel was adjusted to the molecular mass of the protein of interest. For standard operations 12.5% polyacrylamide separation gels were used composed of 12.5% (w/v) acrylamide-bisacrylamide (30%), 375 mM Tris (pH 8.8, RT), 0.1% (w/v) SDS, 0.67% (w/v) ammonium persulfate (APS) (100 g L^{-1}), 0.067% (v/v) N,N,N´,N´-tetramethylethylenediamine (TEMED). The stacking gel was composed of 4.0% (w/v) acrylamide-bisacrylamide (30%), 125 mM Tris (pH 6.8, RT), 0.1% (w/v) SDS, 0.45% (w/v) APS (100 g L^{-1}), 0.15% (v/v) TEMED. The separation gel was poured into the gel casting chamber and overlaid with isopropanol (100%). After polymerization the stacking gel was poured on top, the comb inserted and, if not used immediately, stored at 4°C after polymerization.

Protein samples were mixed with loading buffer (final concentration of 62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) dithiothreitol (DTT), and 0.005% (w/v) Bromophenol blue) and incubated at 95°C for 10 min in order to denature proteins. The samples as well as 5 µL of a protein size marker were loaded per lane (PageRuler Unstained Protein Ladder, Fermentas). Electrophoresis was performed using a Mini-Protean 3-System (Bio-Rad) at constant 25 mA per gel in electrophoresis buffer (25 mM Tris-HCl, 190 mM glycine and 0.1% (v/v) SDS, pH 8.3). For visualization of protein bands, gels were stained with Coomassie Brilliant Blue (section 2.11.3). Gel documentation was performed using the

Molecular Imager Gel Doc XR System (Bio-Rad) and the Quantity One Software Package (Bio-Rad).

2.10.2 2 D gel electrophoresis

Sample preparation

EPS isolated from *S. acidocaldarius* biofilms were analyzed by 2 D gel electrophoresis (2 DE). The EPS required additional clean-up steps for 2 DE to remove interfering DNA, low molecular weight substances and salts. 9 mL EPS solution were mixed with 1 mL Benzonase buffer (500 mM Tris, 10 mM $MgCl_2$, pH 8, filter sterilized) and 10 µL Benzonase (purity > 99%; Novagen) corresponding to a final concentration of 65 U mL^{-1} and the solution was incubated at 37°C for 1 h (180 rpm). The Benzonase treated EPS solution was dialyzed (MWCO of 12-14 kDa, Spectropor) against 3 x 5 L deionized water (2 changes 1 h and 1 change overnight). Protein concentration in the Benzonase-treated EPS solutions was determined using the modified Lowry assay (section 2.8.3). Aliquots of the protein solution (100 µg protein to 400 µg protein) for 2 DE were freeze-dried.

Isoelectric focusing (IEF)

Freeze-dried samples were suspended in 380 µL IEF buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 5 mM tributylphosphine, 0.25% (w/v) Servalyt 3 – 10 ampholyte (Serva), few crystals bromophenol blue prepared in ultrapure water; Ultrapur, Merck). After 60 min incubation at room temperature the samples were transferred to wells of a focusing tray and a thawed IPG strip was added (immobilized pH gradient; pH 3 – 10 linear or nonlinear, Bio-Rad). Each strip was covered with 3 mL mineral oil to avoid desiccation. IPG strips were rehydrated overnight at 20°C. After rehydration, wicks wetted with ultra-pure water, were placed between the gel and the electrodes. Focusing was performed with a maximum current of 75 µA per gel and change of wicks before each step to remove accumulated salts (Table 2.5). After focusing, IPG strips were either directly used for separation in the second dimension or stored at -20°C.

Table 2.5: Steps for isoelectric focusing.

Step	Voltage	Mode	Duration
1	200	Rapid increase	45 min
2	500	Rapid increase	45 min
3	1000	Rapid increase	45 min
4	10,000	Linear increase	4 h
5	10,000	Rapid increase	Approx. 5 h

Preparation of gels for 2 DE

Polyacrylamide gels for separation of the proteins in the 2nd dimension were poured one day prior to the gel run. For one 12% polyacrylamide gel with a size of 20 x 20 cm 17 mL, deionized water were mixed with 12.5 mL resolving gel buffer (1.5 M Tris/HCl pH 8.8), 20 mL 30% (w/v) acrylamide-bisacrylamide solution (ratio 29:1), 0.5 mL 10% (w/v) SDS solution, 25 µL TEMED and 75 µL 40% (w/v) APS and poured into the space between the two glass plates. 3 mL water saturated 2-butanol were used to cover the top of the gel in order to generate an even surface and avoid desiccation. The gels were allowed to polymerize for approximately 4 h at room temperature and then were stored at 4°C overnight.

Gel electrophoresis

Focused IPG strips were thawed and equilibrated on a rocking platform for 15 min in 10 mL equilibration buffer (6 M urea, 30% (w/v) water free glycerin, 2% (w/v) SDS, 0.05 M resolving gel buffer) containing 0.1 g DTT and another 15 min in 10 mL equilibration buffer per strip containing 0.5 g iodoacetamide. 2-butanol was removed from the gels and the top of the gel washed 3 times with 1 x SDS-Tris-glycine running buffer (Rotiphorese® 10x SDS PAGE, Roth). The strip was washed briefly in running buffer and placed on top of the gel. A wick was wetted with 5 µL protein marker (Mark12, Invitrogen or PageRuler™ Plus Prestained Protein Ladder, Fermentas), allowed to dry, and placed next to the IPG strip. 2 mL of 0.5% (w/v) agarose, mixed with bromophenol blue, were used to fix the marker and the strip. Electrophoresis was performed at 20 mA per gel for 45 min, followed by 35 mA per gel for 4.5 h in a Protean II Xi cell with 1 x SDS-Tris-glycine running buffer.

2.11 Gel staining and image acquisition

2.11.1 Silver staining

Silver staining was performed according to the protocol of Blum *et al.* (1987) (Table 2.6). For large gels (20 x 20 cm) 400 mL per gel of each solution were prepared fresh using Milli-Q water while for mini gels (10 x 8.5 cm) 200 mL per gel were used. Gel documentation was performed with the Imaging Densitometer GS-700 (Bio-Rad).

Table 2.6: Silver staining protocol according to Blum *et al.* (1987).

Step	Solution	Duration
Fix	50% (v/v) methanol 12% (v/v) acetic acid 0.5 mL L^{-1} 37% (w/v) formaldehyde	≥ 1 h
Wash	50% (v/v) ethanol	3 x 20 min
Pretreat	0.2 g L^{-1} $Na_2S_2O_3$ x 5 H_2O	1 min
Rinse	Deionized water	3 x 20 s
Impregnate	2 g L^{-1} silver nitrate 0.75 mL L^{-1} 37% (w/v) formaldehyde	20 min
Rinse	Deionized water	2 x 20 s
Develop	50 g L^{-1} Na_2CO_3 0.5 mL L^{-1} 37% (w/v) formaldehyde 4 mg L^{-1} $Na_2S_2O_3$ x 5 H_2O	Approx. 3 min
Wash	Deionized water	2 x 2 min
Stop	50% (v/v) methanol 12% (v/v) acetic acid 50% (v/v) methanol	10 min ≥ 20 min

2.11.2 Enhanced Coomassie Brilliant Blue staining

Coomassie staining was performed at room temperature according to the protocol by Kang *et al.* (2002) using 0.02% (w/v) Coomassie Brilliant Blue (CBB) G-250, 5% (w/v) aluminum sulfate-(16)-hydrate, 10% (v/v) ethanol (96%) and 2% (w/v) orthophosphoric acid in Milli-Q water. Prior to staining overnight, gels were washed in Milli-Q water 3 times for 10 min to remove residual SDS, which would decrease the staining efficiency. Gels were destained in Milli-Q water for up to 24 h at room temperature. Gel documentation was performed with the Imaging Densitometer GS-700 (Bio-Rad).

2.11.3 Rapid Coomassie Blue staining

Rapid Coomassie Blue staining was only applied for mini gels. The staining solution contained 0.05% (w/v) Coomassie Brilliant Blue (CBB) G-250 (B0770 Sigma) 40% (v/v) ethanol and 10% (v/v) acetic acid in Milli-Q water. Gels were stained for 30 min at room temperature. For destaining gels were immersed in water, covered with a folded paper towel to remove the stain, boiled for a few seconds in a microwave and agitated at room temperature until sufficiently destained. Gel documentation was performed either with the Imaging Densitometer GS-700 (Bio-Rad) or with the Molecular Imager Gel Doc XR System (Bio-Rad).

2.11.4 Staining of glycoproteins

Glycoproteins were stained with the Pro-Q®Emerald 300 staining kit (Invitrogen) according to the manufacturer's instructions. Briefly, gels (20 cm x 20 cm) were fixed in 1 L 50% (v/v) methanol and 5% (w/v) glacial acetic acid in Milli-Q water. Gels were washed 2 x 20 min in 1 L 3% (w/v) glacial acetic acid. Oxidizing of carbohydrates was performed for 1 h in 500 mL periodic acid supplied with the kit. Gels were washed again 3 x 20 min in 3% (w/v) glacial acetic acid and stained for 2.5 h in the supplied staining solution. After two additional washing steps fluorescence was detected using the Molecular Imager Gel Doc XR System (Bio-Rad). For mini gels (8.5 cm x 10 cm) the volumes were quartered and the oxidizing and staining times reduced by half.

2.12 Determination of enzyme activities

2.12.1 Fluorimetric screening of enzyme activity

EPS and cells obtained after EPS isolation from *S. acidocaldarius* biofilms were analyzed for enzyme activity of seven different enzyme groups. The activity screening was performed in microtiter plates using the fluorescent 4-methoxy-ß-naphthylamide and methylumbelliferyl (MUF) substrates (Sigma). EPS samples were adjusted to pH 3.5 (corresponding to the environmental pH) while cell suspensions obtained after EPS isolation and resuspension in phosphate buffer were tested at a pH of 7.0 (corresponding to the approximate intracellular pH of 6.5). 2 mM stock solutions in 2-methoxyethanol of the following substrates were prepared:

L-alanine-4-methoxy-β-naphthylamide, 4-MUF-α-D-glucopyranoside, 4-MUF-β-D-glucopyranoside, 4-MUF-N-acetyl-β-D-glucosaminide, 4-MUF-stearate, 4-MUF-butyrate and 4-MUF-phosphate.

10 µL of the respective substrate were mixed with 190 µL cell suspension or EPS solution. Controls without substrate contained 10 µL 2-methoxyethanol instead and 190 µL cell suspension or EPS. In order to evaluate heat stability and possible hydrolysis of the substrates, substrates mixed with phosphate buffer at pH 7.0 and pH 3.5 without addition of EPS or cells were used as negative controls. A 3-point calibration was performed with methylumbelliferone in a range of 0 mM (blank) up to 200 mM (50 mM, 100 mM, 150 mM). For 4-methoxy-ß-naphthylamide a 3-point calibration in the range of 0 mM (blank) to 100 mM (25 mM, 50 mM, 100 mM) was performed.

Fluorescence was measured immediately after the addition of the samples to the substrates using a plate reader (Infinite Pro 200, Tecan). Measurements were performed at an excitation of 360 nm and an emission of 450 nm for MUF substrates and an excitation of 330 and an emission of 420 for 4-methoxy-ß-naphthylamide. Prior to each determination the plate was shaken using the plate reader (5 s, 1.5 mm amplitude). In between measurements the plate was placed into a heating block set to 70°C.

2.12.2 Zymographic analysis of protease activity

For the detection of protease activity polyacrylamide separating gels without SDS (10% (w/v) acrylamide-bisacrylamide (30%), 375 mM Tris-HCl, pH 8.8 (RT), 0.7% (w/v) APS (100 mg mL^{-1}), 0.067% (v/v) TEMED) containing either 0.1% casein (C3400 Sigma) or gelatin (48723 Sigma) were prepared. Stacking gels were composed of 4.0% (w/v) acrylamide-bisacrylamide (30%), 125 mM Tris-HCl, pH 6.8 (RT), 0.45% (w/v) APS (100 mg mL^{-1}), 0.1% (v/v) TEMED. Samples, each containing 5 µg protein, were mixed with non-reducing Roti®-Load 2 (4 x) sample buffer (Roth) and incubated for 15 min at room temperature. 5 µL of the prestained marker PageRuler™ Plus (Fermentas) were applied. Electrophoresis was performed at 125 V for 90 min in 1 x Rotiphorese SDS running buffer (Roth). The gels were gently agitated in 1 x Novex® Zymogram renaturing buffer (Invitrogen) at room temperature 2 x 30 min. Afterwards gels were transferred to 1 x Novex® Zymogram developing buffer (Invitrogen) with a pH of either 3.8 or 7. The gels were incubated for 1 h at room

temperature and, after exchange of buffer, overnight at either 37°C or 70°C. Staining was performed after washing 3 x 5 min in deionized water using Simply Blue Safe Stain (Invitrogen) for 1 h. Destaining was performed in deionized water 2 x for 1 h. Gel documentation was performed with the Imaging Densitometer GS-700 (Bio-Rad).

2 D gels for detection of protease activity were poured and run as described in section 2.10.2 but without addition of SDS and with addition of 0.1% casein to the separating gel and a protein amount of 400 µg. After electrophoresis gels were treated as stated for 1 D gels above. Treatment times were adjusted to the increased size of the gels: Agitation in renaturing buffer: 2 x 1 h, developing buffer: 1 x 1 h, 1 x 48 h, staining: 1 x 2 h.

2.12.3 Zymographic analysis of esterase activity

For the detection of esterase activity 2 D gels as described in section 2.10.2 were poured and run using 400 µg lyophilized protein. After electrophoresis gels were washed 2 x 15 min in water and agitated 2 x 30 min in renaturing buffer (100 mM Tris-HCl, pH 8, 25% (v/v) isopropanol). As substrate 4-methylumbellyferyl-butyrate (Sigma, 5 mM in renaturing buffer adjusted to pH 3 and pH 8) was used. Gels were agitated in 100 mL substrate for 10 min and images were taken using the Molecular Imager Gel Doc XR System (Bio-Rad). Afterwards gels were washed 2 x 15 min in water and stained with the enhanced CBB method after Kang *et al.* (2002)(section 2.11.2).

2.13 Identification of EPS proteins via MALDI-TOF/MS

EPS for protein identification via MALDI-TOF/MS was isolated from unsaturated *S. acidocaldarius* biofilms as stated in section 2.7. The dialyzed EPS proteins were analyzed by Jörg Kahnt at the MPI Marburg. Obtained protein identifications were compared to the arCOG (archaeal cluster of orthologous groups of proteins) functional codes and annotations using the updates *S. acidocaldarius* genome information (Esser *et al.* 2011). Only identifications based on at least 2 unique peptides were considered significant. Subcellular localization was performed using PSORTb (Yu *et al.* 2010).

2.14 Molecular biology methods

2.14.1 Isolation of chromosomal DNA from *S. acidocaldarius*

Chromosomal DNA of *S. acidocaldarius* was isolated from stationary grown cells (OD_{600} of approximately 0.9) using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer´s instructions (Protocol: Pretreatment for Gram-Negative Bacteria section 1., 2., 3., Protocol: Purification of Total DNA from Animal Tissue (Spin-column Protocol) section 2.-8.). DNA was eluted in 100 µL RNase/DNase-free water and stored at -20°C.

2.14.2 Isolation of plasmid DNA from *E. coli* DH5α

For isolation of large amounts of plasmid DNA, the QIAfilter Plasmid MidiKit (Qiagen) was applied, following the manufactures' instructions. For small scale isolation the QIAprep Spin Miniprep Kit (Qiagen) was used according to the manufactures' instructions. Plasmid DNA was stored in RNase/DNase-free water at -20°C.

2.14.3 Agarose gel electrophoresis

DNA yield, purity as well as size determination was performed using agarose gel electrophoresis of linear DNA fragments. 1% (w/v) agarose gels were prepared in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Samples were mixed with loading buffer (6 x DNA Loading Dye, Fermentas), loaded into the wells of the gel next to a DNA size marker (GeneRuler 1 kb Ladder, Fermentas) and electrophoresis was performed at 100 V at room temperature for approximately 45 min. Gels were stained for 20 min in an 500 µg L^{-1} ethidium bromide bath and destained for 2 min in deionized water. Gel documentation was performed under UV light using the Molecular Imager Gel Doc XR System (Bio-Rad).

2.14.4 Purification of DNA fragments

DNA fragments from agarose gels were purified using the Wizard® SV Gel and PCR Clean-Up System. For purification of PCR products the QIAquick PCR Purification Kit was applied. For nucleotide removal (after restriction) the QIAquick Nucleotide Removal Kit was applied. All kits were used following the manufacturer's instructions.

2.14.5 Amplification of *S. acidocaldarius* genomic DNA via polymerase chain reaction (PCR)

PCR amplification was carried out using 100 ng DNA template, 0.2 mM dNTPs (PeqLab), 0.2 µM of each forward and reverse primer, phusion polymerase buffer (5 x Phusion HF Buffer, NEB) and 1 U phusion polymerase in a total volume of 50 µL. Oligonucleotide primers were purchased from Invitrogen (Table 2.7). PCRs were performed using a thermocycler (Bio-Rad; Eppendorf) according to the following cycling protocol:

Initial denaturation step: 10 min, 95°C

Denaturation: 30 sec at 95°C

Annealing: 30 s (T_m; 55°C)

Extension: 25 s per 1,000 bp at 72°C

After 30 cycles, a final extension step was carried out at 72°C for 10 min. The product was stored at 4-8°C until further use.

Table 2.7: Primer for PCR amplification of *S. acidocaldarius* genes. Given are the name of the primer with the respective ORF ID as well as the primer sequence, restriction sites (marked in red) and expected size of the PCR product.

Name/ORF ID	Sequence of primer (5' → 3')	Restriction site	Size [bp]
Saci_1904_F	GCGC**CATATG**GACTTTGTCTTCTTTACAG	*Nde*I	1110
Saci_1904_R	GCGC**GGATCC**TTAACTCTTGACACCACAAC	*BamH*I	
Saci_1907_F	GGCCGG**CATATG**AAGGTTCTCGTCGTAAATC	*Nde*I	1165
Saci_1907_R	GAGGCC**GGATCC**TTAATCGTTTTCGGTAATTCTC	*BamH*I	
Saci_1909_F	GCGCGC**CATATG**CTTAGTGTTGTGATACC	*Nde*I	737
Saci_1909_R	GCGCGC**GGATCC**CTAGGAGTTCAGTCTATTAAATAC	*BamH*I	
Saci_1910_F	GCGC**CATATG**AGATCTGTGATAATTCTGTTG	*Nde*I	993
Saci_1910_R	GCGC**GGATCC**TCACTTTCCTTTTCTCCTATAAATG	*BamH*I	
Saci_1911_F	GCGC**CATATG**TGGTCGATTGAGATCC	*Nde*I	786
Saci_1911_R	GCGC**GGATCC**TCATCCTAATAAGTAGCCTAATATG	*BamH*I	
Saci_1914_F	GCGC**CATATG**CATTATGTGGAGATTAGT	*Nde*I	1068
Saci_1914_R	GCGC**GGATCC**TTACTCTGATTGATGTATATAACTTAAA	*BamH*I	
Saci_1915_F	GCGC**CATATG**CCCAAAGACTTCAGTG	*Nde*I	957
Saci_1915_R	GCGC**GGATCC**TTAACTAAACATCGAATTACTCCTTAAC	*BamH*I	
Saci_1916_F	GCGC**CATATG**ATAATGGAAAAACCGATAGC	*Nde*I	1329
Saci_1916_R	GCGC**GGATCC**TTATACTTTCATTAAATCCTCATATAG	*BamH*I	

Name/ORF ID	Sequence of primer (5' → 3')	Restriction site	Size [bp]
Saci_1917_F	GACCG**CATATG**GACTTTCTCAGGGC	*Nde*I	2374
Saci_1917_R	GCC**GGATCC**TTATTTTTCCCATCTTACATGAC	*BamH*I	
Saci_1917_Out_F	GCGC**CATATG**TCTTATCAACAAATAAAGACC	*Nde*I	956
Saci_1917_Out_R	GCGC**GGATCC**TTACAAAGCGATAACTACTG	*BamH*I	
Saci_1917_CBM_F	CGCG**CATATG**TCCAACAATCTATTGACTG	*Nde*I	380
Saci_1917_CBM_R	GCGC**GGATCC**TTACAAAGCGATAACTACTG	*BamH*I	
Saci_1918 _F	GCGC**CATATG**AAAAAATTTATAATTGAATCCAG	*Nde*I	780
Saci_1918_R	GCGC**GGATCC**TTACCTTTTAAACCTAGCATAAATC	*BamH*I	
Saci_1921_F	GCGC**CCATGG**TTAGTATAACATTCCTTATTG	*Nco*I	1152
Saci_1921_R	GCGC**CTCGAG**TATATCAACTTCAATCTGTTTC	*Xho*I	
Saci_1922_F	GCGC**CATATG**AGTAGAAAAACTAATAAGAACATAAC	NdeI	1314
Saci_1922_R	GCGC**CTCGAG**TTAAGAGGGAGTTGAAGCCAGTT	*Xho*I	
Saci_1923_F	GCGC**CCATGG**GTAAGAGAGGAATAATTTTTATAG	*Nco*I	1110
Saci_1923_R	GCGC**CTCGAG**CATTAAAAACTTATCTAACAACTC	*Xho*I	
Saci_1926_F	GCGC**CCATGG**CTATAAGAAGAGACGTTATC	*Nco*I	558
Saci_1926_R	GCGC**CTCGAG**ATTTGTTTCGAAAAATTTCG	*Xho*I	
Saci_1927_F	GCGC**CATATG**AGCACGGTAAGTGAG	*Nde*I	399
Saci_1927_R	GCGC**GGATCC**TTACGAAAATCTATTATGGTCCATCAC	*BamH*I	

2.14.6 Restriction and ligation of DNA

Digestion of plasmids was performed using 1 µL FastDigest enzymes (Fermentas) and 1 µg plasmid DNA in a final volume of 20 µL. For restriction of PCR products, 1 µL FastDigest enzyme was used to restrict 0.2 µg PCR product in 30 µL final volume. Restriction was performed using FastDigest buffer (Fermentas) at 37°C for 30 min, except for *Nde*I, which, in case of PCR product restriction, required an incubation time of at least 1 h. The applied restriction enzymes are listed in Table 2.7. Restricted plasmid DNA (60 ng) and PCR products were ligated in a molar ratio of 1:3 using 1 U T4 DNA ligase (Fermentas) in the respective buffer in a final volume of 10 µL. Ligation was carried out at 16°C overnight. Afterwards the samples were heated at 65°C for 10 min and used for ligation.

2.14.7 Preparation of chemically competent *E. coli* cells

Chemically competent *E. coli* DH5α and Rosetta(DE3) strains were prepared using the modified calcium chloride method. Briefly, 200 mL LB medium were inoculated with an overnight culture of *E. coli* (1% (v/v)) and incubated at 37°C (180 rpm) until an OD_{600} of 0.4 was reached. The culture was incubated on ice for 10 min and centrifuged (4,000 x g, 7 min, 4°C). The cell pellet was gently suspended in 10 mL ice cold buffer (60 mM $CaCl_2$, 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 15% (v/v) glycerol, pH 7.0) on ice, centrifuged (4,000 x g, 5 min, 4°C) and resuspended in 2 mL of the same buffer. Aliquots of 80 µL were frozen in liquid nitrogen and stored at -80 °C.

2.14.8 Preparation of competent *S. acidocaldarius* MW001 cells for electroporation

S. acidocaldarius MW001 cells were grown to an OD_{600} of 0.1-0.3 in Brock medium (0.2% (v/v) dextrin, 0.1% (v/v) N-Z-amine, 20 µg mL^{-1} uracil) and incubated on ice for 20 min. Cells were centrifuged (2,000 x g, 20 min, 4°C) and suspended 3 x in 20 mM sucrose (50 mL, 25 mL, 1 mL). After centrifugation (2,000 x g, 20 min, 4°C), the cell pellet was suspended in sucrose solution to give a theoretical OD_{600} of 10 and stored at -80°C in 50 µL aliquots.

2.14.9 Transformation of competent *E. coli* cells

Transformation of *E. coli* strains with the recombinant plasmids was performed by adding 10 µL ligation mix or 1 µL plasmid DNA to 80 µL suspensions of competent cells on ice. After 30 min incubation on ice cells were incubated at 42°C for 90 s, transferred back on ice, mixed with 600 µL LB medium and incubated at 37°C for 50 min (180 rpm). 100 µL of the transformed cells were spread on LB agar plates supplemented with the respective antibiotics. The remaining cells were centrifuged (6,000 x g, 2 min, RT) and the pellet resuspended in 100 µL LB medium and also plated on LB agar plates. Plates were incubated overnight at 37°C. Single colonies were transferred to liquid LB medium with the respective antibiotics and used for test restrictions in order to identify positive clones.

2.14.10 Electroporation of competent *S. acidocaldarius* MW001 cells

For the preparation of insertion mutants 50 µL competent MW001 cells were mixed with 600 ng PCR product, incubated on ice for 5 min and transferred to a 0.1 cm electroporation cuvette (Bio-Rad). Electroporation was performed at 1500 V, 600 Ω and 25 µF. Cells were immediately suspended in 2 x regeneration buffer (1% (w/v) sucrose, 10 mM $MgSO_4$ x 7 H_2O, 20 mM ß-alanine-malate-buffer (20 mM ß-alanine, 20 mM malate-solution; pH adjusted to 4.5 using malate solution)). After incubation at 75°C for 30 min cells were plated on gellan gum solidified Brock medium plates and the plates incubated at 78°C for 3 to 5 days.

2.14.11 Identification of positive *E. coli* clones

Clones carrying the recombinant plasmid were identified by restriction analysis. For this each 0.25 µL of the restriction enzymes used for restriction of the plasmid DNA were used to digest 4 µL DNA isolated via the QIAprep Spin Miniprep Kit (Qiagen) (section 2.14.6). For the plasmid isolation a 4 mL overnight culture was used. After 30 min incubation at 37°C samples were mixed with loading dye and agarose gel electrophoresis was performed as stated in section 2.14.3.

2.14.12 DNA Sequencing

Automated DNA sequencing was performed by LGC Genomics. 14 µL plasmid DNA (100 ng μL^{-1}) were sent for one sequencing reaction. For sequencing using primers not available in the LGC Genomics database, 10 µL plasmid DNA (100 ng μL^{-1}) mixed with 4 µL primer (5 µM) were sent.

2.14.13 Deletion of glycosyltransferase genes in *S. acidocaldarius* MW001

Glycosyltransferase (GT) gene deletion mutants (Table 3.3.1) were prepared by Benjamin Meyer (MPI Marburg). The uracil auxotrophic mutant *S. acidocaldarius* MW001 was used due to its lack to produce uracil, which was used as a selective marker (Wagner *et al.* 2012).

2.14.14 Construction of thermopsin insertion mutant *S. acidocaldarius* Δ1714

Disruption of Saci_1714, encoding a protease termed thermopsin, was achieved by insertion of the *pyr*EF cassette in the thermopsin gene. For this primers with flanking regions of the *pyr*EF cassette as well as homologous region within the middle of the thermopsin gene were used to amplify the *pyr*EF cassette from plasmid pSVA406 (FW 5'-ACG TTT ATT GAT AAT GTA TGG AAT TTG ACA GGA AGT TTA TCA ACT TTA AGT TTG AGC AGT TCT AG-3'; RV 5'-GGT TTG TCC ACC ACC TGC GGA TGC AAC TTG CCC ATT ACC TGT TAT TGC ACT TGA AGA CCG GCT ATT TTT TCA C-3'). The result was a PCR product consisting of a homologous region of the thermopsin gene, the *pyr*EF cassette and another homologous region of the thermopsin gene adjacent to the region before. The PCR product was purified by application of the GelEx Kit and electroporated (600 ng PCR product) into competent MW001 cells as stated in section 2.14.10.

2.14.15 Screening for positive *S. acidocaldarius* mutants

Colonies formed on gellan gum-solidified Brock medium plates were transferred to a new plate for backup using a pipette tip. The tip was then placed into a well of a 96-well microtiter plate containing 30 µL 0.2 M NaOH for cell lysis. After 10 minutes the solution was neutralized by adding 70 µL Tris-HCl-buffer (260 mM, pH 7.8). 1-2 µL were used for colony PCR using primers annealing at base pair position 382 and 890 within the thermopsin gene (FW 5'-GAA TTC CTT AAC GTT TAT TGA TAA TG-3'; RV 5'-TTG TTG TGT AGA CGT TAT ATG-3'). PCR conditions were set as described in section 2.14.5. Colonies with an insertion of the PCR fragment display a PCR product with a size of approximately 2000 bp while the non-disrupted gene display a PCR product of 508 bp.

2.14.16 Heterologous expression of *S. acidocaldarius* glycosyltransferases

For heterologous expression of the glycosyltransferases summarized in Table 2.8, *E. coli* Rosetta(DE3) cells were transformed with the respective plasmid and grown as a pre-culture. Expressions were performed in LB medium supplemented with the respective strain (1%-2%) and plasmid specific antibiotics (section 2.5.1). Cells were grown to an OD_{600} of 0.4 at which expression was induced by the addition of IPTG (final concentration of 1 mM). Cultures were further incubated overnight (approximately 17 h) at 20°C or for 3 h at 37°C and then centrifuged (7,000 x g, 20 min, 4°C).

Table 2.8: Heterologous expression of *S. acidocaldarius* target proteins. ORF ID, number of amino acids (aa), expression vectors and hosts (*E. coli*) are given.

ORF ID	Vector	aa	Host
Saci_1907	pET15b^{Amp}	354	Rosetta (DE3)^{Cam}
Saci_1909	pET15b^{Amp}	244	Rosetta (DE3)^{Cam}
Saci_1910	pET15b^{Amp}	330	Rosetta (DE3)^{Cam}
Saci_1911	pET15b^{Amp}	261	Rosetta (DE3)^{Cam}
Saci_1915	pET15b^{Amp}	318	Rosetta (DE3)^{Cam}
Saci_1917	pET15b^{Amp}	758	Rosetta (DE3)^{Cam}
Saci_1917_Out	pET15b^{Amp}	313	Rosetta (DE3)^{Cam}
Saci_1917_CBM	pET15b^{Amp}	119	Rosetta (DE3)^{Cam}
Saci_1918	pET15b^{Amp}	259	Rosetta (DE3)^{Cam}
Saci_1922	pET28b^{Kan}	437	Rosetta (DE3)^{Cam}
Saci_1923	pET28b^{Kan}	369	Rosetta (DE3)^{Cam}
Saci_1926	pET28b^{Kan}	185	Rosetta (DE3)^{Cam}
Saci_1927	pET15b^{Amp}	132	Rosetta (DE3)^{Cam}

2.14.17 Cell disruption and heat precipitation

Cell disruption was performed in 1 x Lysis-Equilibration-Wash buffer (LEW buffer: 50 mM NaH_2PO_4, 300 mM NaCl, pH 8) in a ratio of 1 g cell pellet (wet weight) to 3 mL buffer. Cell disruption was performed with an ultrasonic processor (amplitude 50%, time cycle 0.5) 3 x 10 min on ice. Cell debris was removed by centrifugation (16,000 x g, 45 min, 4°C). The supernatant (crude extract) was diluted 1:1 in LEW buffer and heat precipitations were performed at 60°C, 70°C, 80°C and 90°C for 20 min. Denatured proteins were removed by centrifugation (16,000 x g, 30 min, 4°C). Enrichment of heat stable proteins was monitored by SDS-PAGE (section 2.10.1).

2.14.18 Ni-TED affinity chromatography

Ni-TED chromatography was performed to purify His-tagged proteins. For this purpose the Nucleic Acid and Protein Purification Kit Protino® Ni-TED 150 or 2000 (Macherey-Nagel GmbH & Co.) was used, following the manufacturer´s instructions. Briefly, the Ni-TED column was equilibrated with LEW buffer. Binding of the His-tagged proteins was performed by application of the crude extract or the crude extract after heat precipitation to the

column. Two washing steps with LEW buffer and 3 elution steps with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8) followed. Purification of the protein was monitored by SDS-PAGE (section 2.10.1).

2.14.19 Western blotting and immunodetection

A tank blot system (Bio-Rad) was applied for western blotting. SDS-PAGE was performed using a prestained marker (PageRuler™ (Plus) Prestained Protein Ladder, Fermentas) as described in section 2.10.1. The polyacrylamide gel was equilibrated in transfer buffer (50 mM Tris, 380 mM glycin, 20% (v/v) methanol) for 30 min. A Roti®-PVDF membrane (Roth) was washed in methanol (100%) for 10 min, then washed in water for 2 min and finally equilibrated in transfer buffer for 5 min. The system was assembled as follows: Sponge, one thick Whatman paper, PVDF membrane, polyacrylamide gel, one thick Whatman paper, sponge. The tank blot system was filled with transfer buffer and transfer was performed at 4°C either for 2 h at 60 V or overnight at 20 V.

For detection of the His-tagged proteins the membrane was blocked with 5% (w/v) milk powder (blotting grade, low fat, T145.1, Roth) in TBST (0.3% (v/v) Tween, 1 x TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5)) for 1 h. For antibody binding the membrane was incubated for 1 h with the antibody solution (3% (w/v) milk powder, 1 x TBST, 17 µg His-Tag antibody Ms mAb to 6 x Histag®, abcam). Afterwards the membrane was washed 3 x 15 min in 1 x TBST and 2 x 10 min in 1 x TBS. All steps were performed on a rocking platform at room temperature. For detection of the His-tag antibody with bound alkaline phosphatase, 1 mL CDP-Star® (Sigma) substrate solution was poured onto the membrane and incubated for 10 min before chemiluminescence was detected using the VersaDoc Imaging system (Bio-Rad).

3. RESULTS

3.1 Optimization of growth and general characterization of *S. acidocaldarius* biofilms

The major objective of this study was the characterization of biofilms of the thermoacidophilic Archaeon *S. acidocaldarius*. In comparison to their bacterial counterparts, little is known about archaeal biofilms and their EPS. In order to isolate and analyze EPS produced by *S. acidocaldarius*, sufficient amounts of biofilm were required. Hence, the preliminary aim was to establish a method for cultivation of large amounts of biofilm mass. Diverse methods were adapted or developed considering the harsh growth conditions required for the cultivation (low pH value of 3.5, high temperature of 78°C) of *S. acidocaldarius*.

3.1.1 Cell surface hydrophobicity

Attachment of cells is known to be influenced by the hydrophobicity/hydrophilicity of the substratum and of the cell surface (Rosenberg 2006; Palacio and Bhushan 2012). The MATH test relies on the adherence of cells to the added hydrocarbon, in this case n-hexadecane. Depending on the hydrophobicity index (HPBI), the distribution of cells between the buffer and the hydrocarbon phase, cells are divided into three classes: hydrophobic, intermediary or hydrophilic. The results listed in Table 3.1 show that the surface of *S. acidocaldarius* cells was categorized as hydrophobic with HPBI indices of 87 ± 5% and 63 ± 2% suspended in Brock medium (pH 3.5) and PBS buffer (pH 7.0), respectively.

Table 3.1: Surface hydrophobicity according to MATH test for *S. acidocaldarius* and bacterial reference strains. Test was performed in either PBS (pH 7.0) or Brock medium (pH 3.5). For *S. acidocaldarius* n = 2, for reference strains n = 1.

Strain	Medium	HPBI [%]	Hydrophobicity
S. acidocaldarius	Brock medium (pH 3.5)	86 ± 5	hydrophobic
S. acidocaldarius	PBS (pH 7.0)	63 ± 2	hydrophobic
K. oxytoca RW15	PBS (pH 7.0)	-1	hydrophilic
A. junii RW3	PBS (pH 7.0)	96	hydrophobic

3.1.2 Submersed biofilm formation on solid supports

In order to grow *S. acidocaldarius* as a submersed biofilm on a solid support, different materials, which are known to promote cell attachment, were tested. The first tested substratum was glass in the form of microscope glass slides, which are frequently used for the formation of bacterial biofilms (e.g. Mah and O'Toole 2001; Vallet *et al.* 2001). The advantage of using glass slides is their suitability for subsequent microscopic analysis. In this study microscope glass slides were placed into wide neck Erlenmeyer flasks filled with Brock medium. After inoculation with *S. acidocaldarius*, flasks were incubated at 78°C for 2 or 4 days either with or without shaking at 180 rpm. After 2 or 4 days attachment of single cells to the glass surface was detected using light microscopy. However, cells were scattered over the surface and did not produce a multilayered biofilm required for further analysis.

Aside from glass slides, coupons (72 mm x 26 mm x 2 mm) made of materials shown to promote the formation of biofilms were tested using the same approach as described for the glass slides. First, EPDM, which was previously used in studies for the cultivation of drinking water biofilms (Bressler *et al.* 2009; Michalowski 2012) was tested. Additionally to a hydrophobic surface, which should assist in the attachment of cells, EPDM leaches plasticizers, which can serve as nutrients and thus, should further enhance growth of biofilms. Nevertheless, in this study no biofilm growth was detectable on EPDM coupons using cell staining with SYTO9. Other materials (PVC, PE) also did not reveal any colonization under shaking conditions as well as static conditions. Only a deposit of medium, most likely iron, was visible. Consequently, cultivation on solid supports did not yield enough biomass formation for further analysis of *S. acidocaldarius* EPS.

3.1.3 Biofilm formation on floating polycarbonate membrane filters

For the formation of unsaturated biofilms on black polycarbonate membrane filters an exponentially growing planktonic culture (approximately 10^9 cells mL^{-1}) was diluted 1:20, 1:100 and 1:200 and 1 mL of each dilution was filtered onto a membrane. The membrane was placed on top of liquid Brock medium either with or without dextrin supplementation and incubated for 6 to 14 days at 78°C. After incubation for 6 days single colonies of approximately 1-2 mm in diameter and an average thickness of 110 µm (as estimated by light microscopy and CLSM measurements (section 3.2.3)) were observed on membranes

inoculated with the 1:20 dilution (Figure 3.1). Membranes on medium without dextrin showed less colonies compared to those on medium with dextrin. Colonies on both membrane filters had a slimy appearance. Additionally, a difference in coloration of the colonies was observed. While colonies on medium without dextrin displayed a grey color, colonies on medium with dextrin exhibited a grey-brown coloration. Given the approximate cell number of 5 x 10^7 cells per filter, a confluent biofilm was expected.

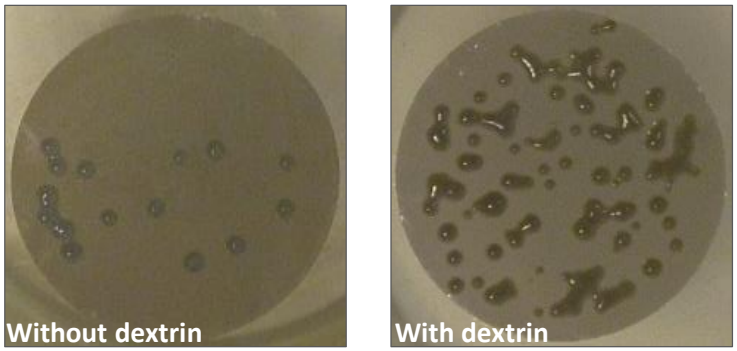

Figure 3.1: *S. acidocaldarius* biofilm growth on polycarbonate membrane filters floating on Brock medium after 6 days. Photos were taken after 6 days of incubation on Brock medium with (right) and without (left) dextrin at 78°C. Membrane filters were inoculated with 1 mL 1:20 diluted exponentially growing planktonic culture (approximately 5 x 10^7 cells mL^{-1}).

On filter membranes inoculated with fewer cells colony formation was observed after 9 ± 2 days leading to large colonies after 14 days (Figure 3.2). While the diameter of the colonies was increased (approximately 4 mm) the thickness of the colonies did not increase compared to the biofilm formed after 6 days in the 1:20 diluted cell suspensions. Similar to the 1:20 diluted cell suspensions, the color of the formed biofilm on medium with dextrin changed over the period of 14 days from dark grey to brown. A color change was also observed in the media showing a clear, slightly white color at the start of the experiment, which changed to a yellow-brownish and clear color in the medium with dextrin (Figure 3.2, right column). This method of biofilm formation was shown to be best suited for the microscopic analysis of biofilms since the filter membranes are easily removed from the medium without destruction of the biofilm architecture. Nevertheless, formation of biofilms was too low for EPS isolation and due to variations of the filter quality in different batches, resulting in discoloration of the medium, extent of biofilm formation was not reproducible.

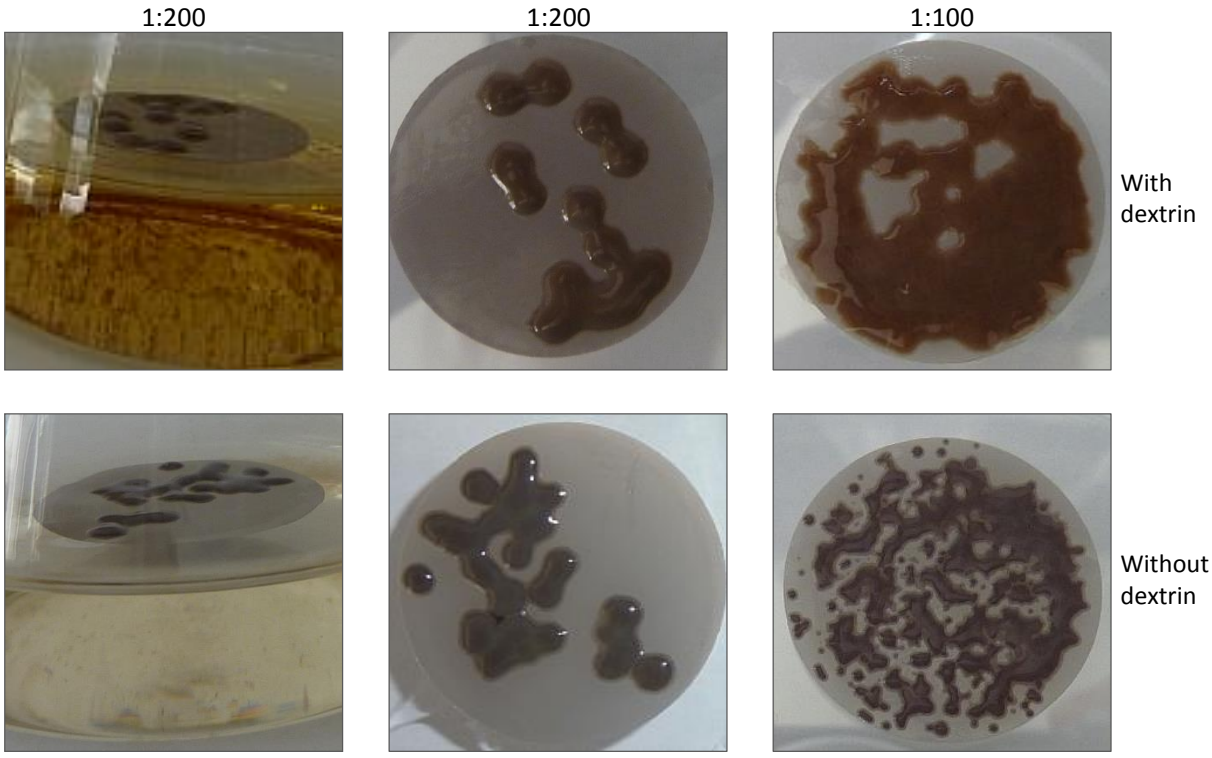

Figure 3.2: *S. acidocaldarius* biofilm growth on polycarbonate membrane filters floating on Brock medium after 14 days. Filters were incubated on Brock medium supplemented with (upper row) and without (lower row) dextrin at 78°C and inoculated with 1 mL 1:100 (approximately 10^7 cells) or 1:200 (approximately 5 x 10^6 cells) diluted exponentially growing planktonic culture. The left column shows the different coloration of Brock medium with or without dextrin.

3.1.4 Biofilm formation on gellan gum-solidified Brock medium

For the formation of an unsaturated biofilm on the surface of Brock medium plates initially the spread plate method was tested. 100 µL of decimal dilutions of cell suspensions were spread on the plates with a Drygalski spatula and the plates were wrapped in wet paper towels, sealed in plastic bags and incubated for 4 days at 78°C. This method, however, often resulted in insufficient biofilm formation or softening and even liquefaction of the gellan gum. To circumvent these effects, membrane filtration of cells was applied. Planktonic cells were filtered onto mixed cellulose ester membranes, which were placed on top of solid Brock medium. This led to formation of a biofilm on top of the membrane, which could easily be scraped off (Figure 3.3a). However, the amount of biofilm obtained from one membrane was too low for sufficient EPS analysis (< 0.05 g wet weight per membrane). The procedure of spreading *S. acidocaldarius* on the solid Brock medium directly was improved by spreading only single lines of the planktonic culture on the plates (Figure 3.3b). It was shown that wrapping each plate with a wet paper towel, as it was commonly performed prior to this study, was not necessary for incubation for up to 7 days but rather seemed to

enhance the softening of the plates due to the increased humidity within the sealed stack of plates.

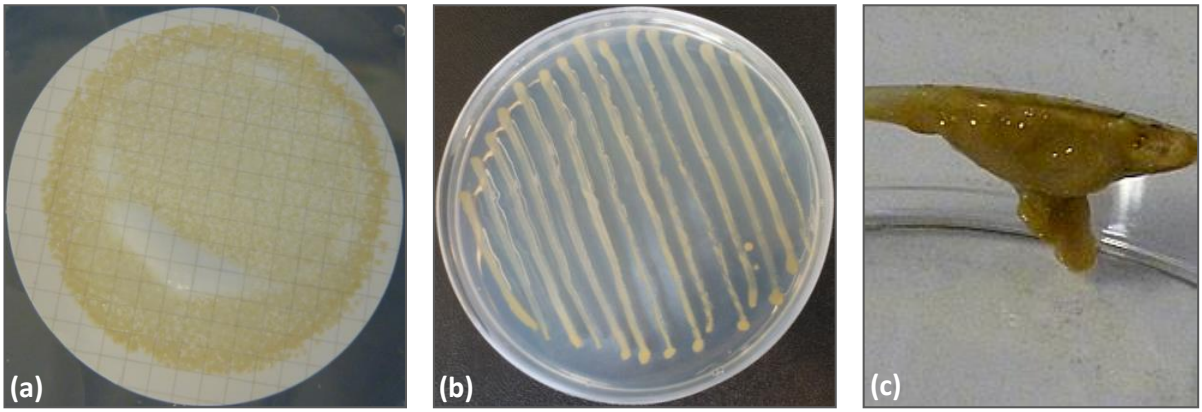

Figure 3.3: *S. acidocaldarius* biofilm formation on gellan gum-solidified Brock medium plates. The images show unsaturated biofilms after 4 days of incubation at 78°C (a) on mixed cellulose ester membrane filters on top of Brock medium plates, (b) on Brock medium plates inoculated by single line streaking of an exponentially growing planktonic culture, and (c) sampled from Brock medium plates shown in (b).

3.1.5 Determination of culturability

Culturability of *S. acidocaldarius* was assessed in terms of colony formation on Brock medium plates. This determination required optimization as the standard spread plate method, plating 100 µL of cell suspensions or decimal dilutions using a spatula, did not lead to reproducible colony counts. Instead, a smear of cells on the plates with only a few single colonies was observed (Figure 3.4b). It appeared that *S. acidocaldarius* cells are very sensitive towards the pressure applied using a spatula. Since the determination of culturability represented an important factor in this study, alternatives to the standard procedures were tested. Spreading of cells by glass beads was applied to improve the distribution of cells on the plate. This attempt, however, also resulted in inhomogeneous and not reproducible colony formation. A third method was the spreading of 1 mL cell suspension by tilting of the plates. This method led to reproducible results and thus, was used for the determination of culturability (Figure 3.4a). An exponentially grown planktonic culture in Brock medium at 78°C after approximately 32 h with an OD_{600} of 0.7 was shown to contain approximately 1.5 x 10^9 cells mL^{-1} with a culturability of 60%.

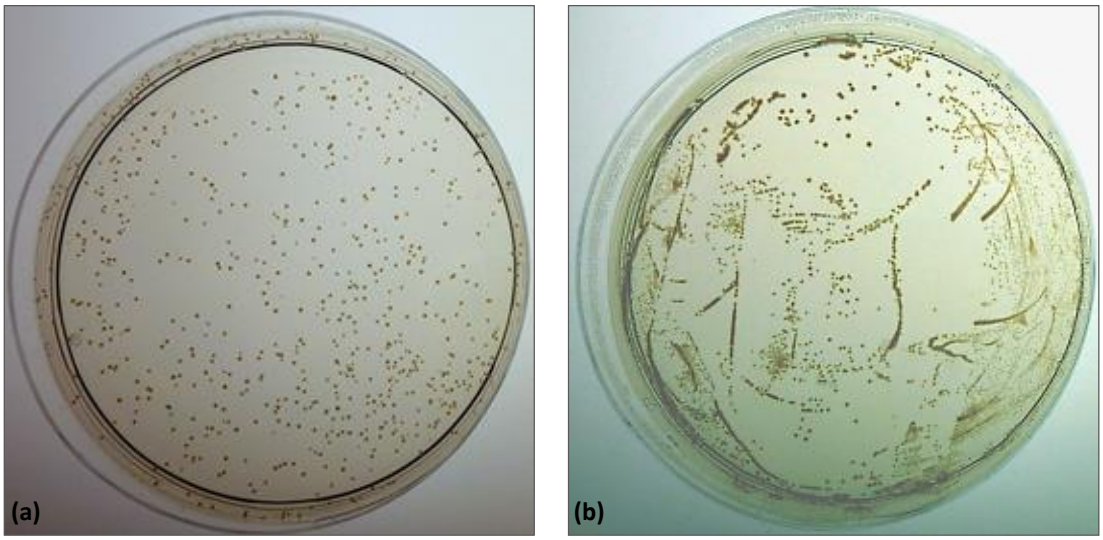

Figure 3.4: *S. acidocaldarius* colony formation on Brock medium plates. For the distribution of the planktonic cell solution (a) tilting and (b) the spread plate method were applied. Plates were incubated for 4 days at 78°C.

3.1.6 Composition and general characterization of biofilms

S. acidocaldarius unsaturated biofilms grown on Brock medium plates were analyzed for their water content, dry weight, loss and residue on combustion as well as the cation composition and total cell count for a general characterization of this type of biofilm. 4 days old *S. acidocaldarius* unsaturated biofilms grown on Brock medium plates showed a water content of 75.1 ± 1.4% (Table 3.2). The dry weight accounted for 24.9 ± 1.4%, of which 97% were organic and 3% were inorganic substances as determined by loss and residue on combustion, respectively. Determination of the total cell count of the applied samples via DAPI staining revealed a total count of $1.2 \pm 0.4 \times 10^{12}$ cells per g biofilm wet weight and a culturability of 29%, which was slightly lower than the culturability of exponentially growing planktonic cultures.

Table 3.2: Composition of *S. acidocaldarius* biofilms. Biofilms were cultivated on Brock medium plates for 4 days at 78°C; n = 3.

Parameter	g per g wet weight
Water content	0.751 ± 0.014
Dry weight	0.249 ± 0.014
Residue on combustion	0.008 ± 0.002
Loss on combustion	0.242 ± 0.013

Multivalent cations, which can be involved in the stability of the EPS matrix via ionic interactions, were determined in unsaturated biofilms grown on Brock medium plates (4 d, 78°C) (Table 3.3). The occurrence of multivalent, especially divalent cations, is of importance for the choice of the EPS isolation method. An accumulation of multivalent cations was observed especially for magnesium (385 ± 7.1 µg), iron (162 ± 56.6 µg) and calcium (125 ± 7.1 µg) in 1 g biofilm wet weight, which are components of the Brock medium.

Table 3.3: Concentrations of multivalent cations in *S. acidocaldarius* biofilms. Biofilms were cultivated on Brock medium plates for 4 days at 78°C. Values are given per g biofilm wet weight as determined via ICP-OES; n = 2.

Cation	Concentration [µg g^{-1} biofilm wet weight]	Cation	Concentration [µg g^{-1} biofilm wet weight]
Ca	125 ± 7.1	**Al**	0.147 ± 0.095
Mg	385 ± 7.1	**Zn**	0.235 ± 0.007
Fe	162 ± 56.6	**B**	0.037 ± 0.001
Cu	5.6 ± 0.8	**Ba**	0.039± 0.004
Cr	0.004 ± 0.001	**Sr**	0.265 ± 0.064
Co	0.003 ± 0	**Mo**	0.031 ± 0.001

3.2 Isolation and characterization of extracellular polymeric substances (EPS)

The type of isolation procedure and its efficiency determine the composition of the isolated fraction and hence, is crucial for all further analyses. Application of too harsh conditions can result in increased cell lysis contaminating the EPS with intracellular and membrane material. Too mild conditions on the other hand, can lead to an underestimation of the actual EPS fraction. Several methods for the isolation of EPS from different bacterial and eukaryotic biofilms have been developed and optimized (Wingender *et al.* 1999), however, so far none was applied for the EPS isolation from archaeal biofilms. Therefore, in this study, five methods commonly applied for isolation of EPS from bacterial biofilms and activated sludge were compared with respect to EPS isolation yield, suitability for further biochemical analysis as well as their impact on cell integrity. The tested EPS isolation methods included isolation via shaking, shaking in presence of a cation exchange resin (CER), as well as stirring of the biofilm solution with either NaOH, EDTA or crown ether.

3.2.1 Comparison and evaluation of EPS isolation methods

EPS isolation efficiency

For the comparison of the five EPS isolation methods, biofilms grown on Brock medium plates were used. The biofilm suspension (termed “biofilm”) as well as the cells after EPS isolation (termed “cells”), the supernatants after EPS isolation procedures containing EPS and low molecular weight substances (termed “supernatant”) and the dialyzed EPS solution (termed “EPS”) obtained with the five isolation methods were subjected to protein, carbohydrate and DNA determinations. All tested methods for isolation of EPS from *S. acidocaldarius* biofilms yielded quantifiable amounts of these EPS components (Figure 3.5-3.6 and Table 3.4).

Isolation of EPS using shaking, CER and EDTA led to similar distributions of carbohydrates among the analyzed fractions, i.e. cells (35 - 40 fg cell^{-1}), supernatant (8 - 9 fg cell^{-1}) and EPS (3.0 - 4.7 fg cell^{-1}) with CER tending to yield the highest EPS carbohydrate concentration of the three methods. The same was observed for the protein concentrations of 121 - 141 fg cell^{-1} in the cell fraction, (2.8 - 18 fg cell^{-1}) in the supernatant and 2.7 - 4.2 fg cell^{-1} in the EPS with CER resulting in the highest yield.

EPS isolation using NaOH led to the highest carbohydrate and protein concentrations of 29 fg cell^{-1}and 122 fg cell^{-1} in the isolated EPS, respectively. However, treatment with NaOH yielded very low carbohydrate (10.5 fg cell^{-1}) and protein (14.8 fg cell^{-1}) concentrations for the cell fraction, corresponding to only 20% and 10.5% of the initial concentrations determined in the biofilm suspension. The very low amounts of carbohydrates and proteins, which remained associated with the cell fraction, may indicate damage and lysis of cells. This was already suspected during the isolation procedure due to the visual appearance of the biofilm suspension, which changed from brownish turbid to clear and viscous.

Crown ether treatment resulted in the second highest concentrations of EPS carbohydrates (11.4 fg cell^{-1}) and proteins (35.3 fg cell^{-1}), however, the yield of carbohydrates in the supernatant was remarkable since it corresponded to 88% of the initial biofilm carbohydrate concentration. Taken together with the concentrations obtained for the cell fraction (33.6 fg cell^{-1}), this concentration exceeded the total initial biofilm carbohydrate concentration by 60% indicating interference of the crown ether with the measurement.

Apart from proteins and carbohydrates, eDNA was detected in quantifiable amounts with concentrations of 0.09 fg cell^{-1} to 1.5 fg cell^{-1}. Highest amounts were detected for crown ether followed by NaOH and CER. The lowest yield was achieved using shaking followed by EDTA.

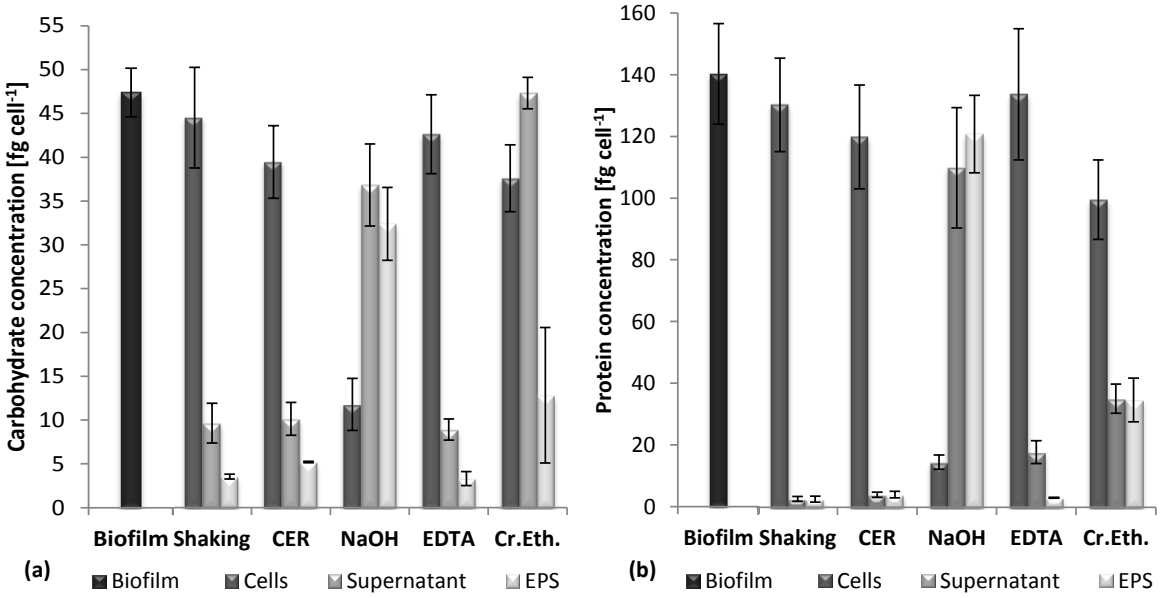

Figure 3.5: Concentrations of a) carbohydrates and b) proteins in the biofilm of *S. acidocaldarius* and different biofilm fractions after EPS isolation with shaking, CER, NaOH, EDTA or crown ether (Cr.Eth.). EPS was isolated from biofilms collected from Brock medium plates after incubation for 4 days at 78°C; n = 3.

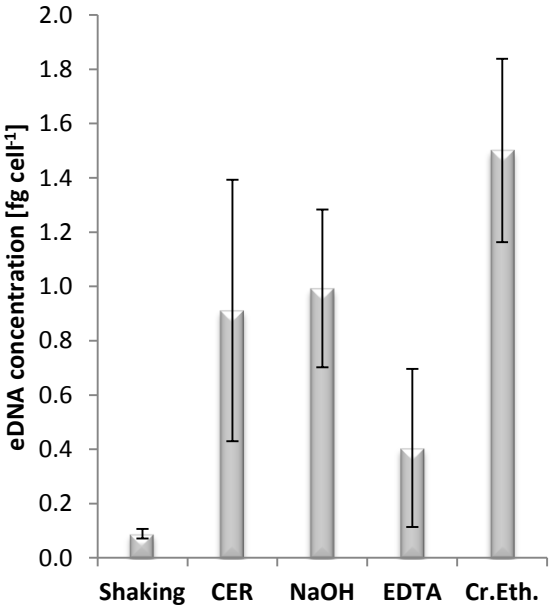

Figure 3.6: Concentrations of eDNA in EPS isolated from *S. acidocaldarius* biofilms by shaking, CER, NaOH, EDTA or crown ether (Cr.Eth.). EPS was isolated from biofilms collected from Brock medium plates after incubation for 4 days at 78°C; n = 3.

The protein to carbohydrate mass ratio, which has been applied in various studies as an indicator of cell lysis, greatly depended on the method applied (Wingender *et al.* 1999) (Table 3.4). The EPS isolated with either shaking or CER contained more carbohydrates than proteins with ratios of 0.86 and 0.90, respectively. With EDTA nearly equal amounts of proteins and carbohydrates were extracted (protein/carbohydrate ratio of 1.07). Significantly more proteins than carbohydrates were determined in EPS isolated with NaOH (protein/carbohydrate ratio of 4.22) or crown ether (protein/carbohydrate ratio of 3.07), similar to the ratio in the total biofilm (protein/carbohydrate ratio of 2.96).

EPS proteins isolated with the different methods were visualized using 2 DE (Figure 3.7). Isolation using shaking resulted in approximately 300 protein spots and thus about 40% less than in EPS isolated with CER (approximately 500 protein spots). NaOH treatment for isolation of EPS resulted in a smear of proteins on the gel without distinct spots, especially in the low molecular mass region. The highest number of spots (approximately 600) was obtained for the isolation using crown ether. For EPS isolation using EDTA no separation of proteins was possible due to an unusual high voltage during isoelectric focusing. In general, most protein spots were detected between 116 kDa and approximately 25 kDa and a pI of 5 to 8 using linear IPG strips. Therefore, in 2 dimensional separations for following

experiments non-linear IPG strips with a wider separation between a pI of 5 to 8 were applied to achieve a better resolution.

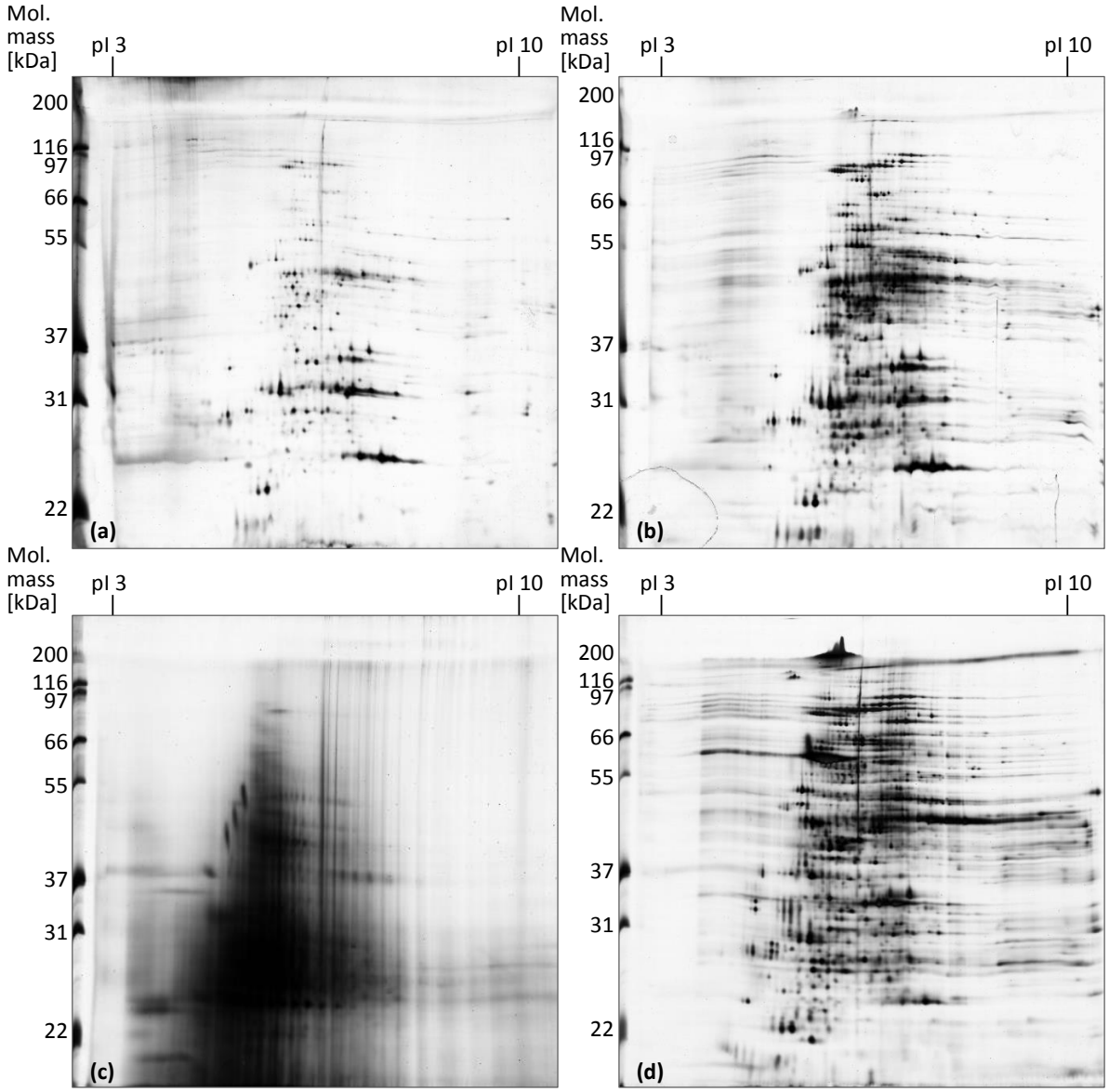

Figure 3.7: Two dimensional gel electrophoresis of EPS proteins of *S. acidocaldarius* isolated with (a) shaking, (b) CER, (c) NaOH and (d) crown ether. 200 µg EPS protein were applied for IEF (linear IPG strips). Polyacrylamide gels were stained with silver according to Blum *et al.* (1987). Molecular mass marker: PageRuler Prestained Protein Ladder (Fermentas).

Table 3.4: Carbohydrate, protein and eDNA concentrations and ratios of the EPS extracted from *S. acidocaldarius* biofilms by shaking, CER, NaOH, EDTA and crown ether. EPS carbohydrates and proteins are also given as % of the respective polymer found within the total biofilm before EPS isolation. Also given is the pH value in biofilms suspended in either phosphate buffer for shaking and CER or in NaOH, EDTA and crown ether after the EPS isolation (before separation of supernatant and cells via centrifugation).

Isolation method applied	EPS carbohydrates		EPS proteins		Ratio (prot./carb.)	eDNA	pH in suspensions after EPS isolation
	[fg cell^{-1}]	% of total biofilm carbohydrates	[fg cell^{-1}]	% total biofilm proteins		[fg cell^{-1}]	
Shaking	3.2 ± 0.3	6.7	2.7 ± 0.9	2.0	0.86	0.09 ± 0.02	7.0
CER	4.7 ± 0.1	9.8	4.2 ± 1.1	3.0	0.90	0.91 ± 0.48	6.8
NaOH	28.9 ± 3.7	61.0	122.0 ± 12.6	87.0	4.22	0.99 ± 0.29	13.3
EDTA	3.0 ± 0.7	6.2	3.2 ± 0.2	2.3	1.07	0.40 ± 0.29	5.1
Crown ether	11.4 ± 6.9	24.2	35.2 ± 7.2	25.1	3.07	1.50 ± 0.34	8.1

Since EPS isolation using CER is based on the removal of multivalent cations from the EPS matrix, concentrations of cations within the biofilm suspension before and after CER treatment as well as in the EPS were determined. A significant reduction of calcium and magnesium below the limit of detection showed that at least 60% and 87%, respectively, bound to the CER (Table 3.5). For iron and copper no reduction after CER treatment was detected.

Table 3.5: Concentrations of calcium, magnesium, iron and copper in EPS and in biofilm suspensions of *S. acidocaldarius* before and after CER treatment. Determination was performed with ICP-OES; n = 2.

Sample	Calcium [mg L^{-1}]	Magnesium [mg L^{-1}]	Iron [mg L^{-1}]	Copper [mg L^{-1}]
Initial biofilm suspension	1.25 ± 0.07	3.85 ± 0.07	1.62 ± 0.57	0.056 ± 0.01
Biofilm suspension after CER treatment	< 0.60	0.65 ± 0.07	1.51 ± 0.50	0.053 ± 0.01
EPS	< 0.50	< 0.50	0.13 ± 0.04	0.004 ± 0.00

Impact of EPS isolation on cell integrity and culturability

Evaluation of cell integrity after EPS isolation was performed by comparison of the total cell count and the culturability of cells before and after each isolation procedure. Total cell counts showed no significant reduction after EPS isolation using shaking, CER, EDTA or crown ether compared to the initial cell counts in the biofilm (Figure 3.8). The plate count showed a similar proportion of culturable cells after EPS isolation using shaking, CER and EDTA. Crown ether treatment led to a reduction of culturability by five orders of magnitude. EPS isolation using NaOH resulted in a complete loss of culturability and a reduction of the cell number below the limit of detection (approximately 1.0 x 10^5 cells mL^{-1}).

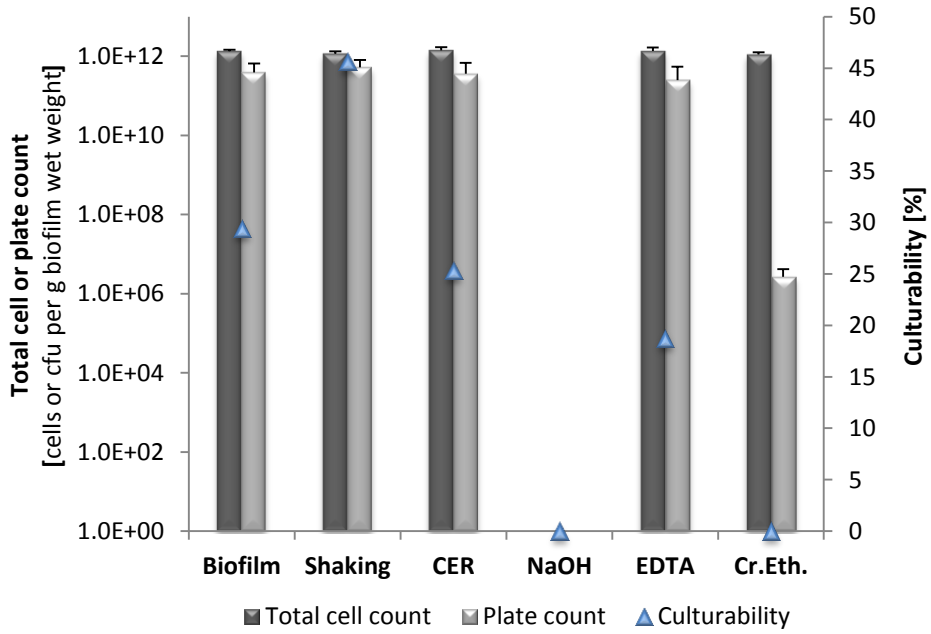

Figure 3.8: Total cell counts, plate counts and culturability of *S. acidocaldarius* cells after EPS isolation using shaking, CER, NaOH, EDTA or crown ether (Cr.Eth.). Measurements were performed using biofilm and cells suspensions in phosphate buffer (pH 7) after EPS isolation with the respective methods. Total cell counts were determined via counting of DAPI stained cells. Colonies formed on Brock medium plates were counted after incubation for 4 days at 78°C; n = 3.

To test the influence of the pH of the isolation medium on culturability, biofilm cells were dispersed in phosphate buffer (pH 7.0) or Brock medium (pH 3.5). Comparison of the culturability for biofilm cells suspended in either phosphate buffer or Brock medium prior to EPS isolation via shaking or CER showed no statistically significant differences ($p > 0.05$, two-tailed paired *student's t-test*) (Figure 3.9). Moreover, stirring of cells at 4°C for 3 h, which is required for isolation of EPS using NaOH, EDTA and crown ether, revealed no decrease of culturability compared to cells immediately spread after suspension for both, phosphate buffer and Brock medium. Hence, suspension of cells in phosphate buffer with a pH of 7.0 for EPS isolation showed no detrimental effect on the culturability of *S. acidocaldarius* for the applied conditions.

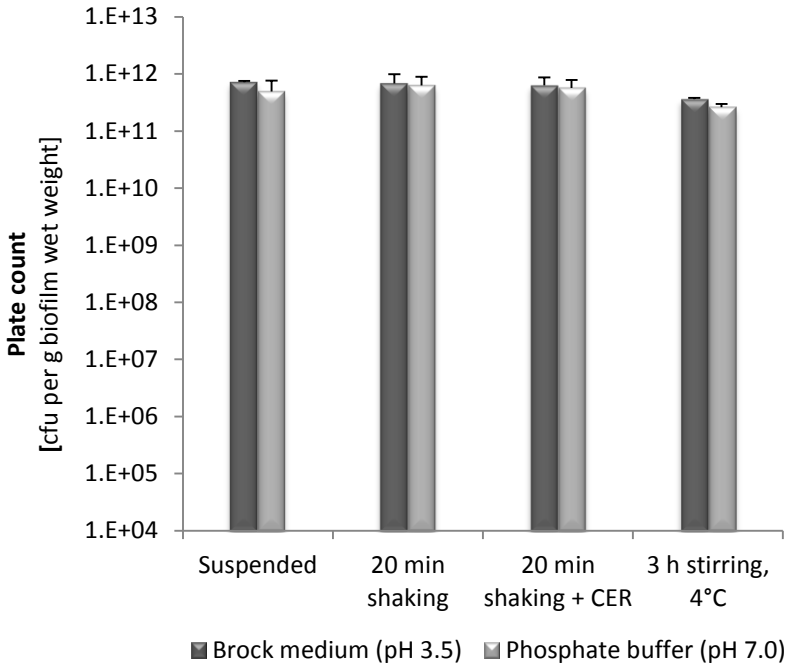

Figure 3.9: Impact of pH on culturability on *S. acidocaldarius*. Impact was tested for *S. acidocaldarius* biofilms suspended in either phosphate buffer (pH 7.0) or Brock medium (pH 3.5), shaken in presence or absence of CER (room temperature) or stirred for 3 h (4°C). Colonies formed on Brock medium plates were counted after incubation for 4 days at 78°C; n = 3.

3.2.2 Characterization of EPS proteins

Proteins within the EPS matrix were shown to possess multiple functions, in particular serving as structural components or as exoenzymes degrading polymers to make them available as nutrients to the cell. Moreover, due to release of proteins by cell lysis, they can also act as nutrient source. The precise identities and roles of proteins within the EPS matrix of *S. acidocaldarius* have not been addressed so far. The protein concentration in the EPS isolated from biofilms cultivated on Brock medium plates (4 days, 78°C) amounted to 4.2 ± 1.1 fg cell^{-1}.

In an initial step the diversity of EPS proteins isolated from biofilms and planktonic cultures using CER was analyzed by 1 D and 2 D SDS-polyacrylamide gel electrophoresis. Biofilms were collected from Brock medium plates after 4 days of incubation at 78°C. EPS from planktonic cultures (exponential phase) were isolated by centrifugation, re-suspension of the cell pellet in phosphate buffer and treatment with CER. EPS from both samples were treated with benzonase, dialyzed against water (MWCO 12 - 14 kDa) and lyophilized.

To obtain maximum sensitivity of separation via SDS-polyacrylamide electrophoresis, protein staining was optimized. Three commonly applied methods for staining of proteins in polyacrylamide gels, namely rapid Coomassie Brilliant Blue (CBB) staining, enhanced CBB staining after Kang *et al.* (2002) and silver staining after Blum *et al.* (1987) were compared. The comparison showed distinctive differences with respect to the amount of protein required for visualization and the number of bands obtained as counted roughly by eye (Figure 3.10).

The best results were obtained with silver staining, showing a maximum of 54 bands, followed by the enhanced CBB staining with 32 bands and the rapid CBB staining with only 24 bands for 2 µg EPS proteins per lane isolated from *S. acidocaldarius* biofilms (Table 3.6). Silver staining was the most sensitive staining method showing a total of 37 bands when 0.5 µg EPS protein were applied. At this protein load enhanced CCG staining revealed 22 lightly stained bands, while no bands were detected using the rapid CBB staining procedure. Additionally to the number and intensity of the bands obtained, also the most focused bands were obtained with silver staining. These results also revealed that more proteins were detected in the EPS of biofilms compared to EPS isolated from an exponentially growing planktonic culture.

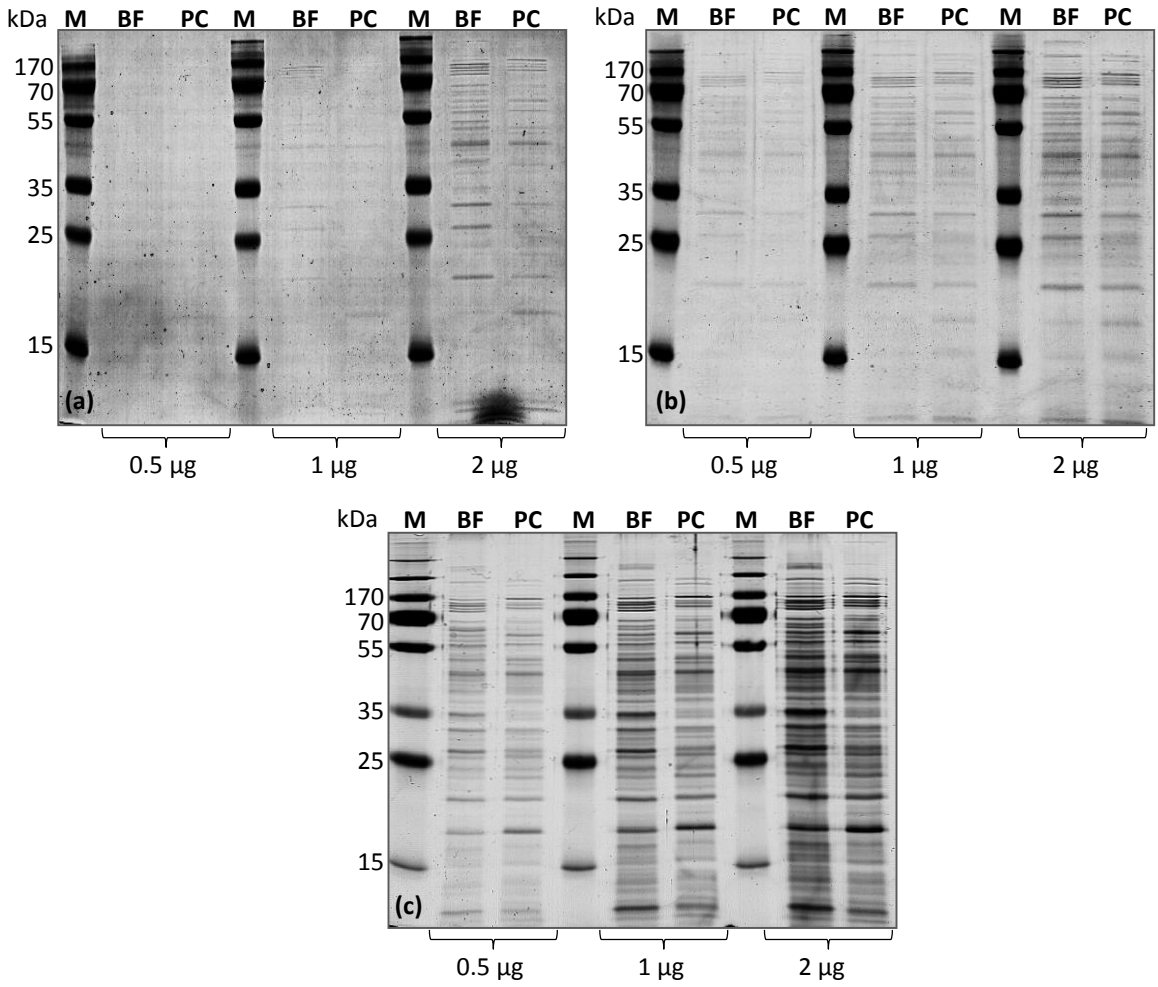

Figure 3.10: Visualization of EPS proteins of *S. acidocaldarius* biofilms (BF) and planktonic cells (PC) on SDS-polyacrylamide gels. Gels were stained with (a) rapid CBB staining, (b) enhanced CBB staining after Kang *et al.* (2002) and (c) silver staining after Blum *et al.* (1987). M: Molecular mass marker PageRuler Prestained Protein Ladder (Fermentas). Protein load per lane given in the figure below the lanes.

Table 3.6: Number of bands as estimated by eye in SDS-polyacrylamide gels obtained with different protein staining techniques for EPS isolated from *S. acidocaldarius* planktonic cultures and biofilms. EPS was isolated from biofilms grown on Brock medium plates after 4 days at 78°C and planktonic cultures in the exponential phase.

Origin of EPS	Amount of EPS protein per lane [µg]	**Silver staining**; Blum *et al.* (1987)	**Enhanced CBB staining**; Kang *et al.* (2002)	Rapid CBB staining
Biofilm/ planktonic cultures	0.5	37/37	22/16	0/0
	1	54/44	26/22	11/7
	2	54/47	32/23	26/19

Enhanced separation of CER-isolated EPS proteins was achieved by 2 DE using non-linear IEF strips, which enhance the resolution in the pH range of 4 - 7 resulting in a sigmoidal pH gradient. This type of IEF strips was applied since previous 2 DE for the comparison of EPS isolation methods revealed that the vast majority of EPS proteins were detected at pI values between 5 and 8 (Figure 3.11). Gels were stained with silver after Blum *et al.* (1987) and compared to the enhanced CBB stain after Kang *et al.* (2002). In comparison to the linear IPG strips used before, about twice the number of spots was obtained due to the better separation between pI values of 5 to 8. While with the silver stain approximately 1000 protein spots were detectable by eye only 505 spots were observed for the enhanced CBB stain. Nevertheless, the enhanced CBB stain showed less streaking and background staining. Furthermore, this staining method allows for further analyses like mass spectrometry without significant further clean-up and is easier to replicate.

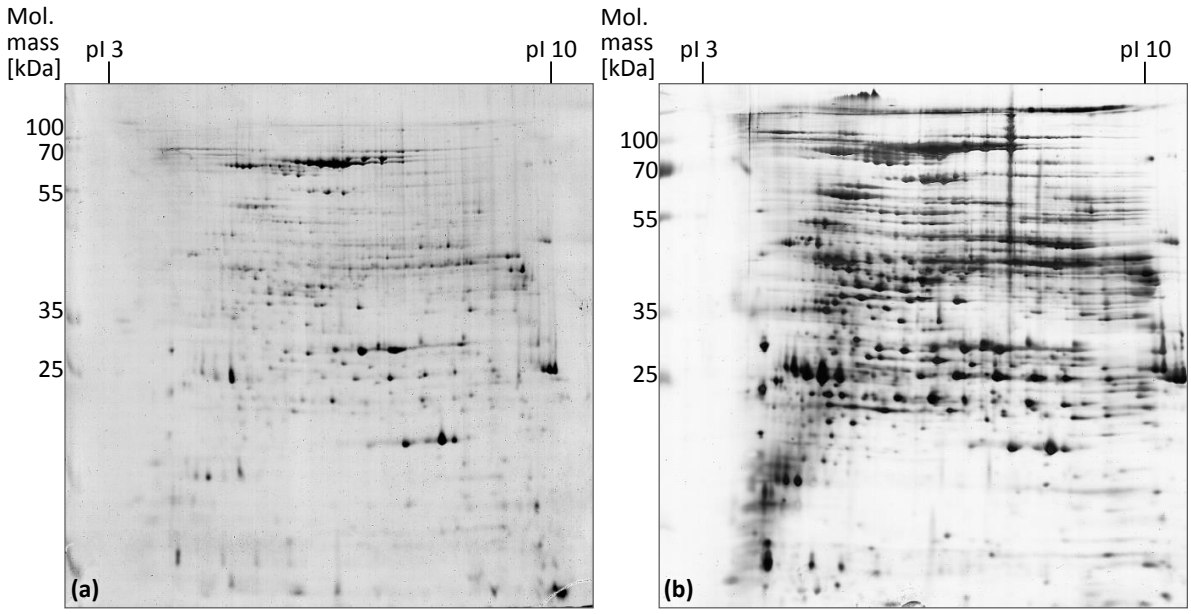

Figure 3.11: EPS proteins of *S. acidocaldarius* biofilms separated by 2 DE and visualized using (a) the enhanced CBB stain and (b) silver stain. EPS was isolated from biofilms collected from Brock medium plates after 4 days of incubation at 78°C. 200 µg EPS protein were applied for IEF (non-linear IPG-strips). Molecular mass marker: PageRuler Prestained Protein Ladder (Fermentas).

Identification of EPS proteins via MALDI-TOF/MS

To obtain insights into the identity of the EPS proteins, trypsin digested EPS proteins, isolated from unsaturated *S. acidocaldarius* biofilms (4 d, 78°C), were analyzed using MALDI-TOF/MS. The resulting protein identities were classified according to the arCOG functional codes. Within the EPS of *S. acidocaldarius* unsaturated biofilms, 85 proteins of 15 functional arCOG categories were detected (Figure 3.12). 53 proteins belonged to arCOG categories corresponding to energy production and conversion (arCOG C), amino acid transport and metabolism (arCOG E), lipid transport and metabolism (arCOG I) and translation, ribosomal structure and biogenesis (arCOG J). Prediction of the subcellular localization of the proteins revealed 73 proteins of intracellular origin. 11 proteins were of unknown origin and one predicted ATPase, involved in biogenesis of archaeal flagella, was predicted to be localized in the cytoplasmic membrane (Table 6.1). No extracellular proteins were detected.

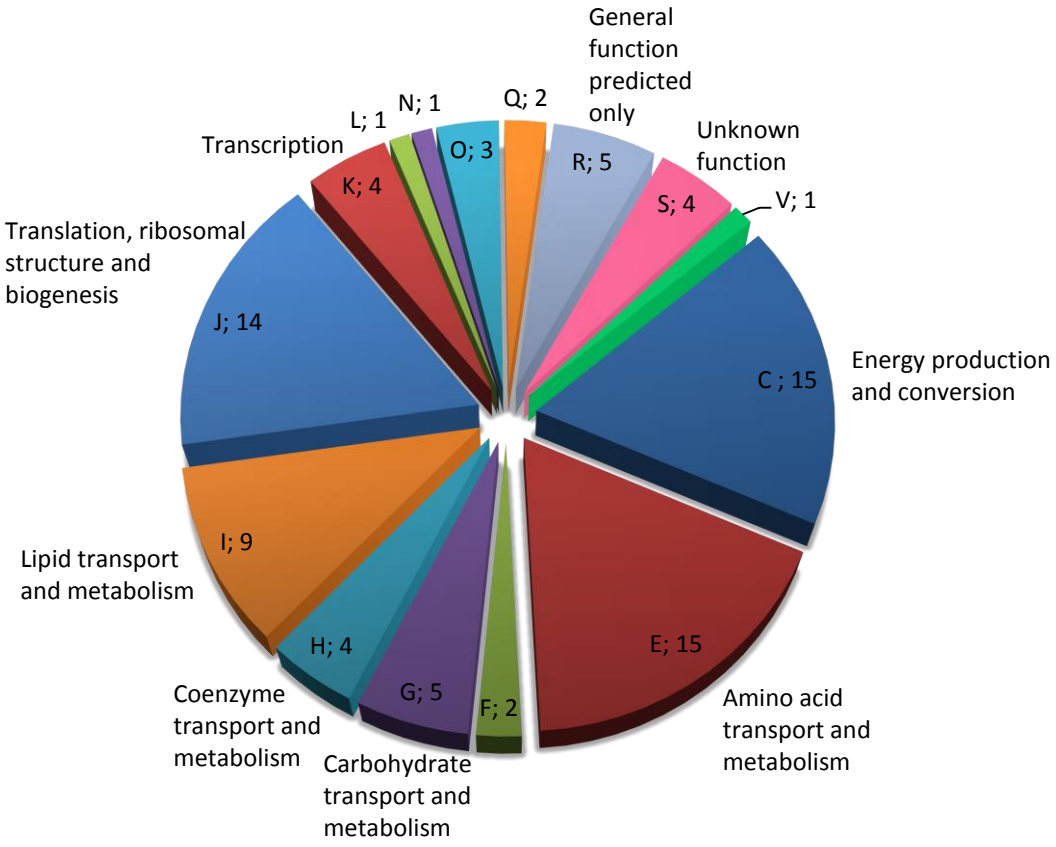

Figure 3.12: Number of *S. acidocaldarius* biofilm EPS proteins in the different arCOG functional categories. For identification EPS isolated from unsaturated *S. acidocaldarius* biofilms (4 d, 78°C) was digested with trypsin and applied for MALDI-TOF/MS analysis. Proteins were assigned to arCOG functional categories (capital letters). F: Nucleotide transport and metabolism, L: Replication, recombination and repair, N: Cell motility, Q: Secondary metabolites biosynthesis, transport and catabolism, V: Defense mechanism.

Screening for extracellular hydrolytic enzyme activity

One major function of proteins within the EPS is their role as hydrolytic enzymes, degrading macromolecules into low molecular weight substances, which can readily be utilized by the cells. In the present study, the hydrolytic potential of EPS proteins secreted by *S. acidocaldarius* biofilms was investigated using fluorimetric assays with MUF substrates.

EPS was isolated from biofilms collected from Brock medium plates after 4 days of incubation at 78°C using CER. For the determination of enzyme activity the dialyzed EPS and disrupted cells (sonication) after EPS isolation suspended in phosphate buffer were applied. Enzyme activities using MUF substrates were determined at pH 3.5 for EPS corresponding to the environmental growth conditions, and thus should enhance activity of extracellular enzymes. Enzyme activity measurement in disrupted cells after EPS isolation was performed at pH 7.0 to determine activity of intracellular enzymes. The highest substrate turnover rate in the EPS was detected for esterases followed by lipases, phosphatases, N-acetyl-β-D-glucosaminidases, β-D-glucosidases and α-D-glucosidases (Figure 3.13 and Table 3.7). No peptidase activity was detected. In contrast, disrupted cells exhibited the following descending order of substrate turnovers: Esterases, α-D-glucosidases, lipases, β-D-glucosidases and peptidases. Lowest turnover rates were detected for phosphatases and N-acetyl-β-D-glucosaminidases.

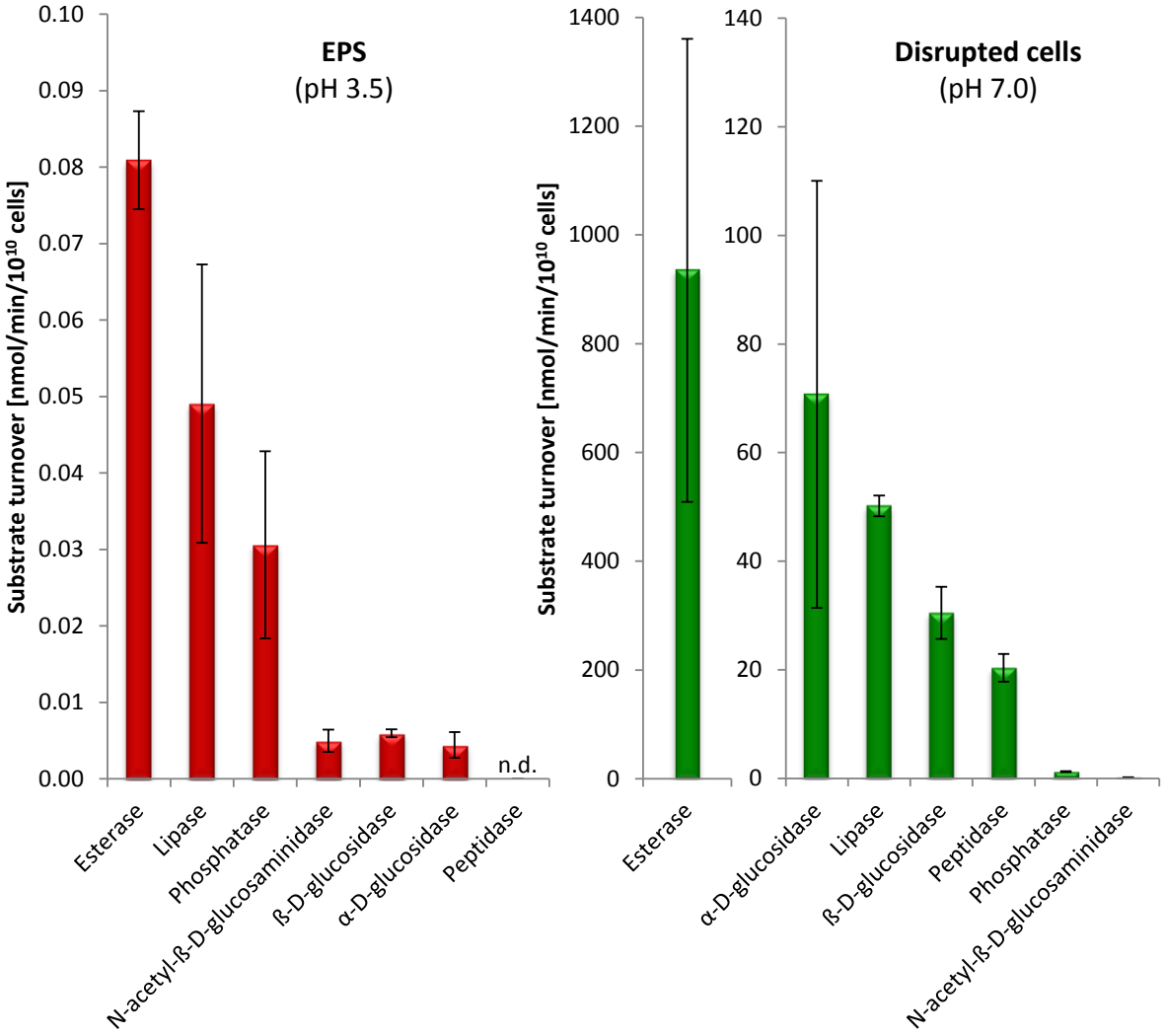

Figure 3.13: Substrate turnover rate for different enzyme classes in EPS and disrupted cells after EPS isolation from *S. acidocaldarius* biofilms. Left: enzyme activities in EPS at pH 3.5; right: enzyme activities in suspensions of disrupted cells at pH 7.0. EPS was isolated via CER from unsaturated biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Enzyme activity was determined for the substrates listed in Table 3.7 using a microtiter plate assay; n.d.: not detected; n = 2.

Table 3.7: Substrate turnover rates in EPS and disrupted cells of *S. acidocaldarius*. EPS was isolated via CER from unsaturated biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Enzyme activity was determined for the substrates listed in the table using a microtiter plate assay; n.d.: not detected; n = 2.

Substrate	Enzyme class	Substrate turnover [nmol/min/10^{10} cells]	
		EPS (pH 3.5)	**Disrupted cells** (pH 7.0)
L-alanine-4-methoxy-β-naphthylamide	Peptidase	n.d.	0.31 ± 0.07
4-MUF-α-D-glucopyranoside	α-D-glucosidase	0.004 ± 0.002	1.03 ± 0.47
4-MUF-β-D-glucopyranoside	ß-D-glucosidase	0.006 ± 0.001	0.45 ± 0.02
4-MUF-N-acetyl-β-D-glucosaminide	N-acetyl-β-D-glucosaminidase	0.005 ± 0.001	0.003 ± 0.000
4-MUF-stearate	Lipase	0.049 ± 0.018	0.75 ± 0.11
4-MUF-butyrate	Esterase	0.081 ± 0.006	14.3 ± 6.7
4-MUF-phosphate	Phosphatase	0.031 ± 0.012	0.019 ± 0.001

Zymography – Protease activity

The occurrence of protease activity in *S. acidocaldarius* biofilms, cells after isolation of EPS by CER treatment, EPS and crude cell extract was evaluated using zymography. Crude extract from biofilm cells after EPS isolation was obtained by sonication of cells suspended in phosphate buffer and subsequent removal of the membrane fraction via centrifugation. 5 µg protein of the respective sample were applied to zymogram gels containing either casein or gelatin as substrate. Two different pH values, 3.8 and 7.8, for development of gels were compared, resembling either the pH of the external environment or the approximate intracellular pH value of 6.5 (Moll and Schäfer 1988).

Development at pH 3.8 led to the formation of a clear band based on the protease activity degrading the casein at a molecular mass of approximately 47 kDa in all fractions, with the EPS fraction giving the highest signal (Figure 3.14a). At pH 7.8 a faint band at the same molecular mass was detected in the EPS fraction while 4 other bands at lower molecular masses (approximately 20 kDa, 25 kDa, 27 kDa and 35 kDa) were observed only in the total biofilm, crude extract and cell fraction (Figure 3.14b). The lower signal of the band at 47 kDa at pH 7.8 and the strong signal at pH 3.8 are an indication for an extracellular enzyme. The 4 additional bands at pH 7.8, which are absent at pH 3.5 may represent intracellular enzymes.

Heat stability of the detected protease was tested by incubation at 100°C for 1 h and by autoclaving (121°C, 20 min) (Figures 3.14 and 3.15). Treatment of all fractions (EPS, crude extract, cells after EPS isolation and total biofilm) for 1 h at 100°C resulted in a disappearance of enzyme activity bands at 47 kDa at pH 7.8, while at pH 3.8 no significant reduction of activity was detectable. An increase in temperature to 121°C resulted in the inhibition of enzyme activity at 47 kDa at pH 3.8 in the biofilm, the crude extract and the cell fraction. The protease activities detectable at 27 kDa and at 25 kDa at pH 7.8 were still detectable after 1 h at 100°C and after 20 min at 121°C. A protease inhibitor cocktail (23 mM AEBSF, 2 mM Bestatin, 100 mM EDTA, 0.3 mM E-64, 0.3 mM Pepstatin A; Sigma) did not significantly reduce the activity of proteases, especially at 47 kDa, as was evident by the formation of activity bands with similar intensities (data not shown).

Comparison of the protease substrates casein and gelatin showed that casein was more efficiently degraded, showing more pronounced cleavage detected via the intensity of clear activity bands (Figure 3.14a and c).

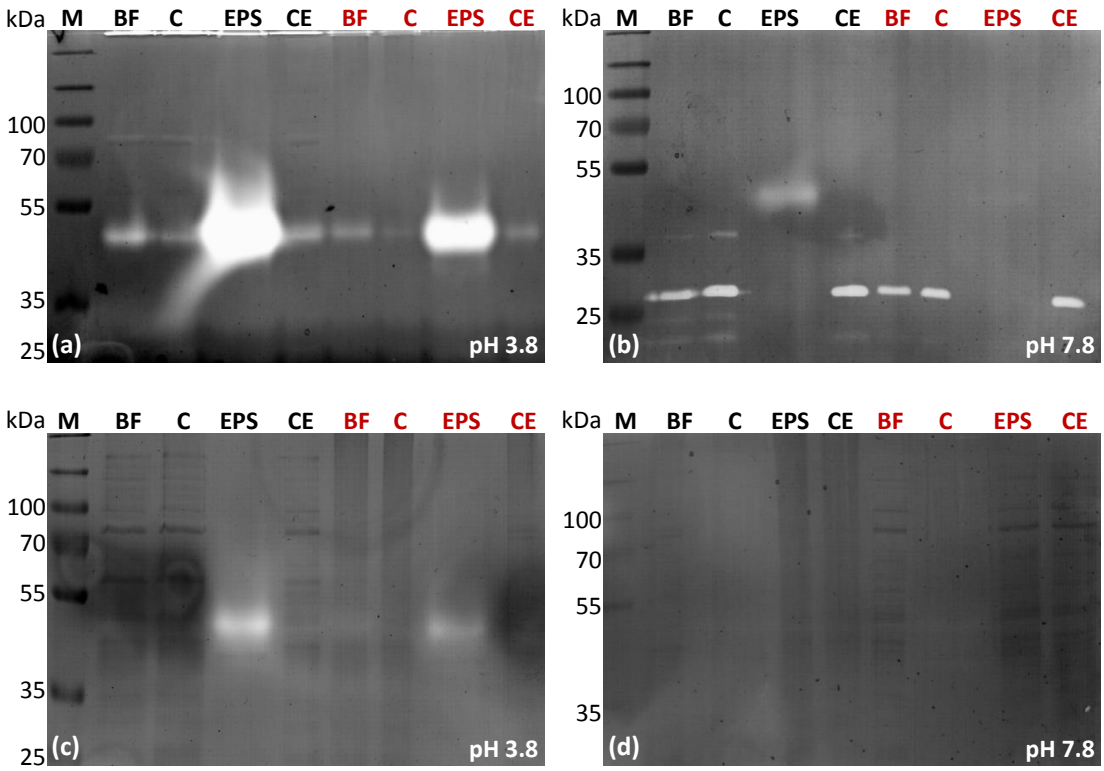

Figure 3.14: Protease activity in biofilm (BF), cells after EPS isolation (C), crude extract (CE) and EPS of *S. acidocaldarius* and effect of temperature (100°C, 1 h) visualized in zymogram gels. Gels were developed at either pH 3.8 or 7.8 with (a,b) casein or (c,d) gelatin as substrate for 24 h at 78°C. 5 µg protein were applied per lane. Black: untreated samples, red: samples heated at 100°C for 1 h. EPS was isolated via CER from biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Molecular mass marker: PageRuler Prestained Plus Protein Ladder (Fermentas); n = 2.

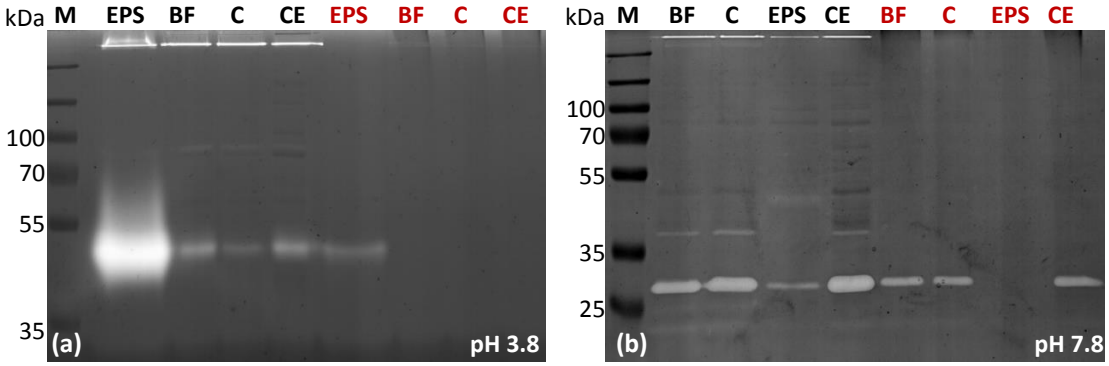

Figure 3.15: Protease activity in biofilm (BF), cells after EPS isolation (C), crude extract (CE) and EPS of *S. acidocaldarius* and effect of temperature (121°C, 20 min) visualized in zymogram gels. Gels were developed at (a) pH 3.8 and (b) pH 7.8 with casein as substrate for 24 h at 78°C. 5 µg protein were applied per lane. Black: untreated samples, red: samples autoclaved (121°C, 20 min). EPS was isolated via CER from biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Molecular mass marker: PageRuler Prestained Plus Protein Ladder (Fermentas); n =2.

To obtain more information about the proteases of *S. acidocaldarius*, EPS proteins were separated using 2 DE zymography with casein as substrate. The analysis showed one distinct spot for gels developed at pH 3.8 at approximately 47 kDa and a pI of 3 - 4 (Figure 3.16a). A spot at the same position was detected on gels developed at pH 7.8, however, with less

pronounced intensity. Additionally, two weaker spots at approximately 27 kDa were observed at this pH (Figure 3.16b). Corresponding gels stained with silver did not exhibit visible spots at the location of the most active protease at 47 kDa at pH 3.8, while the weaker activities at pH 7.8 were detected in the area of highest protein spot density (Figure 3.16c).

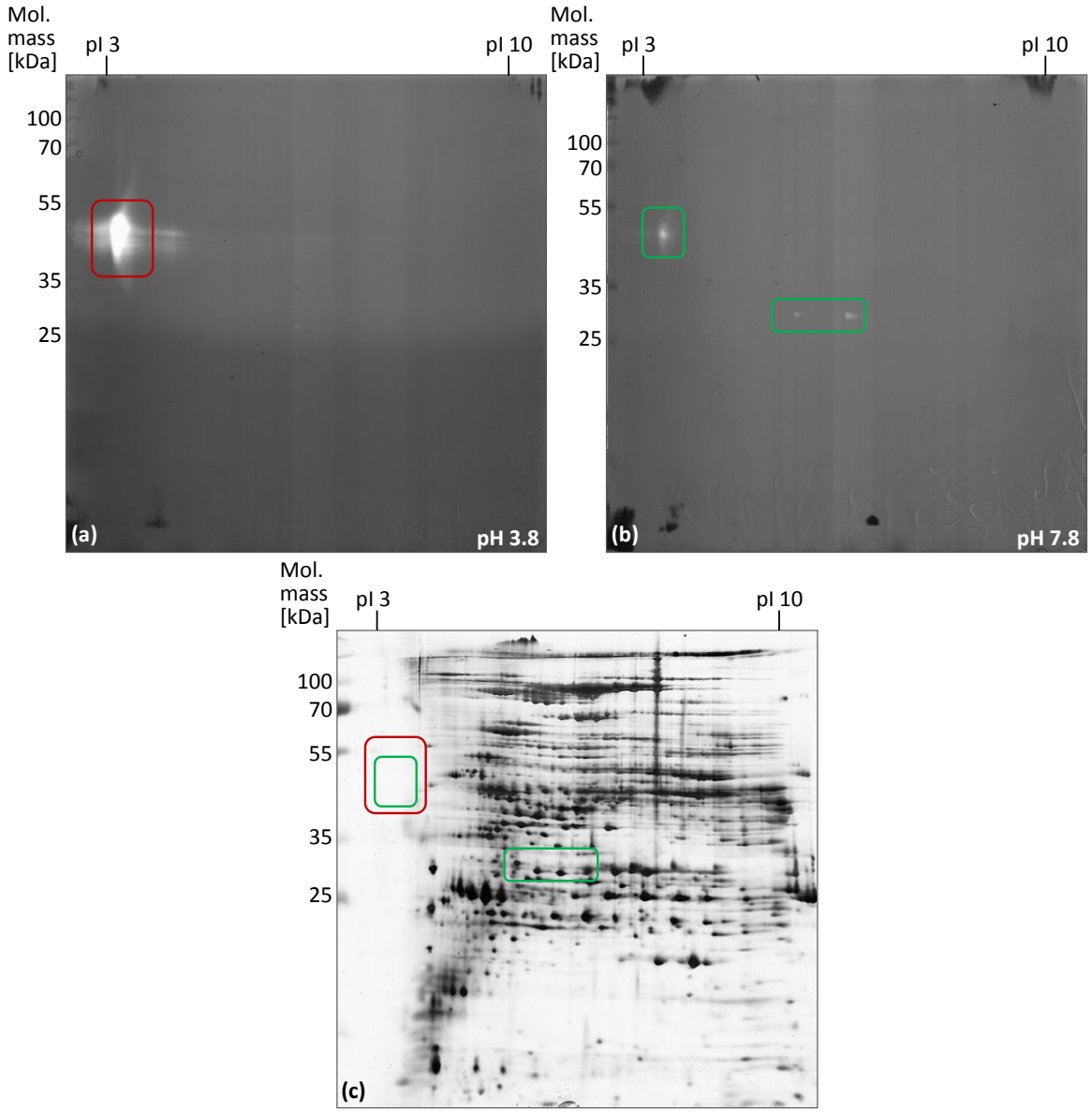

Figure 3.16: Protease activity in EPS of *S. acidocaldarius* biofilms visualized in 2 DE zymogram gels. 400 µg EPS protein were applied for IEF. Casein was used as substrate. Gels were developed at (a) pH 3.8 and (b) pH 7.8 for 48 h at 78°C. Boxes indicate protease activity and corresponding position in (c) reference gel stained with silver. Red: activities at pH 3.8; green: activities at pH 7.8. EPS was isolated via CER from biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Molecular mass marker: PageRuler Prestained Plus Protein Ladder (Fermentas); n = 2.

Activity of proteases in the crude cell extract was detected in casein gels developed at pH 3.8 as well as pH 7.8 (Figure 3.17a and b). At pH 7.8 five distinct spots with a molecular mass of approximately 27 kDa at isoelectric points between 5 and 8 were observed. The three most dominant of these spots were also detected when the gels were developed at pH 3.8. Bands at this molecular mass were previously detected in the EPS, cells, crude extract and biofilm samples on 1 D zymogram gels at pH 7.8. Additionally, a spot at a molecular mass of approximately 47 kDa, as obtained in the EPS fraction, was observed for a developing pH of 3.8. Due to the high occurrence of protein spots in the reference gels stained with either silver or the enhanced CBB stain, no precise correlation to a certain protein spot was possible (Figure 3.17c and d).

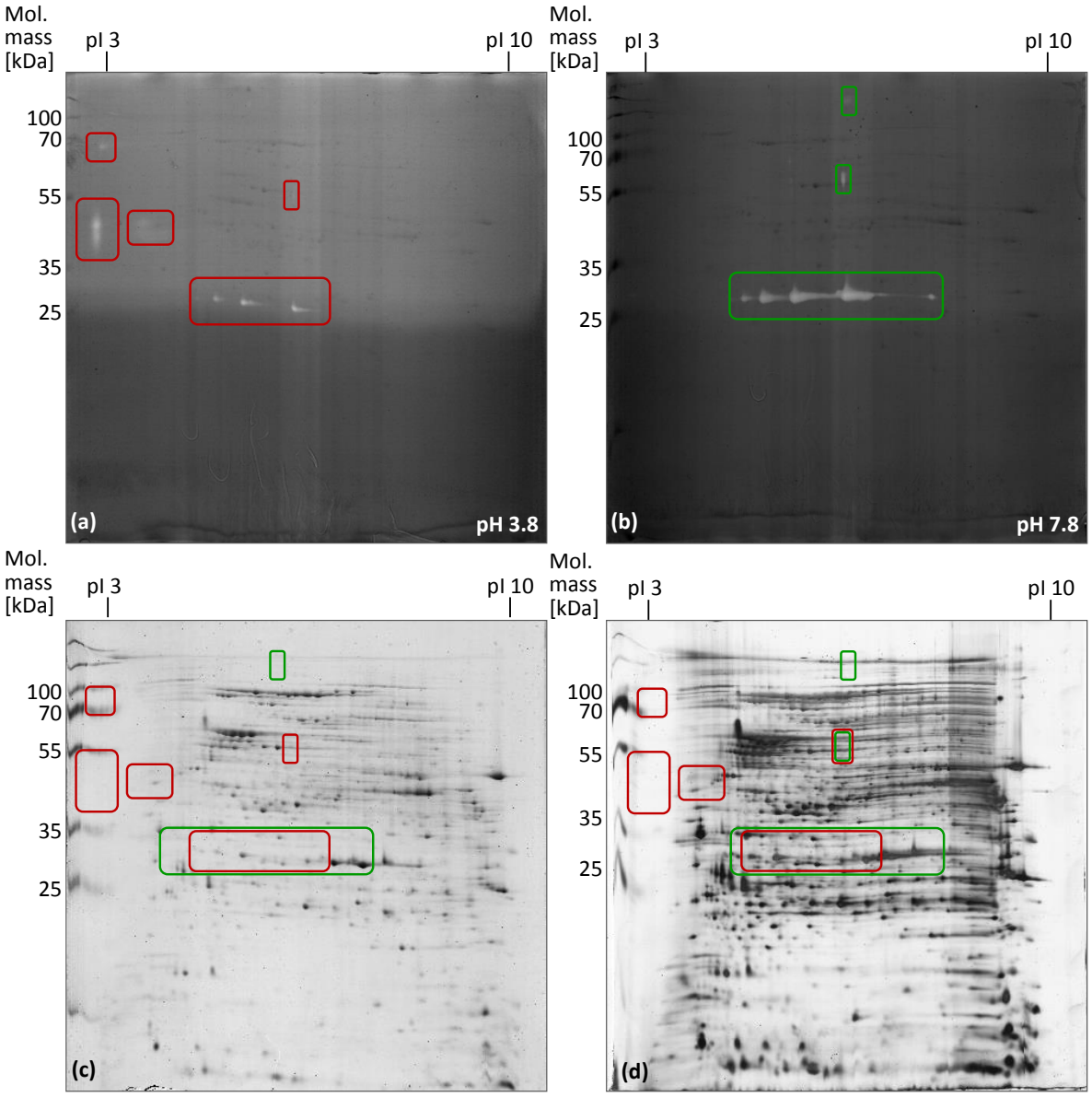

Figure 3.17: Protease activity in crude extract of *S. acidocaldarius* visualized in 2 DE zymogram gels. 400 µg crude extract protein were applied for IEF. Casein was used as substrate. Gels were developed at (a) pH 3.8 and (b) pH 7.8 for 48 h at 78°C. Boxes indicate protease activity and corresponding position in reference gels stained with (c) enhanced CBB and (d) silver. Red: activities at pH 3.8; green: activities at pH 7.8. Molecular mass marker: PageRuler Prestained Plus Protein Ladder (Fermentas); n = 2.

Zymography – Esterase activity

In order to detect activity of esterases in the EPS of *S. acidocaldarius* 2 D gels were incubated with the substrate MUF-butyrate, using developing buffers with a pH of either 3.5 or 8.0. At both pH values similar spot patterns were observed (Figure 3.18). On both gels a cluster of spots at a pI of 3 and a molecular mass of approximately 28 kDa was detected. At a developing pH of 8.0 spots 2 and 4 were indistinguishable and gave a weaker signal compared to the spots at the same position in the gel developed at pH 3.5. Additionally, spots at a higher pI and a molecular mass of approximately 20 and 35 kDa were observed.

One spot at a pI of 10 and a size of approximately 25 kDa was only observed at pH 8.0. Counterstaining of the gels with the enhanced CBB stain showed that for the spots giving the highest signal, no corresponding protein spot was observable in the gels, indicating that the concentration of the respective esterases is rather low while their activity is high. The smaller activity spots cannot be distinctively assigned to specific protein spots due to the high number of spots at this molecular mass and pI range (Figure 3.18c and d).

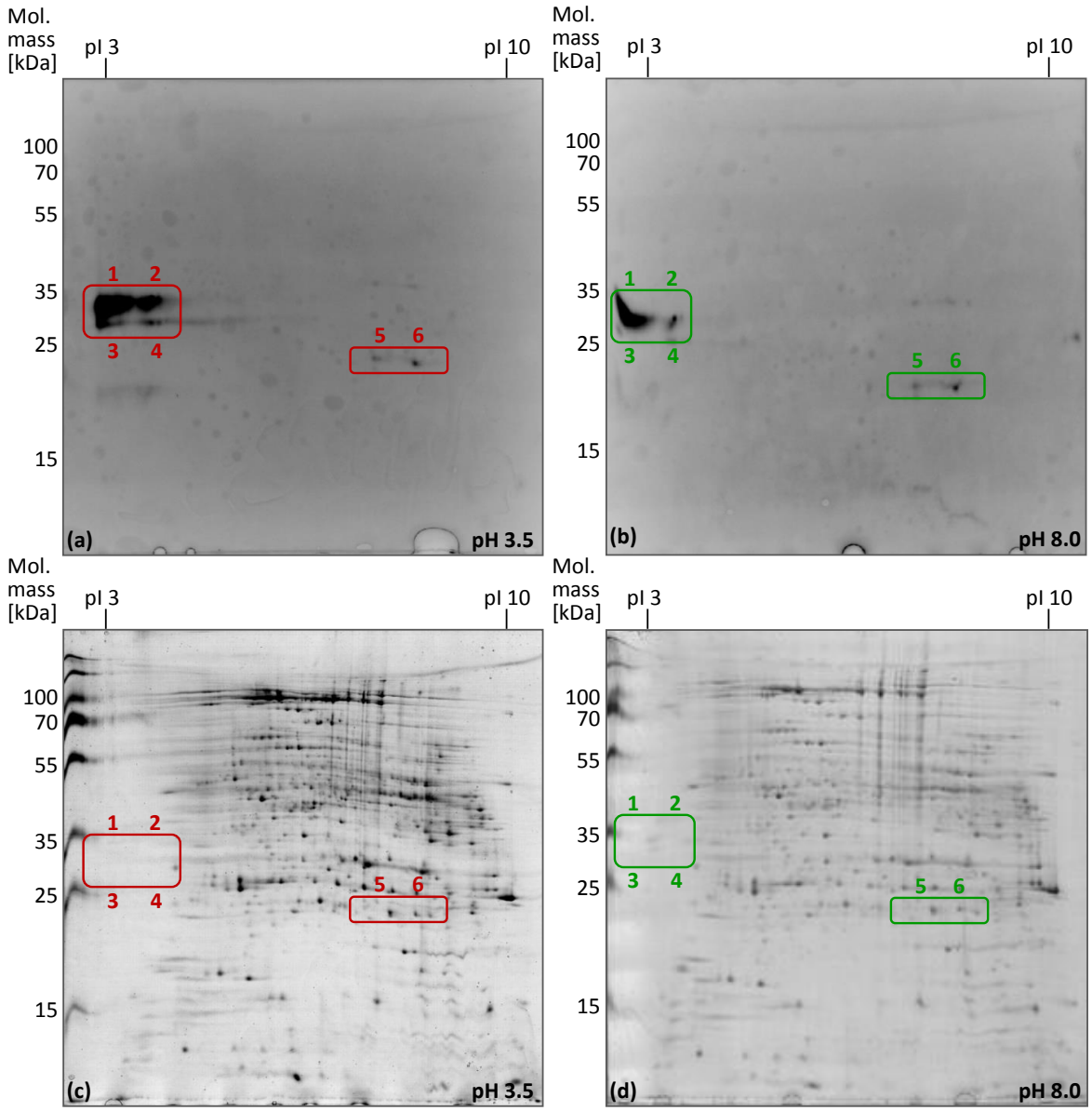

Figure 3.18: Esterase activity in EPS of *S. acidocaldarius* biofilms visualized in 2 DE gels. 400 µg EPS protein were applied for IEF. Gels were developed in MUF-butyrate at (a) pH 3.5 and (b) pH 8.0; (c and d) enhanced CBB counterstained gels. Red: activities at pH 3.5; green: activities at pH 8.0. EPS was isolated via CER from biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Molecular mass marker: PageRuler Prestained Plus Protein Ladder (Fermentas); n = 2.

Visualization of glycosylated EPS proteins

The glycosylation of asparagine residues (N-glycosylation) is one of the most common and complex posttranslational modification of proteins found in Archaea and is involved in the adaptation to extreme environments (Eichler and Maupin-Furlow 2013). For instance in *S. acidocaldarius* the S-layer as well as flagella proteins are known to be glycosylated (Peyfoon *et al.* 2010; Meyer and Albers 2013).

EPS proteins of *S. acidocaldarius* were analyzed for glycosylation, using the Pro-Q Emerald Glycoprotein staining kit. Figure 3.19 illustrates that the EPS contained glycosylated high molecular mass proteins or protein complexes, which did not enter the gel. Furthermore, 6 distinctive bands were observed. The 3 most dominant bands were detected at approximately 250 kDa, 39 kDa and 20 kDa. Weaker bands were observed at approximately 180 kDa, 50 kDa and 75 kDa.

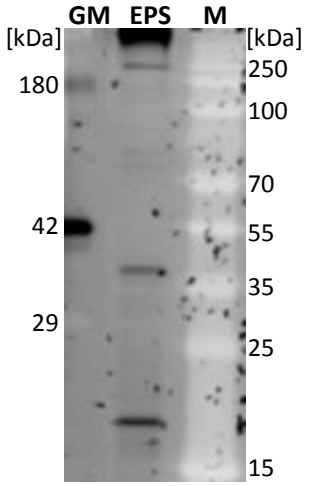

Figure 3.19: SDS-polyacrylamide gel of *S. acidocaldarius* EPS (2 µg) stained with the Pro-Q Emerald Glycoprotein staining kit. GM: CandyCane glycoprotein molecular mass marker [kDa]; M: Molecular mass marker PageRuler Prestained Plus [kDa]. EPS was isolated via CER from biofilms collected from Brock medium plates after 4 days of incubation at 78°C.

Staining of glycosylated EPS proteins on 2 DE gels indicated the occurrence of extracellular proteins possessing sugar residues (Figure 3.20a). The most distinctive spots were assignable to spots in gels stained with silver (Figure 3.20b).

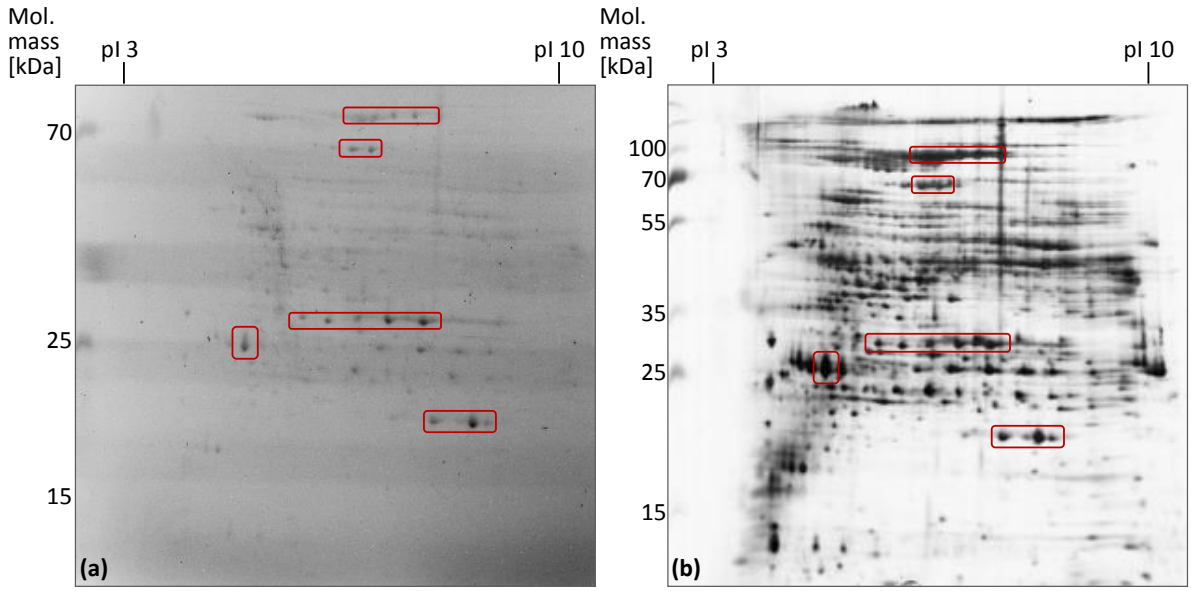

Figure 3.20: 2 DE of *S. acidocaldarius* biofilm EPS proteins stained with (a) Pro-Q Emerald Glycoprotein staining kit and (b) silver stain. 400 µg EPS protein were applied for IEF. Red boxes indicate most distinctive spots visible with both stains. EPS was isolated via CER from biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Molecular mass marker: PageRuler Prestained Plus Protein Ladder (Fermentas).

3.2.3 Characterization of EPS polysaccharides

Polysaccharides are the main components of *S. acidocaldarius* EPS extracted from biofilms grown on Brock medium plates amounting to 4.7 ± 0.2 fg cell^{-1}. Several studies about bacterial biofilms revealed a structural role of polysaccharides within the EPS matrix (Watnick and Kolter 1999; Danese *et al.* 2000; Ma *et al.* 2009). In previous studies of our cooperation group at the MPI Marburg submersed biofilms grown on the bottom of µ-dishes were successfully stained with lectins binding to α-D-mannosyl and α-D-glucosyl residues (ConA) as well as N-acetyl-D-galactosamine end groups and terminal α-D-galactosyl residues (IB4), thus giving a first information about the extracellular polysaccharide composition (Koerdt *et al.* 2010). In this study, colonies grown on polycarbonate filters (section 3.1.3) were stained with the lectin WGA (wheat germ agglutinin), binding to N-acetylglucosamine residues and with the DNA stain SYTO9 (Figure 3.21). A strong signal for WGA surrounding densely packed biofilms cells was detected. Using the light microscopy mode of the CLSM a thickness of the biofilm of 110 µm was measured. However, the biofilm was too dense and only allowed staining with SYTO9 of the upper 60 µm of the biofilm.

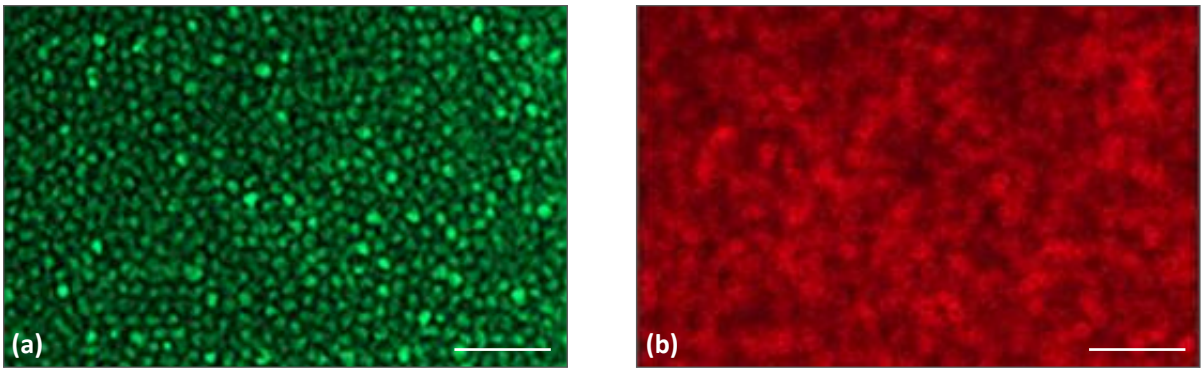

Figure 3.21: *S. acidocaldarius* biofilm stained with (a) SYTO9 and (b) WGA. Biofilms were cultivated on polycarbonate membrane filters floating on top of liquid Brock medium for 6 days at 78°C. The lectin WGA binds to N-acetylglucosamine residues. Scale bar 10 µm.

Since the binding of lectins only gives a hint to the actual polysaccharide composition due to non-specific lectin binding, EPS polysaccharides were hydrolyzed and subjected to thin layer chromatography (TLC). For the applied hydrolysis conditions D-glucose was detected as the main component of the EPS polysaccharides (Figure 3.22). A clear spot (no. 1) of the EPS sample in the same heights and of the same color as D-glucose was observed. A second weaker spot (no. 2) below was detected, which did not correlate to any of the applied sugar standards.

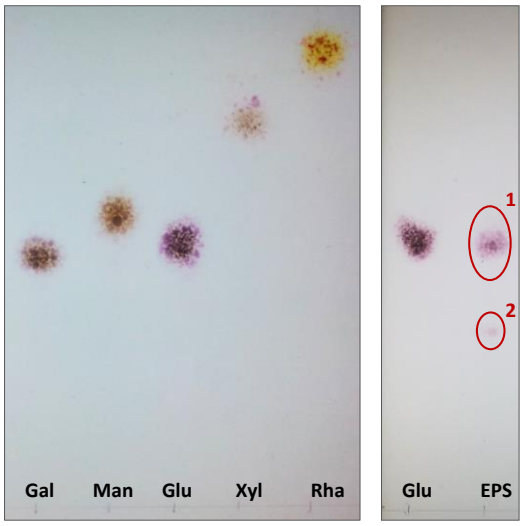

Figure 3.22: TLC of hydrolyzed EPS of *S. acidocaldarius* biofilms. EPS was isolated via CER from biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Lyophilized EPS was hydrolyzed for 48 h using 0.1 M HCl, neutralized with 1 M NaOH and treated with acetone. Applied samples: Hydrolyzed EPS (5 µL) and monosaccharide standards (6 µL of 30 mM stock solution). Samples from left to right: D-galactose, D-mannose, D-glucose, D-xylose, L-rhamnose, D-glucose and hydrolyzed EPS. Red circles and numbers indicate spots in the EPS sample. Samples were applied to Silicagel G60 plates and developed in acetonitrile:1-Pentanol:water (60:20:20, v/v/v) as solvent. Detection was performed by spraying with the derivatization reagent N-(-1-Naphthyl) ethylenediamine-dihydrochloride (3 g L^{-1} in 5% (v/v) methanol) and heating at 100°C for at least 10 min.

3.2.4 Characterization of EPS eDNA

In several preparations of *S. acidocaldarius* biofilms grown on Brock medium plates (4 days, 78°C), macroscopic white filaments within the biofilm suspensions in phosphate buffer were observed, which could not be dispersed by shaking or pipetting. The filaments showed sizes of more than 0.5 cm in length. Staining with DAPI and visualization using an epi-fluorescence microscope revealed that the filaments consisted of densely packed cells sticking to each other (Figure 3.23). CLSM images of filaments stained with SYTO9 displayed a round structure with a diameter of approximately 40 - 60 µm (Figure 3.24). Böckelmann *et al.* (2006) reported similar formation of so called microfilament networks in which bacterial cells were attached to each other with a compound stainable with SYTO9. Using different approaches they identified the compound as eDNA. By now it is well established that eDNA is an important component of the EPS matrix (Whitchurch *et al.* 2002). Considerable amounts of eDNA (0.91 ± 0.48 fg $cell^{-1}$) were quantified in the EPS of *S. acidocaldarius* in the present study (section 3.2.1).

To further investigate the component keeping the cells together in the filaments of *S. acidocaldarius* biofilms, filaments were treated with DNase for 1 h at 37°C. Treatment with DNase led to a nearly instant dispersal of cells (Figure 3.26). Images of the initial filament and the filament 10 min after addition of DNase showed dispersal of cells and detachment of pieces after DNase addition (Figure 3.25). Addition of a drop of water on top of the filament resulted in an instant and total dispersal of the filament. In contrast, RNase and proteinase K treatment and control treatments using only the respective enzyme buffers led to no dispersal or difference in the appearance of the filaments.

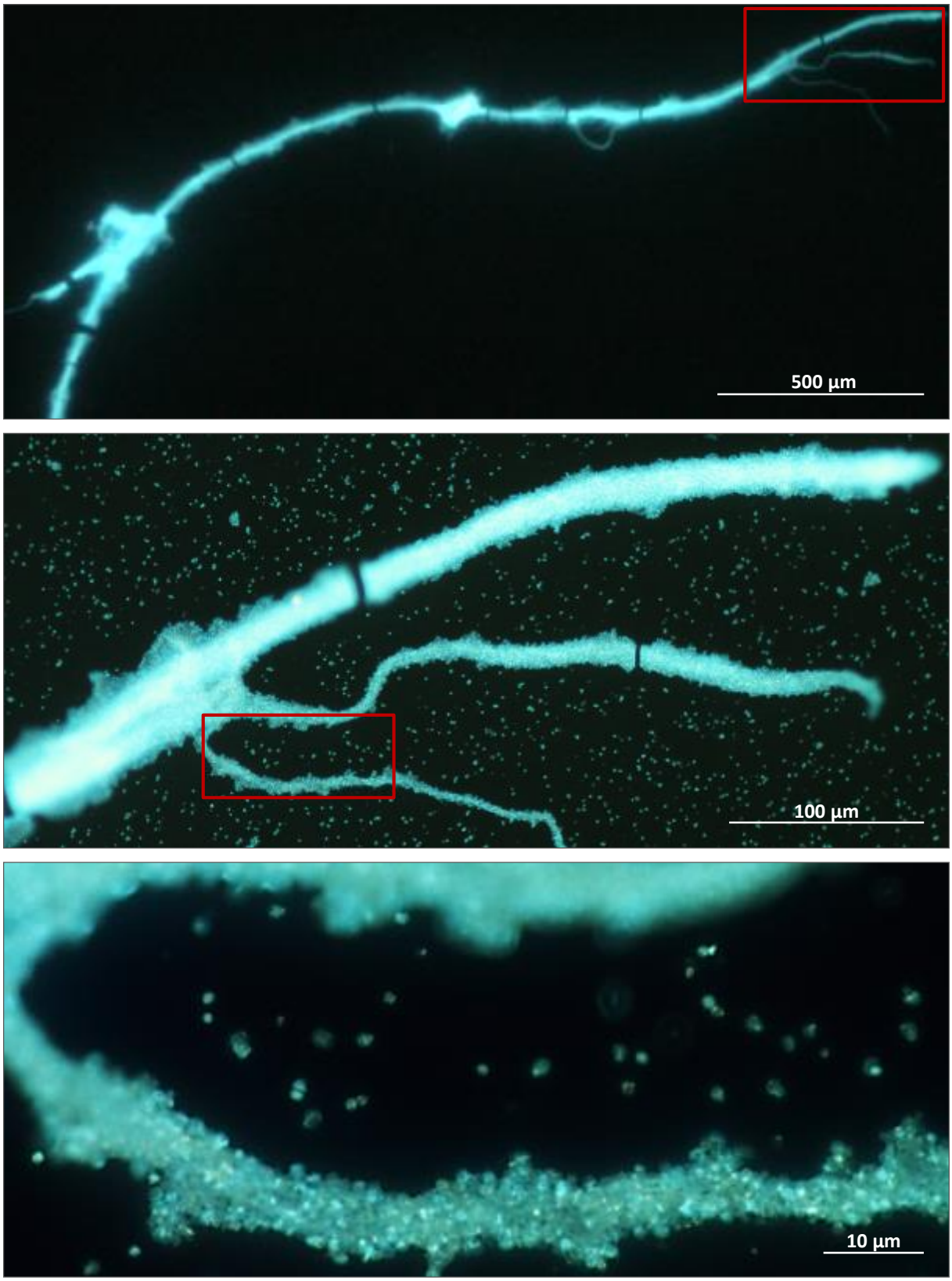

Figure 3.23: Epi-fluorescence images of *S. acidocaldarius* filaments stained with DAPI. Filaments were obtained from biofilms grown on Brock medium plates (4 days, 78°C). Filaments were stained with DAPI and filtered onto black polycarbonate membrane filters. Images were taken at different magnifications. Red boxes indicate sections enlarged in image below.

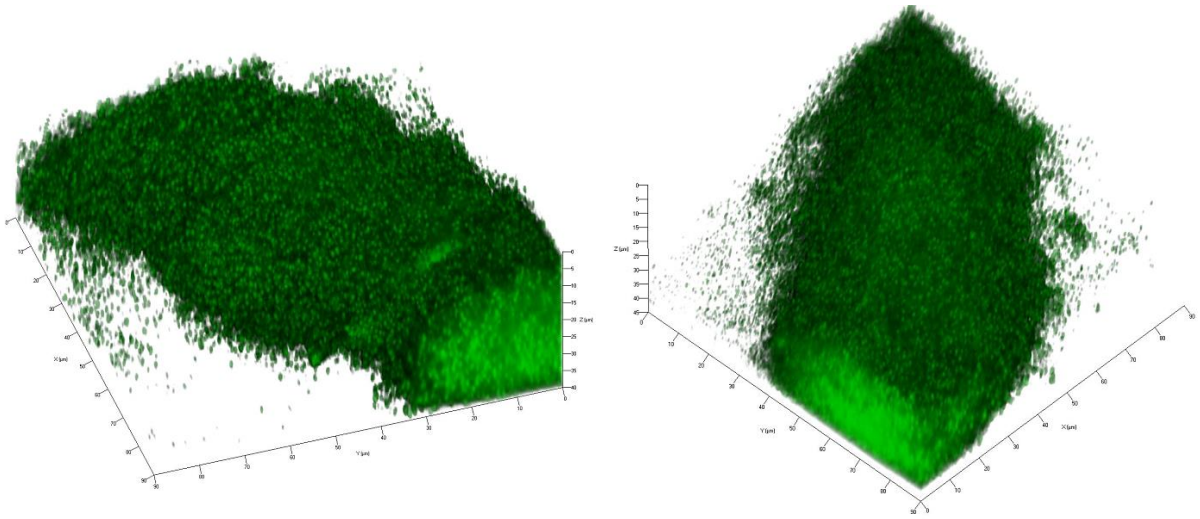

Figure 3.24: CLSM images of *S. acidocaldarius* filaments stained with SYTO9. Filaments were obtained from biofilms grown on Brock medium plates (4 days, 78°C). X- and Y-axis: 90 µm; Z-axis: 40 µm.

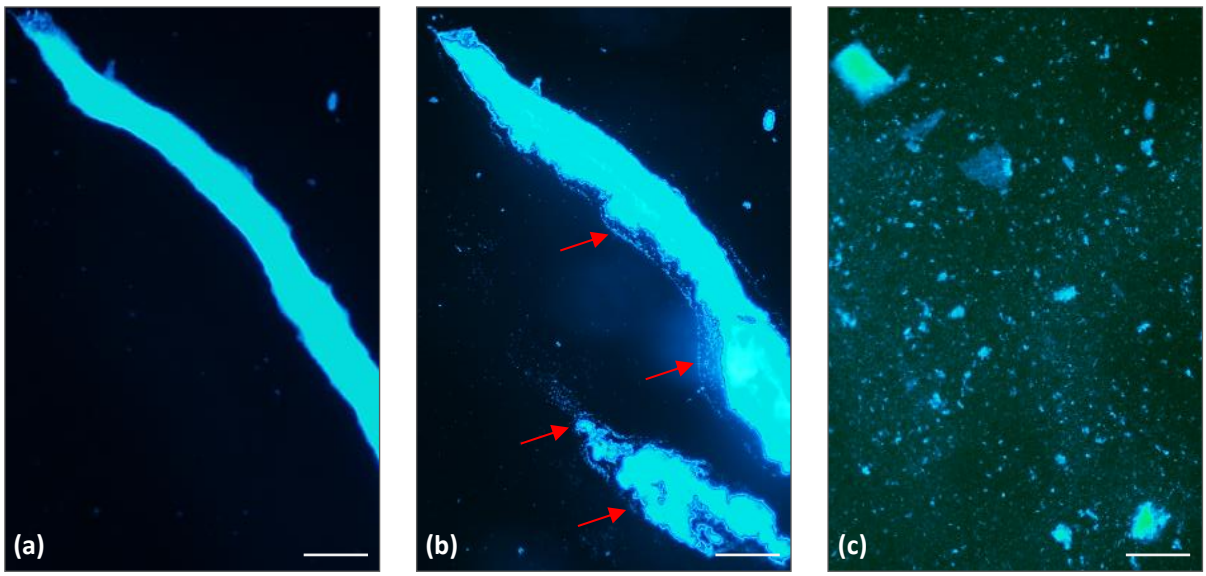

Figure 3.25: Epi-fluorescence images of *S. acidocaldarius* filaments before and during treatment with DNase. Filaments were obtained from biofilms grown on Brock medium plates (4 days, 78°C). Filaments were fixed on a microscope slide and stained with DAPI. Images show filaments (a) before addition of DNase, (b) 10 min after addition of DNase and (c) 1 h after addition of DNase and addition of a drop of water. Arrows indicate dispersing cells and detached pieces. Scale bars 10 µm.

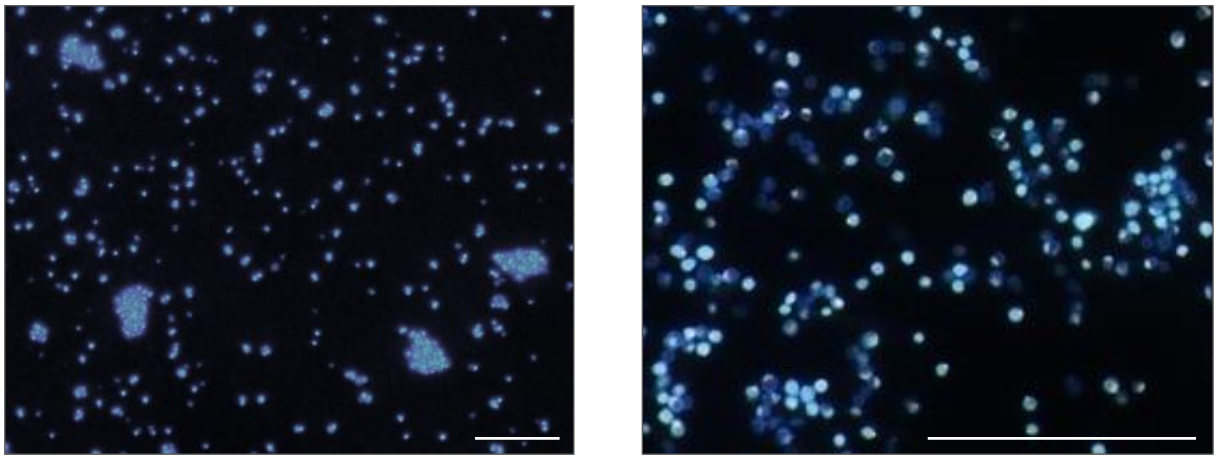

Figure 3.26: Epi-fluorescence images of *S. acidocaldarius* filaments stained with DAPI after treatment with DNase. Filaments were obtained from biofilms grown on Brock medium plates (4 days, 78°C). After DNase treatment, samples were filtered onto black polycarbonate membrane filters; scale bars 20 µm.

The eDNA of the filaments was further characterized by isolation of the DNA from the biofilm suspension as described by Böckelmann *et al.* (2006). Briefly, the biofilm suspension was centrifuged and the pellet resuspended in NaCl (0.85% (w/v)). After sterile filtration (pore size 0.2 µm) the Blood and Tissue kit was applied to isolate and concentrate DNA of the filtrate. The isolated DNA was analyzed by agarose gel electrophoresis and stained with ethidium bromide (Figure 3.27). A distinct band above the 10,000 bp band of the bp size marker was detected, running at the same heights as chromosomal DNA of *S. acidocaldarius*. Additionally, a DNA smear was observed between 1,000 bp and 1,500 bp for both, the isolated DNA and the chromosomal DNA.

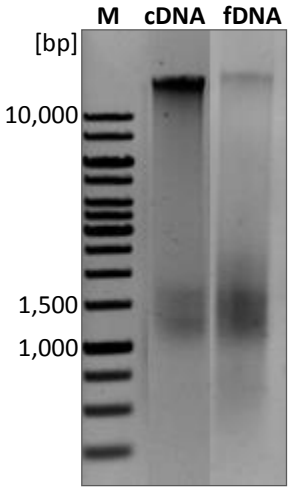

Figure 3.27: Agarose gel electrophoresis of DNA isolated from *S. acidocaldarius* biofilm filaments (fDNA) and chromosomal DNA (cDNA). M: Base pair size marker; 1 kb Gene Ruler (Fermentas).

In conclusion dispersion of cells after addition of DNase suggests that also in *S. acidocaldarius* biofilms eDNA can act as a structural component responsible for the

maintenance of filaments. This is in coherence to previous studies by Böckelmann *et al.* (2006) and Whitchurch *et al.* (2002) demonstrating the structural role of eDNA in biofilms of *P. aeruginosa* and a bacterial isolate from river snow of the South Saskatchewan River.

In order to replicate the occurrence of the filaments and determine their cause, parameters, which might differ between individual cultivations of *S. acidocaldarius* biofilms, were modified. The most significant difference in biofilm appearance was observed for shifts of the pH value. Values lower than the standard pH of 3.5 resulted in softening of the gellan gum and the formation of a very soft and watery biofilm that was easily suspended in phosphate buffer. A pH above 5.5 led to very firm plates and firm and sticky biofilms. The single stripes of biofilm could be removed in one piece by pulling one end of the stripe up with a spatula. Those biofilms were difficult to suspend. On several occasions formation of filaments was observed at elevated pH values, however, this phenomenon was not reproducible. In general, determination of the pH after incubation showed that for any plate the pH increased by 0.5 - 1 units.

Due to the long incubation times of 4 days at a temperature of 78°C the loss of water from the plates was also presumed to be of importance due to possible evaporation variations depending on the position within the incubator and within the stack of plates. However, a water loss of merely 6% on average was determined. Addition of different concentrations of gellan gum to the medium to adapt to possible water loss did not result in reproducible formation of filaments. Since evaporation was also assumed to be lower in plates with a high volume of solid medium, plates with different volumes were poured. To exclude impact by higher or lower evaporation due to the location of the plates within the stack, two stacks were prepared; one with the lowest volume at the bottom and one with the lowest volume on top. On the plates with the lowest volumes of medium (20 mL and 25 mL) lower biofilm formation was observed independent of the location within the stack. It appeared that the volume did not influence the occurrence of filaments. Moreover, culture age and also a higher/lower growth temperature itself did not influence the occurrence of filaments.

Taken together, none of the tested conditions repeatedly led to the formation of filaments even though the water content and the pH value of plates after incubation seemed to play a crucial role. Instead, it appeared to be a combination of factors leading to the formation of filaments and no reproducible conditions could be established.

3.3 Role of glycosyltransferases in exopolysaccharide synthesis

3.3.1 Glycosyltransferase gene cluster of *S. acidocaldarius*

In bacteria glycosyltransferases (GTs) were shown to be involved in biofilm formation as well as in the synthesis of exopolysaccharides. In the archaeal model strain *S. acidocaldarius* a cluster of genes was detected encoding gene products, which show high similarities to enzymes previously determined to be involved in bacterial exopolysaccharide biosynthesis as well as in exopolysaccharide secretion (Orell *et al.* 2013b) (section 1.3.4). Within this cluster comprising 24 genes, 12 genes encoding GTs were predicted (Table 3.8).

Table 3.8: Overview of GT genes within the predicted gene cluster for exopolysaccharide biosynthesis and excretion in *S. acidocaldarius*. Listed are characteristics of the GTs within the cluster consisting of Saci_1904 up to Saci_1927. Annotations of proteins were performed using the updated *S. acidocaldarius* genome annotation based on arCOGs and available functional information (Esser *et al.* 2011) as well as BLASTp. GT families were classified based on the CAZy database. Availability of deletion mutants or prediction of transmembrane domains identified via SMART (Schultz *et al.* 1998; Letunic *et al.* 2012) within the proteins is indicated by crosses.

S. acidocaldarius ORF ID	Mutant constructed	GT family	Prediction of transmembrane domains
Saci_1904	+	4	-
Saci_1907	+	4	-
Saci_1909	+	2	-
Saci_1911	-	2	+
Saci_1914	+	4	-
Saci_1915	+	2	-
Saci_1916	+	4	-
Saci_1921	+	4	-
Saci_1922	+	4	+
Saci_1923	-	4	-
Saci_1926	-	-	-
Saci_1927	-	2	-

Interestingly, structural predictions carried out for one of the genes annotated to encode an uncharacterized conserved protein using HHpred (Söding *et al.* 2005) indicated the occurrence of a carbohydrate binding module (CBM) within the extracellular part of the encoded membrane protein Saci_1917, which was predicted to comprise 11 transmembrane helices (Figure 3.28). CBMs are usually found adjacent to carbohydrate active enzymes and assist in the enzymatic process by binding of the substrate. The here described putative CBM was detected within a membrane protein without any obvious similarity to carbohydrate utilizing enzymes.

The complete gene Saci_1917, the extracellular region as well as that part of the extracellular region showing structural homology to CBMs, were cloned for expression in *E. coli*. However, no expression of the His-tagged protein was detected via western blotting using the expression strains Rosetta(DE3), Lemo21(DE3) or BL21-CodonPlus(DE3)-RIL (Bernds 2011).

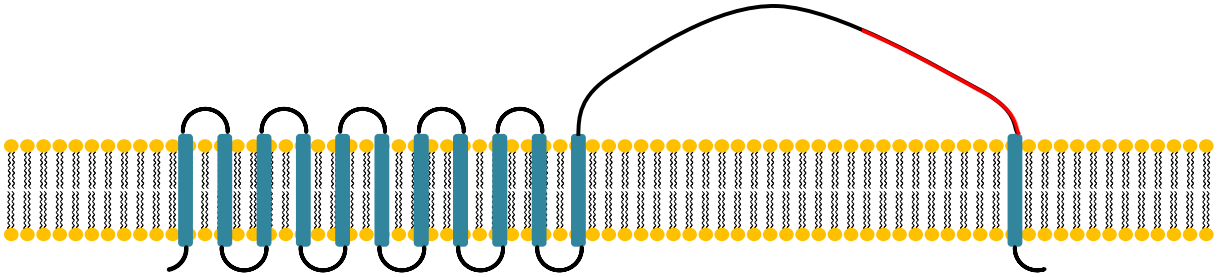

Figure 3.28: Schematic overview of the localization of Saci_1917 in the membrane. Red indicates the region with high structural homology to known CBM structures detected using HHpred. Prediction of transmembrane helices performed using SMART (Schultz *et al.* 1998).

3.3.2 Quantitative EPS analysis of glycosyltransferase gene deletion mutants

To investigate the role of GTs of the gene cluster in *S. acidocaldarius* single gene deletion mutants were constructed (see Table 3.8). Additionally, a mutant lacking membrane protein Saci_1917, which carries the putative CBM was constructed as well as mutants lacking the membrane protein Saci_1908 and the methyltransferase Saci_1918. The impact of the deletion was studied by comparing the quantity of EPS proteins and carbohydrates. EPS from biofilms grown on Brock medium plates (4 d, 78°C) were isolated using the CER method and their composition was determined in terms of carbohydrate and protein concentration and compared to the reference strain MW001 (parental strain) (Figure 3.29, Table 3.9).

The determination of the carbohydrate concentrations in the EPS of the GT deletion mutants and MW001 showed that the highest formation of polysaccharides was found in the MW001 EPS with 5.89 ± 0.74 fg cell^{-1}. Mutants Δ1904 and Δ1914 possessed the lowest carbohydrate concentration corresponding to 33% (1.94 ± 0.19 fg cell^{-1}) and 30% (1.76 ± 0.09 fg cell^{-1}) of the concentrations of MW001, respectively.

In contrast to the carbohydrate concentrations, the protein concentration of MW001 was significantly exceeded by two deletion mutants. MW001 showed a protein concentration of 2.47 ± 0.43 fg cell^{-1} while mutants Δ1908 (membrane protein) and Δ1916 (GT) exceeded this concentration by 149% (3.67 ± 0.26 fg cell^{-1}) and 137% (3.39 ± 0.51 fg cell^{-1}), respectively. The lowest protein concentrations were detected for GT mutants Δ1914 and Δ1915 with 1.33 ± 0.15 fg cell^{-1} and 1.24 ± 0.07 fg cell^{-1}, respectively, corresponding to approximately 50% of the protein concentration of MW001. Nevertheless, in general the protein concentrations for most GT deletion mutants revealed only slight variances compared to the reference strain MW001 in contrast to the significantly more pronounced differences in carbohydrate concentrations.

Comparison of the ratio of proteins to carbohydrates showed that even though the amount of protein and carbohydrates within the EPS of GT deletion mutant Δ1915 was drastically decreased compared to MW001, the ratio was similar to that of MW001 (i.e. 0.42). Methyltransferase mutant Δ1918 and GT mutant Δ1921 also revealed ratios similar to MW001 EPS (Table 3.9). Highest ratios of 1.05 and 0.87 were determined for GT mutants Δ1904 and Δ1922, respectively, due to the significantly lower carbohydrate concentrations and simultaneously similar protein concentrations.

The mutant Δ1917 lacking the membrane protein Saci_1917, which carries the putative CBM, showed only 56% of the carbohydrate concentration of MW001, while the protein concentration remained equal resulting in a protein to carbohydrate ratio of 0.75.

In general the results indicate an involvement of the deleted GTs in the exopolysaccharide synthesis but also in the occurrence of EPS proteins since several mutants showed altered EPS protein concentrations.

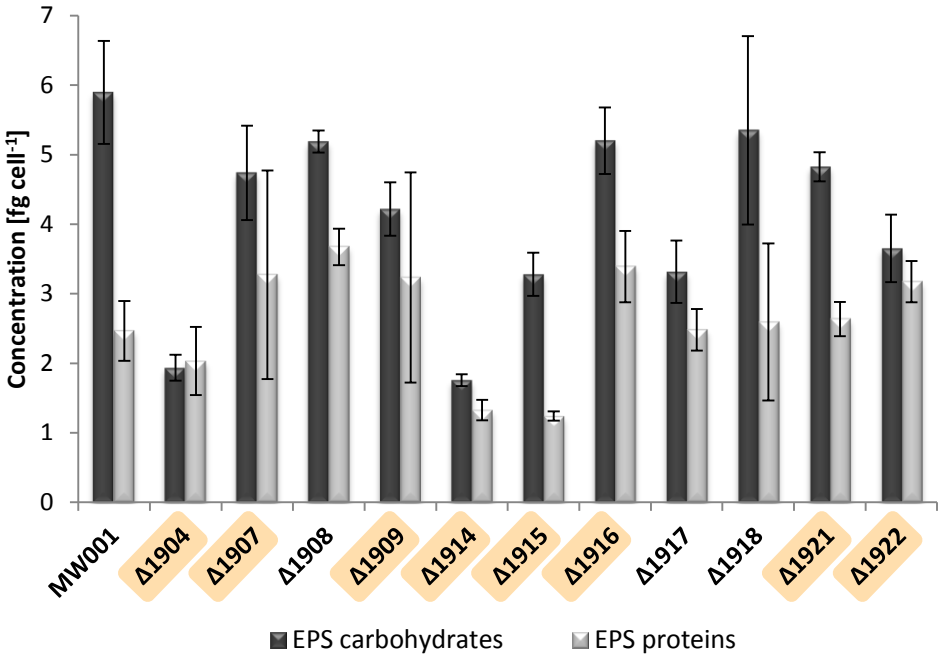

Figure 3.29: Concentrations of EPS carbohydrates and EPS proteins in gene deletion mutants of the GT gene cluster and reference strain MW001. EPS was isolated from biofilms of *S. acidocaldarius* reference strain MW001, GT deletion mutants, membrane protein deletion mutant Δ1917, which carries a putative CBM domain as well as membrane protein deletion mutant Δ1908 and the methyltransferase deletion mutant Δ1918. Biofilms were cultivated on Brock medium plates with supplementation of uracil for 4 d at 78°C. GT deletion mutants are highlighted in orange; n = 3.

Table 3.9: EPS protein and carbohydrate concentrations of *S. acidocaldarius* gene deletion mutants in % of the concentrations of reference strain MW001. Amount of proteins and carbohydrates are given in % of the concentrations of reference strain MW001. Ratios of proteins to carbohydrates are listed for the reference strain MW001, GT deletion mutants, mutant Δ1917 lacking the gene encoding membrane protein Saci_1917, which carries a putative CBM domain as well as mutants with deletion of the gene encoding membrane protein Saci_1908 (Δ1908) and deletion of the methyltransferase Saci_1918 (Δ1918). EPS was isolated from biofilms cultivated on Brock medium plates with supplementation of uracil for 4 days at 78°C; n = 3.

S. acidocaldarius deletion mutant	% protein of reference strain MW001	% carbohydrates of reference strain MW001	Protein/carbohydrate ratio
MW001	-	-	0.42
Δ1904	82	33	1.05
Δ1907	133	80	0.69
Δ1908	149	88	0.71
Δ1909	131	72	0.77
Δ1914	54	30	0.76
Δ1915	50	56	0.38
Δ1916	137	88	0.65
Δ1917	101	56	0.75
Δ1918	105	91	0.48
Δ1921	107	82	0.55
Δ1922	129	62	0.87

3.3.3 Heterologous expression of *S. acidocaldarius* glycosyltransferases

For the heterologous expression of the GTs found in the cluster presented before (section 1.3.4) the *E. coli* expression strain Rosetta(DE3) was used. After induction of expression of the His-tagged proteins with IPTG, cells were cultivated overnight at 20°C, centrifuged, and the cells were disrupted using sonication (section 2.14.17). The resulting membrane fraction and the soluble fraction (crude extract) were separated by centrifugation and recombinant proteins were partially enriched from crude extracts by heat precipitation at 60°C, 70°C, 80°C and 90°C. Protein expression and enrichment was analyzed by SDS-PAGE as well as western blotting and immuno-detection of the His-tagged proteins. Expression was tested for GTs Saci_1907, Saci_1909, Saci_1911, Saci_1915, Saci_1918, Saci_1922, Saci_1923 and Saci_1927 (supplementary material Figure 6.1-6.7). Except for Saci_1909 and Saci_1911, which were only very weakly expressed, overexpression was visible in all membrane fractions on CCB stained polyacrylamide gels after SDS-PAGE (Table 3.10). Detection of expressed GTs in the soluble fraction could only be determined using western blotting and immunological detection of the His-tag present in all expressed GTs. Heat precipitations of the crude extract at 60°C for 20 min resulted in co-precipitation of GTs Saci_1907, Saci_1909, Saci_1911 and Saci_1922 as was evident by the lack of a signal in this fraction in the immuno-blot.

Table 3.10: Overview of detected expression of *S. acidocaldarius* GTs in *E. coli* Rosetta. Expression of GTs was performed in *E. coli* Rosetta(DE3) at 20°C over night. Expression in the membrane fraction (MF), crude extract (CE) or heat precipitation of crude extract at 60°C (HP60°C) was examined via SDS-PAGE and His-tag immunological detection after western blotting. Confirmed expression is designated with a cross, absence of clearly detectable expression with a dash. IB: Expression in inclusion bodies; n.d.: not determined.

Glycosyltransferase	SDS-PAGE			Western blot and His-tag immunological detection		
	MF	CE	HP60°C	MF	CE	HP60°C
Saci_1907	+ (IB)	-	-	+	+	-
Saci_1909	-	-	-	+	+	-
Saci_1911	-	-	-	+	Very faint	-
Saci_1915	+	-	-	+	+	Very faint
Saci_1918	+ (IB)	-	-	+	+	+
Saci_1922	+ (IB)	-	-	+	+	Very faint
Saci_1923	+	-	n.d.	n.d.	n.d.	n.d.
Saci_1927	+	-	n.d.	n.d.	n.d.	n.d.

Due to the lack of solubility none of the tested GTs was purified in amounts sufficient for characterization of enzyme activity and substrate specificity. In Figure 3.30 expression and purification of GT Saci_1909 is illustrated, showing the highest solubility of the tested GTs. Like listed in Table 3.10, overexpression of GT Saci_1909 was not clearly detectable on polyacrylamide gels, however, His-tag detection revealed clear bands in the membrane fraction as well as in the crude extract (Figure 3.30). Heat treatment at 60°C of the crude extract led to precipitation of GT Saci_1909.

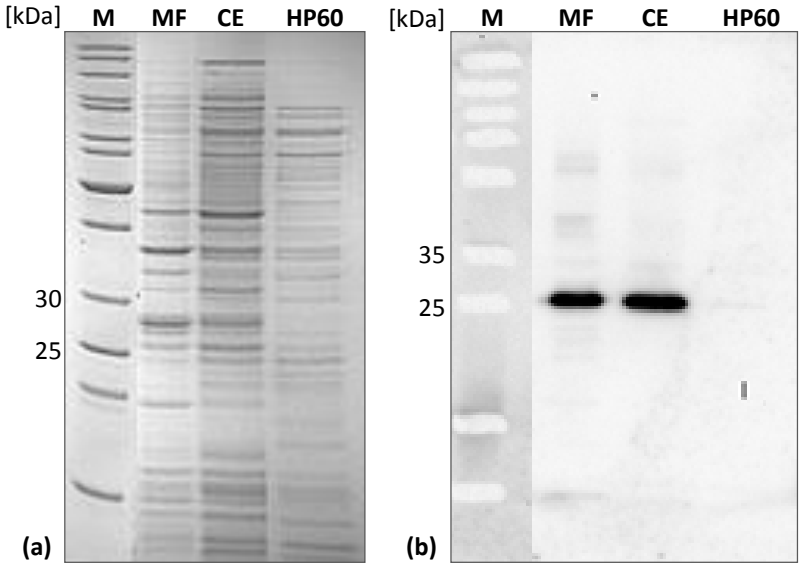

Figure 3.30: Examination of expression of *S. acidocaldarius* GT Saci_1909 in *E. coli* Rosetta(DE3). Expression was examined in the membrane fraction (MF), crude extract (CE) and heat precipitation of crude extract at 60°C (HP60). Visualization by (a) SDS-PAGE (12.5% (w/v) polyacrylamide) stained with CBB and (b) immuno-detection of His-tagged protein after western blotting (Antibody: Ms mAb to 6 x Histag®, abcam). (a) M: PageRuler Protein Ladder, (b) M: PageRuler Prestained Protein Ladder; enhanced by separate decrease of image contrast. Applied protein concentrations: 5 µg CE, 2.5 µg HP60.

Since the heat precipitation of *E. coli* proteins resulted in the co-precipitation of the protein of interest, for the purification of GT Saci_1909 via Ni-TED affinity chromatography, crude extract was used. After western blotting and immuno detection a strong band at the expected molecular mass of GT Saci_1909 (28 kDa) was detected in the flow through as well as in the first washing step (Figure 3.31). Elution 1 and 2 showed a weaker band at the same heights. However, protein concentrations in these fractions were below the detection limit.

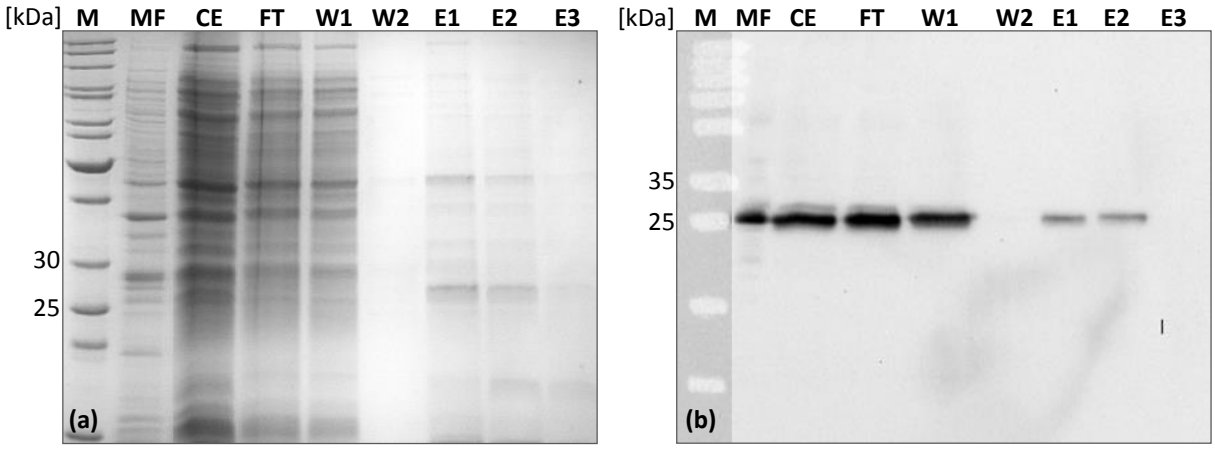

Figure 3.31: Purification of GT Saci_1909 via affinity chromatography. 800 µg crude extract were used for purification of the His-tagged protein Saci_1909 using a nickel TED column. Visualization by (a) SDS-PAGE (12.5% (w/v) polyacrylamide) stained with CBB and (b) immuno-detection of His-tagged protein after western blotting (Antibody: Ms mAb to 6 x Histag®, abcam) and immunological detection in membrane fraction (MF), crude extract (CE; 13 µg), flow through (FT; 13 µg), washing step 1 (W1; 7 µg) washing step 2 (W2; protein concentration below the detection limit) as well as elution fractions 1-3 (E1-E3; protein concentration below the detection limit) was visualized using western blotting and immuno-detection. M: PageRuler Prestained Protein Ladder; enhanced by separate decrease of image contrast.

Of the expressed GTs only Saci_1911 and Saci_1922 were predicted to contain a transmembrane domain (Table 3.8). Nevertheless, under the tested conditions, also Saci_1907, Saci_1909, Saci_1915, Saci_1923 and Saci_1927 were detected mainly in the insoluble fraction (supplementary material Figure 6.1-6.7).

3.4 Involvement of Lrs14 regulators in EPS composition of *S. acidocaldarius* biofilms

In a study performed in collaboration with the group of Dr. S.-V. Albers (MPI Marburg) (Orell *et al.* 2013b) the involvement of the archaeal Lrs14-like regulator Saci_0446 on biofilm formation was determined. Lrs-like proteins are transcriptional regulators and members of this protein family were shown to be involved in biofilm formation (Koerdt *et al.* 2011). Hence, absence of the Lrs14 regulator Saci_0046 due to gene deletion was assumed to alter the formation and composition of the biofilm. The reference strain MW001, the gene deletion mutant ΔSaci_0446 as well as the same mutant complemented with plasmid pSVA2026 (named pSVASaci_0446 in this study) carrying the deleted gene Saci_0446 for overexpression (ΔSaci_0446 pSVASaci_0446) were cultivated as submersed biofilms in Brock medium (0.1% (w/v) N-Z-amine, 10 mg mL^{-1} uracil) at the bottom of Petri dishes (3 d, 76°C, static). Since the overexpression of Saci_0446 in the complemented strain ΔSaci_0446 pSVA2026 was induced by the addition of maltose (0.4% (w/v)), the reference strain MW001 was grown in absence as well as in presence of maltose to exclude its influence on EPS formation and composition.

The results of the quantitative EPS protein and carbohydrate determinations showed that the mutant lacking the Lrs14 regulator produced 4.7- and 2.7-fold higher concentrations of EPS proteins and carbohydrates, respectively, compared to the reference strain MW001 (Figure 3.32). Complementation of the mutant with the Lrs14 gene on a plasmid and induction of expression with maltose restored the EPS component quantities to the level of the reference strain. No difference in EPS quantity was observed in biofilms of the reference strain MW001 cultivated in absence or presence of maltose, hence, excluding an effect of maltose on the EPS composition. These results were further confirmed by staining of EPS compounds of submersed biofilms and visualization via CLSM imaging by the cooperation partner at the MPI Marburg. Application of the lectin ConA resulted in an enhanced signal of the Lrs14 mutant biofilm compared to the reference strain. The reference strains cultivated in presence or absence of maltose showed similar ConA signals. The Lrs14 mutant complemented with the Lrs14 regulator by overexpression showed even slightly lowered signals compared to the reference strain.

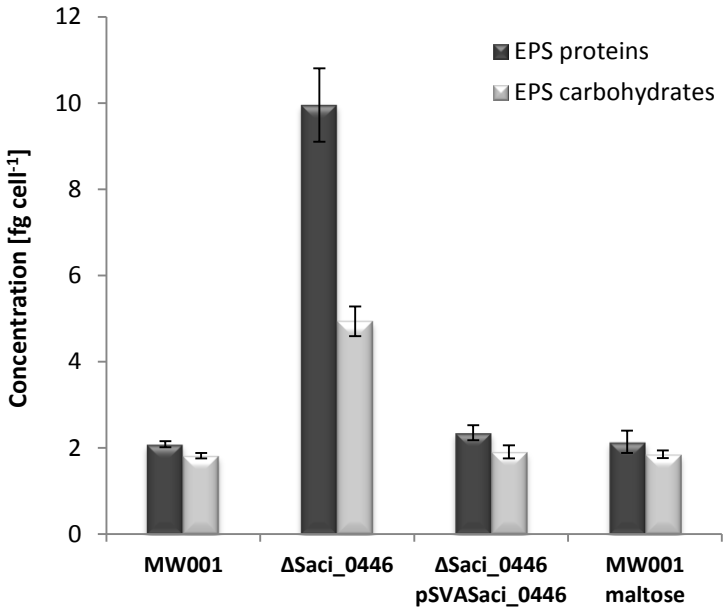

Figure 3.32: Concentration of EPS proteins and carbohydrates isolated from biofilms of MW001 (presence/absence of maltose), ΔSaci_0446 and ΔSaci_0446 pSVASaci_0446. EPS were isolated from submersed biofilms cultivated on the bottom of large Petri dishes using CER (3 d, 78°C); n = 3.

Transcriptional analysis performed by the collaboration partners of the Lrs14 mutant Δ0446 showed altered regulation for the genes encoding GT Saci_1909 as well as for the membrane protein Saci_1908, both belonging to the previously described GT cluster (Figure 1.2)(Orell *et al.* 2013b). While transcript levels of Saci_1909 were down-regulated, transcription of Saci_1908 was up-regulated. Analysis of double mutants lacking the Lrs14 regulator and either Saci_1909 or Saci_1908 showed that mutants lacking the Lrs14 regulator and Saci_1908 showed a phenotype similar to the reference strain MW001. Deletion of the GT Saci_1909 as well as combined deletion of this GT and the Lrs14 regulator, however, led to an overproduction of exopolysaccharides as evident by CLSM imaging.

3.5 Effect of an α-mannosidase on EPS composition of *S. solfataricus* biofilms

The EPS isolation method established for *S. acidocaldarius* in this study was also applied for EPS isolation from unsaturated biofilms of *S. solfataricus* in collaboration with the group of Dr. S.-V. Albers (MPI Marburg) (Koerdt *et al.* 2012). The isolated EPS was quantitatively analyzed to evaluate the involvement of the α-mannosidase SSO3006 in the biofilm formation of *S. solfataricus.* The α-mannosidase is found in the wild type strain P2 but not in the natural mutant strain PBL2025, which lacks a genome region of approximately 50 genes (SSO3004 – SSO3050), including the β-galactosidase lacS (SSO3019) and the gene encoding the α-mannosidase (SSO3006, Ssα-man). To test the influence of the enzyme on biofilm formation and EPS composition a virus-based vector system was used to complement PBL2025 with a plasmid encoding either the α-mannosidase, LacS or ABCE1 (a cytoplasmic protein involved in ribosome recycling used as control). *S. solfataricus* P2, PBL2025 and PBL2025 complemented with one of these plasmids were cultivated as unsaturated biofilms on Brock medium plates (4 d, 78°C). EPS was isolated using CER and quantified with respect to EPS protein and carbohydrate concentrations (Figure 3.33).

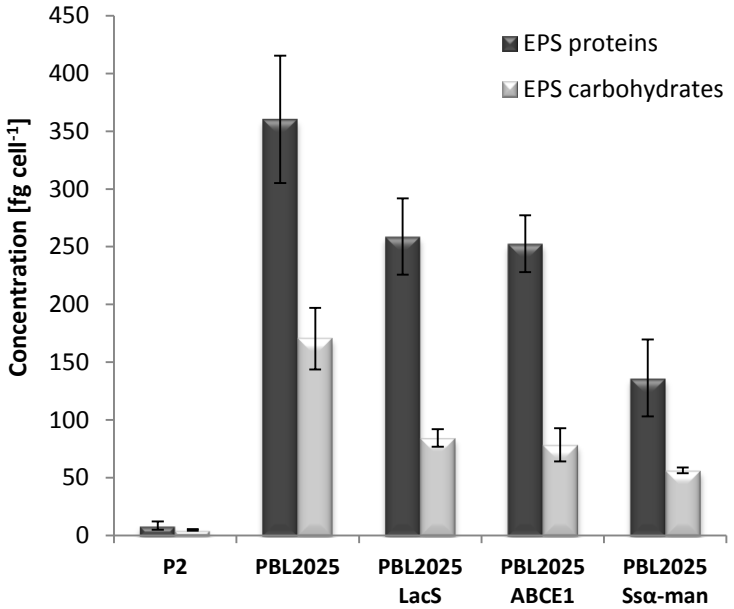

Figure 3.33: Protein and carbohydrate concentrations in the EPS of *S. solfataricus* unsaturated biofilms. EPS was isolated from unsaturated biofilms of *S. solfataricus* P2, PBL2025, PBL2025 LacS PBL2025 ABCE1 and PBL2025 Ssα-man cultivated on Brock medium plates (4 d, 78°C) using CER; n = 3.

Biofilms of the *S. solfataricus* natural mutant PBL2025 revealed a 35-fold increased EPS carbohydrate production and 42-fold increased protein production per cell compared to the wild type strain P2. The complementation of PBL2025 with the α-mannosidase still resulted in an 11-fold increased EPS carbohydrate concentrations and 16-fold increased EPS protein concentrations compared to P2, however, compared to the natural mutant lacking the α-mannosidase, the concentrations of both EPS components were significantly reduced. The complementation of PBL2025 with LacS or ABCE1 also reduced the concentration of EPS proteins and carbohydrates compared to PBL2025, however, in a less pronounced way as did the complementation with the α-mannosidase.

The results obtained for the quantities of the EPS components are consistent with CLSM images of ConA stained submersed biofilms of the respective strains performed by the cooperation partners at the MPI Marburg (Koerdt *et al.* 2012). The microscopic images indicated an increased occurrence of glucose and mannose residues in PBL2025 compared to P2. Complementation of PBL2025 with the α-mannosidase, however, led to similar ConA signals as observed for P2. An influence of LacS or ABCE1 complementation was not observed.

4. DISCUSSION

4.1 Thermoacidophilic biofilms – Challenging cultivation

The major aim of this study was the analysis of biofilms of the thermoacidophilic Archaeon *Sulfolobus acidocaldarius* and their EPS. This aim required cultivation methods, which allowed for growth of large amounts of *S. acidocaldarius* biofilms in order to obtain enough biomass for the isolation of EPS and subsequent quantitative and qualitative analysis of their composition. Cultivation of thermoacidophilic organisms represents a special challenge due to the high temperatures and low pH values required for optimal growth.

Only very limited information about the cultivation of *Sulfolobus* spp. biofilms exists in the literature (Table 4.1). Previous studies involving the cultivation of biofilms were focused on the initial steps during biofilm formation (e.g. Zolghadr *et al.* 2010). So far, no study aimed at the production of elevated biofilm mass for biochemical analysis.

In the presented study cultivation of *S. acidocaldarius* biofilms on glass slides was tested for its suitability to produce high amounts of biofilm mass. This approach was previously applied for the investigation of the involvement of *Sulfolobus* spp. cell surface structures in the initial attachment of cells (Zolghadr *et al.* 2010; Henche *et al.* 2012b; Henche *et al.* 2012a). These studies demonstrated the formation of microcolonies on glass slides incubated for one to three days. Attachment of planktonic *S. acidocaldarius* cells to glass slides was also demonstrated in the current study, however, microscopic analysis showed only single cells, which were scattered throughout the surface and even prolonged incubation and exchange of media over time to promote further growth did not result in a confluent biofilm. Initial attachment to the substratum is a profound step in the formation of biofilms involving e.g. cell surface structures like archaella and pili, or secreted polymers like polysaccharides, proteins and DNA. Also the hydrophobicity of the cells and of the substratum are of importance regarding this fundamental stage of biofilm formation (Donlan 2002). Cell surface hydrophobicity was addressed in the present study in order to increase the rate of initial attachment of *S. acidocaldarius* to solid supports, and thus to promote biofilm formation. The cell surface hydrophobicity of *S. acidocaldarius* cells was determined using the MATH test and indicated a hydrophobic cell surface of this microorganism. Hence, it was hypothesized that other hydrophobic materials could enhance the formation of multilayered

biofilms. Nevertheless, none of the tested substrata (e.g. EPDM) allowed for growth of thick biofilms, and therefore, this method was not applicable for the cultivation of high biofilm mass, as required for subsequent EPS isolation and analysis.

Due to the lack of methods yielding substantial biofilm mass, new cultivation methods had to be established in this study (for an overview see Table 4.1). *S. acidocaldarius* was grown as unsaturated biofilm on Brock medium plates. Since the standard spread-plate method for cultivation of unsaturated biofilms, as described by Bressler *et al.* (2009) for the cultivation of *P. aeruginosa* biofilms, yielded limited colony growth and caused liquefaction of the gellan gum this method was optimized by growing the cells in single lines. This allowed for the formation of a relatively large biomass of approximately 0.6 g biofilm wet weight per conventional Petri dish (100 mm in diameter), suitable for subsequent EPS isolation and analysis. However, this method was not suited for microscopic analysis of the biofilms, since colonies had to be transferred to a solid support, which would destroy the architecture of the biofilm. To overcome this problem, an alternative method was established, growing unsaturated biofilms on floating polycarbonate filters. Though the extent of biofilm growth was limited and did not yield sufficient amounts for EPS isolation, this method allowed for simple transfer of the unsaturated biofilm onto a solid support, and microscopic analysis. Brown coloration of colonies grown on floating filters in the presence of dextrin occurred, which is assumed to be caused by the Maillard reaction.

In parallel to the presented study, the group of S.-V. Albers at the Max-Plank-Institute (Marburg, Germany) developed a method to produce submersed *S. acidocaldarius* biofilms in µ-dishes as well as large Petri-dishes (Koerdt *et al.* 2010; Koerdt *et al.* 2012; Orell *et al.* 2013b). In collaboration with this group, the EPS content of genetically modified *S. solfataricus* strains (Koerdt *et al.* 2012) as well as gene deletion mutants of *S. acidocaldarius* (Orell *et al.* 2013b) was analyzed, once a method for the isolation of EPS was established in the presented study (see section 3.4, 3.5 and 4.5, 4.6). The results of the study showed, that biofilms of *Sulfolobus* formed at the bottom of large Petri dishes led to biofilm mass sufficient for isolation of low amounts of EPS. However, biofilms from a large number of Petri dishes had to be pooled to obtain sufficient amounts for EPS isolation and analysis, and yielded concentrations slightly above the limit of detection of the quantitative analysis of polysaccharides and proteins in several samples. More detailed analyses via for

instance 2 DE or TLC, however, require a higher amount of biomass than can be obtained by this cultivation method.

Table 4.1: Methods for the investigation of attachment and biofilm formation of *Sulfolobus* species. Applications, advantages and disadvantages are outlined for each cultivation method.

Methods	Applications/Advantages	Disadvantages for application in this study	References
Glass slides (1 – 3 d, shaking)	• Attachment of cells and formation of micro-colonies • CLSM imaging of attached cells after staining with different lectins and DNA stains	• No formation of a confluent biofilm	Henche *et al.* 2012a Henche *et al.* 2012b Koerdt *et al.* 2010 Zolghadr *et al.* 2010 This study
Carbon-coated gold grids (2 d, shaking)	• Attachment of cells and formation of micro-colonies • Scanning electron microscopy for visualization of cell appendages	• No formation of a confluent biofilm	Zolghadr *et al.* 2010
96-well polystyrene plate (2 d, static)	• Fast screening of different environmental factors on extent of biofilm formation	• Evaporation of medium • Low biofilm formation of certain strains (e.g. *S. acidocaldarius* MW001 and certain deriving from this strain)	Koerdt *et al.* 2010 This study (data not shown)
µ-dishes (35 mm) or polystyrene Petri dishes (100 – 150 mm) (3 d, static)	• CLSM imaging of 3D biofilm after staining with different lectins and DNA stains	• Evaporation of medium • Exchanges of medium required every 24 h resulting in stress due to shear forces, temperature and pH shifts • Settling of planktonic cells to the bottom • Several large Petri dishes required for sufficient biofilm material for EPS isolation of one sample (only quantitative determinations)	Henche *et al.* 2012a Koerdt *et al.* 2010 Koerdt *et al.* 2012
Brock medium plates (100 – 150 mm) (4 d, static)	• Sufficient biofilm mass for quantitative and qualitative EPS analysis	• Limited microscopic analysis	This study
Floating polycarbonate membrane filters (6 – 12 d, static)	• CLSM imaging of 3D biofilm after staining with different lectins and DNA stains	• Extent of biofilm formation on membranes not reproducible • Change in filter quality between batches	This study

For the high EPS amounts required for this study, the single line streaking method on Brock medium plates was considered the most suitable method. Similar methods are constantly applied for bacterial biofilms since they represent a system mimicking the natural environment of certain organisms, which inhabit water limited environments e.g. soil or plants (Holden 2001). The cultivation of unsaturated biofilms represents a system different from the cultivation in an aqueous phase (submersed biofilms). Due to the lower water content of the EPS matrix diffusion rates are expected to be lower and thus processes in unsaturated biofilms should differ from saturated biofilms (Holden *et al.* 1997). The determination of the biodegradation rates of volatile hydrocarbons for instance suggests that the degradation rates vary as a function of EPS water potential (Holden *et al.* 1997).

4.2 EPS isolation from the thermoacidophilic *Sulfolobus*

One of the major objectives of this study was the quantitative and qualitative analysis of EPS. In general, methods to study EPS can be divided into destructive and non-destructive methods. A commonly applied non-destructive method also applied in this study is the staining and visualization of certain EPS components like proteins or sugar residues using CLSM. Several destructive methods to analyze EPS have been developed in recent years including the isolation of EPS and subsequent chemical and biochemical characterization of the components. In the present study the focus was set on the isolation of EPS from the cells and a quantitative and qualitative analysis of the proteins, polysaccharides and DNA within the EPS. The EPS isolation is a critical step for all further analyses of EPS. Too mild conditions isolate only a fraction of EPS, while too harsh conditions may lead to cell lysis contaminating the EPS with intracellular and membrane material. Several studies found in literature deal with the development and comparison of various EPS isolation techniques to determine the most efficient technique for a respective sample (e.g. Comte *et al.* 2006; Park and Novak 2007; Tapia *et al.* 2009; Aguilera *et al.* 2008). The number of studies and the diversity of methods indicate that there is no universal method capable to isolate EPS completely from any given sample. Studies of the composition of EPS of activated sludge even demonstrated that several methods have to be applied in order to extract different fractions of the EPS, e.g. iron linked EPS, magnesium and calcium linked EPS or aluminum linked EPS of activated sludge (Park and Novak 2007). The aim of this study was to establish a method for the isolation of EPS from *S. acidocaldarius* biofilms. The method of choice should be capable of

isolating high amounts of EPS components, without causing damage to biofilm cells and without causing significant changes to the biochemical properties of the EPS. Five EPS isolation methods previously applied for EPS isolation from bacterial biofilms or sludge were compared for their suitability to isolate EPS from *S. acidocaldarius* biofilms with respect to EPS isolation yield, which varied significantly depending on the applied method. Furthermore, the impact of the procedure on cell viability as well as impact on subsequent analysis was analyzed. The methods tested were shaking on an orbital shaker, shaking with addition of CER, as well as treatment with EDTA, NaOH or crown ether. The biofilm mass cultivated on solidified Brock medium plates (4 d, 78°C) was suspended in phosphate buffer (biofilm suspension) and subjected to the EPS isolation methods. After centrifugation and dialysis of the supernatant (MWCO 3500 kDa) the final EPS solution was obtained.

Shaking on an orbital shaker was applied as reference method. This simple and gentle physical method should isolate only the loosely bound and water soluble EPS fraction without causing cell lysis or influencing subsequent EPS analysis. Isolation of EPS by shaking of the biofilm suspension for 20 min led to the lowest amounts of isolated EPS carbohydrates, proteins and eDNA among the tested methods. The visualization of the extracellular proteome via 2 DE revealed the lowest amount of protein spots (approximately 300 spots) for this method compared to the other tested EPS isolation methods. No reduction of culturability was determined. The method of EPS isolation via shaking was improved by adding CER. The mechanism of EPS isolation using CER relies on the removal of multivalent cations by the resin. Since the EPS is known to be stabilized by multivalent cations via for instance cross-linking of polysaccharides and/or proteins, removal of these cations should result in a destabilization of the matrix and hence, increase its solubility (Jahn and Nielsen 1995; Frølund *et al.* 1996; Park and Novak 2007; D'Abzac *et al.* 2010).

In this study multivalent cations added to the growth medium were detected in the biofilm, indicating an accumulation of cations within the EPS matrix. Removal of calcium and magnesium from the EPS matrix was confirmed in this study, showing a reduction of at least 60% and 80% after CER treatment, respectively. These reductions are similar to results stated in a study by Park and Novak (2007). In three activated sludge samples they determined cation removal efficiencies of CER of 80% for calcium and 60% for magnesium. In accordance to their results, no reduction in aluminum or copper cations was observed in EPS

isolated from *S. acidocaldarius* biofilms using CER. Determination of total cell count before and after EPS isolation using CER in the present study revealed a recovery of cells from the CER solution of 98 % ± 8 %. These results exclude an attachment of cells to the CER. The addition of CER led to an enhanced yield of extracted carbohydrates, proteins and DNA by factors of 1.47, 1.53 and 2, respectively, compared to shaking without CER.

The application of CER to isolate EPS from *S. acidocaldarius* did not lead to a loss of culturability. Hence, contamination of the EPS with intracellular and membrane material due to the isolation procedure is considered to be insignificant. Previous studies testing the suitability of CER for isolation of EPS from bacterial biofilms and sludge also showed that CER represents a mild method not causing significant cell lysis. Frølund *et al.* (1996) for instance tested CER for a pure culture of *Pseudomonas putida* and activated sludge and determined cell lysis by release of the intracellular enzyme G6PDH. They showed that enhanced activity and thus lysis was only detected for high concentrations of CER and high stirring magnitudes for isolation times of over 1 h. Jahn and Nielsen (1995) observed a reduction in cell viability of *P. putida* and two sewer biofilms determined via colony counts after 30 min of CER treatment. After prolonged treatment of 2 h 20% of the bacteria remained culturable. Nevertheless, nearly no activity of G6PDH was detectable after 2 h. Chen *et al.* (2013) tested CER, sonication, heating and steaming for EPS isolation from sludge and biofilms. CER was proven to be most efficient without causing cell lysis.

Similar to CER, isolation of EPS via treatment with EDTA or crown ether relies on the removal of cations, thus, destabilizing the EPS matrix. Isolation using NaOH treatment is based on a rapid pH shift resulting in the destabilization of the EPS network. Compared to shaking in presence or absence of CER, the chemicals used for isolation in this study were not completely removed from the EPS solution even after dialysis as was previously stated by Comte *et al.* (2006b). This led to interferences with certain subsequent biochemical analysis. Crown ether interfered with the sulfuric acid/phenol carbohydrate assay leading to concentrations exceeding the total concentrations within the biofilm. EDTA chelates copper ions, which are required for the protein concentration determination using the modified Lowry assay (Peterson 1979) and thus, falsified protein concentrations. Moreover, it interfered with the isoelectric focusing for visualization of the extracellular proteome with 2 D gel electrophoresis. In contrast to isolation of EPS from *Acinetobacter* (Wu and Xi 2009),

no reduction in culturability after EPS isolation using EDTA was observed in the present study.

The increased pH value of NaOH (pH 13.3) is likely to cause damage to biofilm cells or extracellular polymers. Thus, subsequent analyses like determinations of enzyme activities are precluded. Induction of cell lysis due to NaOH or crown ether treatment was demonstrated by the decrease in culturability. For NaOH a reduction of turbidity of the biofilm sample during EPS isolation was observed, further indicating cell lysis. Visualization of the extracellular proteome of NaOH-isolated EPS proteins revealed a smear of proteins in the low molecular mass region suggesting degradation of high molecular mass proteins.

A major challenge in the evaluation of an isolation method is the detection of cell lysis. In the presented study the culturability of biofilm cells after EPS isolation was used to determine cell damage induced by the tested EPS isolation methods and conditions. Comparison of colony counts of cells shaken, shaken with CER or merely suspended in either Brock medium (pH 3.5) or phosphate buffer (pH 7.0) revealed no statistically significant differences demonstrating the high tolerance of *S. acidocaldarius* cells towards pH stress. No influence on culturability was detected for EDTA treatment while crown ether treatment significantly reduced the culturability. NaOH treatment led to a complete loss of culturability.

In several studies methods to detect cell lysis included for example the ratio between proteins and carbohydrates as well as the determination of DNA (Brown and Lester 1980; Comte *et al.* 2006a; Karapanagiotis *et al.* 2007; D'Abzac *et al.* 2010). Nowadays, it is well established that proteins and eDNA represent crucial components of the EPS matrix of microorganisms and sometimes even are required for initial cell attachment and biofilm formation (Whitchurch *et al.* 2002; Flemming and Wingender 2010). The ratio of proteins to carbohydrates can vary significantly between EPS of different microorganisms or different growth conditions. Other methods to detect cell lysis are for instance the activity screening of strictly intracellular enzymes, like G6PDH (Frølund *et al.* 1996) or application of viability stains (e.g. Live/Dead kit) (Wu and Xi 2009). The Live/Dead kit is composed of two fluorescent nucleic acid dyes (Stocks 2004). The first stain, the green fluorescent SYTO9, stains all cells; however, it is displaced by the red fluorescent dye propidium iodide in cells with an impaired membrane. Thus, cells with an impaired membrane appear red and cells with an intact membrane appear green. However, this viability stain cannot be applied to

any sample due to several drawbacks: For once the concentration of the propidium iodide must be adjusted to each strain and condition (Stocks 2004) and thus, cannot be applied to mixed species biofilms. Additionally, membrane impaired cells stained with propidium iodide can recover and thus, cannot necessarily be regarded as dead or lysed. So far, viability staining using the Live/Dead kit could not be established for *S. acidocaldarius* due to the complete staining of planktonic cells with propidium iodide irrespectively of growth phase or propidium iodide concentration (results not shown).

The results of this study suggest that the CER method was best suited for the isolation of EPS from *S. acidocaldarius* biofilms resulting in high yields of EPS without causing damage to biofilm cells or the EPS components and without interference of any analytical method. The here described CER method was already successfully applied in a cooperation project for EPS isolation from *S. solfataricus* biofilms as well as from different *S. acidocaldarius* gene deletion mutant biofilms (Koerdt *et al.* 2012; Orell *et al.* 2013b).

4.3 Functions of extracellular polymeric substances

Exopolysaccharides have been acknowledged as actively secreted parts of the biofilm matrix for decades. In recent years also proteins as well as DNA have been found to be substantial secreted components of the extracellular matrix (Platt *et al.* 1985; Jahn and Nielsen 1995; Frølund *et al.* 1996; Michalowski 2012). In the present study, all three components have been quantified in the EPS of *S. acidocaldarius* biofilms. Apart from quantitative determinations the functions of each component were addressed in this study.

4.3.1 Proteins

Proteins can enter the EPS matrix via secretion, membrane vesicles or cell lysis. These extracellular proteins are frequently shown to have two main functions either as structural components of the EPS matrix or as enzymes. The EPS of *S. acidocaldarius* biofilms contained a considerable concentration of proteins (4.2 ± 1.1 fg cell^{-1}), which was only slightly lower compared to polysaccharides (4.7 ± 0.1 fg cell^{-1}). The diversity of EPS proteins was analyzed using 2 DE (Figure 3.11). The analysis of the EPS proteins isolated with CER revealed approximately 1000 protein spots from the EPS of *S. acidocaldarius* unsaturated biofilms. The presence of the large diversity of extracellular proteins suggests a potential function within the EPS of *S. acidocaldarius* biofilms.

Analysis of EPS proteins via MALDI-TOF/MS directly after EPS isolation revealed the occurrence of approximately 200 proteins of which 85 proteins could be identified. The identified proteins belonged to 15 functional arCOG categories. The majority of these proteins was involved in energy production and conversion, amino acid transport and metabolism, lipid transport and metabolism and translation, as well as ribosomal structure and biogenesis. Prediction of the subcellular localization showed that 73 proteins were of intracellular origin. The origins of 11 proteins were not predictable by the PSORTb subcellular prediction program and only one protein was predicted to originate from the cytoplasmic membrane. No protein with extracellular localization was identified. The results show, that the vast majority of identified proteins were involved in intracellular processes and/or adapted to the intracellular milieu, in particular the pH of 6.5, within *S. acidocaldarius* cells. Therefore, it is unlikely that the assigned functions are indeed performed by the respective proteins within the EPS matrix.

Since cell lysis due to the isolation of EPS with CER was excluded by the unchanged culturability of *S. acidocaldarius* biofilm cells before and after CER treatment, these results indicate that, like in bacterial biofilms, cell lysis represents a common process during maturation of a biofilm (Rice *et al.* 2007). The role of these proteins in archaeal biofilms remains unknown so far. They might, however, represent a nutrient, in particular amino acid, reservoir for other inhabitants of the biofilm.

Comparison of the identification results obtained with MALDI-TOF/MS with analysis by 2 DE showed that only 9% of proteins detected by 2 DE could be identified by MALDI-TOF/MS. It is likely that the concentration of proteins originating from cell lysis is much higher than that of actively secreted proteins and hence, leads to a masking of the secreted protein fraction. This assumption is further underlined by the results of the zymogram gels. Zymography performed in this study showed that extracellular hydrolytic enzymes, in particular esterases and proteases, are indeed present and active within the EPS, however, in relatively low concentrations, which could not be detected by MALDI-TOF/MS and were not even visible on 2 D gels stained with silver.

Recently, the secretion of proteins in *Sulfolobus* spp. was analyzed in two different studies using *in vivo* and *in silico* analysis. The presence of signal sequences was employed to reveal the number and identity of secreted proteins, since proteins, which are secreted into the growth medium or the biofilm matrix, are expected to carry signal sequences marking them for transport across the membrane (see section 1.3.3). Signal sequences required for secretion via the Tat and Sec pathways in bacteria and eukaryotes have also been detected in archaeal proteins. Ellen *et al.* (2010) used mass spectrometry to identify proteins carrying signal peptides or N-terminal transmembrane segments in glycosylated proteins obtained from the supernatant as well as the purified glycosylated membrane fractions of planktonic cultures of *S. tokodaii, S. solfataricus* and *S. acidocaldarius* at different growth stages. They identified 64 proteins of which 29 were shown to carry a signal peptide using an archaea-specific prediction program (PRED-SIGNAL). Three extracellular proteases (thermopsins) in *S. tokodaii* were identified but none in *S. solfataricus* or *S. acidocaldarius* even though the expression of the glycosylated extracellular protease thermopsin was previously described in *S. solfataricus* as well as in *S. acidocaldarius* (Lin and Tang 1990; Lin *et al.* 1992; Cannio *et al.* 2010). Saleh *et al.* (2010) used *in silico* analyses to analyze 24 archaeal genomes using the

signal peptide prediction program ExPort and predicted 258 exported proteins in *S. acidocaldarius*, 12% of the total identified proteins.

The different results obtained with these *in vivo* and *in silico* analyses as well as the results obtained in the current study for the extracellular proteins visualized via 2 DE might indicate the lack of sensitivity of *in vivo* experiments to detect the low amounts of extracellular proteins. The ExPort prediction program is based on signal peptides known for Gram-positive bacteria (Saleh *et al.* 2001). Even though these signal peptides are assumed to be closely related to archaeal signal peptides experimental proof is required. Furthermore, some secreted proteins produced by Archaea were demonstrated to lack known identifiable signal sequences (Cannio *et al.* 2000; Ellen *et al.* 2010), and thus, would not be detected by this approach. Moreover, an underestimation of secreted proteins in the *in vivo* study of Ellen *et al.* (2010) might be attributed to the sample preparation. Ellen *et al.* (2010) only used the supernatant and the purified glycosylated membrane fraction of planktonic cultures for analysis obtained by centrifugation. Comparison of EPS proteins extracted with either CER or only shaking in the current study demonstrated that twice as many proteins were obtained with CER. Hence, a large diversity of proteins is closely attached to the cell surface, which was not addressed by the study of Ellen *et al.* (2010). Additionally, it is possible that certain proteins are only released in the biofilm growth state which, due to the formation of the EPS matrix, retains secreted molecules (Flemming and Wingender 2001). Cell lysis may also be a factor influencing the diversity of proteins on 2 D gels in the present study as well as proteolytic degradation of proteins. The modified Lowry assay, which was applied for protein quantification as well as 2 DE detects the sum of all proteins within a given sample. Within the EPS matrix this includes secreted extracellular proteins, glycoproteins and proteins released via cell lysis or membrane vesicles as well as cell appendages released into the matrix. Cell lysis is a common element during the life cycle of a biofilm (Rice *et al.* 2007), hence, intracellular proteins as well as membrane proteins are expected to be found within the biofilm matrix and may be responsible for the high diversity of proteins in this study.

4.3.1.1 Determination of extracellular enzyme activity

In general, proteins found in the EPS of microbial biofilms have two main functions, a) as hydrolytic enzymes, and b) as structural components, providing mechanical stability for the biofilm. Hydrolytic and other enzymes within the EPS matrix can be considered as an external digestive system of biofilm cells, allowing for the degradation of complex macromolecules into smaller molecules that can be readily taken up by the cell (Wingender and Jaeger 2003). The hydrolytic ability of proteins within the EPS matrix was further investigated in the present study. A screening of *S. acidocaldarius* EPS using substrates for different enzyme classes revealed activity of esterases, lipases, phosphatases, N-acetyl-ß-D-glucosaminidases, β-D-glucosidases and α-D-glucosidases within the EPS. These groups of enzymes have also been detected in diverse naturally occurring biofilms, as well as in biofilms from technical systems (Wingender and Jaeger 2003). Activity of these enzymes within the EPS of *S. acidocaldarius* suggests the potential of the EPS to modify and even to degrade diverse biopolymers, such as lipids, polysaccharides, or nucleic acids and indicates their importance for *S. acidocaldarius* biofilms. Two enzyme classes, proteases and esterases, were further analyzed using in gel activity staining.

Protease activity

S. acidocaldarius as well as *S. solfataricus* can utilize peptide sources such as yeast extract or tryptone for growth. These peptides are organic substances, which require degradation into smaller molecules before they can be taken up and utilized by the cell. Hence, activity of extracellular proteases was expected to be detected within the EPS. Screening for protease activities in the EPS of *S. acidocaldarius* using 2 DE zymogram gels with casein as substrate revealed 1 activity spot when gels were developed at pH 3.8 and 3 activity spots at a developing pH of 7.8. The pH values for development of the gels of pH 3.8 and 7.8 were selected to mimic the acidic environmental conditions encountered by *S. acidocaldarius* in its natural environment, and to simulate the approximate intracellular pH value, respectively. The activity spot obtained at pH 3.8 at a molecular mass of approximately 47 kDa is expected to be of extracellular origin due to its lowered activity at pH 7.8. Its high residual activity after heating for 1 h at 100°C or autoclaving (20 min, 121°C), as well as its residual activity at developing temperatures of 37°C (not shown) suggest that the spot corresponds to a protease named thermopsin, previously described by Lin and Tang (1990). In their study

thermopsin (Saci_1714) activity was detected in the growth medium as well as in the cells of *S. acidocaldarius*. Its activity was shown to be exceptional being active at temperatures of 5°C to 100°C and pH values of 0.5 to 5 with residual activity up to pH 11 (Lin and Tang 1990; Lin *et al.* 1992). In the genome of *S. acidocaldarius* DSM 639 seven genes show similarities to thermopsin (EC 3.4.23.42). Thermopsin belongs to the peptidase family A5 preferring bulky hydrophobic side-chains on either side of the cleavable bond. The calculated molecular mass of thermopsin is 36 kDa. The protease in the current study showed a molecular mass of approximately 47 kDa. The same discrepancy was stated by Lin and Tang (1990), who assumed that thermopsin is glycosylated at up to 11 sites resulting in the mass difference. Recently, several archaeal proteins were shown to be glycosylated, including extracellular proteins confirming this assumption (for an overview see Meyer and Albers 2013).

In frame disruption of gene Saci_1714, coding for thermopsin, was attempted to confirm that the detected activity spot indeed corresponds to thermopsin (data not shown). However, after various independent efforts no disruption mutant was obtained, while a control disruption mutant (Δ2140), produced in parallel, was obtained in the second experiment. The difficulty in disrupting the thermopsin gene might indicate the substantial requirement of this protease for the degradation of macromolecules for nutrient acquisition in *S. acidocaldarius*. Even though growth solely on certain sugars without addition of proteinaceous substances has been reported (Joshua *et al.* 2011), *S. acidocaldarius* would require prolonged adaption over weeks to these conditions with a slow reduction of the organic compound.

In addition to the activity spot in the EPS fraction at both tested pH values, two further spots were obtained at pH 7.8 and a molecular mass of approximately 27 kDa. Since these spots only appear at this pH it is assumed that their origin is from the cytoplasm of lysed cells. Analysis of the crude extract of cells after EPS isolation indeed revealed the same spots after developing at pH 7.8. Interestingly, the spot assumed to be caused by thermopsin, was also detected in the crude extract developed at pH 3.8. This further hints to its identity being thermopsin since Lin and Tang (1990) also obtained activity within the cell fraction.

Esterase activity

Esterases represent a large family of enzymes splitting ester bonds and possess a considerable potential for industrial applications (Panda and Gowrishankar 2005). So far three esterases from *S. acidocaldarius* have been characterized (Sobek and Görisch 1988; Arpigny *et al.* 1998; Porzio *et al.* 2007). Two of the esterases (Saci_1116 and Saci_2140) were allocated as intracellular enzymes. Only the esterase Saci_2213 was determined to possess a signal peptide found in extracellular amylolytic enzymes of *Pyrococcus furiosus* and thus, might be secreted into the growth medium. However, secretion of the enzyme was not detected when expressed in *E. coli* (Arpigny *et al.* 1998). In the current study a pI of 4.81 and a molecular mass of 34 kDa for this esterase were determined using ExPASy (SIB Swiss Institute of Bioinformatics, Artimo *et al. 2012)*. Determination of esterase activity on 2 D gels using MUF-butyrate revealed four activity spots in the low pI region and molecular weights between 30 kDa and 35 kDa at a developing pH of 3.8. Only two spots were also obtained in similar intensity at developing pH of 8.0 hinting to an extracellular origin of the other two esterases. One of these esterases might correlate to the afore-mentioned esterase described by Arpigny *et al.* (1998). However, due to the similar molecular masses in the range of 33 kDa to 35.3 kDa of all three detected esterases the identity of the respective esterases on the activity stain gel needs to be further evaluated by e.g. mass spectrometry. Moreover, it needs to be considered that MUF-butyrate can also be degraded by other hydrolytic enzymes (Jones and Lock 1989). Nevertheless, the activity staining using MUF-butyrate gives a first hint towards the esterases found in the EPS (Emtiazi *et al.* 2004; Niemi and Vepsäläinen 2005).

Glycosylation

The majority of the secreted proteins of *S. acidocaldarius* detected by 2 DE were stainable with a glycosylation stain hinting to a high abundance of glycosylated proteins within the EPS matrix (Figure 3.20). Glycosylation of cell-surface and extracellular proteins is the most common posttranslational modification in Archaea and plays a crucial role in physiological processes like attachment to substrata, correct protein folding as well as protection against proteolytic activity and certain environmental stresses (Herrmann *et al.* 1996; Kuo *et al.* 1996; Lindenthal and Elsinghorst 1999; Goon *et al.* 2003; Chaban *et al.* 2006; Meyer and Albers 2013). In contrast to Eukaryotes the glycan trees of glycosylated proteins in Bacteria

and Archaea are mainly linear with a diverse composition (Dell *et al.* 2010). In Archaea, extracellular surface structures, e.g. archaella, pili or S-layer proteins, have been shown to be glycosylated (Ng *et al.* 2006; Peyfoon *et al.* 2010; Meyer and Albers 2013). The number of glycosylated sites is higher in hyperthermophilic than in mesophilic organisms and assumed to be a response to the harsh environmental conditions. Glycosylation of the S-layer protein of *H. salinarum* was shown to be slightly modified in response to different salt concentrations in the growth medium (Guan *et al.* 2012). The structures of several glycan trees in Archaea have been identified and revealed variations in the composition between the different species. In *S. acidocaldarius* the S-layer protein was shown to be glycosylated at 9 sites, showing a highly branched hexasaccharide chain containing mannose, glucose, N-acetylglucosamine and a sulfoquinovose (Peyfoon *et al.* 2010). In the current study, staining of unsaturated biofilms formed on polycarbonate filters using the fluorescently labeled lectin WGA, specific for binding to N-acetylglucosamine residues, gave a strong signal tightly surrounding the cells (Figure 3.21). Therefore, it is assumed that it primarily binds to the N-acetylglucosamine residue found within the glycan tree of the S-layer. In contrast to this, other lectins like ConA, binding to glucose and mannose residues, led to a signal in the space between the cells (Koerdt *et al.* 2010). This indicates the difficulty to distinguish between sugar residues of a glycoprotein or a free polysaccharide using only lectin staining.

4.3.2 Polysaccharides

Apart from the glycan trees found in the EPS matrix attached to proteins, which are also determined using the sulfuric acid/phenol method in this study, free polysaccharides are found. Polysaccharides represent one of the main components in most EPS matrices. Polysaccharides were demonstrated to have several functions within the biofilm. They form the three-dimensional structure of biofilms and provide mechanical stability in particular in the presence of crosslinking multivalent cations (Wingender *et al.* 1999, Mayer *et al.* 1999). Moreover, they are involved in the retention of e.g. water, enzymes and DNA and even were shown to stabilize enzymes like lipases (Flemming and Wingender 2010; Tielen *et al.* 2013).

In the EPS of *S. acidocaldarius* biofilms polysaccharides represent a major component amounting to 4.7 ± 0.1 fg cell^{-1}. In the presented study the isolated exopolysaccharides were hydrolyzed and the monosaccharides analyzed using TLC. The main component was identified as D-glucose but also another spot, which did not correlate to any of the applied

standards, was detected in much lower intensity. D-mannose, D-galactose, D-xylose and L-rhamnose were not detected via TLC. Indication of the presence of glucose in *Sulfolobus* spp. biofilms was previously reported by Koerdt *et al.* (2010) using fluorescently labeled ConA. In their study the lectin ConA was considered to bind to glucose and mannose residues. However, staining with lectins only gives first hints to the composition of a polysaccharide since the binding of the lectin to the polysaccharide is not always specific. Unspecific binding was for instance shown for ConA, which bound to bacterial alginate which consists of (1 → 4)-linked β-D-mannuronate and α-L-guluronate residues but does not contain glucose and mannose residues (Strathmann *et al.* 2002). Only in a limited number of Archaea exopolysaccharides have been described and analyzed with respect to their composition and structure. In two strains of *S. solfataricus*, an exopolysaccharide was found to be secreted into the growth medium of planktonic cultures (Nicolaus *et al.* 1993). The purified polysaccharides were determined to be composed of glucose/mannose/ glucosamine/galactose with a molar ratio of 1.2 : 1.0 : 0.18 : 0.13 in strain MT4 and a ratio of 1.2 : 1.0 : 0.77 : 0.73 in strain MT3. In different species of *Haloferax* exopolysaccharides were also isolated from planktonic cultures and analyzed according to their composition and structure (Antón *et al.* 1988; Paramonov *et al.* 1998; Parolis *et al.* 1999). The identified sugars in *Haloferax* included galactose, glucose, glucuronic acid, glucosamine, N-acetylglucosamine, 2-acetamido-2-deoxyglucuronic acid, 2,3-diacetamido-2,3-dideoxy-D-glucopyranosiduronic acid, mannose and rhamnose. However, the functions of polysaccharide within the biofilm matrix stated above have up to date not been investigated and confirmed in archaeal biofilms.

4.3.3 eDNA

In several bacterial biofilms eDNA represents an important component of the EPS matrix (Whitchurch *et al.* 2002). Quantification of eDNA within the EPS isolated from *S. acidocaldarius* biofilms in this study confirmed the presence of eDNA also in this type of biofilm and showed a concentration of 0.91 ± 0.02 fg cell^{-1}.

eDNA can enter the environment by either active secretion or by active and passive lysis of cells (Hara and Ueda 1981; Lewis 2000; Webb *et al.* 2003). Several bacterial species were shown to actively secrete DNA into the environment, which can then be taken up via natural transformation by competent recipient cells (e.g. Lorenz *et al.* 1991; Whitchurch *et al.* 2002; Webb *et al.* 2003).

eDNA is known to have several functions within biofilm formation and maintenance including initial attachment and structural stability (Whitchurch *et al.* 2002; Molin and Tolker-Nielsen 2003). Nevertheless, a structural role in unsaturated biofilms of *Pseudomonas* spp. was opposed by Steinberger and Holden (2005) due to the absence of hydrodynamic shear forces under this cultivation conditions. In *Sulfolobus* spp. submersed biofilms cultivated in µ-dishes, the role of eDNA in attachment and maintenance was tested by the addition of a DNase to established biofilms of different growth stages (Koerdt *et al.* 2010). The authors reported that even though the applied DNase was functional under the tested conditions (not specified) no influence on biofilm structure was observed. However, a reduced DDAO signal intensity was stated after treatment of the biofilm with DNase. DDAO was used to stain eDNA and thus, a reduced signal intensity might indicate a degradation of eDNA.

Additionally to structural functions, eDNA can be used as nutrient source during starvation (Finkel and Kolter 2001). This function has not been addressed in Archaea so far. Moreover, eDNA was shown previously to enhance gene transfer via natural transformation, which can enhance the fitness of a biofilm (Molin and Tolker-Nielsen 2003). Natural transformation in Archaea was detected in *Pyrococcus furiosus* (Lipscomb *et al.* 2011), *Methanobacterium thermoautotrophicum* (Worrell *et al.* 1988), *Methanococcus voltae* (Bertani and Baresi 1987; Patel *et al.* 1994)*, Thermococcus kodakarensis* (Sato *et al.* 2005) as well as in the haloarchaea *Haloferax* and *Halorubrum* (Chen *et al.* 2012). For *Haloferax* and *Halorubrum* natural transformation was induced in response to low salinity as may occur in natural environments

due to heavy rainfall. Further details influencing natural transformation in Archaea, however, remain mostly unexplored.

In addition to the conjugation process, which was confirmed for *Sulfolobus,* Schleper *et al.* (1995) tested the potential of *Sulfolobus* for natural transformation. However, free DNA incubated for 1 h in growth medium at pH 3 and 80°C, resembling the growth conditions of *Sulfolobus* was completely degraded after 1 h. Plasmid DNA added to a liquid culture was not spread, either confirming the instant degradation of the DNA or suggesting a lack of natural competency in the culture. Nevertheless, so far the pH value within the EPS matrix of *S. acidocaldarius* biofilms was not determined. Thus, it is possible that DNA released into the *S. acidocaldarius* EPS matrix is more stable due to a pH above the environmental pH or the DNA can be stabilized by other EPS components.

In the present study unsaturated biofilms of *S. acidocaldarius* grown on Brock medium plates occasionally exhibited the formation of macroscopically visible, approximately 0.5 cm long filaments comprised of densely packed cells (Figure 3.23). Attempts to reproduce the formation of these filaments could not be achieved, despite application of diverse stress factors separately or in combination. The factors influencing the formation seemed to involve the water content of the plates and the biofilm, the pH and the nutrient availability.

Böckelmann *et al.* (2006) observed the formation of a filamentous network, comprised of thin strings, to which cells of a river snow bacterial isolate attached. Using different staining techniques as well as DNase treatment, the filaments were shown to be made of eDNA. The study demonstrated that the DNA isolated from filaments of a bacterial isolate resembled the chromosomal DNA, however, also revealed several differences in band patterns using endonuclease cleavage and random amplified polymorphic analysis of isolated DNA, as well as differences in size. This led to the assumption that the eDNA is actively constructed and released into the environment. In *Sulfolobus* cell aggregation due to stress by UV radiation was reported by Fröls *et al.* (2008). However, in their study the aggregates were not held together by eDNA but by pili (Fröls *et al.* 2008).

Results of the present study showed that proteins, polysaccharides as well as eDNA are abundant in the EPS of *S. acidocaldarius*. Hence, it was speculated that either of these polymers or their combination could be responsible for the occasional filament formation. In an attempt to identify the nature of the agent responsible for the cohesion of

S. acidocaldarius cells in filaments, the samples were incubated with different classes of hydrolytic enzymes, i.e. proteases, DNase, RNase, aiming at the degradation of the respective polymers, and thus, destabilization of the filament and release of cells into the bulk solution. Microscopic analysis revealed that the addition of DNase resulted in instantaneous and complete dispersion of cells from the filaments, while none of the other enzyme classes had an effect on the structural stability of the filaments. Hence, the filaments in the present study were kept together by eDNA, which contributed to the structure of the filament, keeping the cells closely attached to each other in this kind of biofilm. This emphasized the structural importance of eDNA for the formation of such filaments.

eDNA from *S. acidocaldarius* filaments was further analyzed by agarose gel electrophoresis. It was shown that undigested eDNA isolated from the filaments of *S. acidocaldarius* in the presented study revealed the same size pattern on agarose gels as chromosomal DNA of *S. acidocaldarius*. Similar results were obtained for eDNA isolated from unsaturated *P. aeruginosa* and *P. putida* biofilms (Steinberger and Holden 2005). Terminal restriction fragment length polymorphism revealed that the isolated DNA corresponded to the cellular DNA. In contrast to this, Böckelmann *et al.* (2006) showed that the eDNA isolated from a filamentous network of cells of a river snow bacterial isolate was larger than chromosomal DNA and showed slightly altered band patterns after random restriction analysis.

For several bacterial species, e.g. *Salmonella typhimurium* and *E. coli*, a change in cell morphology depending on nutrient availability has been well documented (Schaechter and Kjeldgaard 1958; Van de Merwe *et al.* 1997). Also the formation of filaments is known for several biofilms under nutrient limited conditions (Wright *et al.* 1988). A *Pseudomonas* isolate was shown to elongate when grown under nutrient deprived conditions, which was not observed for growth under nutrient rich conditions. Filamentous growth has been observed for *P. putida* and *P. fluorescens* when grown attached to a solid support in a bioreactor with low molecular weight aromatics, while cells in liquid cultures are short rods (Shim and Yang 1999; Steinberger *et al.* 2002). Apart from the nutrient limitations, also water deprivation, found in unsaturated biofilms, was shown to influence biofilm formation and cell morphology (Chang and Halverson 2003). A *P. putida* strain was demonstrated to show altered cell morphologies in unsaturated biofilms with reduced water availability. The cells were smaller, surrounded by a thicker layer of EPS at the air interface and formed

filamentous structures. Hence, the occurrence of filaments in the *S. acidocaldarius* strain might indeed be related to the availability of nutrients and the degree of desiccation due to the elevated temperatures.

Taken together, two phenotypical specifications were observed for *S. acidocaldarius* biofilms; the formation of homogenous biofilms and the formation of biofilms presenting filamentous structures, which are held together by eDNA. The occurrence of DNA rich *S. acidocaldarius* filaments was hypothesized to be caused by slightly altered cultivation conditions e.g. low water concentration, pH value and nutrient limitation in the dense and thick biofilm. This assumption, however, could not be verified in this study.

4.4 Involvement of glycosyltransferases in exopolysaccharide synthesis

Glycosyltransferases (GTs) are involved in the synthesis of exopolysaccharides found in the extracellular matrix of biofilms. In *S. acidocaldarius* a cluster of 24 genes was detected, comprising 12 genes with homologies to GTs (Orell *et al.* 2013b). Previously the cluster was assumed to be involved in the N-glycosylation of the *S. acidocaldarius* S-layer protein (Dr. S.-V. Albers, personal communication). However, this theory was not confirmed. A mutational approach showed that none of the GTs of this cluster was involved in the N-glycosylation of the S-layer protein, but instead the GT Agl16 (Meyer *et al.* 2013).

Involvement of GT rich clusters in biofilm formation and exopolysaccharide production have been stated for several bacterial strains and thus, a similar role was predicted for the cluster found in *S. acidocaldarius* (Orell *et al.* 2013b). In *P. aeruginosa* for instance the *psl*ABCDEFGHIJKLMNO cluster, required for the formation of the polysaccharide Psl, comprises 6 putative GTs (*psl*ACFHIK) (Byrd *et al.* 2009). Single deletion of the GT genes resulted in a lowered exopolysaccharide production and decreased cell attachment. In *Pseudomonas gingivalis* a cluster comprising 16 genes has been identified, which was predicted to be involved in the synthesis and in the transport of a capsular polysaccharide (K-antigen) (Aduse-Opoku *et al.* 2006). Deletion of PG0106, a gene showing high sequence similarity to a GT led to an altered biofilm phenotype of *Porphyromonas gingivalis* (Davey and Duncan 2006). Mutants were no longer capable of capsule formation and instead increased biofilm formation was observed.

In the here presented study involvement of 9 GTs was evaluated by comparison of EPS carbohydrate and protein concentrations of single GT gene deletion mutants. The results give first insights into the impact of deletions on EPS quantity; however, influences on biofilm architecture still need to be examined.

Most significant reductions in exopolysaccharide quantity of 67%, 70% and 44% compared to the reference strain MW001 were observed for gene deletion mutants Δ1904, Δ1914 and Δ1915, respectively, hinting to a crucial involvement of the respective GTs in the production of exopolysaccharides. Deletion of GT Saci_1915 led to a limited production of EPS compared to the parent strain; however, the ratio of proteins to carbohydrates remained nearly equal. This indicates that the same amount of biofilm cells possesses a lowered EPS matrix with respect to proteins and carbohydrates, or an altered EPS composition, which is

less prone to EPS isolation by CER. Within the GT cluster other proteins putatively involved in exopolysaccharide synthesis or transport were identified using BLASTp. For two of these proteins, gene deletion mutants were obtained and their EPS analyzed for carbohydrate and protein concentrations (Table 3.9). The deletion of the proposed methyltransferase Saci_1918 did not result in a significantly altered EPS composition with respect to carbohydrate and protein concentrations. The membrane protein Saci_1917 was shown to carry a putative carbohydrate binding module (CBM) hypothesized to be involved in the attachment of exopolysaccharides to the cell surface. The lowered EPS carbohydrate composition in the Saci_1917 deletion mutant, further hints to its involvement in sugar binding since no enzymatic activity, as usually found in proteins carrying a CBM, was detected.

Future experiments aim to solve the structure of the exopolysaccharides in the matrix of *S. acidocaldarius* to determine influences of the respective GTs not only on the quantity but also on the structure of the biopolymer.

Table 4.1: Overview of annotations and characteristics of proteins other than GTs within the GT cluster. Listed are characteristics of the GT cluster consisting of Saci_1904 up to Saci_1927. Annotations of proteins were performed using the updated *S. acidocaldarius* genome annotation based on arCOGs and available functional information (Esser *et al.* 2011) as well as BLASTp. Prediction of transmembrane domains identified via SMART (Schultz *et al.* 1998; Letunic *et al.* 2012) within the proteins is indicated by crosses. EPS was analyzed for gene deletion mutants Δ1908, Δ1917 and Δ1918.

S. acidocaldarius protein number	Mutant available	ArCOG code	Transmembrane domains	Annotation
Saci_1905	-	R	+	Polysaccharide biosynthesis protein, wzx-flippase
Saci_1906	-	S	+	Sugar acetylase
Saci_1908	+	-	+	Predicted membrane protein
Saci_1910	-	S	+	Uncharacterized conserved protein
Saci_1912	-	S	+	Polysaccharide biosynthesis protein, wzx-flippase
Saci_1913	-	-	-	Hypothetical protein
Saci_1917	+	S	+	Uncharacterized conserved protein
Saci_1918	+	QR	-	SAM-dependent methyltransferase
Saci_1919	-	S	+	Predicted membrane protein
Saci_1920	-	S	+	Predicted membrane protein
Saci_1924	-	-	-	SAM-dependent methyltransferase, fragment
Saci_1925	-	EH	-	3'-phosphoadenosine 5'-phosphosulfate sulfotransferase (PAPS reductase)/FAD synthetase or related enzyme

4.4.1 Heterologous expression of glycosyltransferase

The deletion of single glycosyltransferase genes revealed an impact of deletion of certain GTs on the production of EPS carbohydrate as well as EPS protein concentration. To obtain further insights into the role of glycosyltransferase, in this study, genes of 8 GTs (Saci_1907, Saci_1909, Saci_1911, Saci_1915, Saci_1922, Saci_1923, Saci_1926, Saci_1927), one membrane protein (Saci_1917), one putative methyltransferase (Saci_1918) and one hypothetical protein with unknown function (Saci_1910) were cloned and first test expressions were performed using the expression host *E. coli* Rosetta(DE3). The initial experiments showed that several GTs were only weakly expressed and only detectable in the soluble fraction via western blot and immunological detection of the His-tag. The main fraction of expressed proteins was found in the membrane fraction for all expressed GTs. Saci_1907, Saci_1918 (methyltransferase) and Saci_1922 seemed to be expressed in inclusion bodies. Moreover, a reduction by precipitation of heterologously expressed GTs at 60°C was observed for Saci_1907, Saci_1909, Saci_1911, Saci_1915 and Saci_1922. Since the proteins originate from a thermophilic organism they are expected to be stable at these temperatures.

In general, the expression of hyperthermophilic enzymes in *E. coli* offers several advantages like the enhancement of expression by application of strong promoters and the simple purification via heat precipitation of host proteins (Adams *et al.* 1995; Schiraldi and De Rosa 2002). One of the main drawbacks in heterologous expression is the different codon usage of *E. coli* and *S. acidocaldarius* and thermophiles in general. This codon bias can lead to low expression levels due to e.g. frame shifts, early termination or amino acid misincorporation (Kim and Lee 2006). Hence, for enhanced expression levels, strains carrying plasmids encoding the respective tRNAs for the rare codons are required (Wakagi *et al.* 1998; Wu *et al.* 2004). The *E. coli* strain Rosetta(DE3) applied in the current study carries the plasmid pRARE, which encodes tRNAs for the codons AUA, AGG, AGA, CUA, CCC, and GGA, representing rare codons in *E. coli* (Novagen). Usually with application of such plasmids expression can be optimized. Nevertheless, for some heterologously expressed proteins differences to the native protein have been observed and it is assumed that not all proteins can be sufficiently expressed. However, unsuccessful expression usually is not published but is likely to occur due to a lack of correct posttranslational modifications, e.g.

phosphorylation, glycosylation or methylation (Vieille and Zeikus 2001). Incorrect folding for instance was reported for heterologously expressed methylthioadenosine phosphorylase of *S. solfataricus*. Due to the formation of incorrect disulfide bridges the enzyme was less heat stable than the native enzyme (Cacciapuoti *et al.* 1999).

Further research is required applying *E. coli* expression strains, which are optimized for expression of proteins found in inclusion bodies. Moreover, a homologous expression in *S. acidocaldarius* should be contemplated to circumvent incorrect protein folding.

4.5 Lrs14 regulator effects EPS composition in *S. acidocaldarius* biofilms

In collaboration with the MPI Marburg, EPS isolation using CER was applied to isolate EPS from submersed biofilms of *S. acidocaldarius*. In the study, the involvement of an Lrs14-like protein (Saci_0446) on biofilm formation and cell motility was determined. Lrs14-like proteins are related to the Lrp-AsnC family, which is involved in bacterial gene regulation. Members of this protein family were shown to be involved in biofilm formation of various *Sulfolobus* species (Koerdt *et al.* 2011). EPS isolation and quantification of EPS proteins and carbohydrates revealed an increased amount of EPS proteins and carbohydrates in the gene deletion mutant Saci_0446 lacking the Lrs14 regulator compared to the reference strain MW001. The MW001 EPS protein and carbohydrate quantities, however, were restored when the mutant was complemented with a plasmid for overexpression of the Lrs14 regulator. These results confirmed an involvement of this regulator in the EPS synthesis and were further confirmed by CLSM imaging performed by the cooperation partners at the MPI Marburg. Submersed biofilms of ΔSaci_0446 showed an increased binding of ConA to the EPS matrix compared to MW001. The complementation of the mutant with the Lrs14 regulator restored the MW001 phenotype. Gel shift assays performed at the MPI Marburg further revealed an involvement of the regulator in cell motility due to binding of the regulator to the promoter regions of the genes involved in the synthesis of the archaellum and adhesive pili.

A combination of mutational analysis and determination of transcription levels showed an up-regulation of the gene encoding the membrane protein Saci_1908 and a down-regulation of the gene encoding GT Saci_1909 in *S. acidocaldarius* Δ0446. CLSM imaging of biofilms of double mutants Δ0446-1908 and Δ0446-1909 showed altered productions of exopolysaccharides. While in biofilms of Δ0446-1908 the MW001 biofilm phenotype was restored, biofilms of Δ0446-1909 and Δ1909 showed an increased synthesis of exopolysaccharides. These results suggest that both proteins are involved in exopolysaccharide synthesis but only Saci_1908 is regulated by the Lrs14 regulator. Based on these results, the name abfR1 (for Archaeal Biofilm Regulator 1) was proposed for the Lrs14 regulator Saci_0446.

4.6 An α-mannosidase influences the EPS composition of *S. solfataricus* biofilms

In a further study performed in collaboration with the group of Dr. S.-V. Albers (MPI Marburg) the involvement of an α-mannosidase, found in *S. solfataricus* P2 but not in *S. solfataricus* PBL2025, in EPS composition and quantity was demonstrated. *S. solfataricus* PBL2025 is a natural spontaneous mutant lacking 50 genes of the genome of P2 including the α-mannosidase and the β-galactosidase LacS. The quantitative analyses of EPS isolated from unsaturated biofilms of P2, PBL2025 and PBL2025 complemented with either the α-mannosidase, LacS, or ABCE1 showed a significantly higher concentration of EPS carbohydrates and proteins in PBL2025 compared to P2. Complementation of PBL2025 with the α-mannosidase significantly reduced the concentrations of EPS proteins and carbohydrates and hence, was hypothesized to be involved in the composition or modulation of EPS. The EPS quantification also showed a reduced EPS quantity for PBL2025 complemented with LacS or ABCE1, which, however, was less pronounced than with complementation of the α-mannosidase. In combination with the CLSM imaging of submersed biofilms of *S. solfataricus*, performed at the MPI Marburg, an involvement of this α-mannosidase in the modulation of the EPS composition or the de-mannosylation of the glycan tree of extracellular glycosylated proteins was suggested (Koerdt *et al.* 2012).

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6. APPENDIX

6.1 Glossary

2DE	two-dimensional gel electrophoresis
aa	amino acid
Amp	ampicillin
APS	ammonium persulfate
arCOG	archaeal cluster of orthologous genes
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
BSA	bovine serum albumin
bp	base pair
Cam	chloramphenicol
CE	crude extract
CER	cation exchange resin
CDP-star	disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-5 chlorotricyclo[3.3.1.13.7]decan])-4-yl]-1-phenyl phosphate
cfu	colony forming units
CHAPS	[3-(3-Cholanamidopropyl)dimethylammonio]-1-propansulfonate
CLSM	confocal laser scanning microscopy
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
DNA	deoxyribonucleic acid
EC	enzyme commission number
EDTA	ethylene diamine tetra-acetic acid
EPDM	ethylene propylene diene monomer
EPS	extracellular polymeric stubstances
G6PDH	glucose-6-phosphate dehydrogenase
GT	glycosyltransferase
HP	heat precipitation
HPLC	high performance liquid chromatography
ICP	inductively coupled plasma
IEF	isoelectric focusing
IPG	immobilized pH gradient
IPTG	isopropyl-β-thiogalactopyranoside
kDa	kilo Dalton
LB	Luria-Betani
Kan	kanamycin

MeOH	methanol
MF	membrane fraction
MS	mass spectrometry
MUF	methylumbelliferyl
MW	molecular weight
MWCO	molecular weight cut-off
NCBI	national center for biotechnology information
OD	optical density
ORF	open reading frame
p. a.	per analysis
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	polyethylene
pI	isoelectric point
PI	propidium iodide
PVC	polyvinylchloride
RNA	ribonucleic acid
RT	room temperature
Saci	*Sulfolobus acidocaldarius*
SDS	sodium dodecyl sulfate
SSO	*Sulfolobus solfataricus*
TAE	Tris/acetic acid/EDTA
TE	Tris/EDTA
TCC	total cell count
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)-aminomethane
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

6.2 Supporting information

Table 6.1: Identification of *S. acidocaldarius* EPS proteins using MALDI-TOF/MS. EPS was isolated from *S. acidocaldarius* unsaturated biofilms (4 d, 78°C) and digested with trypsin. Proteins were categorized according to arCOG functional codes and annotations using the updated genome information (Esser *et al.* 2011). Subcellular localizations were performed with PSORTb (Yu *et al.* 2010).

Gene ID	arCOG assignment	arCOG functional code	arCOG annotation	Subcellular localization
Saci_0243	arCOG04237	C	Citrate synthase	Cytoplasmic
Saci_0315	arCOG00448	C	Electron transfer flavoprotein, alpha and beta subunits	Cytoplasmic
Saci_0916	arCOG01926	C	Aerobic-type carbon monoxide dehydrogenase, middle subunit CoxM/CutM homolog	Cytoplasmic
Saci_1009	arCOG01167	C	Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homolog	Cytoplasmic
Saci_1020	arCOG01167	C	Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homolog	Cytoplasmic
Saci_1214	arCOG01697	C	Aconitase A	Cytoplasmic
Saci_1265	arCOG01337	C	Succinyl-CoA synthetase, beta subunit	Unknown
Saci_1742	arCOG01167	C	Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homolog	Unknown
Saci_2117	arCOG01167	C	Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homolog	Unknown
Saci_2154	arCOG00853	C	Malic enzyme	Cytoplasmic
Saci_2269	arCOG01926	C	Aerobic-type carbon monoxide dehydrogenase, middle subunit CoxM/CutM homolog	Cytoplasmic
Saci_2271	arCOG01167	C	Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homolog	Cytoplasmic
Saci_0227	arCOG01252	CH	Lactaldehyde dehydrogenase, NAD-dependent aldehyde dehydrogenase	Cytoplasmic
Saci_1738	arCOG01252	CH	Lactaldehyde dehydrogenase, NAD-dependent aldehyde dehydrogenase	Cytoplasmic
Saci_1857	arCOG01252	CH	Lactaldehyde dehydrogenase, NAD-dependent aldehyde dehydrogenase	Cytoplasmic
Saci_0253	arCOG01698	E	Homoaconitate hydratase/3-isopropylmalate dehydratase large subunit family protein	Cytoplasmic
Saci_0372	arCOG02969	E	Aminopeptidase N	Cytoplasmic
Saci_0798	arCOG01678	E	Archaeal S-adenosylmethionine synthetase	Cytoplasmic

Gene ID	arCOG assignment	arCOG functional code	arCOG annotation	Subcellular localization
Saci_0827	arCOG01877	E	Methionine synthase II (cobalamin-independent)	Unknown
Saci_0828	arCOG01876	E	Methionine synthase II (cobalamin-independent)	Cytoplasmic
Saci_1358	arCOG00070	E	Glycine/serine hydroxymethyltransferase	Cytoplasmic
Saci_1412	arCOG00861	E	Aspartokinase	Cytoplasmic
Saci_1428	arCOG01130	E	Aspartate/tyrosine/aromatic aminotransferase	Cytoplasmic
Saci_1483	arCOG01909	E	Glutamine synthetase	Cytoplasmic
Saci_1617	arCOG00112	E	Argininosuccinate synthase	Unknown
Saci_1620	arCOG01594	EF	Carbamoylphosphate synthase large subunit	Cytoplasmic
Saci_1715	arCOG04045	EG	Dihydroxyacid dehydratase/phosphogluconate dehydratase	Unknown
Saci_2255	arCOG02001	EH	Acetolactate synthase large subunit or other thiamine pyrophosphate-requiring enzyme	Cytoplasmic
Saci_2281	arCOG03657	EH	Acetolactate synthase large subunit or other thiamine pyrophosphate-requiring enzyme	Cytoplasmic
Saci_1079	arCOG01459	ER	Threonine dehydrogenase or related Zn-dependent dehydrogenase	Cytoplasmic
Saci_0707	arCOG04346	F	5-formaminoimidazole-4-carboxamide-1-beta-D-ribofuranosyl 5'-monophosphate synthetase (purine biosynthesis)	Unknown
Saci_0789	arCOG04048	F	Deoxycytidine deaminase	Unknown
Saci_0671	arCOG04180	G	Archaeal fructose 1,6-bisphosphatase	Cytoplasmic
Saci_0806	arCOG00767	G	Phosphomannomutase	Cytoplasmic
Saci_0854	arCOG00574	G	Ribulose 1,5-bisphosphate synthetase, converts PRPP to RuBP, flavoprotein	Cytoplasmic
Saci_1356	arCOG00493	G	Glyceraldehyde-3-phosphate dehydrogenase/erythrose-4-phosphate dehydrogenase	Cytoplasmic
Saci_0059	arCOG04435	GC	Phosphoenolpyruvate carboxylase	Cytoplasmic
Saci_0396	arCOG00977	H	Precorrin-6B methylase 2	Cytoplasmic
Saci_0646	arCOG04137	H	S-adenosylhomocysteine hydrolase	Cytoplasmic
Saci_0720	arCOG00972	H	Nicotinamide mononucleotide adenylyltransferase	Cytoplasmic
Saci_1555	arCOG04075	H	Pyridoxine biosynthesis enzyme	Cytoplasmic
Saci_0306	arCOG04201	I	Acyl-coenzyme A synthetase/AMP-(fatty) acid ligase	Cytoplasmic
Saci_1033	arCOG01707	I	Acyl-CoA dehydrogenase	Cytoplasmic

Gene ID	arCOG assignment	arCOG functional code	arCOG annotation	Subcellular localization
Saci_1071	arCOG05965	I	Acyl-CoA dehydrogenase	Cytoplasmic
Saci_1123	arCOG01707	I	Acyl-CoA dehydrogenase	Cytoplasmic
Saci_2232	arCOG01280	I	Acetyl-CoA acetyltransferase	Cytoplasmic
Saci_2233	arCOG01279	I	Acetyl-CoA acetyltransferase	Cytoplasmic
Saci_2288	arCOG01278	I	Acetyl-CoA acetyltransferase	Cytoplasmic
Saci_1122	arCOG00856	IQ	Acyl-CoA synthetase (AMP-forming)/AMP-acid ligase II	Cytoplasmic
Saci_0199	arCOG01262	IQR	Short-chain alcohol dehydrogenase	Cytoplasmic
Saci_0081	arCOG04239	J	Ribosomal protein S4 or related protein	Cytoplasmic
Saci_0082	arCOG04240	J	Ribosomal protein S11	Cytoplasmic
Saci_0585	arCOG04093	J	Ribosomal protein S4E	Cytoplasmic
Saci_0596	arCOG04071	J	Ribosomal protein L4	Unknown
Saci_0597	arCOG04070	J	Ribosomal protein L3	Cytoplasmic
Saci_0620	arCOG04186	J	Ribosomal protein S3AE	Cytoplasmic
Saci_0685	arCOG01561	J	Translation elongation factor EF-1alpha (GTPase)	Cytoplasmic
Saci_0758	arCOG04154	J	Ribosomal protein S8E	Cytoplasmic
Saci_0768	arCOG00406	J	Aspartyl/asparaginyl-tRNA synthetase	Cytoplasmic
Saci_0853	arCOG04182	J	Ribosomal protein S24E	Cytoplasmic
Saci_1261	arCOG00401	J	Threonyl-tRNA synthetase	Cytoplasmic
Saci_1347	arCOG01923	J	Protein implicated in ribosomal biogenesis, Nop56p homolog	Cytoplasmic
Saci_1544	arCOG00810	J	Methionyl-tRNA synthetase	Cytoplasmic
Saci_0692	arCOG04257	K	DNA-directed RNA polymerase subunit A'	Cytoplasmic
Saci_0834	arCOG00675	K	DNA-directed RNA polymerase, subunit E'	Cytoplasmic
Saci_1874	arCOG03482	K	Predicted transcriptional regulator associated with CRISPR system, contains COG1517 family domain	Cytoplasmic
Saci_1507	arCOG00492	KE	Transcriptional regulators containing a DNA-binding HTH domain and an aminotransferase domain (MocR family)	Cytoplasmic
Saci_1629	arCOG04298	L	Predicted adenosine-specific kinase	Cytoplasmic
Saci_1174	arCOG04148	NU	Predicted ATPase involved in biogenesis of archaeal flagella	Cytoplasmic membrane
Saci_0335	arCOG02062	O	Predicted redox protein, regulator of disulfide bond formation	Cytoplasmic
Saci_1401	arCOG01257	O	Chaperonin GroEL (HSP60 family)	Cytoplasmic
Saci_2276	arCOG00636	O	Hydrogenase maturation factor	Cytoplasmic
Saci_1103	arCOG02143	Q	Aromatic ring hydroxylase	Cytoplasmic
Saci_2294	arCOG02143	Q	Aromatic ring hydroxylase	Cytoplasmic
Saci_0331	arCOG01064	R	NAD(FAD)-dependent dehydrogenase	Cytoplasmic

Gene ID	arCOG assignment	arCOG functional code	arCOG annotation	Subcellular localization
Saci_0336	arCOG02064	R	Peroxiredoxin family protein	Cytoplasmic
Saci_0998	arCOG00040	R	Predicted phosphoribosyltransferase	Unknown
Saci_2205	arCOG01455	R	Zn-dependent alcohol dehydrogenase	Cytoplasmic
Saci_2230	arCOG01288	R	Predicted nucleic-acid-binding protein containing a Zn-ribbon	Cytoplasmic
Saci_0064	arCOG05888	S	Chromosomal protein Sac7d	Cytoplasmic
Saci_0333	arCOG02114	S	Uncharacterized conserved protein	Unknown
Saci_0386	arCOG02738	S	NifX family protein	Cytoplasmic
Saci_1678	arCOG07227	S	Uncharacterized conserved protein	Cytoplasmic
Saci_0274	arCOG00790	V	CRISPR-associated protein Cas4 (RecB family exonuclease)	Cytoplasmic

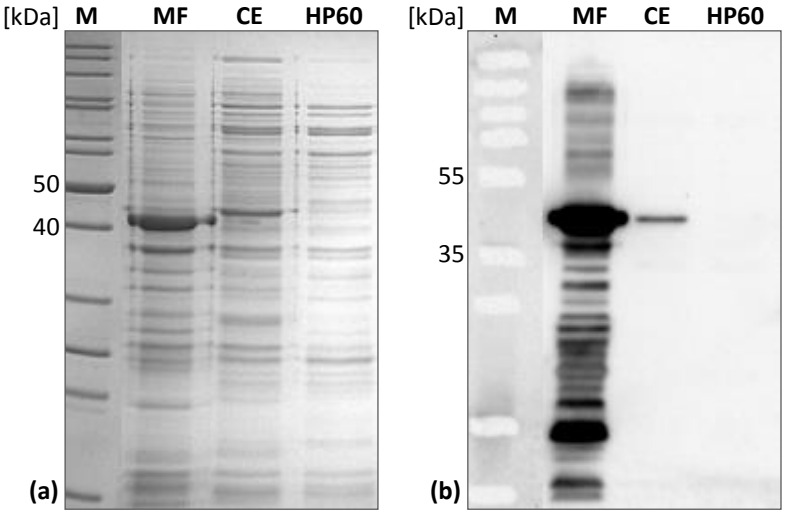

Figure 6.1: Examination of expression of GT Saci_1907 in *E. coli* Rosetta(DE3). Expression was examined in the membrane fraction (MF), crude extract (CE) and heat precipitation of crude extract at 60°C (HP60). Visualization by (a) SDS-PAGE (12.5% (w/v) polyacrylamide) stained with CBB and (b) immuno-detection of His-tagged protein after western blotting (Antibody: Ms mAb to 6 x Histag®, abcam). (a) M: PageRuler Protein Ladder (Fermentas), (b) M: PageRuler Prestained Protein Ladder (Fermentas); enhanced by separate decrease of image contrast.

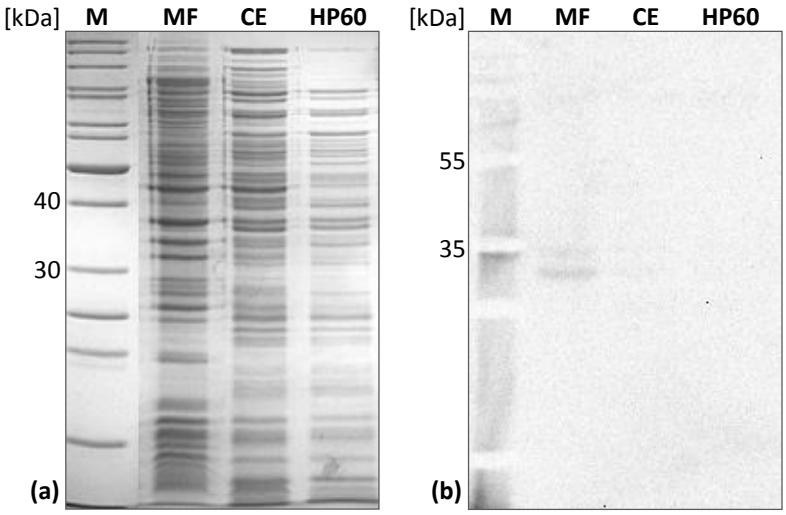

Figure 6.2: Examination of expression of GT Saci_1910 in *E. coli* Rosetta(DE3). Expression was examined in the membrane fraction (MF), crude extract (CE) and heat precipitation of crude extract at 60°C (HP60). Visualization by (a) SDS-PAGE (12.5% (w/v) polyacrylamide) stained with CBB and (b) immuno-detection of His-tagged protein after western blotting (Antibody: Ms mAb to 6 x Histag®, abcam). (a) M: PageRuler Protein Ladder (Fermentas), (b) M: PageRuler Prestained Protein Ladder (Fermentas); enhanced by separate decrease of image contrast.

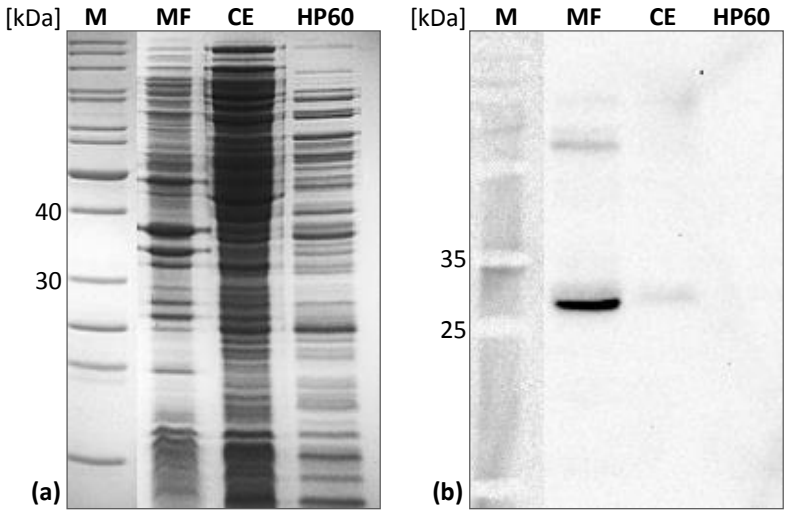

Figure 6.3: Examination of expression of GT Saci_1911 in *E. coli* Rosetta(DE3). Expression was examined in the membrane fraction (MF), crude extract (CE) and heat precipitation of crude extract at 60°C (HP60). Visualization by (a) SDS-PAGE (12.5% (w/v) polyacrylamide) stained with CBB and (b) immuno-detection of His-tagged protein after western blotting (Antibody: Ms mAb to 6 x Histag®, abcam). (a) M: PageRuler Protein Ladder (Fermentas), (b) M: PageRuler Prestained Protein Ladder (Fermentas); enhanced by separate decrease of image contrast.

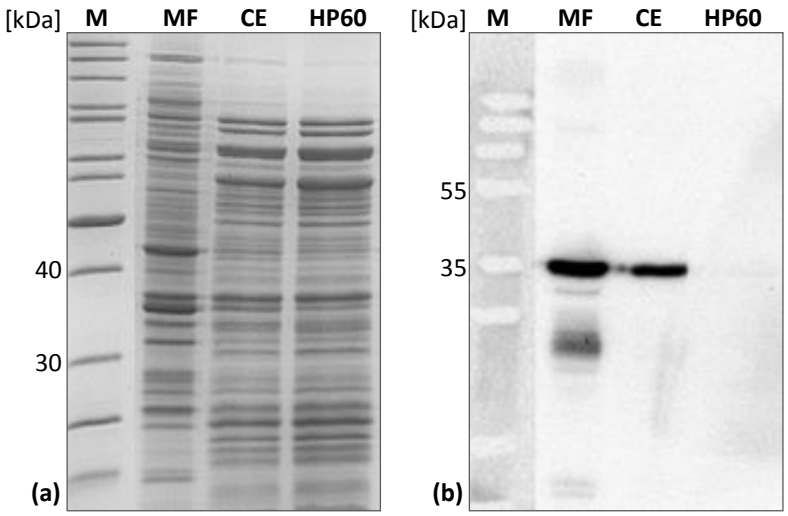

Figure 6.4: Examination of expression of GT Saci_1915 in *E. coli* Rosetta(DE3). Expression was examined in the membrane fraction (MF), crude extract (CE) and heat precipitation of crude extract at 60°C (HP60). Visualization by (a) SDS-PAGE (12.5% (w/v) polyacrylamide) stained with CBB and (b) immuno-detection of His-tagged protein after western blotting (Antibody: Ms mAb to 6 x Histag®, abcam). (a) M: PageRuler Protein Ladder (Fermentas), (b) M: PageRuler Prestained Protein Ladder (Fermentas); enhanced by separate decrease of image contrast.

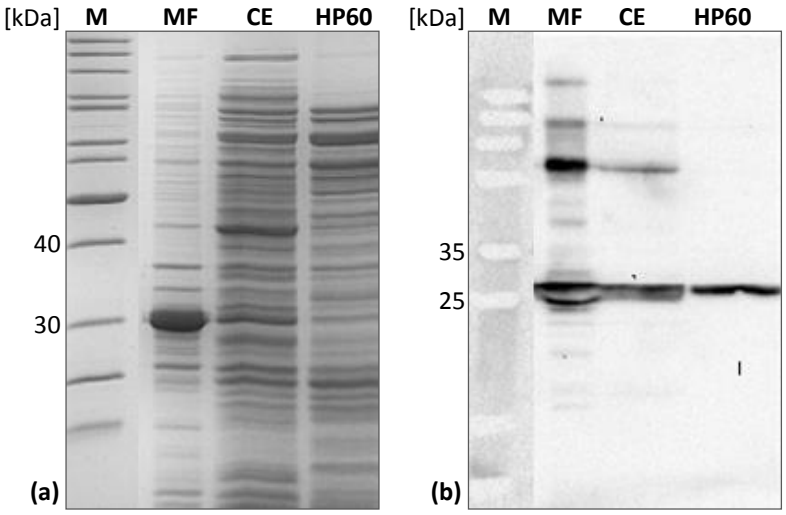

Figure 6.5: Examination of expression of Saci_1918 in *E. coli* Rosetta(DE3). Expression was examined in the membrane fraction (MF), crude extract (CE) and heat precipitation of crude extract at 60°C (HP60). Visualization by (a) SDS-PAGE (12.5% (w/v) polyacrylamide) stained with CBB and (b) immuno-detection of His-tagged protein after western blotting (Antibody: Ms mAb to 6 x Histag®, abcam). (a) M: PageRuler Protein Ladder (Fermentas), (b) M: PageRuler Prestained Protein Ladder (Fermentas); enhanced by separate decrease of image contrast.

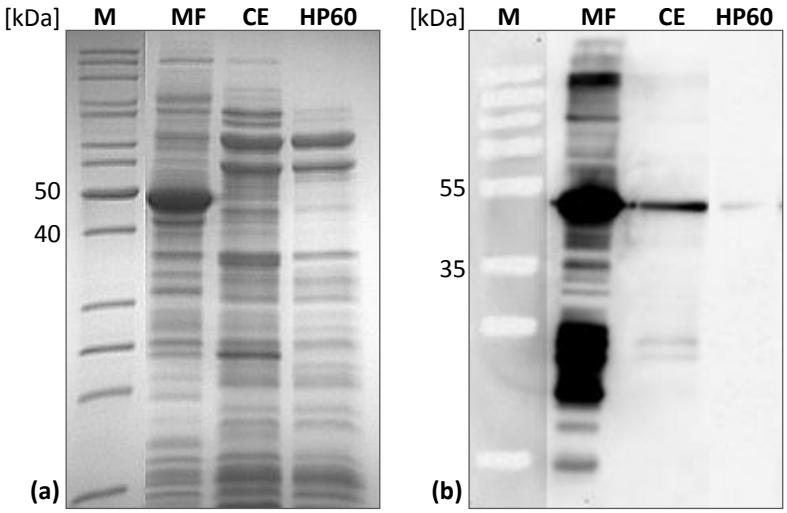

Figure 6.6: Examination of expression of GT Saci_1922 in *E. coli* Rosetta(DE3). Expression was examined in the membrane fraction (MF), crude extract (CE) and heat precipitation of crude extract at 60°C (HP60). Visualization by (a) SDS-PAGE (12.5% (w/v) polyacrylamide) stained with CBB and (b) immuno-detection of His-tagged protein after western blotting (Antibody: Ms mAb to 6 x Histag®, abcam). (a) M: PageRuler Protein Ladder (Fermentas), (b) M: PageRuler Prestained Protein Ladder (Fermentas); enhanced by separate decrease of image contrast.

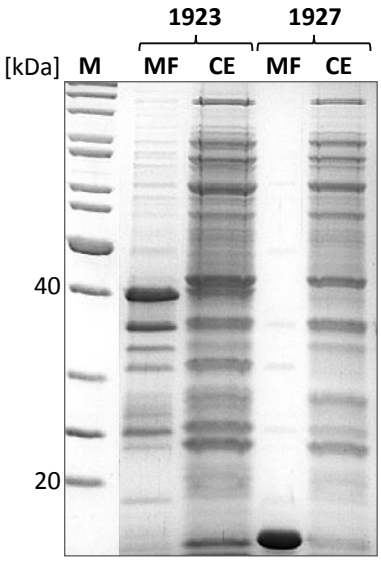

Figure 6.7: Examination of expression of GT Saci_1923 and Saci_1926 in *E. coli* Rosetta(DE3). Expression was examined in the membrane fraction (MF) and crude extract (CE). Visualization on SDS-PAGE (12.5% (w/v) polyacrylamide) stained with CBB; M: PageRuler Protein Ladder (Fermentas).

Liste der Veröffentlichungen

Referierte Publikationen

2013	**Jachlewski, S.**, Michalowski, W.D., Wingender, J., & Siebers, B. (2013). Comparison of methods for the EPS isolation from biofilms of the thermo-acidophilic Archaeon *Sulfolobus acidocaldarius*. (in Vorbereitung)
	Jachlewski, S., Botes, M., & Cloete, T.E. (2013). The effect of ultrasound at 256 KHz on *Microcystis aeruginosa*, with and without gas vacuoles. *Water SA 39*(1), 171–174.
	Orell, A., Peeters, E., Vassen, V., **Jachlewski, S.**, Schalles, S., Siebers, B., & Albers, S.-V. (2013). Lrs14 transcriptional regulators influence biofilm formation and cell motility of Crenarchaea. *The ISME journal*, 1–13.
2012	Koerdt, A., **Jachlewski, S.**, Ghosh, A., Wingender, J., Siebers, B., & Albers, S.-V. (2012). Complementation of *Sulfolobus solfataricus* PBL2025 with an α-mannosidase: effects on surface attachment and biofilm formation. *Extremophiles: Life under extreme conditions*, *16*(1), 115–25.

Vorträge

05/2012	Stress response in biofilms of the thermoacidophilic Archaeon *Sulfolobus acidocaldarius* (Mercur Projekt Meeting, Dortmund)
09/2011	Archaeal biofilms – Biofilm formation and EPS analysis of *Sulfolobus acidocaldarius* (Archaea Tagung, Wiesbaden)
03/2011	Biofilm formation and EPS analysis of the Archaeon *Sulfolobus acidocaldarius* (Glyco Meeting, Marburg)

Poster

07/2012	„Hot biofilms – EPS composition and exopolysaccharide synthesis in the Archaeon *Sulfolobus acidocaldarius*", **S. Jachlewski**, W.D. Michalowski, B. Meyer, S.-V. Albers, J. Wingender, B. Siebers (Molecular Biology of Archaea 3, Marburg)
10/2011	„A novel system to transform *Acinetobacter baylyi* ADP1 biofilms", Y. Jin, **S. Jachlewski**, S. Wuertz (IWA Conference on biofilms, Shanghai, China)
10/2011	„Hot biofilms – EPS composition and exopolysaccharide synthesis in the Archaeon *Sulfolobus acidocaldarius*", **S. Jachlewski**, W.D. Michalowski, C. Schäffer, B. Meyer, S.-V. Albers, J. Wingender, B. Siebers (IWA Con-ference on biofilms, Shanghai, China)
09/2010	„Hot biofilms – EPS analysis of the Archaeon *Sulfolobus acidocaldarius*", **S. Jachlewski**, W.D. Michalowski, J. Wingender, B. Siebers (Biofilms 4 International Conference, Winchester, UK)
09/2008	„Ultrasonic treatment of a natural and a laboratory culture of *Microcystis aeruginosa*", **S. Jachlewski**, and T.E. Cloete (IWA World Water Congress, Wien, Österreich)

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Biofilm formation and EPS analysis of the thermoacidophilic Archaeon *Sulfolobus acidocaldarius*"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im September 2013

Silke Jachlewski

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Last but not least I am very grateful for the constant support of my lovely family and all my friends who never stopped believing in me.