The effect of organic solvents on biofilm formation of the thermoacidophilic Archaeon *Sulfolobus acidocaldarius*

Dissertation

zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

– Dr. rer. nat. –

vorgelegt von

Jens Carlo Benninghoff

geboren in Dortmund

Biofilm Centre

Molekulare Enzymtechnologie und Biochemie

der

Universität Duisburg-Essen

2016

Die vorliegende Arbeit wurde im Zeitraum von April 2012 bis Dezember 2015 im Arbeitskreis von Prof. Dr. Bettina Siebers am Biofilm Centre der Universität Duisburg-Essen durchgeführt.

Tag der Disputation: 20.12.2016

Gutachter: Prof. Dr. Bettina Siebers

Prof. Dr. Hans-Curt Flemming

Vorsitzender: Prof. Dr. Maik Walpuski

DOI: 10.17185/duepublico/43311
URN: urn:nbn:de:hbz:464-20201012-114150-1

Für meine Familie

Table of Contents

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Sulfolobus acidocaldarius is a thermoacidophilic Crenarchaeon (78°C and pH 2-3) with established genetic systems. With these properties, *S. acidocaldarius* offers high potential for biotechnological applications as alternative host for the production of bulk and fine chemicals, solvents and second generation biofuels, which are, however, often toxic to cells. Biofilms, the dominant lifestyle of microorganisms, were shown to confer higher resistance towards solvent exposure and hence turn more and more into research focus for biotechnological applications. Although *S. acidocaldarius* is known to form biofilms, the development of suitable incubation systems is still in its beginning and also the influence of organic solvents on *S. acidocaldarius* has only rarely been studied.

In this work, incubation systems for the cultivation of submerse biofilms of *S. acidocaldarius* cells on glass and polystyrene surfaces in microtiter plates, µ- and Petri-dishes as well as a flow-through system were successfully developed and optimized. These newly developed methods enabled detailed microscopic analyses and also yielded sufficient amounts of biofilms for isolation and analyses of extracellular polymeric substances (EPS) as well as for application of various -omics methods. The analyses were performed in presence and absence of organic solvents (with special respect to 1-butanol) and the results were compared to shaking cultures.

S. acidocaldarius formed biofilms even in shaking cultures in Erlenmeyer flasks at the liquid/glass/air interface as a (stress)response to sublethal concentration of different solvents. In accordance with shaking cultures, in a 96-well plate static incubation system, the tendency of enhanced biofilm formation as a response to sublethal concentrations of 1-butanol (<1.5% (v/v)) was visible. By the use of an adapted resazurin assay, indicating respiratory activity, the viability of biofilm cells was confirmed colorimetrically. Furthermore, in static incubation systems e.g. on glass slides, *S. acidocaldarius* formed monolayered biofilms of homogenously distributed cells even in the absence of solvents as shown by crystal violet staining and subsequent light microscopy. In the presence of solvents these biofilms appeared thicker and showed an altered morphology with a heterogenous distribution and cell aggregate formation, indicated by enhanced crystal violet binding. Additionally, visualised by scanning electron microscopy, under 1.5% (v/v) 1-butanol exposure the aggregated cells were surrounded by extracellular material, probable EPS. Furthermore, the cell morphology was

altered compared to the control experiments showing a diversified cell envelope with holes. In the presence of 1% (v/v) 1-butanol, less changes in biofilm morphology and no changes in cell morphology were observed by the application of atomic force microscopy. Lectin binding and subsequent confocal laser-scanning microscopy of submersed biofilms showed an increased amount of carbohydrate structures, especially α-mannopyranosyl- and α-glucopyranosyl residues. The enhanced EPS formation was also confirmed by EPS isolation and quantitative determination using colorimetric assays, which showed a 5-fold increase of carbohydrates within the extracted EPS. The amount of proteins was even 19-fold higher compared to the control without butanol, indicating that EPS formation in biofilms is significantly increased in the presence of toxic solvent concentration.

Beside the influence of 1-butanol on changes in biofilm morphology and EPS formation, the response of *S. acidocaldarius* towards changes in lifestyle and 1-butanol was investigated by transcriptomic and proteomic studies. For this, *S. acidocaldarius* was cultivated as shaking culture in Erlenmeyer flasks without 1-butanol exposure and statically in Petri-dishes for biofilm and planktonic growth in the absence and presence of 1-butanol. Statically grown biofilm and planktonic cells in Petri-dishes showed only less differentially regulated genes and proteins. Lots of differentially regulated genes and proteins were found in cells of shaking cultures and statically incubated planktonic cells. In the presence of 1% (v/v) 1-butanol, genes and proteins are predominantly down-regulated, especially proteins containing transmembrane helices, confirmed by transcriptomic analyses. In planktonic as well as biofilm cells in the presence of 1-butanol, the archaellum operon, responsible for cell motility, was down-regulated. Generally, in response to 1-butanol and different lifestyles, changes in cell division and vesicle formation, stress response systems like the CRISPR-Cas and toxin-antitoxin system, transcriptional regulators and protein kinases and phosphatases (signal transduction) were observed.

In conclusion, this study gives an overview of the stress response of *S. acidocaldarius* towards organic solvents and 1-butanol in detail. For the first time the influence of organic solvents on biofilm formation was described and investigated by various microscopic methods and EPS isolation and quantification. In addition, transcriptomic and proteomic studies were performed to investigate genome-wide and cellular responses towards different lifestyles and 1-butanol.

1. INTRODUCTION

1.1. Archaea

Based on comparison of rRNA sequences, Carl Woese and co-workers discovered that Archaea, next to Eukaryotes and Bacteria, represent the third domain of life (Woese et al., 1990). Currently, the archaeal domain is represented by two superphyla (DPANN and TACK) and the phylum Euryarchaeota (Guy et al., 2011). The superphyla are further devided into the following phyla: Crenarchaeota, Thaumarchaeota, Korarchaeota, Lokiarchaeota, Woesearchaeota, Nanoarchaeota, Geoarchaeota and Altiarchaeota (Auchtung et al., 2006; Castelle et al., 2015; Cavicchioli, 2011; Kozubal et al., 2013; Pester et al., 2011; Probst et al., 2015; Spang et al., 2015). Deep metagenomic sequencing and the recent discovery of the archaeal group of Lokiarchaeota changed the view on the phylogenetic tree and domains of life (Embley et al., 2015). The change of the classical three-domain phylogenetic tree to a two-domain eocyte tree is currently under discussion (Koonin, 2015; Spang et al., 2015). In the three-domain of life tree, Archaea and Bacteria are primary branches directly derived from the cenancestor and Eukaryotes evolved secondarily (Fig 1-1 A). By discovery of the Lokiarchaeota the hypothesis was supported that the eukaryotic cell evolved from an archaeal ancestor (Spang et al., 2015)(Fig 1-1 B).

Figure 1-1: Schematic phylogenetic trees by 16S rRNA sequences representing the classical three-domain archaeal tree (A) and the two-domain eocyte tree (B) (modified from (Koonin, 2015)).

Initially, Archaea were isolated mainly from extreme environments with low pH (acidophile), high temperatures (thermophile), low temperature (psychrophile), acidic and hot environments (thermoacidophile), high salt concentrations (halophile) or anaerobic

conditions (mostly methanogens). However, continued analysis of environmental samples showed an ubiquitous occurrence of Archaea also in mesophilic habitats (DeLong, 1998). Detailed analysis showed that Archaea harbour some bacterial and eukaryal as well as unique archaeal features. Like Bacteria, Archaea harbour circular chromosomes without a nuclear membrane and plasmids. Archaeal genes are organized in operon structures and devoid of cell organelles. In contrast, the archaeal translation and transcription machinery as well as DNA repair mechanisms harbour Eukaryal-like mechanisms (Kelman et al., 2005).

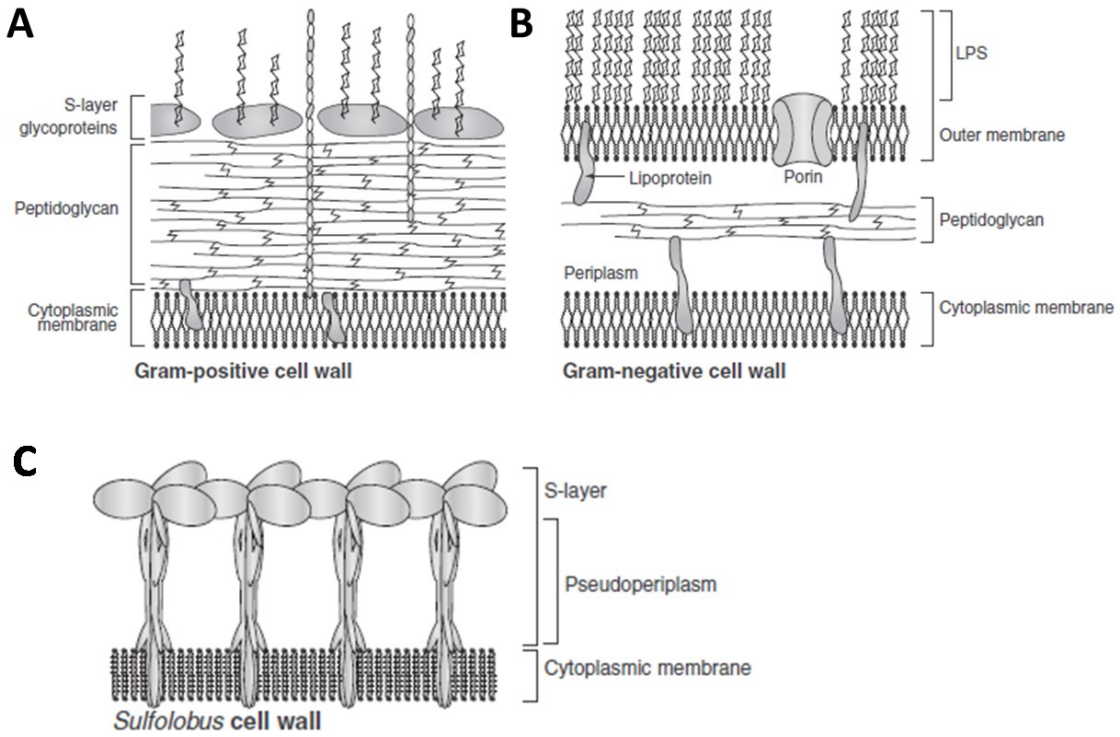


Figure 1-2: Schematic views of cell wall profiles of Gram-positive and Gram-negative bacterial cells and the cell wall of *Sulfolobus*. Figure taken from (Oger et al., 2013), redraw from (Albers et al., 2011).

Unique to Archaea is the cell envelope, which differs from Bacteria and Eukarya. The cell wall of Gram-positive and -negative Bacteria consists of a thick (Gram-positive, Fig. 1-2 A) or thin (Gram-negative, Fig. 1-2 B) peptidoglycan layer, respectively, whereas in Archaea like *Sulfolobus* spp. (Fig 1-2 C) the peptidoglycan layer is missing. Some Archaea contain cell walls consisting of pseudomurein or S-layer proteins or harbour no cell envelops (Albers et al., 2011). The archaeal cell membranes, like in Bacteria, can either consist of a lipid bilayer or of a monolayer with hydrophobic headgroups on both ends (Albers et al., 2011). The lipids of the bacterial membrane are glycerol-ester-linked whereas in Archaea the lipids are glycerol-ether

linked (Albers et al., 2011; Kandler et al., 1978). *Sulfolobus spp*. show a cell wall and the acyl chain composition consists of membrane-spanning tetraether lipids and cyclopentane rings (Brock et al., 1972). Its S-layer consists of a branched, six-membered glycan tree and the S-layer proteins are mostly N- or O-glycosylated (Albers et al., 2011; Peyfoon et al., 2010). Since the cell wall represents the first barrier between the cells and the often harsh environment, the glycoproteins can be changed via post-translational modifications as response to environmental changes (Kandiba et al., 2014). Beside the S-layer, Archaea can have diverse cell appendages like different pili, the archaellum (same function as bacterial flagella, but different in structure), cannulaea, hami and bindosome (Jarrell et al., 2013; Ng et al., 2008). For *Sulfolobus spp.*, S-layer proteins are the sole constituent of the cell envelope (Albers et al., 2011). *S. acidocaldarius* possesses different types of cell surface structures like archaella with a curved appearance necessary for motility. Furthermore, aap pili (archaeal adhesive pili, mostly straight) and ups pili (UV-induced pili, shortest pili) were characterized (Henche et al., 2012b). These surface structures play an important role during planktonic lifestyle as well as for attachment and biofilm formation. For *S. solfataricus*, the presence of archaella and pili is essential for initial attachment on surfaces (Zolghadr et al., 2010).

The metabolism of Archaea is as complex as those of Bacteria and lower Eukaryotes but differ especially in the central carbohydrate metabolism. “Classical” pathways are missing but modified variants of these pathways like the Entner-Doudoroff (ED) or the Embden-Meyerhof-Parnas (EMP) pathway were identified (Bräsen et al., 2014). The pentose phosphate pathway differs significantly from the bacterial pentose degradation and is present only partly (Bräsen et al., 2014). As a consequence of differences in the central carbohydrate metabolism, in Archaea new enzyme families were found which often share no similarity to the bacterial and eukarayal counterparts (Bräsen et al., 2014). A detailed overview about the central carbohydrate metabolism was reviewed in Bräsen et al. (Bräsen et al., 2014).

1.2. Bacterial and archaeal biofilms

As Archaea, biofilms are ubiquitous as well. Biofilms represent the dominant lifestyle of prokaryotic as well as eukaryotic microorganisms under natural conditions (Donlan et al., 2002). Since growth as biofilm is the predominant lifestyle of all prokaryotes, also Archaea can form biofilms. Biofilms usually occur in nature as multispecies biofilms rather than as single

species biofilms (Elias et al., 2012). Therefore, environmental biofilms consist predominantly of Bacteria and Archaea as multispecies biofilm. Bacterial biofilms are studied for decades not only because of their ecological importance but also for application in biotechnology and wastewater treatment as well as their role as reservoir for pathogens in the environment and infections in humans (Hall-Stoodley et al., 2004). Archaeal biofilms are studied only since recently, probably because no pathogenic Archaea are known and the relevance for medicine and industry is little so far.

For investigations of bacterial biofilms, various systems for cultivation under static and flow-through conditions are established (Crusz et al., 2012; Merritt et al., 2005). These may be applied for mesophilic Archaea as well (Di Meglio et al., 2014; Fröls et al., 2012). Incubation systems for biofilm formation of thermoacidophilic strains are adapted and optimized but still have limitations, especially on medium evaporation (Koerdt et al., 2010). The requirements of capable incubation systems especially for the cultivation of thermophilic Archaea is a reason for limited information on their biofilm formation. Therefore, research on development of capable incubation systems is necessary. The microscopic methods for investigation of archaeal biofilms are not influenced by the harsh cultivation temperatures and can be applied as already for Bacteria. The formation of archaeal biofilms, especially those of Euryarchaeota and Crenarchaeota, was predominatly analysed by microtiter plate assays and DNA and lectin staining with subsequent confocal laser scanning microscopy (Fröls et al., 2012; Koerdt et al., 2010). In some studies, the biofilm morphology as well as cell morphology was further visualized by scanning electron microscopy or atomic force microscopy (Koerdt et al., 2010; R. Y. Zhang et al., 2015).

The development of biofilms was found to be comparable for Bacteria and Archaea: The irreversible bound cells lose their mobility, naturally mediated by cell appendages like flagella (Bacteria) or archaella (Archaea), respectively. When the biofilm maturates, mobile cells harbouring flagella/archaella are released and can form biofilm themselves (Fig. 1-3, (Sauer, 2003; Stoodley et al., 2002). In Fig. 1-3, only motile and non-motile cells are shown, but the cell heterogeneity inside a developed biofilm is more complex. Under laboratory conditions most of the investigated biofilms are single species biofilms and, therefore, genotypically identical. However, biofilms were found to consist of phenotypically distinct subpopulations (Lopez et al., 2010). Gradients of e. g. oxygen, nutrients and electron acceptors within the

biofilm creates microenvironments and therefore differences in cell differentiation (Spormann, 2008). Furthermore, the physiology of biofilm cells might be different to planktonic cells by unique gene-expression pattern (Davies, 2003).

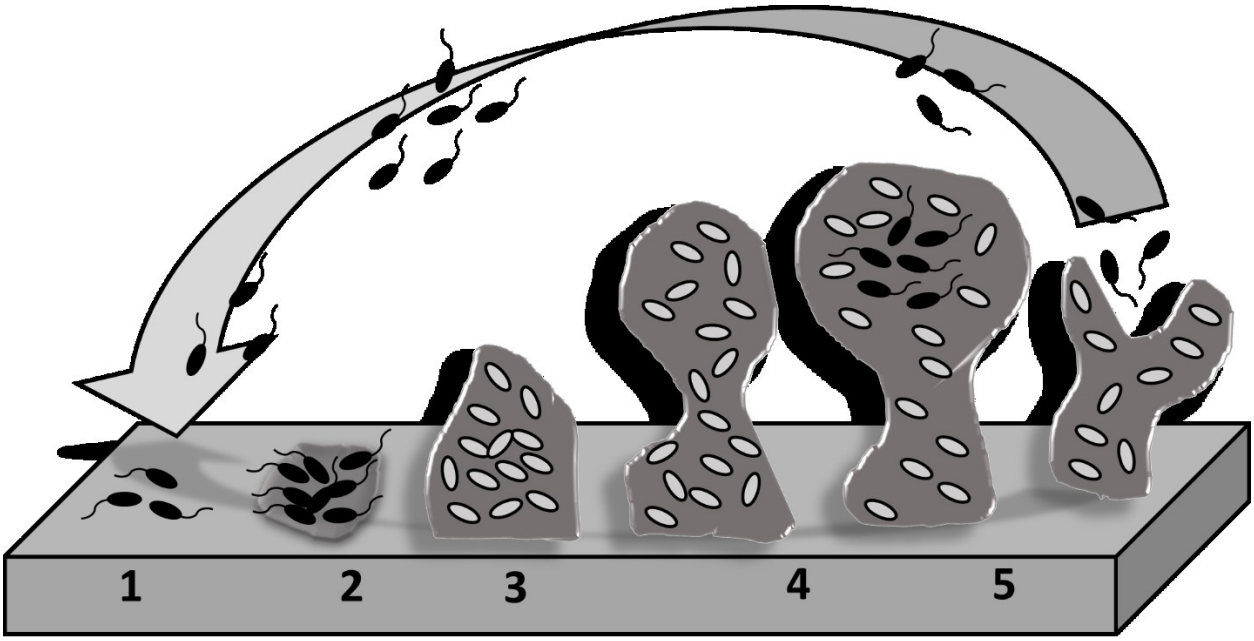


Figure 1-3: Stages of biofilm development. Biofilm formation is initiated with reversible attachment of planktonic cells to the surface (1) followed by irreversible cell attachment and production of extracellular polymeric substances (2). Process of biofilm maturation (3) ends with fully maturated biofilm with complex architecture (4). Finally, in the dispersion stage (5), single motile cells (black) disperse from the microcolonies and can attach on surfaces and form new biofilm themselfes. Modified from (Sauer, 2003; Stoodley et al., 2002).

Lopez et al. reviewed different factors inducing biofilm formation (Lopez et al., 2010). Communication between cells is an important factor for biofilms in Bacteria and probably for Archaea as well. The release and sensing of molecules (autoinducer signals) or peptides (pheromons) is called quorum sensing and is a change in gene expression in a regulated and coordinated way, resulting in a synchronized cell response to environmental conditions (Camilli et al., 2006; Fuqua et al., 1994). High concentrations of autoinducer, molecules produced and secreted by the bacterial population, trigger multicellular responses. For Bacteria, molecular mechanisms and of quorum sensing molecules are known (Dickschat, 2010). In Gram-negative Bacteria, acyl homoserine lactones (AHLs) often act as autoinducers, whereas in Gram-positive Bacteria often peptides are found to act as autoinducers (Lopez et al., 2010). However, molecules not involved in quorum sensing can induce biofilm formation as well, e. g. antibiotics, pigments and siderophores (Calderon et al., 2013). The antibiotic tobramycin induced biofilm formation in *Pseudomonas aeruginosa* and *Escherichia coli* at

sublethal concentrations (Hoffman et al., 2005). Therefore, biofilm formation is induced by quorum sensing and autoinducer molecules as well as in toxic environments as a protective mechanism of cells. So far, only less information is known about archaeal cell-cell communication.

The molecular mechanisms of archaeal biofilm formation are so far undiscovered. In studies with *S. acidocaldarius* it was found that Lrs14-like proteins, archaea-specific transcriptional regulators, are a key regulatory factor during biofilm development of *Sulfolobus* (Orell et al., 2013b).

Biofilms are generally defined as microbial aggregates accumulated usually at a solid-liquid interphase embedded in a matrix of extracellular polymeric substances (EPS) (Flemming et al., 2010). The EPS matrix is highly hydrated and stabilizes the biofilm, act as nutrient source and digestion compartment of exogenous macromolecules or as diffusion barrier for toxic compounds (Flemming et al., 2010). Main components of an EPS matrix of Bacteria and Archaea are biomolecules like proteins, carbohydrates, nucleic acids and lipids (Flemming, 2011; Fröls, 2013; Orell et al., 2013a). So far, lipids have not been analysed in archaeal biofilms but have been identified in bacterial biofilms. As cells embedded in EPS look macroscopically like a thin layer of slime, they are called biofilms. Biofilm development is initiated with reversible and subsequent irreversible attachment of cells to surfaces. The irreversible attachment of cells in this process is mediated by EPS (Flemming et al., 2010). By secretion of EPS, a cohesive, three-dimensional network from polymers is formed and stabilizes the biofilm (Flemming et al., 2010).

One advantage of cells being embedded in biofilms is an enhanced resistance against toxic environmental conditions. Compared to planktonic cells 1000-fold higher resistance towards antibiotic compounds can be achieved in biofilms (Venkatesan et al., 2015). Factors enhancing the resistance of Bacteria embedded in biofilm are categorized in biochemical factors (exo-polysaccharides, eDNA, degrading enzymes, efflux pumps and quorum sensing), molecular mechanism (gene transfer and mutation) and altered host factors (e. g. nutrients, temperature, pH, osmolarity, toxin-antitoxin modules) (Venkatesan et al., 2015). Resistance to chemicals, antibiotics and toxins is suggested to be generated by thick biofilm layers or EPS matrix (Mah et al., 2001; Stewart, 2002). Since the EPS is highly hydrated and molecules can pass the matrix by diffusion, the EPS may not act as a diffusion barrier but can quench toxic

compounds, e. g. by complex formation or enzymatic degradation. Different factors promoting a higher tolerance of biofilm cells towards toxic compounds are recently reviewed and discussed (Flemming et al., 2016).

EPS extraction and quantification was only done for *Sulfolobus* species grown as submerged and unsaturated biofilms on solid media (Jachlewski et al., 2015; Koerdt et al., 2012; Orell et al., 2013b). EPS extraction and quantification of single EPS components from *S. acidocaldarius* indicated the presence of different biomolecules like carbohydrates, proteins and eDNA (Jachlewski et al., 2015). Carbohydrates are the main component of EPS followed by proteins and eDNA. Further, hydrolytic enzyme activity and a high number of cytosolic proteins were observed (Jachlewski et al., 2015).

So far, only one enzyme involved in archaeal EPS formation is reported. An α-mannosidase from *S. solfataricus* PBL2025 was found to have an influence on EPS formation (Koerdt et al., 2012). Studies of archaeal biofilms under laboratory conditions showed that Archaea form complex multilayer three-dimensional biofilms embedded in EPS (Chimileski et al., 2014; Koerdt et al., 2010). Most analysed archaeal biofilms are monospecies-biofilms and only some bispecies-biofilms were investigated so far (Castro et al., 2016; Di Meglio et al., 2014; Schopf et al., 2008).

As for biofilm formation of Bacteria, cell surface structures facilitate the initial attachment of cells on surfaces (Jarrell et al., 2013; Pohlschröder et al., 2015). For *M. maripaludis* and *S. solfataricus* it was found that pili and archaella are necessary for attachment of cells on abiotic surfaces (Jarrell et al., 2011; Zolghadr et al., 2010). For *S. acidocaldarius* archaella are proposed to be important for cell release from biofilms and pili are important for attachment and biofilm formation (Henche et al., 2012b).

For Haloarchaea, biofilm formation on different substrates like plastic and glass surfaces was observed (Chimileski et al., 2014; Fröls et al., 2012). Fröls et al. observed different types of biofilm structures (Fröls et al., 2012). *Halobacterium salinarum* and Antarctic isolate t-ADL strain DL24 were found to grow as carpet-like, multi-layered biofilms of up to 50 µm in height containing micro-and macrocolonies. *Haloferax volcanii* DSM 3757T and *Halorubrum lacusprofundi* DL28 form large aggregates of cells. Staining of haloarchaeal biofilms indicated the presence of extracellular DNA and glycoconjugates (Fröls et al., 2012). Investigations of biofilms formed by *Halorubrum* sp. and *Halobacterium* sp. showed that the three dimensional

biofilm structure is microorganism dependent (Di Meglio et al., 2014). Surface coverage as well as biofilm thickness was found to be different. Biofilms of *Halobacterium* sp. developed biofilms with 86 ± 4 µm in thickness whereas biofilms of *Halorubrum* sp. only reached 41 ± 1 µm (Di Meglio et al., 2014).

Haloferax volcanii was found to form biofilm phenotypes comparable to bacterial biofilms, showing a complex three dimensional structure (Chimileski et al., 2014). Formation of multi-layer towers with a maximum height of 148 µm were observed by scanning electron microscopy. The changes in cellular morphology and an unusual form of social motility (biological movement of static cells) were proposed to be strain or domain specific (Chimileski et al., 2014).

The most studied biofilms in Crenarchaeota are those of *Sulfolobus* species (Henche et al., 2012b; Jachlewski et al., 2015; Koerdt et al., 2010; Koerdt et al., 2012; Orell et al., 2013b; R. Zhang et al., 2015; R. Y. Zhang et al., 2015; Zolghadr et al., 2010). As discovered for euryarchaeal biofilms, Crenarchaeota form biofilms on different surfaces like plastic, glass and carbon (Koerdt et al., 2010; Zolghadr et al., 2010). Characterization of biofilms of *Sulfolobus acidocaldarius*, *S. solfataricus* and *S. tokodaii* showed that these strains form multi-layered biofilms with different architecture and carbohydrate quantity as well as composition (Koerdt et al., 2010). Biofilms formed by *S. tokodaii* and S*. acidocaldarius* showed the presence of extensive cell-cell connections, possibly formed by strings of sugars or surface appendages (Koerdt et al., 2010). Comparative growth studies of *S. acidocaldarius* in submerged and solidified substrate cultures analysed the growth in different cultivation systems with different carbon sources. Biomass yields and growth rates of biofilms were higher on solidified medium compared to submerged cultures (Hatzinikolaou, 2001).

Investigations of bi-species biofilms of *Acidianus* spp. and *Sulfolobus metallicus* showed that physical contact between both species occur, indicated by specific lectin binding of single species within mixed cultures (Castro et al., 2016). Both species form heterologously distributed micro-colonies on pyrite surfaces. Biofilms of both species are substrate- and species-dependent and negatively influence each other during pyrite leaching (Castro et al., 2016).

1.3. Applications of biofilms and Archaea for industrial processes

Most industrial processes are based on planktonically grown production strains in fermenters. Whole cell biocatalysis of cells grown as biofilm has some important advantages compared to planktonically grown cells: Higher process stability and long-term activity as well as higher tolerance of cells towards toxic compounds (Halan et al., 2012; Rosche et al., 2009). Bacterial biofilms for whole-cell biocatalysis were successfully used in lab-scale for production of fine chemicals (Gross et al., 2010; Halan et al., 2014). Biotechnological production of chemicals is often limited by substrate toxicity on the production strain. Biofilms are formed spontaneous by self-immobilization of cells, are stable for long-term cultivation and offer an increased tolerance against toxic chemicals. Various systems for biofilm growth are applicable and ensure capable systems for different applications (Cheng et al., 2010; Halan et al., 2012; Rosche et al., 2009). Examples for biofilm reactors are e. g. trickle bed, packed bed, membrane and tubular membrane reactors (Rosche et al., 2009). Biofilms are used for synthesis of fine and bulk chemicals. Fine chemicals are produced in smaller amount and higher prices compared to bulk chemicals, which are cheap in price and produced in large quantities. Production of vinegar using a 60 m^3 trickle-bed reactor by acetic acid Bacteria is one exception for large-scale processes (Crueger, 1990). Studies for biofilm catalysed ethanol production as bulk-chemical are done by immobilized *Saccharomyces cerevisiae* or *Zymomonas mobilis* (Demirci et al., 1997; Kunduru et al., 1996; Najafpour et al., 2004). The recombinant *E. coli* B strain KO11 was successfully applied for ethanol production and increasing ethanol tolerance was shown (Zhou et al., 2008). The effect of different compounds on a continuous biofilm reactor for butanol production was characterized (Qureshi et al., 2005). Formation of L(+)-lactic acid was characterized from immobilized *Rhizopus oryzae* in a fibrous bed reactor (Tay et al., 2002). Immobilized *Propionibacterium acidipropionici* cells were used for propionic acid fermentation continuously for 4 months (Lewis et al., 1992). The applied bioreactor is scalable and is suitable for industrial applications.

Application of biofilms for production of fine chemicals is promising and conducted in lab-scale by a wide variety of microorganisms and substrata. Formation of alcohols like benzyl alcohol (*Zymomonas mobilis*) and 1-octanol (*Pseudomonas putida*) are successfully produced by immobilized cells (Gross et al., 2010; X. Z. Li et al., 2006). Other examples for fine-chemicals produced by biofilms are dihydroxyacetone (*Gluconobacter oxydans*), gibberellic acid

(*Fuscarium moniliforme*), (S)-styrene oxide and pyruvic acid (recombinant *Acetobacter xylinum*) (Gross et al., 2007; Halan et al., 2014; Hekmat et al., 2003; Meleigy et al., 2009).

Various bacterial and eukaryal species grown as biofilms were demonstrated to be applicable in production of chemicals. Therefore, also archaeal species have potential in biotransformation processes.

Commonly microorganisms are cultivated planktonically for biotechnological processes, avoiding long-term stability. The biofilm mode of life, however, provides a stable environment for the cells and characteristics like self-regeneration provide a long-term stability, which possibly also offers potential for industrial application of biofilms (Rosche et al., 2009). *Zymomonas mobilis* biofilms showed to be more tolerant against toxic benzaldehyde compared to planktonic cells (X. Z. Li et al., 2006). This increased tolerance of biofilm cells was found to be not due to conversion of toxic benzaldehyde into less toxic compounds, but rather potentially caused by the biofilm mode of life. In biotechnological applications the microorganisms are cultivated under harsh incubation conditions and the tolerance of the organisms is often the limiting factor. Application of organisms naturally adapted to harsh environmental conditions grown as biofilm may increase the tolerance of the cells compared to current systems. Organisms adapted to extreme temperature, pH or osmolarity, respectively, belong most often to the domain of Archaea.

Biotechnological applications of thermophilic organisms

The potential of Archaea for industrial application is discussed since several years (Egorova et al., 2005; Schiraldi et al., 2002). To date, biotechnological processes predominantly utilize eukaryotic and bacterial microorganisms. Advantages of these organisms are the availability of established genetic systems, knowledge of molecular mechanisms for strain optimization and often experienced use due to application for decades. However, since the knowledge of archaeal strains constantly increases, these organisms may be a promising alternative to Bacteria and Eukaryotes as production strains for biotechnological processes. Genetic systems and expression vectors are under constant development (Choi et al., 2014; Hwang et al., 2015; Wagner et al., 2014). However, for industrial applications chromosomal manipulations are preferred over plasmid based expression with respect to selection pressure and process stability (Zeldes et al., 2015). Most industrial processes are performed with mesophilic strains under moderate temperature conditions. Compared to mesophilic organisms, however, the

application of thermophilic organisms lowers the risk of contaminations or phage infections, distillation of highly volatile compounds can be performed during cultivation, the substrate solubility is improved, unfavoured thermodynamic reactions can be improved and the cooling costs can be reduced (Frock et al., 2012; Zeldes et al., 2015). Viruses from *Sulfolobus* are known but none is lytic (Prangishvili et al., 2001).

Biofuel and hydrogen production by hyperthermophiles as well as synthesis of thermostable hydrogenases are examples for the use of Archaea for industrial processes (Atomi et al., 2011). Müller *et al.* described the biocatalytic production of bioalcohol using a hyperthermophilic Archaeon via a new synthetic route (Müller, 2014). For the thermophilic archaeon *Pyrococcus furiosus* it was shown that a single gene insertion of a bacterial alcohol dehydrogenase drives the bioalcohol production (Basen et al., 2014).

Application of thermophilic organisms for biocatalysis can circumvent the accumulation of toxic products and toxicity at low solvent titer by constant removal of toxic compounds e.g. by distillation or increased tolerance of the production strain embedded in a biofilm. Since a wide variety of different cultivation systems are established, setups can be optimized for archaeal strains (Qureshi et al., 2005).

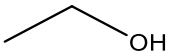
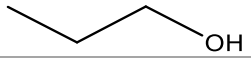
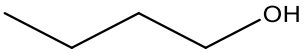
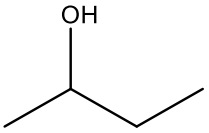
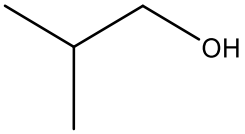
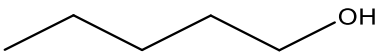
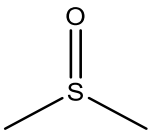
Organic solvents with industrial relevance

As described above, thermophilic Archaea offer potential for the synthesis of chemicals like organic solvents whose synthesis is limited to mesophilic strains. The interest in production of second generation biofuels is of great interest because the resources of fossil energy is limited. Second generation biofuels are produced from cellulose, hemicellulose or lignin, which are not used for food production. Solvents of industrial relevance are e. g. bulk-chemicals like second generation biofuels (ethanol, 1-butanol, 2-butanol and isobutanol), which can be produced in large scale by microorganisms as alternative to the synthesis via chemical production routes. Additionally, fine chemicals with insufficient synthesis routes are of industrial relevance for production by microorganisms.

Ethanol, isobutanol, 1-butanol and 2-butanol are potential second generation biofuels produced by microorganisms via whole cell biocatalysis (Akita et al., 2015; Z. Chen et al., 2015; Lan et al., 2013). The production of alcohols from (lignocellulosic) biomass as alternative for fossil fuels are of increasing importance and research on alternative production methods by microorganisms are ongoing (Kang et al., 2014; Westman et al., 2015). Since hydroxylation for

2-butanol synthesis by chemical methods leads to racemic products, regioselective hydroxylation by microorganisms offers a possible solution. Dimethylsulfoxide (DMSO) is a commonly used solvent and potentially be used as organic phase for 2-phase biocatalysis systems. The solvents used in this study are listed in Tab. 1-4.

Table 1-4: Overview of important parameters of organic solvents used in this study. All data from National Center for Biotechnology Information and PubChem Compound Database.

Solvent	Formula Structure	Molecular weight Density (20 °C)	Boiling point Solubility (water, 25 °C)	logP
Ethanol	C_2H_6O 	46.068 g/mol 0.789 g/cm³	78.29 °C Miscible	-0.31
1-Propanol	C_3H_8O 	60.095 g/mol 0.805 g/cm³	97.2 °C Miscible	0.25
1-Butanol	$C_4H_{10}O$ 	74.122 g/mol 0.810 g/cm³	117.6 °C 68 g/L	0.88
2-Butanol	$C_4H_{10}O$ 	74.122 g/mol 0.806 g/cm³	99.5 °C 181 g/L	0.61
Isobutanol	$C_4H_{10}O$ 	74.122 g/mol 0.802 g/cm³ (24 °C)	108 °C 66.5 – 90.9 g/L	0.76
1-Pentanol	$C_5H_{12}O$ 	88.150 g/mol 0.815 g/cm³	137.5 °C 22 g/L	1.51
Dimethyl sulfoxide (DMSO)	C_2H_6OS 	78.133 g/mol 1.100 g/cm³	189 °C Miscible	-1.35

The toxic effect of solvents on cells is thought to be a result of incorporation of solvent into the membrane lipid bilayer resulting in disruption of essential membrane function (Sikkema et al., 1995). Furthermore, solvents can lead to denaturation or inactivation of proteins. The toxicity and water solubility of molecules is defined by the octanol:water coefficient, logP and is the logarithm of the partition coefficient of a solvent in a standard octanol:water mixture (Inoue. A; Horikoshi, 1991). Compounds with low logP value are good miscible in water (e. g.

ethanol ad DMSO), whereas compounds with higher logP (e. g. 1-pentanol) are less miscible in water but more miscible in unpolar environments like unpolar solvents or lipid membranes. The toxicity of solvents increases with higher logP value, since a higher polarity leads to higher toxicity due to elevated accumulation into the membrane (Sardessai et al., 2002). The cytotoxicity of 1-butanol is based on interference of the alcohol with the phospholipid membrane (O. Simon et al., 2015). Due to high 1-butanol concentration the membrane fluidity is increased and the transmembrane ion gradient cannot be maintained (Isken et al., 1998; Osborne et al., 1990). Furthermore, the polarity properties of solvents have an influence on toxicity by affecting the cell-surface characteristics (Inoue. A; Horikoshi, 1991). Experiments showed that enzyme activity and the solvent parameter logP correlate directly and can therefore serve as a quantitative index for effects of solvents on enzymes. Experiments with different Bacteria showed that growth limitation correlates with logP, the solvent parameter. The solvent tolerance of Gram-negative Bacteria was found to be higher compared to Gram-positives.

1-Butanol as second generation biofuel

The search for alternatives of petrochemical energy sources with respect to the limitations of fossil fuels is of increasing importance. Currently, only ethanol is used as fuel additive. Compared to ethanol, 1-butanol offers some great advantages. Since one molecule of butanol contains twice as much carbon atoms compared to ethanol, butanol contains higher energy density per molecule. Additional advantages are less corrosiveness, lower volatility and hygroscopy and the possibility to use the existing infrastructure for storage (Durre, 2007). 1-butanol is of increased industrial interest since it offers a wide spectrum of applications, e. g. as bulk chemical for industrial processes, in plastic manufacturing or as second generation biofuel (Durre, 2007; Green, 2011). The production of 1-butanol can be performed either by chemical synthesis or by microbes through fermentation (“biobutanol”). To date, 1-butanol is mainly synthetic and produced via a petrochemical route, which is based on propylene oxo synthesis (Jiang et al., 2014). The production of biobutanol by microbes is performed by ABE (acetone, butanol, ethanol) fermentation with acetone and ethanol as by-products but is is limited by the toxicity of the end-products on the production strain (Durre, 2007). Additional limitations of Bacteria for industrial biosynthesis are spore formation, strain degradation and loss of plasmids (Antoni et al., 2007; Borden et al., 2007; Cornillot et al., 1997).

1.4. *Sulfolobus acidocaldarius* as archaeal model organism

One of the best studied Archaea is the thermoacidophilic strain *Sulfolobus acidocaldarius. S. acidocaldarius* belongs to the Crenarchaeota and was first isolated from acidic hot springs at the Yellowstone National Park (USA) (Brock et al., 1972). Optimal growth occurs at a temperature of approximately 78 °C and a pH of pH 2 – 3.5. *S. acidocaldarius* cells were described as irregular cells with distinct lobes and 0.8 – 1 µm in size (Brock et al., 1972). The *S. acidocaldarius* mutant strain MW001, lacking the *pyrEF*-cassette enabling for selection, showed pronounced effects on cell density and cluster formation after the deletion of adhesive pili and UV-induced pili (Henche et al., 2012b). The aap pilus (archaeal adhesive pilus) is the most abundant surface structure of *S. acidocaldarius* and proposed to be unique (Henche et al., 2012a). The chromosome of *S. acidocaldarius* was sequenced in 2005 and enabled the establishment of chromosomal manipulations like gene insertions or gene deletions as well as the development of expression vectors (Atomi et al., 2012; Berkner et al., 2008; Berkner et al., 2010; L. Chen et al., 2005; Wagner et al., 2009; Wagner et al., 2012; Wagner et al., 2014). The genetic manipulation is performed within the uracil auxotrophic strain *S. acidocaldarius* MW001, lacking the pyrEF-cassette, required for uracil biosynthesis.

The advantages and disadvantages of different potentially producers of biofuels are summarized in Fig. 1-4.

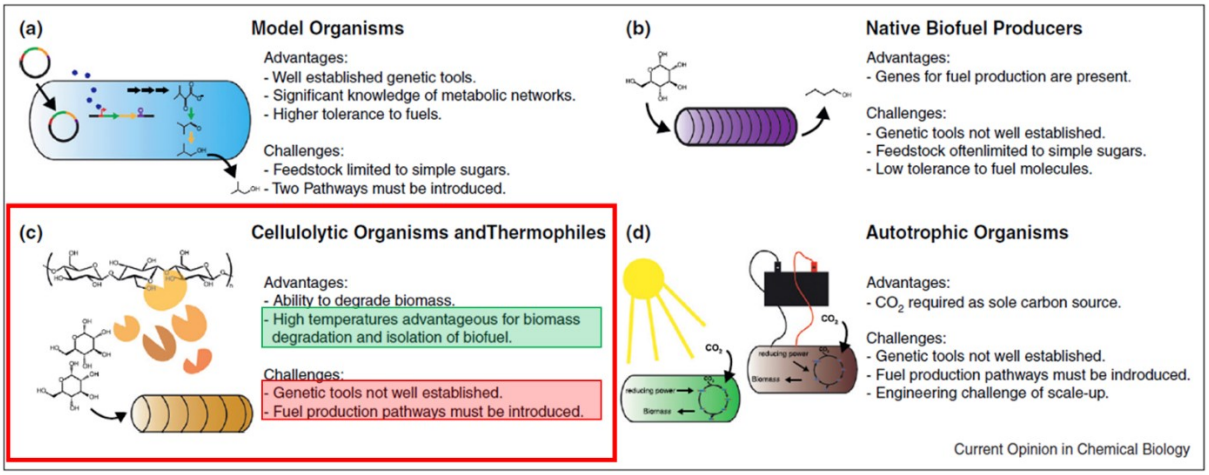


Figure 1-4: Approaches for producers of next generation biofuels. The thermoacidophilic Archaeon *S. acidocaldarius* can be grouped to the producers of category (c). Figure modified from (Gronenberg et al., 2013)

As shown in Fig. 1-4, *S. acidocaldarius*, as hyperthermophilic organism, offers the advantage of biomass degradation at high temperatures. The degradation of biomass at low pH and high temperature is possible as one-pot strategy with *S. acidocaldarius* as production strain. The

challenge of establishing genetic tools for thermophiles is solved for *S. acidocaldarius,* since chromosomal manipulations like gene insertion (knock-in) and gene deletion (knock-out) as well as expression plasmids are well established methods (Wagner et al., 2012). In industrial applications, chromosomal manipulations are especially preferred over plasmid based expression with respect to selection pressure and process stability (Zeldes et al., 2015). Information of the central carbohydrate metabolism and pathways of *Sulfolobales* are under constant research and offer the introduction of production pathways (Bräsen et al., 2014; Wolf et al., 2016).

In conclusion, *S. acidocaldarius* fulfils basic requirements for this study like its comparatively good characterization and advantageous growth requirements with respect to temperature and pH, its ability to form biofilms and its genetically tractability. The above mentioned advantages of biofilms and thermoacidophilic host strains for industrial applications makes *S. acidocadarius* a promising organism for biocatalytical production processes.

1.5. Aim of this work

Sulfolobus acidocaldarius is a thermoacidophilic Crenarchaeon with growth optimum at 78°C and pH 2-3. With these extreme growth requirements combined with the available genetic systems the organism offers high potential for biotechnological applications like the production of bulk and fine chemicals, solvents and second generation biofuels. However, such compounds are known to cause stress to biocatalysts. Therefore, production strains that form biofilms, which were shown to confer higher resistance towards solvent exposure and other stress conditions in Bacteria, turn more and more into research focus. Although the Archaeon *S. acidocaldarius* is known to form biofilms, the development of suitable incubation systems is still in its beginning and also the influence of organic solvents on Archaea in general and on *S. acidocaldarius* in particular has only rarely been studied. Thus, to further elucidate the application potential of *S. acidocaldarius* biofilms shall be comparatively analysed in the presence and absence of organic solvents and the results shall be compared to shaking cultures of the organism.

Therefore, (i) incubation systems for the cultivation of submerse biofilms of *S. acidocaldarius* cells on glass and polystyrene surfaces in microtiter plates, µ- and Petri-dishes have to be developed enabling qualitatively and quantitatively analyses using various microscopic techniques (like light- and epifluorescence, atomic force, scanning electron and confocal laser-scanning microscopy) and yielding sufficient amounts of biofilms for isolation and quantification of extracellular polymeric substances (EPS) as well as for application of omics methods. (ii) Using these newly developed methods, the influence of organic solvents like the industrially highly relevant 1-butanol as well as of ethanol, 1-propanol, 2-butanol, isobutanol and DMSO on the formation of biofilms, their structure and morphology as well as on EPS content and composition will be analysed. (iii) To further understand the genome wide molecular and regulatory mechanisms of *S. acidocaldarius* grown in different lifestyles and in the presence of 1-butanol, transcriptomic and proteomic approaches like iTRAQ und illumina sequencing will be applied.

2. MATERIALS AND METHODS

2.1. Chemicals

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.

Table 2-1: Overview of organic solvents applied for stress tests.

Solvent	Manufacturer	Purity
Ethanol	Fisher Scientific	99.9% GC
1-Propanol	Merck	≥ 99.5% GC
1-Butanol	Roth	≥ 99.5% p. a.
2-Butanol	Honeywell Riedel de Haën	99% GC
Isobutanol	Honeywell Riedel de Haën	≥ 99% GC
Dimethyl sulfoxide (DMSO)	Roth	≥ 99.8% p. a.

2.2. Materials

Table 2-2: List of consumables.

Consumable	Specification	Manufacturer	Application
6-Well Microtiter Plate	6 well cell culture plate, cellstar	Greiner Bio-one	Biofilm formation
96-Well Microtiter Plate	Sarsted Cell+	Sarstedt	Biofilm formation
96-Well Microtiter Plate	Mikrotestplatte 96 Well, F	Sarstedt	ADH-assay
µ-Dish	35 mm; high, ibiTreat	IBIDI	Biofilm-formation
Aluminium Foil	Alu-sealing tape, pierceable	Roth	Sealing of 6-well and 96-well microtiter plates, µ-dish
Breathe Easy Foil	Breathe-Easy® sealing membrane	Roth	Sealing of 96-well microtiter plates
Petri-dish	Petri-dish 92 x 16 mm, without cams	Sarstedt	Formation of submersed biofilms
Glass Coverslips	18 mm x 18 mm	Roth	Biofilm formation

2.3. Instruments

Table 2-3: Laboratory equipment.

Instrument	Specification	Manufacturer
Atomic force microscope	NanoWizard II	JPK Instruments
Analytical scales	TE124S TE601	Sartorius Sartorius
Autoclaves	H+P Varioclav 25T H+P Varioclav 75S	Federgari Autoklav, Integra Bioscience (IBS)
Centrifuges	5810 R Sorvall Centrifuge RC26	Eppendorf Kendro
Concentrator	Concentrator plus	Eppendorf
Clean bench	HERAsafe® KSP Class II Biosafety Cabine	Kendro Laboratory Products GmbH
Epifluorescence / phase contrast microscope	Eclipse Ni	Nikon
Epifluorescence microscope	AxioImager A1m	Zeiss
Incubator (static, 78 °C)	Heraus T20	Kendro
Incubator (static, 78 °C) (Flow-through experiments)	UL30ü	Memmert
Incubator, shaking, 78 °C)	Thermotron	Infors
Peristaltic pump	IPC, 8-Channel, ISM936	Ismatec
pH meter	WTW Series inoLab pH 720	WTW GmbH
Photometer	BioPhotometer Plus	Eppendorf AG
Plate reader	Infinite Pro M200	Tecan
Scanning electron microscope	QUANTA 400 FEG	FEI Company
Sonicator	UP 2008	Hielscher Ultrasonics GmbH
Speed Vac	Concentrator plus	Eppendorf

2.4. Software

Table 2-4: Software and databases.

Software	Version or link	Manufacturer	Application
BioCyc	http://biocyc.org/	SRI International	General protein information
ChemDraw	Professional 15.0.0.106	PerkinElmer	Drawing of structural formula
Confocal laser scanning microscope	LSM 510 Axiovision rel. 4.8.	Zeiss	Confocal laser scanning microscope
Imaris	8.1.2	Bitplane	Optimization of CLSM images
i-control	1.8.50.0	Tecan	Plate reader
JPK SPM Software		JPK Instruments	Atomic force microscope
KEGG	http://www.genome.jp/kegg/	Kanehisa Labs	Information about gene and protein sequences
HHPred	https://toolkit.tuebingen.mpg.de/hhpred	Dept. of Protein Evolution at the Max Planck Institute for Developmental Biology, Tübingen	Protein structure prediction
HSDB	http://www.ncbi.nlm.nih.gov/pccompound	U. S. National Library of Medicine	Information about chemicals
PubChem Compound	http://toxnet.nlm.nih.gov/newtoxnet/hsdb.htm	National Centre for Biotechnology Information (NCBI)	Information about chemicals
STRING	http://string-db.org/	SIB (Swiss Institute of Bioinformatics), CPR - NNF Center for Protein Research, EMBL (European Molecular Biology Laboratory)	Protein-protein interaction networks
Uniprot	http://www.uniprot.org	Collaboration platform of European Bioinformatics Institute (EMBL-EBI), the SIB Swiss Institute of Bioinformatics and the Protein Information Resource (PIR)	Protein sequence and annotation

2.5. Cultivation of *Sulfolobus acidocaldarius* DSM639

2.5.1. Medium composition

Sulfolobus acidocaldarius DSM 639 was received from the German Collection of Microorganisms and Cells (DSMZ). *S. acidocaldarius* DSM639 was cultivated in minimal Brock medium supplemented with 0.1% (w/v) N-Z-Amine (EZMix™ N-Z-Amine®, a casein enzymatic hydrolysate, from bovine milk, C4464, Sigma) and 0.2% (w/v) glucose, if not stated otherwise.

Table 2-5: Composition of single Brock solutions and final Brock medium according to *Brock et al.* (1972).

	Constituents	Amount per L stock solution	Amount per L final Brock medium
Brock I (1000x)	$CaCl_2$ x 2 H_2O	70 g	0.07 g
Brock II (100x)	$(NH_4)SO_4$	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 (200x)	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 µg
	$CoSO_4$ x 7 H_2O	6 mg	0.03 mg

50% (v/v) sulfuric acid and Brock I, II and III (Tab. 2.6) were autoclaved. Glucose and N-Z-Amine as well as glycerol and $FeCl_3$ x 6 H_2O were filter sterilized. For preparation of 1 L Brock minimal medium, autoclaved H_2O was used. All components were stored at room temperature (RT).

Table 2-6: Composition of 1 L of minimal Brock medium according to Brock *et al.* (1972).

Component	Volume for 1 L Brock medium
Brock I	1 mL
Brock II	10 mL
Brock III	5 mL
$FeCl_3$ x 6 H_2O (20% (w/v))	1
H_2SO_4 (50 % (v/v))	Adjust to pH 3
H_2O	Ad 1 L

Solid medium was prepared by addition of 6 mL 0.5 mM $CaCl_2$ (3 mM) and 10 mL 1 M $MgCl_2$ (10 mM) as well as 6 g L^{-1} gellan gum (Gelzan™, Sigma-Aldrich) to 1 L of liquid Brock medium.

For preparation of glycerol stocks, 40 ml of exponentially grown *S. acidocaldarius* were harvested (5,000 xg, 10 min, 4 °C), carefully resuspended with 2 mL glycerol-Brock solution and 200 µL aliquots were stored at -80 °C.

Table 2-7: Composition of glycerol Brock solution for glycerol cultures.

Component	Volume for 50 mL glycerol Brock solution
Brock I	50 µL
Brock II	500 µL
Brock III	250 µL
Glycerol (50% (w/v))	49,20 mL

2.5.2. Planktonic growth curves of *S. acidocaldarius* DSM639

Brock medium supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose was inoculated with a glycerol stock and incubated for two days. Subsequently, the culture was transferred into new medium and cultivated until reaching the exponential phase. This pre-culture was used for inoculating fresh medium to a start OD_{600} of 0.05 or 0.1, respectively (technical replicates). Generally, cultivation was performed at 78 °C with agitation of 180 rpm. Growth was monitored photometrically at 600 nm (BioPhotometer, Eppendorf). For alcohol quantification, 1 ml samples were taken at different time points, centrifuged (8,000 rpm, 3 min) and the supernatant was stored at -20 °C until quantification.

2.5.3. Standardized planktonic *S. acidocaldarius* pre-cultures for biofilm formation

An exponential grown pre-culture was prepared as described above and diluted with Brock medium containing 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose. In each experiment a start OD_{600} of 0.1 was used.

2.6. Static biofilm formation of *S. acidocaldarius*

2.6.1. Biofilm formation in 96-well microtiter plates

For the characterization of *S. acidocaldarius* planktonic and biofilm growth in 96-well microtiter plates an exponentially grown pre-culture was diluted to a final OD_{600} of 0.1. Cavities of 96-well microtiter plates (TC Plate 96 Well, Cell+, F from Sarstedt) were filled with either 150 µl of Brock medium containing 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose (abiotic control cavities A1-12, B1-12) or diluted *S. acidocaldarius* culture (OD_{600} 0.1). OD_{600} was measured prior to sealing the plate with foil. If not stated otherwise, the microtiter plates were sealed with aluminium foil (alu-sealing tape, pierceable, Roth). The plates were then incubated for four days at 78°C within a closed metal box containing a water reservoir to reduce evaporation of medium.

Determination of the effect of solvents on biofilm formation

Diluted *S. acidocaldarius* cultures and different solvents in different concentrations were mixed in 2 mL microcentrifuge vials. A total volume of 2 mL of each solvent concentration was prepared, mixed and 150 µl filled in each cavity. The schema is shown in Fig. 2-1. After sealing, the incubation was done for four days at 78°C inside a closed metal box with a water reservoir for reduction of medium evaporation.

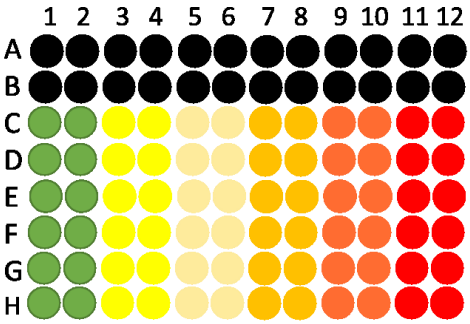


Figure 2-1: Pipetting schema of a 96-well microtiter plate. Black (A 1-12, B1-12): Medium (abiotic control). Green: Control without solvent, yellow to red: Increasing solvent concentrations. Two rows for each concentration.

Determination of the effect of solvents on established biofilms

To evaluate the effect of solvents on established biofilms, wells C 1-12 to H 1-12 were filled with *S. acidocaldarius* culture (OD_{600} 0.1) and incubated for four days. After incubation, the planktonic cells were transferred into a new 96-well microtiter plate and the absorbance was measured. The empty wells were directly filled with 150 µl new Brock medium, supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose, containing the different solvent concentrations. After exchanging the liquid phase, the plate was sealed again and incubated for one to four days at 78 °C as described before.

2.6.2. Static biofilm formation on glass surfaces in 6-well microtiter plates

For biofilm formation on glass surfaces, autoclaved glass coverslips (18 mm x 18 mm; Roth, Karlsruhe [0657]) were placed on the cavity bottoms of a 6-well microtiter plate (6 well cell culture plate, cellstar, Greiner bio-one, [657 160]). 4.5 ml of an exponentially grown *S. acidocaldarius* pre-culture (diluted to an OD_{600} of 0.1) supplemented with and without different solvent concentrations were applied for biofilm growth. The wells were sealed with aluminum foil (alu-sealing tape, pierceable, Sarstedt, Nürnbrecht [ref 95.4995]) and incubated for four days at 78°C inside a closed metal box with a water reservoir to prevent evaporation.

2.6.3. Cultivation of submersed biofilms in µ-dish

µ-Dishes (IBI-treat, 35 mm, high, IBIDI) were filled with 4 mL of an exponentially grown S. acidocaldarius pre-culture (diluted to an OD_{600} of 0.1). The dishes were sealed with

aluminum foil (alu-sealing tape, pierceable, Sarstedt, Nürnbrecht [ref 95.4995]) and placed into a big Petri-dish. The dishes were incubated for four days at 78 °C inside a closed metal with a water reservoir for reduction of medium evaporation. Since the submersed biofilms are quite instable, the dishes were treated very carefully.

2.6.4. Biofilm formation in Petri-dishes

For EPS extraction as well as for proteome and transcriptome studies, biofilm formation in Petri-dishes (Sarstedt) was performed. For this, an exponentially grown *S. acidocaldarius* pre-culture (diluted to a final OD_{600} of 0.1) was mixed with different concentrations of solvent to a final volume of 250 mL each. A sample of 1 mL for alcohol quantification was taken. 25 mL of diluted *S. acidocaldarius* cultures were filled in ten polystyrene Petri-dishes each. The-Petri dishes were placed into an anaerobe box to reduce the evaporation of medium and incubated for four days at 78°C.

2.7. Analysis of static biofilms

2.7.1. Characterization of biofilm formation in 96-well microtiter plates

Quantification of total absorbance and planktonic cells

Before sealing the plates, the absorbance at 600 nm was measured using a plate reader (Tecan, Switzerland). After incubation, the plate was cooled down to room temperature, the sealing foil was replaced by a plastic lid and the absorbance inside the cavities was measured again giving information about biofilm as well as planktonic growth. For quantification of only planktonic cells, the suspension inside the cavities was measured photometrically at 600 nm by transferring it to cavities of a new microtiter plate.

Quantification of biofilm

After removal of the planktonic cells, the biofilm was quantified by crystal violet staining. For this, 175 µL of 0.1% (w/v) crystal violet was added to each well, incubated for 20 min at RT and subsequently each well was washed three times with 200 µL water. The water was removed and the wells were dried completely at room temperature. After complete drying

200 µl of 95% (v/v) ethanol was added and incubated for 30 min. The absorbance was measured at 570 nm for biofilm quantification.

Resazurin viability assay

S. acidocaldarius was grown in 96-well microtiter plates as described before (section 2.6.1.). After removal of planktonic cells, the cavities were washed with 175 µL minimal Brock medium (pH 3). A resazurin stock solution (0.1% (w/v) in H_2O) was diluted in minimal Brock medium (pH 3) to a final concentration of 0.005 % (w/v). The Brock medium used for washing was discarded of each well and directly exchanged by 200 µl of the 0.005% (w/v) resazurin solution. The plates were closed with a plastic lid and incubated at 78°C inside a closed metal box with a water reservoir for three to six hours until the conversion of resazurin was completed. At neutral pH resazurin appears blue, which changes to violet in an acidic milieu. By metabolic respiratory activity of *S. acidocaldarius* the resurufin is converted to the colourless dihydroresurufin. The formation of colourless dihydroresurufin, as a measurement of respiratory activity, was measured photometrically at 520 nm, the maximum of resurufin at acidic pH.

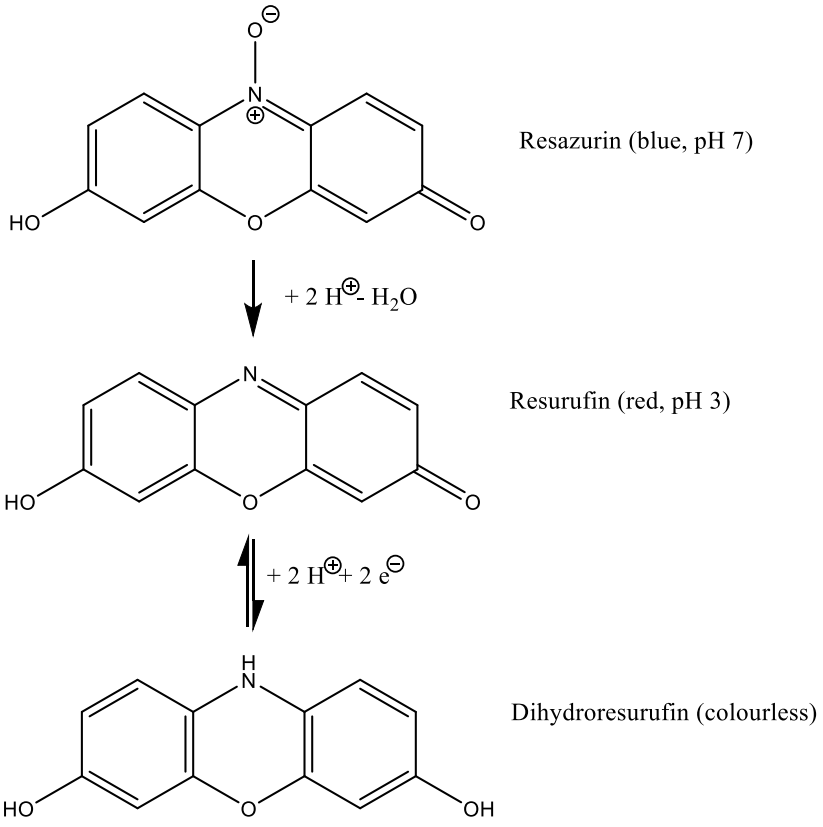


Figure 2-2: Conversion and colour change of resazurin.

2.7.2. Characterization of *S. acidocaldarius* biofilms grown in Petri-dishes

Determination of biofilm wet weight

For determination of biofilm wet weight, biofilms of at least three Petri-dishes were washed with 25 mL minimal Brock medium after removal of the cell suspension. Biofilm cells were isolated by using a cell scraper and transferred into microcentrifuge vials. The biofilms were harvested by centrifugation (5 min, 8,000 rpm, RT) and the supernatant was discarded. Biofilm wet weight was determined by using a fine balance.

Determination of total cell count

The total cell count (TCC) was determined by the DAPI (4',6-diamidino-2-phenylindole) - method. A decimal dilution series of a cell suspension was performed in minimal Brock medium (pH 3). 4.5 mL cell suspension were mixed with 1 ml 4',6-diamidino-2-phenylindole (DAPI, 25 µg/ml in 2% (v/v) formaldehyde; D9542 Sigma-Aldrich) and incubated for 20 min in the dark. Afterwards the stained cells were filtered onto black polycarbonate membrane filters (pore size 0.2 µm, 30 mm diameter, Millipore). Filters were either stored protected at 4 °C (protected from light) or counted immediately using an Epi-fluorescence microscope. For this, the filters were fixed on an objective slide with CitiFluor AF2 (CitiFluor Ltd, London, GB) and covered with a coverslide. 20 grids with 20-200 cells/grid were counted for statistic validity.

2.7.3. Microscopic analysis of biofilms grown on glass slides

Visualization of biofilms using scanning electron microscopy (SEM)

After incubation, the planktonic cells were discarded and the cavities washed with 5 ml minimal Brock medium (pH 3). Cells were fixed by addition of 2% (v/v) glutardialdehyde in Brock medium (pH 3) and incubated for 2 hours at 4°C. Afterwards, the glass slides were transferred into acetone for around 30 min at RT. Biofilms were dried by critical point drying and sputtered with Au/Pd (80 %/20 %) for 15 to 30 s, resulting in a metal layer of 2.5 nm to 5.5 nm in thickness. Images were taken using a scanning electron microscope (QUANTA 400 FEG, FEI company).

Visualization of biofilms by light- and epifluorescence microscopy

After incubation the planktonic cells were discarded and the biofilm was stained with 0.01% (w/v) crystal violet for 20 min. Remaining crystal violet was removed by washing three times with 5 ml of ddH_2O. The glass slides were dried and used for microscopy. For detection of cells by fluorescence, cells were additionally stained using DAPI (see section 2.7.2.). Images were taken using 4x, 40x and 100x air objective and Nikon light- and epifluorescence microscope (Eclipse Ni, Nikon).

Visualization by atomic force microscopy (AFM)

Two different incubation conditions were used for visualization of biofilms grown on coverslips. In both cases the coverslips were incubated in the presence of diluted *S. acidocaldarius* with an OD_{600} of 0.1. For microscopy of biofilms grown in the presence of 1% (v/v) 1-butanol, *S. acidocaldarius* was diluted to an OD_{600} of 0.1 and incubated with Brock medium supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose. The 6-well microtiter plate was sealed with aluminum foil. After incubation slides were washed with 5 mL minimal Brock medium (pH 3) and incubated with 2 mL minimal Brock medium (pH 3) and 1 mL DAPI solution (25 µg/ml) for 30 min at RT. Finally, the slides were washed with 5 mL minimal Brock medium (pH 3), dried and used for microscopy. Biofilms were first examined by epifluorescence microscopy (Zeiss, AxioImager A1m) and afterwards analysed by Atomic Force Microscope (NanoWizard II, JPK Instruments) with cantilever CSC37 by using the intermittent contact mode. Target amplitude was set to 8 V and the scan velocity was 0.3 Hz.

2.7.4. Microscopic analysis of submerse biofilms

After incubation as described above (section 2.6.3.) the planktonic cells were discarded and 1 mL minimal Brock medium (pH 7) was added. The following steps were performed by using minimal Brock medium pH 7 for compatibility with the applied dyes and lectins. For staining of the submersed biofilms, 1 ml of a fluorescence mix containing the DNA binding dye SYTO9 and the lectins Concanavali (ConA) and IB4 was prepared. All dyes were purchased from Invitrogen (Karlsruhe, Germany) and diluted according to the instructions. ConA, preferred binding of α-mannopyranosy- and α-glucopyranosyl residues was labelled with Alexa-633 (excitation: 632 nm, emission: 647 nm). Isolectin GS-IB4 (from *Griffonia simplicifolia*, IB4), favoured binding of α-D-galactosyl and N-acetyl-D-galactosamine residues, was labelled with

Alexa-568 (excitation: 578 nm, emission: 603 nm). For each fluorescence mix 0.5 mM SYTO9 was applied (excitation: 483 nm, emission: 503 nm). The fluorescence mix for staining with the lectins IB4 and ConA in parallel contained 15 µg ConA and 30 µg IB4. For staining of cells as well as carbohydrates, 1 ml of a mix containing SYTO9, ConA and IB4 in Brock medium was added and incubated for 30 min. After staining, the supernatant was removed, the biofilm was washed twice with 1 ml minimal Brock medium (pH 7) and finally 2 ml of minimal Brock medium (pH 7) was added. The visualization was performed by using a Zeisss LSM 510 microscope with 100x oil objective. The data was processed with Imaris 8.1.2 software (Bitplane AG, Zürich, Switzerland).

2.8. Cultivation and analysis of *S. acidocaldarius* under flow-through conditions

Flow-through systems were incubated within an incubator (Memmert) at 75 °C. Flow-through systems were adapted for incubation at high temperature in terms of tubing, bubble-trap and biofilm compartment. The bubble trap was designed to have the in- and outlet connection on different positions: the inlet was at the upper and the outlet at the lower position. The volume of the bubble trap was 27.5 ml. Tubing were chosen with regard to temperature and pH stability as well as gas diffusion. All tubing were tested whether the material inhibits or promotes growth of *S. acidocaldarius* by release of compounds from the tubing material. Tubing tested were Norprene tubing, silicone platinum, C-Flex Clear and Viton tubing. For all tubing it has been necessary to boil the tubing in water for 15 to 30 min. Before use, the tubing system was autoclaved or sterilized by incubation in 70 % (v/v) ethanol. Flow was generated by using a peristaltic pump (Ismatec IPC, ISM936). Tubings were connected with Luer connectors and connectors (straight) with 1.6 x 1.6 mm in size.

Table 2-8: Overview of the applied tubings.

Tubing	Manufacturer	Size
C-Flex Clear	Cole-Palmer	1.6 x 3.2 mm
Silicone Platinum	Cole-Palmer	1.6 x 3.2 mm
Norprene Alim	Cole-Palmer	1.6 x 4.8 mm
FDA Viton Prec Tubing	Masterflex	Size 14
PharMed BPT	Masterflex	1.52 x 3.02 mm

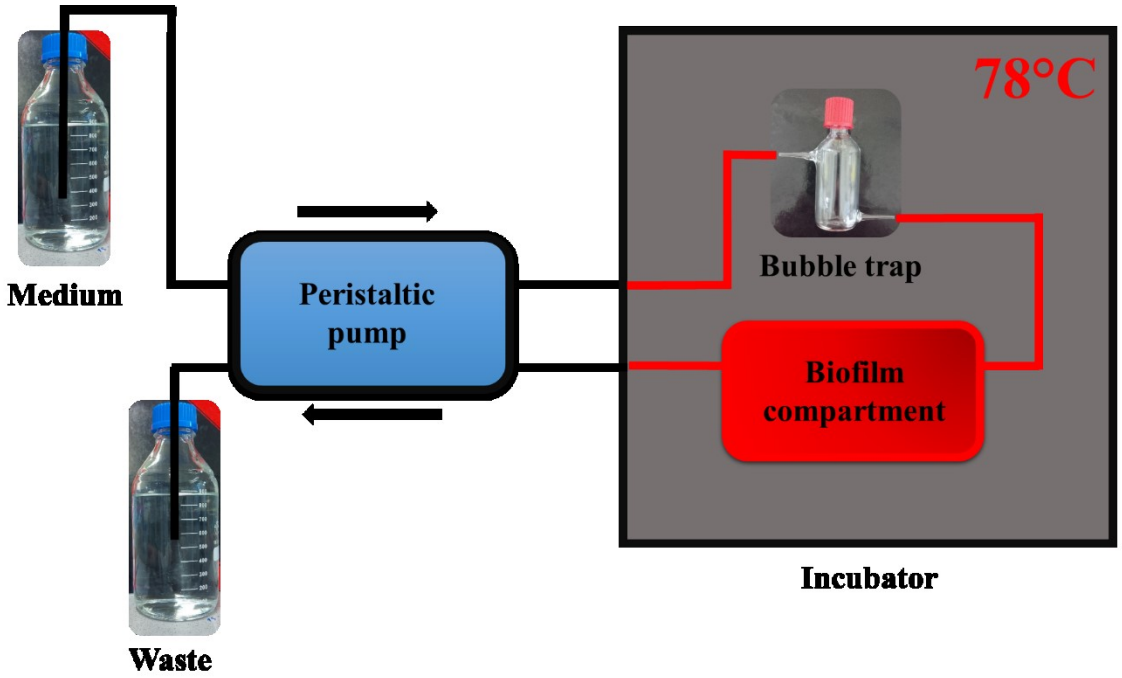


Figure 2-3: Schematic overview about the incubation of the flow-through systems.

2.8.1. Tubing systems

For biofilm formation within tubings the Norprene tubing were chosen. *S. acidocaldarius* was grown in Brock medium (pH 3) containing 0.1% (w/v) N-Z-Amine and 0.2% (w/v) dextrin. Norprene tubing was incubated over night with a pre-culture of *S. acidocaldarius* under static conditions. Afterwards the tubing was inserted into the flow system and incubated for two weeks with Brock medium (pH 3) supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) dextrin with a flow rate of 200 µl/min.

After incubation the biofilm compartment was cut into half, dried over night at 55°C. For scanning electron microscopy, the tubing piece was sputtered with Au/Pd for 4 min resulting in 52 nm coating with metal. Images with scanning electron microscope were performed as described before.

2.8.2. µ-Slide

The µ-slide (ibi-treat, Luer, 0.8 mm, IBIDI) was inserted into the flow system as a biofilm compartment. Elbow luer connectors (1.6 mm, IBIDI) were applied for connecting the µ-slide to the systems. The connectors were sealed with silicone (Pattex Express Silikon, Henkel) and placed into a water bath to generate constant incubation temperature inside the incubator. The µ-slide was incubated statically overnight with an undiluted *S. acidocaldarius* pre-culture

in the late exponential phase grown in Brock medium (pH 3) supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose. For this, the µ-slide was placed in a metal-box containing a water reservoir for prevention of evaporation. Subsequently, the µ-slide was inserted into the flow system and Brock medium (pH 3) containing 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose was pumped through the system with a flow-rate of 200 µL/min. Incubation time was three days. Finally, the biofilm was stained with SYTO9 (2.5 µM in ddH_2O) for 30 min, washed with millipore water and visualized by confocal laser scanning microscopy.

2.9. EPS extraction

For EPS extraction, submersed biofilms of *S. acidocaldarius* were grown and isolated as described in section 2.6.4. The weight of the isolated biofilm suspension was determined and 6 mM phosphate buffer (10 ml per 1 mL suspension; 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) was added to the biofilm suspension. EPS extraction was performed using the cation exchange resin Dowex® (CER, Dowex® Marathon® C sodium form, Sigma-Aldrich). For hydration, Dowex was washed twice with phosphate buffer (15 min, 1 g Dowex per 10 mL phosphate buffer). Afterwards 1 g hydrated Dowex and 10 ml biofilm-suspension were mixed and shaken at highest capacity on a shaker (Vortex Genie®2, Scientific Industries) for 20 min. Afterwards the samples were centrifuged (20 min, 20,000 x g, 4 °C) and the supernatants were filter-sterilized (pore size 0.22 µm, Rotilabo®, Roth). A sample was taken containing high- and low molecular weight EPS components. To remove low molecular weight components, the filter-sterilized supernatant was dialyzed against millipore water (3,5 kDa molecular weight cut-off (MWCO; Spectrum Laboratories)).

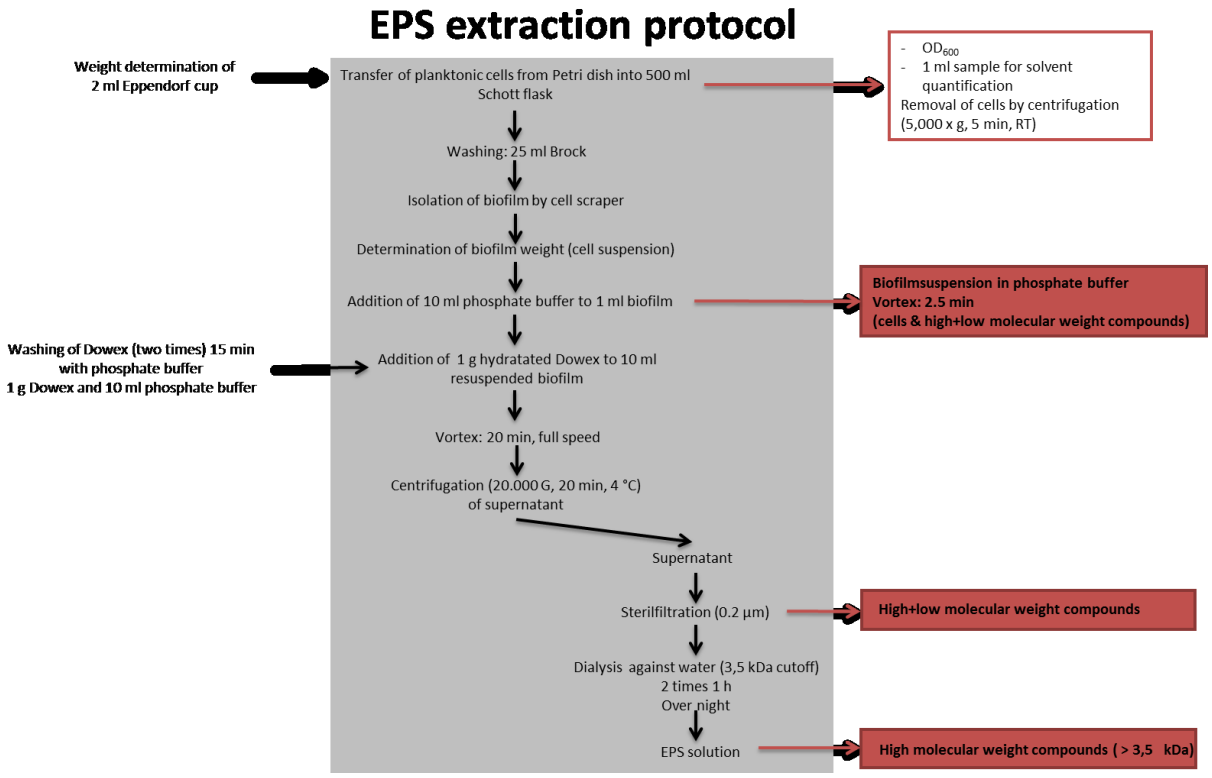


Figure 2-4: EPS extraction protocol. The EPS extraction was performed by using the cation exchange resin DOWEX. The samples used for cell and EPS quantification are shown in red colour.

2.10. Biochemical analysis of EPS carbohydrates and proteins

Quantification of carbohydrates and proteins were performed by colorimetric assays.

2.10.1. Quantification of Carbohydrates

The quantification of neutral carbohydrates was performed according to DuBois (Dubois et al., 1956). In 2 mL microcentrifuge cups (triplicates) a three-point calibration using D-glucose as standard with concentrations of 0, 25, 50 and 75 µg/mL was performed. 250 µL phenol (0.5% (w/v)) and 1.25 mL sulfuric acid (95%-97%) were added to 250 µL sample or water (blank) and mixed immediately. Samples were first incubated for 10 min at room temperature, subsequently for 15 min at 30 C in a water bath and finally for 5 min again at room temperature. 250 µL were then transferred into a 96-well microtiter plate and absorbance was measured at 490 nm using a plate reader.

2.10.2. Quantification of Proteins

Lowry-method:

For protein quantification of EPS samples, a modified Lowry assay was applied (Peterson 1997). Lowry reagent (Sigma-Aldrich L3540) was dissolved in 40 mL deionized water.

Folin-Ciocalteu's phenol reagent (Sigma-Aldrich F9252) was diluted with deionized water in a ratio of 1:5.

Bovine serum albumin powder (BSA, Sigma-Aldrich P5619; 2 mg) was dissolved in 5 mL deionized water to a final concentration of 400 µg/mL.

Protein quantification assay was performed in 2 mL microcentrifuge vials. 250 µL sample (or standard or water (blank), respectively) were mixed with 250 µL Lowry reagent and incubated for 20 min at room temperature. Subsequently, 125 µL diluted Folin-Ciocalteu´s phenol reagent was added, mixed and incubated for 30 min at room temperature. Finally, 250 µL of each sample/standard/blank were transferred to a 96-well microtiter plate and absorbance was measured at 750 nm.

A calibration using bovine serum albumin as protein standard was performed using concentrations of 0, 20, 40 and 60 µg/mL. Protein quantification was done in triplicates.

Bradford-method:

For protein quantification of concentrated EPS samples and *S. acidocaldarius* crude extract the Bio-Rad protein assay based on the method of Bradford was applied (Bradford, 1976). A standard calibration was performed using BSA in a concentration range from 0 – 10 µg/mL. Standards and samples were diluted in ddH_2O to a final volume of 600 µL and 400 µL of Bradford reagent (Bradford Quick Start, Bio-Rad) was added. After incubation in the dark for 5 min at room temperature absorbance was measured at 595 nm using a photometer (BioPhotometer, Eppendorf).

2.11. Sodium dodecyl-sulfate-polyacrylamide gelelectro-phoresis (SDS-PAGE)

2.11.1. Lysis and separation of *S. acidocaldarius* membrane fraction and crude extract

Exponentially grown *S. acidocaldarius* cells were harvested (9,000 rpm, 3 min, RT) and resuspended in Lysis-Equilibration-Wash buffer (LEW-buffer: 50 mM NaH_2PO_4, 300 mM NaCl, pH 8). After sonication (amplitude 50%, time cycle 0.5, 4 times 2 min on ice) membrane fraction and crude extract were separated by centrifugation (13,300 rpm, 45 min, 4 °C).

2.11.2. Concentration of EPS protein

Since the protein concentration of EPS proteins after isolation was too low for visualization via SDS-PAGE, the volume of the EPS fraction was reduced to increase the protein concentration. For this, a concentrator (Concentrator plus, Eppendorf) was used.

2.11.3. Sodium dodecyl-sulfate-polyacrylamide gelelectrophoresis of EPS proteins (SDS-PAGE)

EPS proteins were separated by SDS-PAGE using 12.5% (w/v) polyacrylamide separation gels. The lower separation gel (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) and 0.067% (v/v) N,N,N´,N´-tetramethylethylenediamine (TEMED)) was poured into the casting chamber and overlaid with isopropanol (100%). Polymerization time was 20 min, isopropanol was then removed and the stacking gel (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) was poured on top. The comb was inserted and after 20 min of polymerization the gel was stored at 4°C or used directly.

Protein samples were mixed with loading buffer (final concentration: 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 for 10 min at 95°C. Approximately 10 µg protein samples and 5 µl protein size marker (PageRuler Unstained Protein Ladder, Fermentas) per lane were loaded onto the gel. Electrophoresis was performed within the Mini-Protean 3-System (Bio-Rad) in electrophoresis buffer (Rotiphorese® 10x SDS-PAGE, Roth). For focussing the proteins

within the stacking gel a voltage of 100 V was applied. Subsequently, protein separation was performed at 200 V.

Finally, protein bands were stained with Coomassie Brilliant Blue solution (0.05% (w/v) Coomassie Brilliant Blue (CBB) G-250 (B0770 Sigma) 40% (v/v) ethanol and 10% (v/v) acetic acid) for 20 min at room temperature and destained with water. Gel documentation was performed using a flatbed scanner (LIDE110, Canon).

2.12. Transctriptomics

The transcriptomic analyses were performed by Andreas Albersmeier in the working group of Prof. Dr. Jörn Kalinowski at the University of Bielefeld.

2.12.1. RNA isolation

Cells grown in 7 different conditions including Biofilm-Control (BF0), Biofilm-0.5% Butanol (BF05), Biofilm-1% Butanol (BF1), Planktonic-Control (PL0), Planktonic-0.5% Butanol (PL05), Planktonic-1% Butanol (PL1) and Planktonic-Shaking culture (SC0) were harvested and immediately frozen. Several biological replicates were pooled for each condition for sufficient cell material and to minimize biological variation within a single sample.

RNA was isolated using Trizol (ThermoFisher, Waltham, USA) as described earlier (Hottes et al., 2004). The obtained RNA samples were treated with RNase-free DNase (Qiagen) and afterwards purified with ethanol precipitation. Ribosomal RNA was depleted using a RiboZero magnetic kit for Bacteria (Epicentre, Madison, USA) with a modified protocol. Briefly, 90 µl magnetic beads were used and for the rRNA removal reaction 1 µg RNA was mixed with 4 µl removal solution in a total volume of 20 µl.

2.12.2. RNA sequencing and data analysis

Sequencing libraries for all samples were prepared with the TruSeq® Stranded mRNA HT kit (Illumina, San Diego, USA) starting with the RNA fragmentation step after elution of precipitated RNA in 19 µl of the Fragment-Prime-Mix.

Sequencing libraries were quantified with a High-Sensitivity Chip on a Bioanalyzer (Agilent, Böblingen, Germany) and a measurement with a Quant-iT PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, USA) on a Microplate Reader Tecan Infinite 200 (Tecan, Männedorf,

Switzerland). Sequencing was performed on a MiSeq instrument (Illumina) using v3 chemistry with a read length of 2x76 nt. Sequencing reads were mapped with Bowtie2 (Langmead et al., 2012) against the reference genome *Sulfolobus acidocaldarius* DSM639. Mapped reads were counted and normalized as RPKM values (Mortazavi et al., 2008) using the software ReadXplorer (Hilker et al., 2014). In contrast to the original value, only reads mapping to coding sequences were considered for the calculation of the total number of mapped reads.

For identification of differentially transcribed genes, the ratios between the RPKM values obtained in different conditions for a single gene were calculated. Additionally, an A-value was determined for all genes in each comparison ($0.5 * Log_2$(RPKM Condition1 * RPKM Condition2)). Only genes with a minimum ratio of 4 (or 2, depending on the comparison) and an A-value ≥ 2 were considered as differentially transcribed.

2.13. Proteomics

The proteomic analyses were performed by Trong Khoa Pham in the working group of Prof. Dr. Philip Wright at the University of Sheffield (UK).

2.13.1. Protein extraction and iTRAQ labelling

Frozen cells of *Sulfolobus acidocaldarius* grown in 6 different conditions including biofilm control (incubated without 1-butanol, BF0), biofilm-0.5% (v/v) 1-butanol (BF05), biofilm-1% (v/v) 1-butanol (BF1), planktonic-control (PL0), planktonic-0.5% (v/v) 1-butanol (PL05) and planktonic Shaking-Culture (SC0) (see Tab. 2-09) were washed twice with ice-cold water before being resuspended in 1 mL of protein extraction buffer containing 0.05% SDS (w/v) in 500 mM triethylammonium bicarbonate (TEAB) pH8.5. Protein extraction was performed using an ultra sonicator (Sonifier 450, Branson) for 8 times at 70% duty cycle (alternatively 45 sec of sonication and 45 sec on ice). Proteins were then collected by centrifugation at 21,000 g at 4°C for 30 min, and protein concentrations were determined using a Bradford assay (Sigma, UK).

A total of 100 µg proteins from each phenotype was used for an 8-plex iTRAQ analysis with 6 iTRAQ tags used, and the analysis was performed based on the manufacturer's instruction. Briefly, these proteins were reduced by 2 µl of 50 mM tris- (2-carboxyethyl) phosphine (TCEP)

at 60°C for 1h, and alkylated by 1 µl of 200 mM methyl methanethiosulfonate (MMTS) for 10 min at room temperature before being digested by trypsin MS grade (Promega, UK) with the ratio of trypsin:proteins 1:20. Digested proteins from 6 different growth conditions were then labelled with iTRAQ reagents as shown in Table 1, and incubated at room temperature for 2 hours. All labelled peptides were then combined before being dried in a vacuum concentrator (Eppendorf Concentrator 5301, Germany).

Table 2-9: The iTRAQ labelling of samples

No	Sample	iTRAQ reagent
1	Biofilm-Control (BF0)	113
2	Biofilm-0.5% Butanol (BF05)	114
3	Biofilm-1% Butanol (BF1)	115
4	Planktonic-Control (PL0)	116
5	Planktonic-0.5% Butanol (PL05)	119
6	Planktonic-Shaking-Control (SC0)	121

2.13.2. Hydrophilic Interaction Chromatography (HILIC) for fractionation of labelled peptides

Dried iTRAQ labelled peptides were resuspended in 100 µl of HILIC buffer A containing 10 mM ammonium formate in 80% acetonitrile (ACN) pH3.0 before being loaded onto a 4.6 x 200 mm Poly Hydroxymethyl-A column (5µm, 200Å, Hichrom Limited, UK) coupled with an uHPLC 3000 system (Dionex, Germany). An UV detector was used to monitor peptides' abundance at a wavelength of 280 nm. Peptides were fractionated at a flow rate of 0.5 ml/min using a gradient with HILIC buffer B containing 10 mM ammonium formate in 5% ACN pH5.0: 10 min of buffer B before ramping up to 20% of buffer B for 5 min, then up to 60% of buffer B for 50 min, then ramped up to 100% of buffer B for 10 min and kept for 10 min, and finally 0% of buffer B for 5 min. Eluted peptides were collected every two minutes, then dried in a vacuum concentrator before being cleaned using C18 spinning tips (Nest Group, USA) before submitting to a mass spectrometer (MS).

2.13.3. Nano LC-MS/MS analysis

Selected cleaned peptides (from different fractions) were redissolved in 20 µl of buffer A consisting of 0.1% formic acid (FA) in 3% ACN then combined into 6 different fractions before

3 µl of sample was withdrawn and submitted onto a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo, Germany) coupled with a nano uHPLC 3000 system (Dinonex, UK) operated at a flow rate of 0.3 µl/min. Peptides were separated using a C18 column with a 105 min gradient of buffer B (0.1% FA in 97% ACN) as follow: 3% for 5 min, then ramped up to 10% for 5 min, 50% for 75 min, 90% for 1 min, then kept at 90% for 4 min before ramped back to 3% buffer B for 1 min then maintained at 3% for 14 min.

The MS was operated in positive mode with resolutions of full MS and ddMS2 set at 60,000 and 15,000 respectively. AGC targets were set at 3.106 and 5.104 for full MS and ddMS2 respectively; maximum IT times were set at 100 and 20 ms for full MS and ddMS2 respectively; a full mass scan ranging from 375 to 1500 m/z was applied for MS while the mass scan of 100 - 1500 m/z was applied for ddMS2; default charge state of ion was set at 2.

2.13.4. Identification and quantitation of peptides/proteins

All raw data files from MS analysis were submitted to MaxQuant version 1.5.3.8 for protein identification against *S. acidocaldarius* database (consisting of 2,222 entries) downloaded in August 2015 from Uniprot (http://www.uniprot.org). Modifications of iTRAQ reagents (on N-terminal and Lysine residue) and MMTS were set as fixed modifications while methionine oxidation was set as variable modification; trypsin digestion used with max missed cleavages of 2; minimum peptide length of 6 and maximum peptide mass of 4600 Da were set; tolerances of 20 and 4.5 ppm were applied for MS and MS/MS, respectively. A False Discovery Rate (FDR) of 0.01 was used for identification of both peptides and proteins; a minimum score of 40 was used for modified peptides.

All peptides containing intensities of iTRAQ reagents, from MaxQuant, were then submitted to an in-house proteomic pipeline for quantitation of protein and determination of regulated proteins (Bewley et al., 2011; Pham et al., 2010). Firstly, data imported from MaxQuant was filtered to remove both reversed and potential commination peptides, then proteins identified/quantified by a single peptide were removed before intensities of iTRAQ reporters of peptides being transformed into ln form for further analyses. The quantitation was also done using mean and isobaric corrections. T-tests (α = 0.01) were then performed at the peptide level (peptides corresponding to an identified protein) to determine regulated

proteins for each phenotype comparison. Proteins with their p-value $\leq$ 0.01 from the t-tests were then considered as regulated proteins.

3. RESULTS

In the course of the present study the response of *Sulfolobus acidocaldarius* towards different organic solvents was investigated with a major focus on biofilm formation. Therefore, different incubation systems were established and optimized for the growth of *S. acidocaldarius* challenged by the extremophilic growth conditions and the loss of medium as well as solvent due to evaporation. Different incubation systems such as 96- and 6- well microtiter plates, µ-dishes and Petri-dishes were used to study static cultivation and biofilm formation of *S. acidocaldarius*. In addition, since no flow-through incubation system for biofilm formation of thermoacidophilic organisms has been established so far, different systems were investigated for suitability for the analysis of biofilm formation in thermoacidophiles.

Biofilms were visualized by different microscopic methods such as light- and epifluorescence microscopy, confocal laser scanning microscopy, atomic force microscopy and scanning electron microscopy. Furthermore, EPS was isolated and quantified in respect to carbohydrate and protein content as reported previously by Jachlewski *et al.* (Jachlewski et al., 2015). Further on, the cellular response of *S. acidocaldarius* in respect to different lifestyles (shaking culture and static planktonic and biofilm) as well as the effect of 1-butanol exposure was examined by transcriptomic and proteomic analyses.

3.1. Incubation systems

3.1.1. Establishment of incubation systems

For all experiments, *S. acidocaldarius* was cultivated in Brock-medium (pH 3) supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose at 78°C. Experiments with shaking cultures revealed that N-Z-Amine as sole carbon source leads to an increase in pH over time and therefore a pH-stress for the cells. Since only the stress response on organic solvents should be investigated, glucose as second carbon source was added which keeps the pH constant. In previous studies the medium was partly exchanged on a daily basis (Koerdt et al., 2010). However, since medium exchange disturbs the biofilm formation, constitutes a physiological stress to the cells and causes medium as well as solvent evaporation, a long-term stable system is required for this study. By excess of nutrients and a stable pH over the cultivation

time, the established systems were stable and optimized to investigate the stress response on one single factor like solvent stress.

All incubation systems were investigated with respect to medium and solvent loss as well as growth of *S. acidocaldarius*. The evaporation of medium and solvent 1-butanol in 96-well microtiter plates could be minimized by application of gas-impermeable aluminum foil instead of gas-permeable foil (breathe easy). The 1-butanol loss after incubation for 4d at 78°C was reduced from 97% to 7% (v/v) and the reduction of total volume from 58% to 7% (v/v). However, the evaporation of 1-butanol was strongly influenced by the volume of the gas phase inside the cultivation compartment since the size of the gas phase determines the saturation capacity of solvent. Moreover, the material like glass or polystyrene has an important influence on the experiments. Since various parameters have an influence on the cultivation system each system was optimized separately leading to differences in the applied 1-butanol concentrations. Incubation of *S. acidocaldarius* in glass Petri-dishes revealed increased planktonic growth of cells, whereas polystyrene Petri-dishes enabled increased biofilm growth. Since in this study the focus was on biofilm formation, polystyrene Petri-dishes were applied for later experiments.

3.1.2. Overview about different incubation systems

In general, static cultivation for biofilm formation and static planktonic cells was performed for 4d at 78°C. Shaking cultures were incubated with agitation at 78°C until the growth curves reached late log phase. For static cultivation, Brock medium was inoculated with *S. acidocaldarius* to an OD_{600} of 0.1, whereas the medium for shaking cultivation was inoculated to an OD_{600} of 0.05.

S. acidocaldarius was cultivated in static and shaking incubation systems. For biofilm formation, planktonic *S. acidocaldarius* cells were incubated in 6- and 96-well microtiter plates, µ-dishes and Petri-dishes on polystyrene and glass surfaces for 4d at 78°C. The side and bottom surfaces of wells and dishes as well as inserted glass slides on the bottom served as substrata for biofilm formation. After incubation, the attached cells were defined as biofilm cells and the suspended, not-attached cells, were defined as planktonic cells. Planktonic cells grown with agitation in Erlenmeyer flasks at 78°C until growth curves reached the late stationary phase were defined as shaking culture.

The influence of organic solvent exposure on *Sulfolobus acidocaldarius* cells was investigated in different incubation systems and on different substrata. The cultivation of *S. acidocaldarius* in 96-well microtiter plates is most applicable for the screening of different solvent concentrations and the effect on biofilm and planktonic growth as well as cell viability based on detection of respiratory activity by resazurin. Light-, epifluorescence-, atomic force- and scanning electron microscopy were applied for detailed visualization of single cells as well as biofilms tightly attached on glass surface after growth for 4d at 78°C. To gain sufficient amount of biomass for EPS-isolation and quantification as well as transcriptomic and proteomic analyses, *S. acidocaldarius* was incubated in Petri-dishes. To prevent medium as well as solvent evaporation at incubation temperatures of 78°C, the Petri-dishes were placed inside anaerobe boxes. A schematic overview of the different cultivation conditions is shown in Fig. 3-1. Since the different incubation systems and methods have individual advantages, e. g. application of microscopic methods or biofilm amount, the combination of the different methods allows a broad insight into the cellular response upon solvent exposure.

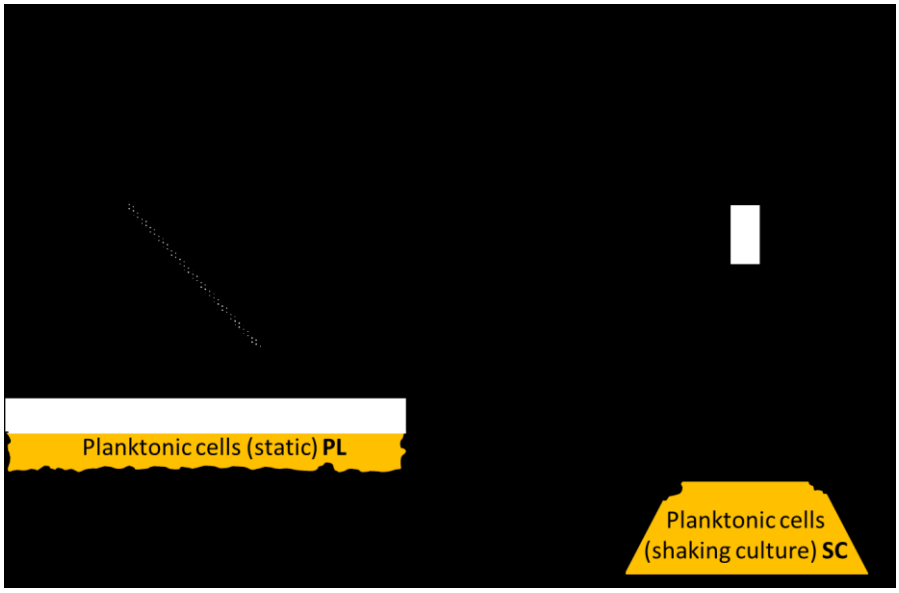


Figure 3-1: Overview of different systems for cultivation of *S. acidocaldarius*. Planktonic (PL) and biofilm (BF) cells were observed in static systems and shaking systems (SC). For analysis of biofilm formation, the static incubation systems with biofilm and planktonic cells was applied. Shaking cultures were applied to address changes of different lifestyles.

3.1.3. Flow-through systems

Since no flow-through incubation systems are published for thermo(acido)philic Archaea or Bacteria, a capable system for cultivation of *S. acidocaldarius* under flow-through conditions was newly developed. A schematic overview of the setup is given in the materials and methods, section 2-8. Shortly, medium was pumped through acid- and thermostable tubing by a peristaltic pump into a biofilm compartment placed inside an incubator with an incubation temperature of around 78°C. In this study the inner wall of a tubing and a commercial flow-cell (IBIDI) was applied for biofilm formation.

Since flow-through systems offer various advantages over static systems like continuous medium exchange and long term stability, its application for thermoacidophilic Archaea is highly necessary. In this study, different systems and cultivation methods were tested. Major obstacles for the establishment of a flow-through systems were medium evaporation, thermostability of materials, biocompatibility and heating. For this, applicable tubing as well as biocompartments were tested. For heating of the biofilm-compartment a modified incubator harbouring an opening for tubing transfer was used. The systems applicable for cultivation of *S. acidocaldarius* are shown in the following section. However, the cultivation system can be further improved with respect to the biocompartment. For all systems, the outflow contained *S. acidocaldarius* cells, indicating planktonic growth or release of biofilm cells to the medium. However, this observation showed that the tubing are biocompatible and allow for growth of *Sulfolobus*.

3.1.4. Biofilm formation in tubing as biofilm compartment

Cultivation of *S. acidocaldarius* under flow-through conditions was done using Brock-medium supplemented with 0.1% (w/v) N-Z-amine and 0.2% (w/v) dextrine. For biofilm formation within tubes, *Sulfolobus* was first statically incubated at 78°C in Norprene tubes for cell attachment. Afterwards the tubie was inserted into a flow-through system and further incubated at 78°C inside an incubator. The medium was pumped through the tubing system with a flow rate of 200 µl/min. After incubation for two weeks the tubing was cut in half and biofilm was visualized by scanning electron microscopy (Fig. 3-2).

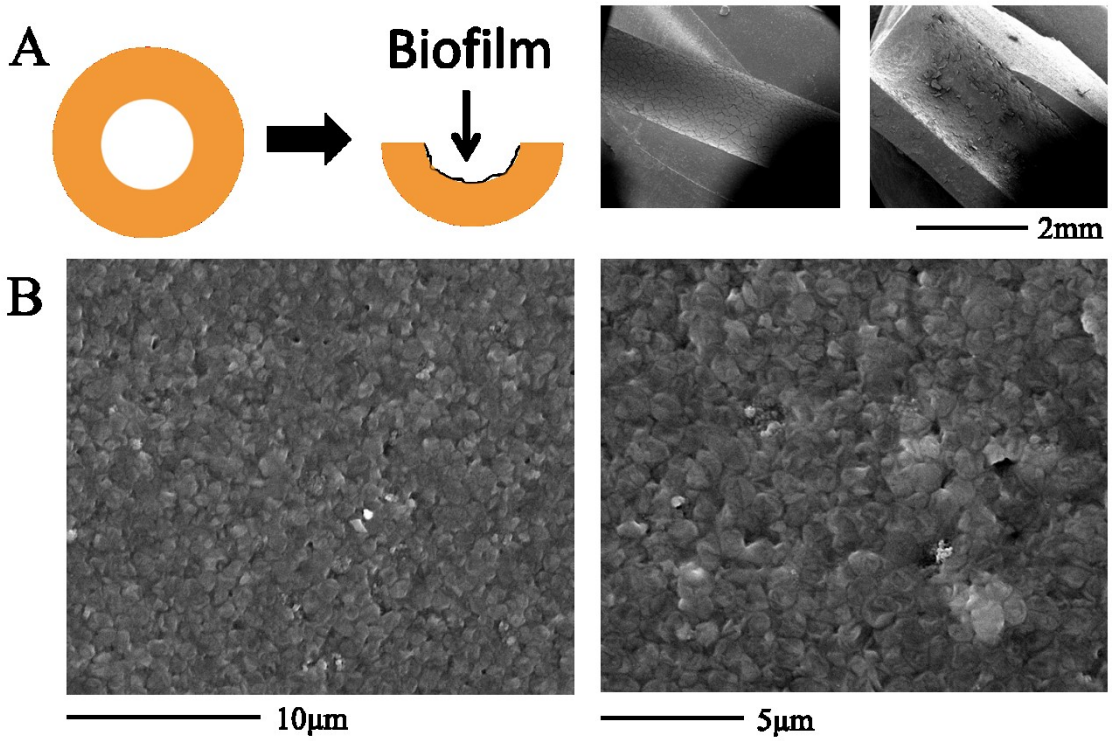


Figure 3-2: Schematic overview of biofilm formation in tubing and scanning electron microscopy images of *S. acidocaldarius* biofilm formed inside the Norprene tube under flow-through conditions. After static incubation of exponentially grown cells inside the tube over night at 78°C for cell attachment, the tube was inserted into the flow-through setup. The biofilm was grown for two weeks at 78°C with 200 µl/min Brock medium containing 0.1% (v/w) N-Z-Amine and 0.2% (v/w) dextrin. After incubation, the tubing was cut in half, sputtered with metals and biofilm was visualised by scanning electron microscopy. **A**, schematic and microscopic image of divided tubing piece containing biofilm. **B**, detailed images of biofilm

Macroscopic images by scanning electron microscopy of the inner tubing side showed plate like structures of different sizes. Since the biofilm had to be dried for microscopy but the tube itself was still flexible, the biofilm was broken into plate like structures. However, higher magnification of these structures showed lobe-shaped cells which have the characteristic morphology of *Sulfolobus*. Therefore, *S. acidocaldarius* biofilm grew inside the Norprene tubing under flow-through conditions.

3.1.5. Biofilm formation in µ-slide as biofilm compartment

The µ-slide (IBIDI) is a commercial flow-system, which is made of the same surface material as the µ-dish (IBIDI) used for static biofilm formation in confocal laser scanning microscopy (Fig 3- 3 A). Since the µ-slide is designed for mesophilic organisms, the system was modified for incubation at 78°C by sealing with silicone and incubation inside a water reservoir. After static incubation with *S. acidocaldarius* overnight at 78°C for cell attachment, macroscopic

aggregates of biofilms were visible after three days of incubation at 78°C with a constant medium flow rate of 200 µl/min (Fig 3-3 B). The biofilm formation was not homogeneous, but rather heterogeneous, with single aggregates, dependent on the medium flow. The macroscopic colonies were bigger in size at the inlet side.

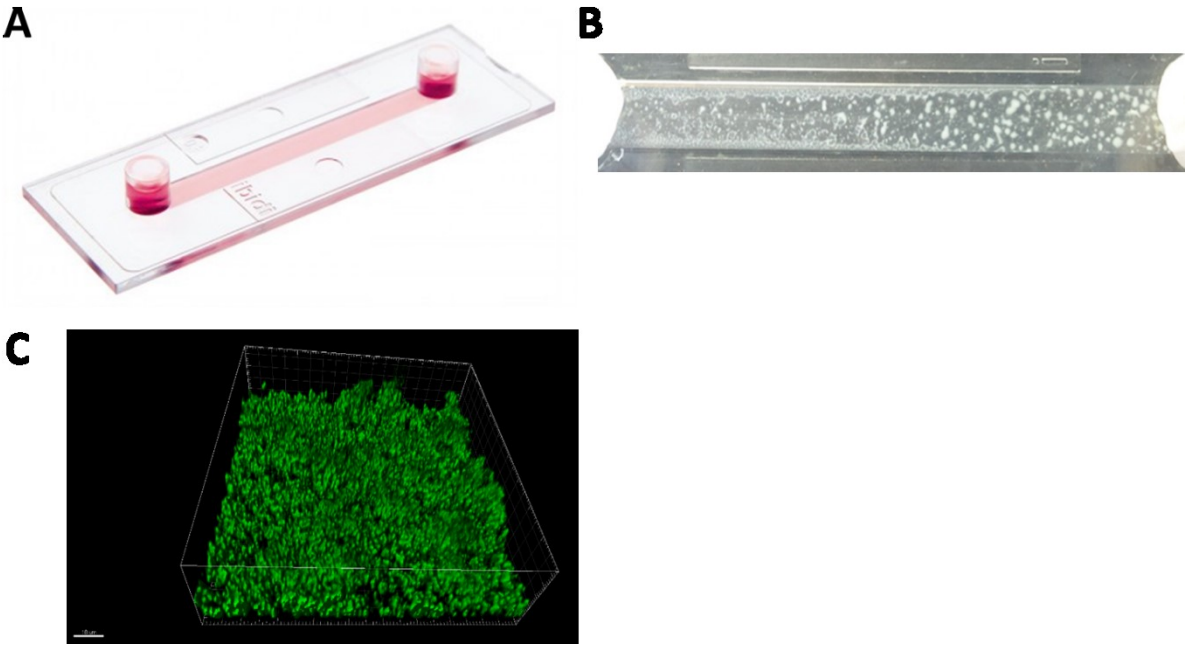


Figure 3-3: Image of the µ-slide (IBIDI) incubation systems for biofilm formation under flow-through conditions.
A, µ-slide (IBIDI, http://ibidi.com/xtproducts/media/images/category/popup/S_8017X_Slide_I_Luer_RGB.jpg). **B**, biofilm formation inside µ-slide (IBIDI) after incubation for three days with constant medium flow (200 µl/min). **C**, confocal laser scanning microscopy image of a three days old biofilm of *S. acidocaldarius* grown in the µ-slide (IBIDI) stained with SYTO9. Scale bar 10 µm.

After incubation the cells were stained with SYTO9 and biofilm formation was visualized by confocal laser scanning microscopy (Fig. 3-3 C). The visualized biofilm cells were comparable to static grown biofilms in size and biofilm architecture.

In this study, biofilm formation was documented for the first time for a (hyper)thermopilic organism in different biofilm compartments under flow-through incubation conditions. Although biofilms were successfully formed, the incubation systems need to be further optimized for reproducible biofilm formation.

3.2. Effects of organic solvents on shaking cultures

Commonly, Archaea like *S. acidocaldarius* are grown under laboratory conditions aerobically as shaking cultures with agitation. Therefore, cultivation of *S. acidocaldarius* cells performed in Erlenmeyer flasks with agitation (180 rpm) is defined as standard cultivation. Standard cultivation was performed first to investigate the influence of different organic solvents on growth of *S. acidocaldarius*. The primary alcohols ethanol, 1-propanol, 1-butanol and 1- pentanol, a secondary alcohol (2-butanol) and a branched alcohol (isobutanol) as well as DMSO as a non-alcohol solvent were applied.

S. acidocaldarius cells were cultivated in Brock medium (pH 3) supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose (Erlenmeyer glass flasks, 100 ml "long-neck" flasks, 50 ml culture volume, 180 rpm, 78°C) in the presence of different concentrations of organic solvents. The pre-cultures for inoculation were grown with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose in the absence of organic solvent. When reaching late log-phase after two days of growth, the pre-cultures were used for inoculation of culture medium supplemented with organic solvents. Growth curves were monitored by determination of the optical density at 600nm (OD_{600}). After cultivation the Erlenmeyer flasks were incubated with crystal violet for visualization of biofilms. Growth curves and biofilm formation are discussed for each solvent independently.

3.2.1. Ethanol

The initial growth rate of cultures grown in the presence or absence of ethanol concentrations of up to 2% (v/v) was comparable (Fig. 3-4 A). In the presence of 1% and 2% (v/v) ethanol the maximal OD_{600} was increased to 2.5 compared to OD_{600} of 2.1 without ethanol and an extended stationary phase was observed. Without ethanol, the OD decreased significantly after reaching the maximal OD. In the presence of 3% (v/v) ethanol a significant reduction of the growth rate and maximal OD was observed. Addition of 4% (v/v) ethanol resulted in a delayed growth, starting only eight days after inoculation and reaching an OD_{600} of 1.25 at the end of incubation. In the presence of 5% (v/v) ethanol no growth was observed over 15 days. The determination of the residual ethanol concentration in the culture medium revealed that cells do not metabolize the ethanol because the reduction level of ethanol concentration is comparable for all initial solvent concentrations tested.

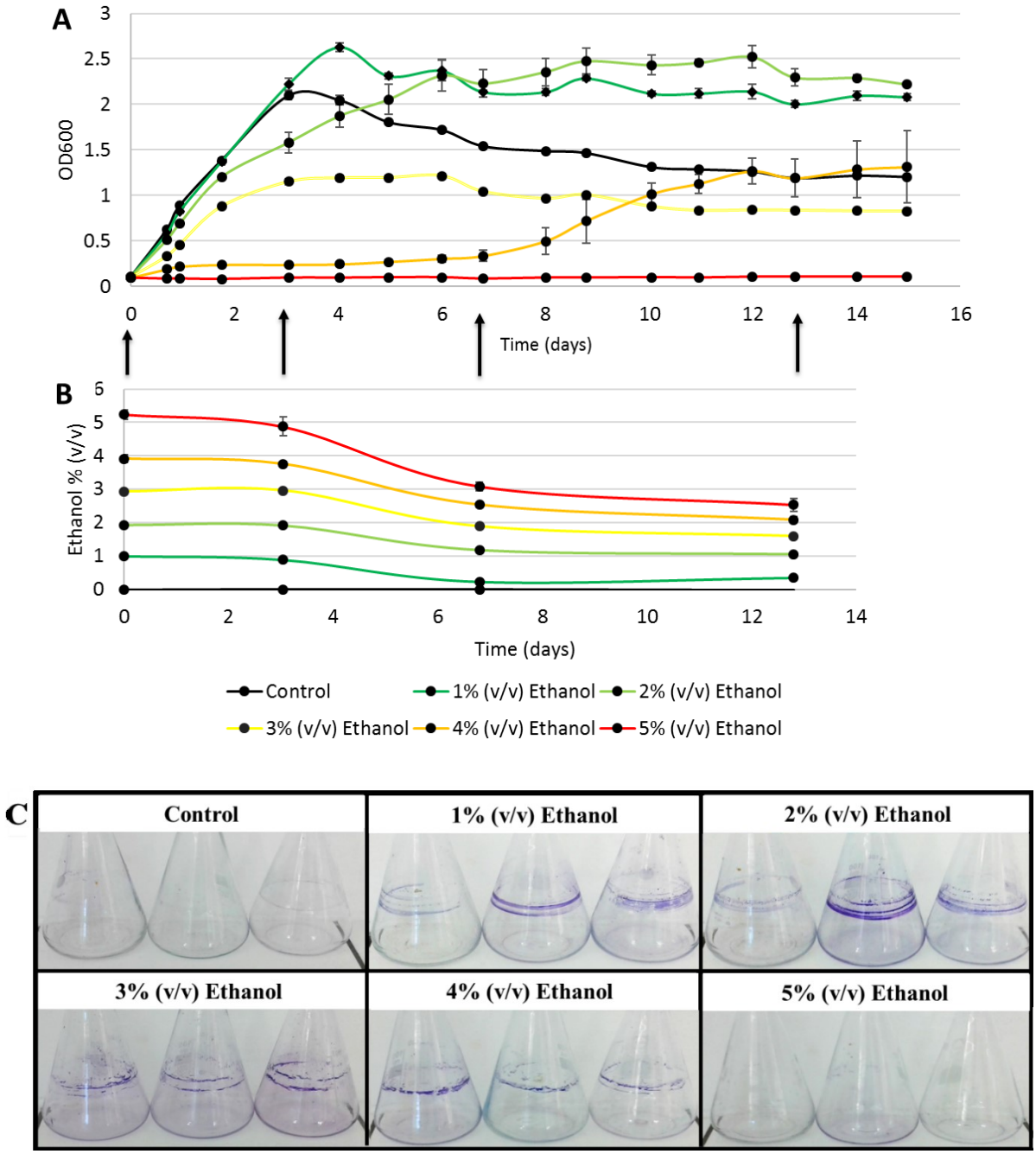


Figure 3-4: *S. acidocaldarius* shaking cultures (long-neck flasks, 78°C, 180rpm) in the presence of different ethanol concentrations (0% to 5% (v/v)). **A**, Growth curves (OD_{600}); **B**, Ethanol quantification by the alcohol dehydrogenase assay; **C**, Shaking flasks after cultivation stained with crystal violet for visualization of biofilm formation. Experiments were performed in triplicates.

Cells incubated with initial 4% (v/v) ethanol started growth after eight days of incubation when the ethanol concentration decreased to 2.5% (v/v) (Fig. 3-4 B). In cultures exposed to 1% to 4% (v/v) ethanol, biofilm formation was observed at the liquid-gas interphase on the glass surface of the Erlenmeyer flasks (Fig. 3-4 C). Without ethanol exposure and in the presence of 5% (v/v) ethanol, no biofilm formation was observed. By eye inspection it was observed, that

cultures incubated in the presence of 3% (v/v) ethanol form most biofilm compared to other cultures.

3.2.2. 1-Propanol

Growth rates of cultures grown in the presence of 0% up to 1% (v/v) 1-propanol were similar (Fig. 3-5 A).

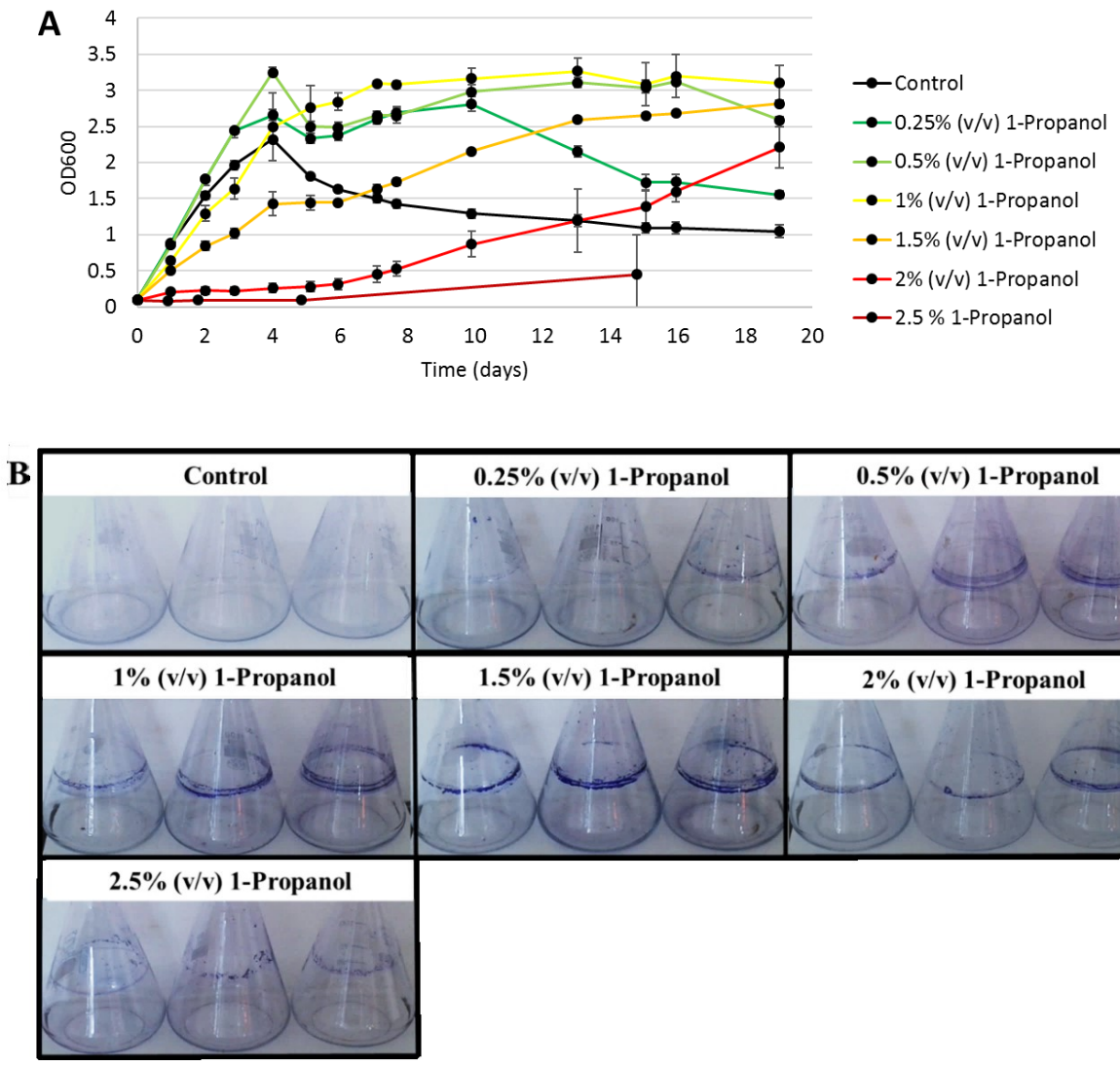


Figure 3-5: *S. acidocaldarius* shaking cultures (long-neck flasks, 78°C, 180rpm) in the presence of different 1-propanol concentrations (0% to 2.5% (v/v)). A, Growth curves (OD_{600}), **B**, Shaking flasks after cultivation stained with crystal violet for visualization of biofilm formation. Experiments were performed in triplicates.

In the presence of higher 1-propanol concentration, the growth rate was significantly reduced. Compared to the control, addition of 1-propanol led to increased OD_{600} values and at 0.25%, 0.5% as well as 1%(v/v) 1-propanol an extended stationary phase was observed. The maximal OD_{600} of the control was 2.3, whereas cultures grown in the presence of 0.25% (v/v), 0.5%

(v/v) as well as 1% (v/v) 1-propanol reached OD_{600} 3.3 and 2.8, respectively. The growth rate of cultures incubated with 1.5 and 2% (v/v) 1-propanol was significantly reduced but the final OD_{600} of the cultures grown with 1.5% (v/v) was comparable to the cultures grown with lower 1-propanol concentrations. After cultivation of cells and crystal violet staining of the Erlenmeyer flasks, significant biofilm formation was observed for cultures exposed with 0.25% - 2% (v/v) 1-propanol and higher (Fig. 3-5 B). Presence of 2.5% (v/v) 1-propanol reduced growth and resulted only in slight biofilm formation. Only slight biofilm formation was also observed with 0.25% (v/v) 1-propanol.

3.2.3. 1-Butanol

The growth rates for cells in the presence of 0% and 0.5% (v/v) 1-butanol were similar (Fig. 3- 6 A).

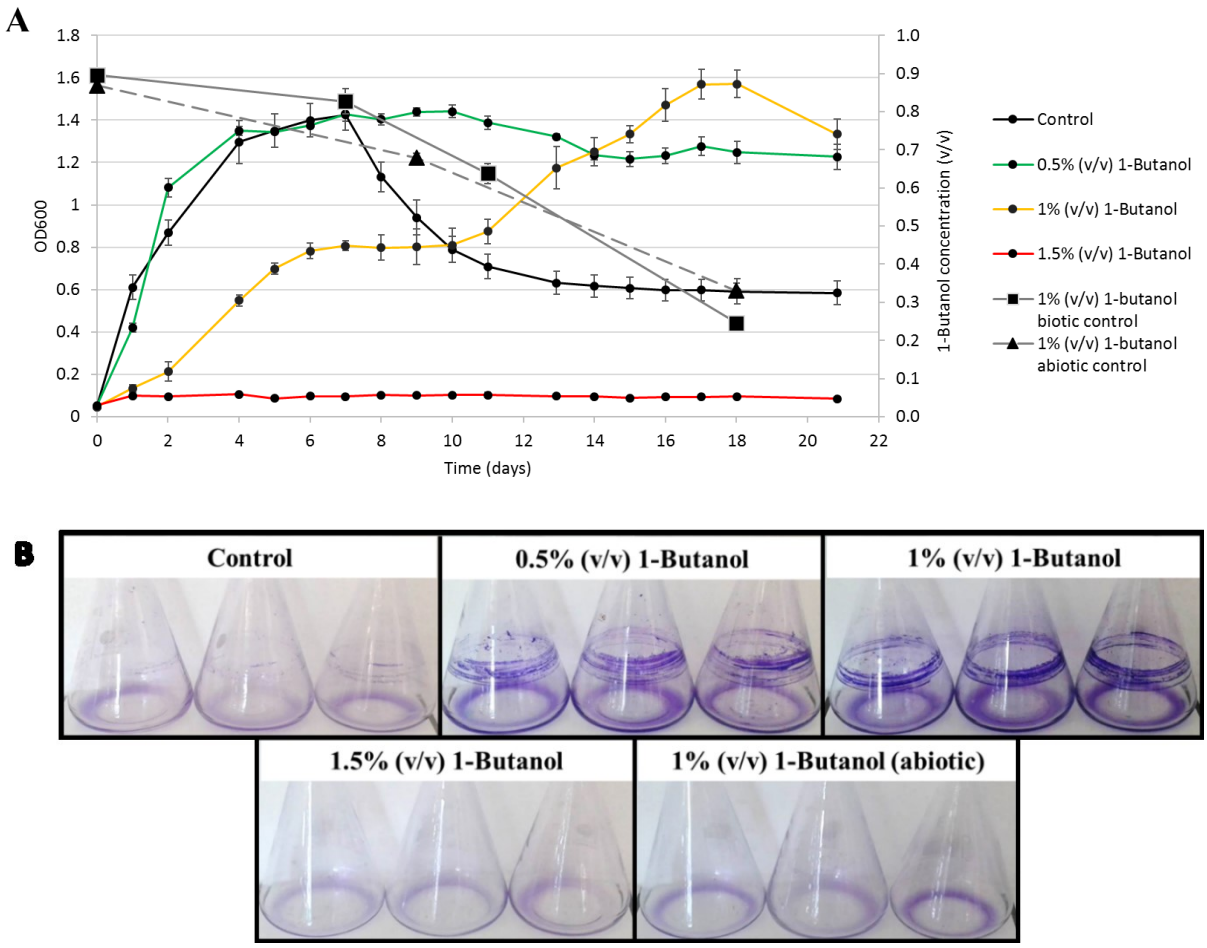


Figure 3-6: *S. acidocaldarius* shaking cultures (long-neck flasks, 78°C, 180rpm) in the presence of different 1-butanol concentrations (0% to 1.5% (v/v)). A, Growth curves (OD_{600}) and 1-butanol quantification by the alcohol dehydrogenase assay, **B**, Shaking flasks after cultivation stained with crystal violet for visualization of biofilm formation. Experiments were performed in triplicates.

After reaching the maximal OD_{600}, the OD in the control decreased, whereas for cultures grown with 0.5% (v/v) 1-butanol an expanded stationary phase was observed. 1-Butanol quantification in Brock medium with cells (biotic) and without cells (abiotic) showed no biotic 1-butanol consumption. Addition of 1% (v/v) 1-butanol led to a biphasic growth curve. After reaching OD_{600} of 0.8 during the first exponential growth phase, the OD kept constant for 4 days (stationary phase) and then, during the second exponential growth phase, further increased up to OD_{600} 1.6. This maximal OD was higher compared to the control experiments without 1-butanol (maximal OD_{600} 1.4). Addition of 1.5% (v/v) 1-butanol resulted in growth inhibition. Biofilm formation was only observed in flasks exposed to 0.5% or 1% (v/v) 1- butanol, respectively (Fig. 3-6 B).

3.2.4. 2-Butanol

For the solvent 2-butanol the addition of 0.25%(v/v) 2-butanol already showed an inhibitory effect and no growth and biofilm formation was observed (Fig. 3-7).

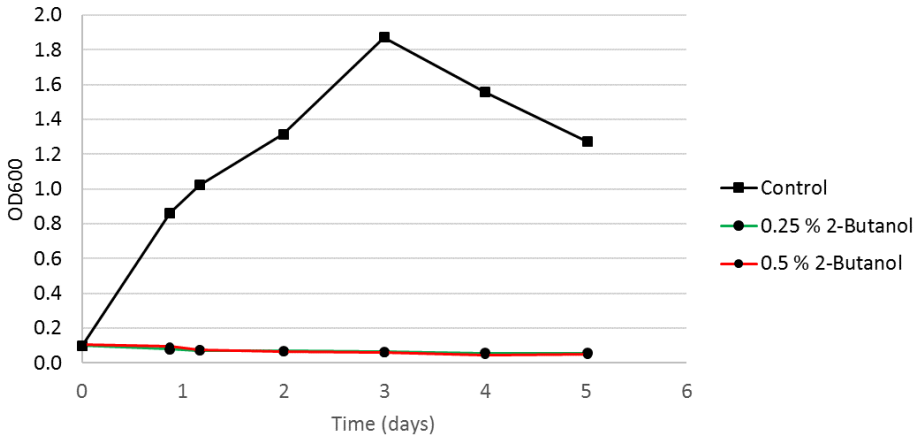


Figure 3-7: *S. acidocaldarius* shaking culture (long-neck flasks, 78°C, 180rpm) in the presence of different 2-butanol concentrations (0% to 0.5% (v/v)). Growth curves were determined at OD_{600} (single measurement).

3.2.5. Isobutanol

In the absence and presence of 0.5% (v/v) isobutanol *S. acidocaldarius* cells showed a similar growth rate, but the maximal OD was increased from 2 to 2.4 and the stationary phase was prolonged with 0.5%(v/v) isobutanol exposure before the OD decreased (Fig. 3-8 A). Addition of 1% (v/v) isobutanol resulted in a reduced growth rate but a slightly higher OD (OD_{600} of 2.7) compared to lower isobutanol concentration and a prolonged stationary phase compared with the control from day 11 to day 15 before the OD decreased. In the presence of 1.5% (v/v)

isobutanol no cell growth was observed. Biofilm formation was visible after crystal violet staining in flasks of cultures grown in the presence of 0.5% and 1% (v/v) 1-butanol.

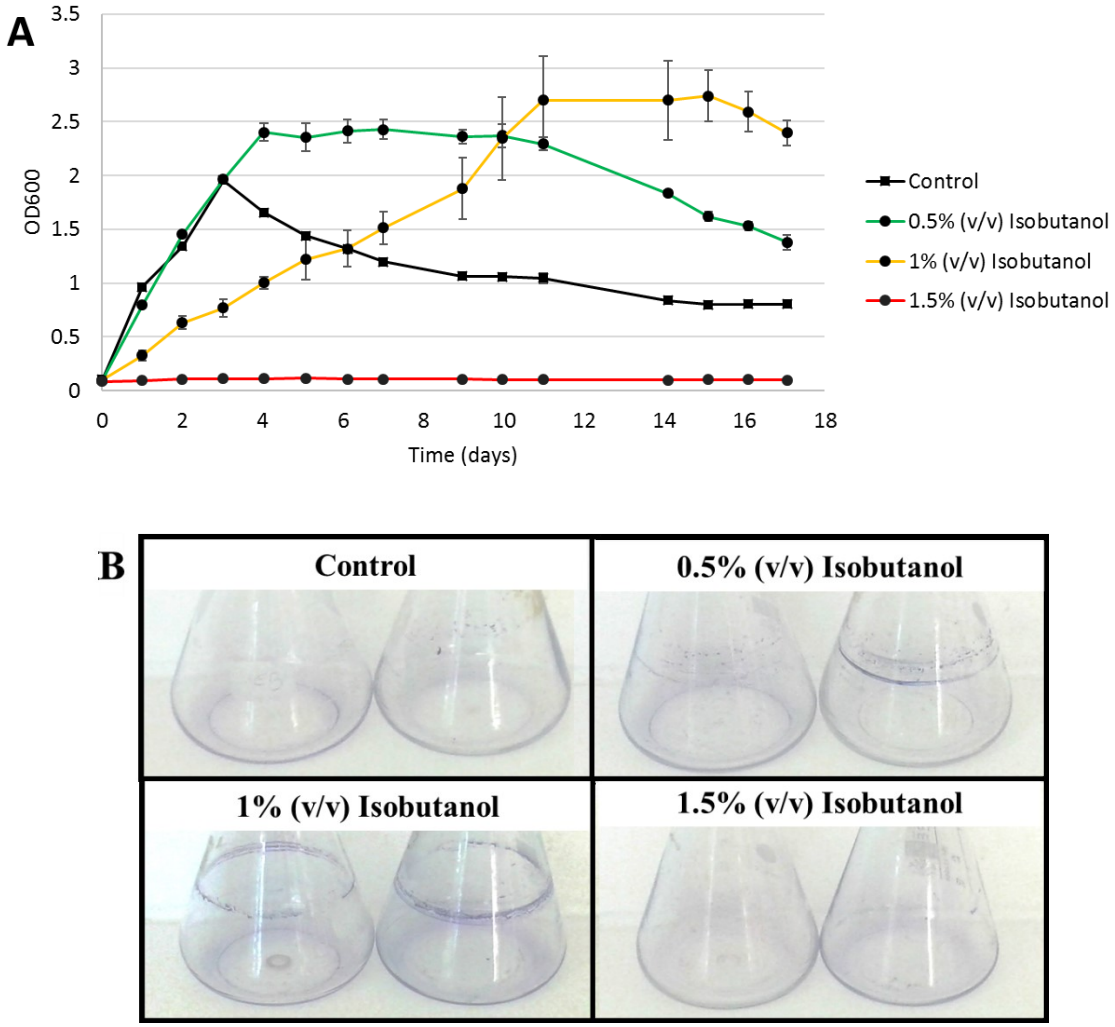


Figure 3-8: *S. acidocaldarius* shaking cultures (long-neck flasks, 78°C, 180rpm) in the presence of different isobutanol concentrations (0% to 1.5% (v/v)). A, Growth curves (OD_{600}), **B**, Shaking flasks after cultivation stained with crystal violet for visualization of biofilm formation. Experiments were performed in triplicates.

3.2.6. DMSO

S. acidocaldarius cells grown in the presence of 0%, 1% and 2% (v/v) DMSO showed comparable growth rates and reached a maximal OD_{600} of 1,5-1,8. However, in the presence of 3% (v/v) DMSO, the growth rate was significantly inhibited and a maximal OD_{600} of 0.5 was observed. After 2d the OD_{600} already decreased. Addition of 4% (v/v) DMSO resulted in complete growth inhibition. No biofilm formation was observed in flasks after crystal violet staining in the presence of any DMSO concentration.

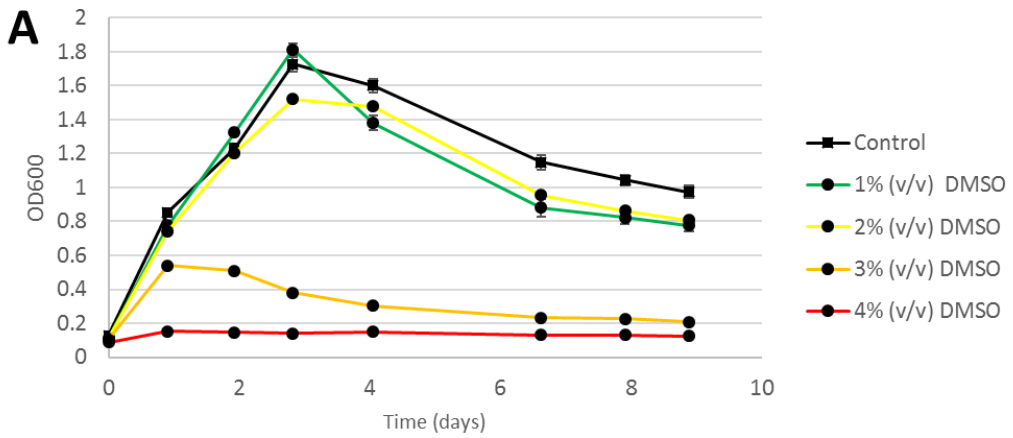


Figure 3-9: *S. acidocaldarius* shaking culture (long-neck flasks, 78°C, 180rpm) in the presence of different DMSO concentrations (0% to 4% (v/v)). A, Growth curves (OD_{600}), B, Shaking flasks after cultivation stained with crystal violet for visualization of biofilm formation. Experiments were performed in triplicates.

3.2.7. Overview of planktonic growth experiments

The overview of maximal OD_{600}, prolonged stationary phase and biofilm formation of *S. acicocaldarius* grown as shaking cultures in Erlenmeyer flasks are summarized in Tab. 3-1. For growth in the presence of ethanol, 1-propanol, 1-butanol, isobutanol and DMSO an increase in maximal OD_{600} compared to the control experiments without solvents was measured. All applied alcohols induced biofilm formation and growth curves showed a prolonged stationary phase, except 2-butanol, which led to growth inhibition already at low concentration. The organic solvent DMSO induced neither biofilm formation nor an elongated stationary phase. Therefore, the response of planktonic growth of *S. acidocaldarius* is influenced by the chemical structure of the organic solvent.

Table 3-1: Overview of maximal OD_{600}, observed elongated stationary phase and biofilm formation of *S. acidocaldarius* shaking cultures grown in 100ml long neck Erlenmeyer flasks at 78°C. - represents no elongated phase and no biofilm formation, + and ++ represents the magnitude of elongated stationary phase and biofilm formation.

Solvent	Concentration % (v/v)	OD_{600} (max)	Prolonged stationary phase	Biofilm formation observed
Ethanol	0	2.1	-	-
	1	2.6	+	+
	2	2.5	+	+
	3	1.2	+	+
	4	1.3	Delayed growth	+
	5	0.1	No growth	No growth
1-Propanol	0	2.3	-	-
	0.25	2.8	+	(+)
	0.5	3.3	+	+
	1	3.3	++	++
	1.5	2.8	Delayed growth	+
	2	2.2 (exp. Phase)	Delayed growth	+
	2.5	0.5	Delayed growth	(+)
1-Butanol	0	1.4	-	-
	0.5	1.4	+	+
	1	1.6	Biphasic growth	++
	1.5	0.1	No growth	-
2-Butanol	0	1.9	-	-
	0.25	0.1	No growth	-
	0.5	0.1	No growth	-
Isobutanol	0	1.9	-	-
	0.5	2.4	+	+
	1	2.7	+	+
	1.5	0.1	No growth	-
DMSO	0	1.7	-	-
	1	1.8	-	-
	2	1.5	-	-
	3	0.5	-	-
	4	0.2	-	-

3.3. Investigation of *S. acidocaldarius* biofilms by microscopic methods

3.3.1. Effect of solvents on cell distribution and biofilm morphology analysed by light microscopy

The effect of different solvent concentrations on biofilm formation and cell morphology was investigated by using crystal violet staining and light microscopy. For the experiments, *S. acidocaldarius* cells were incubated for 4 days in 6-well microtiter plates with a glass slide

as substratum for biofilm formation. The incubation was performed in Brock medium (pH 3) supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose in the absence or presence of different sublethal concentrations of different solvents. Afterwards the glass slides were stained by crystal violet in order to visualize changes in cell distribution on the glass surface by light microscopy. Images were taken of dried biofilms. Cell aggregation was found for all applied solvents, however, for each solvent at different concentrations. The concentrations with the most obvious phenotype are shown in Fig 3-10.

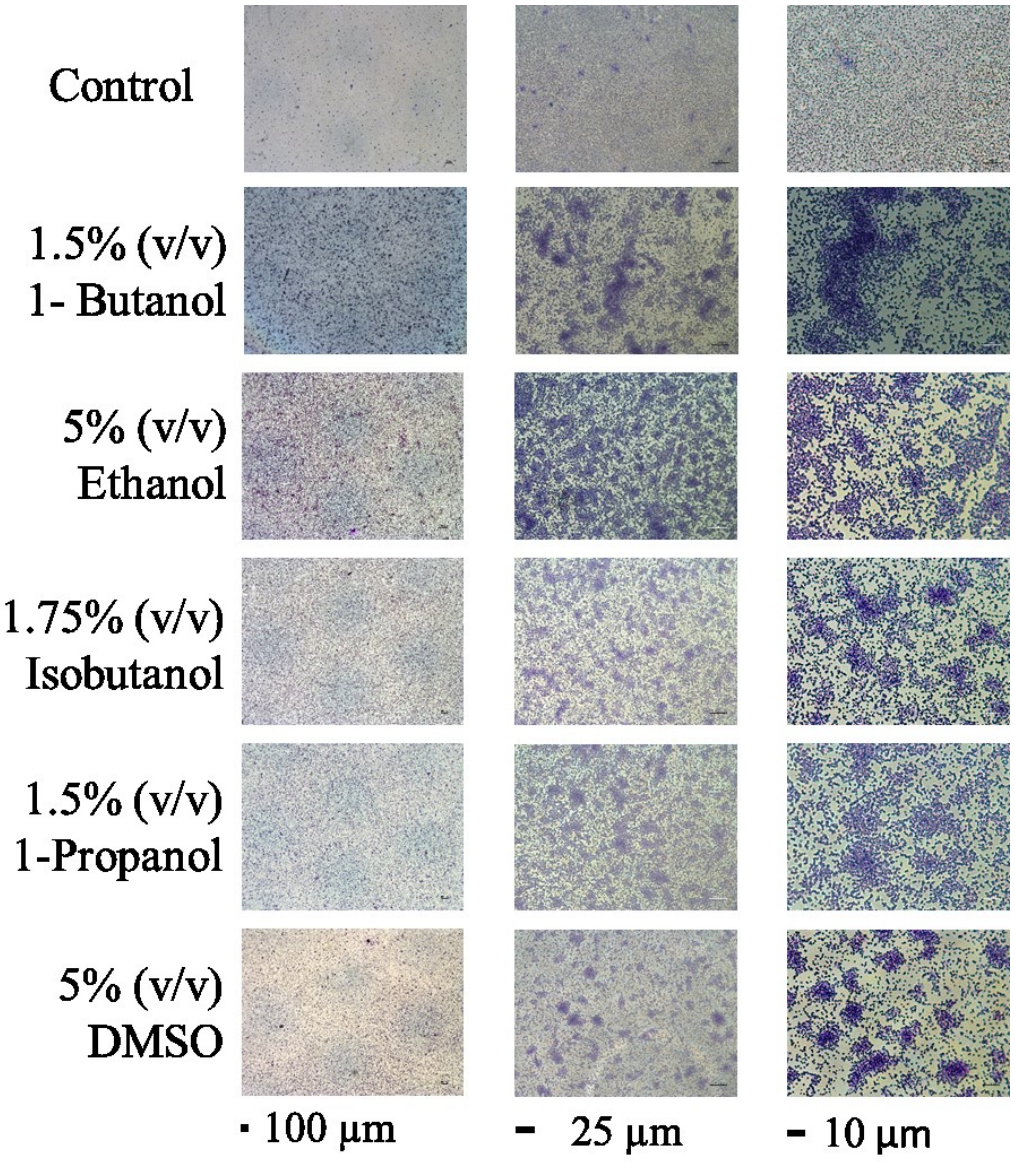


Figure 3-10: Effect of organic solvents on *S. acidocaldarius* cell distribution. *S. acidocaldarius* biofilms were grown on glass surfaces for 4d at 78°C in presence and absence of sublethal concentrations of different organic solvents. Biofilms were stained by crystal violet for subsequent analysis by light microscopy.

S. acidocaldarius cells appeared distributed as a homogeneous layer in the control without the addition of solvents. In the presence of solvents, the cell distribution on the glass surface

changed to a more heterogeneous phenotype with the development of cell aggregates. The effect of 1-butanol was studied in more detail and two different concentrations were tested (Fig. 3-11).

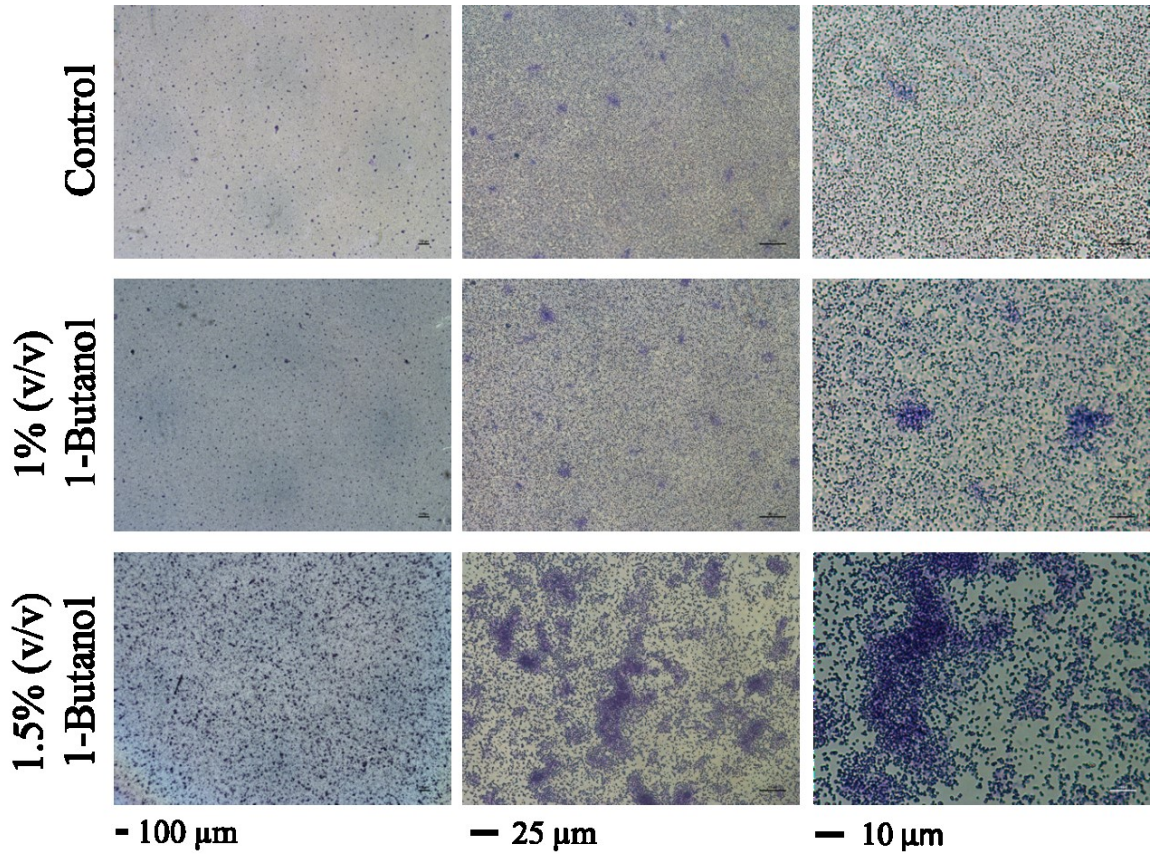


Figure 3-11: Effect of 1-butanol on *S. acidocaldarius* cell distribution. *S. acidocaldarius* biofilms were grown on glass surfaces for 4d at 78°C in the absence and presence of 1% and 1.5% (v/v) of 1-butanol. Biofilms were stained by crystal violet and dried for subsequent analysis by light microscopy.

3.3.2. Effect of 1-butanol on biofilm formation and cell morphology analysed by scanning electron microscopy

Since crystal violet not only binds to cells but also other components rather unspecifically, the resulting biofilms were analysed by scanning electron microscopy (SEM). For biofilm formation *S. acidocaldarius* was incubated as described before for light microscopy but no staining with crystal violet was performed. After cultivation, the slides were carefully washed with minimal Brock-medium (pH 3), fixed by 2% (v/v) glutardialdehyde in minimal Brock medium (pH 3) and incubated with acetone for subsequent critical point drying and sputtering with Au/Pd. After metallization the biofilm was documented by using a scanning electron microscope. Images show dried biofilm samples.

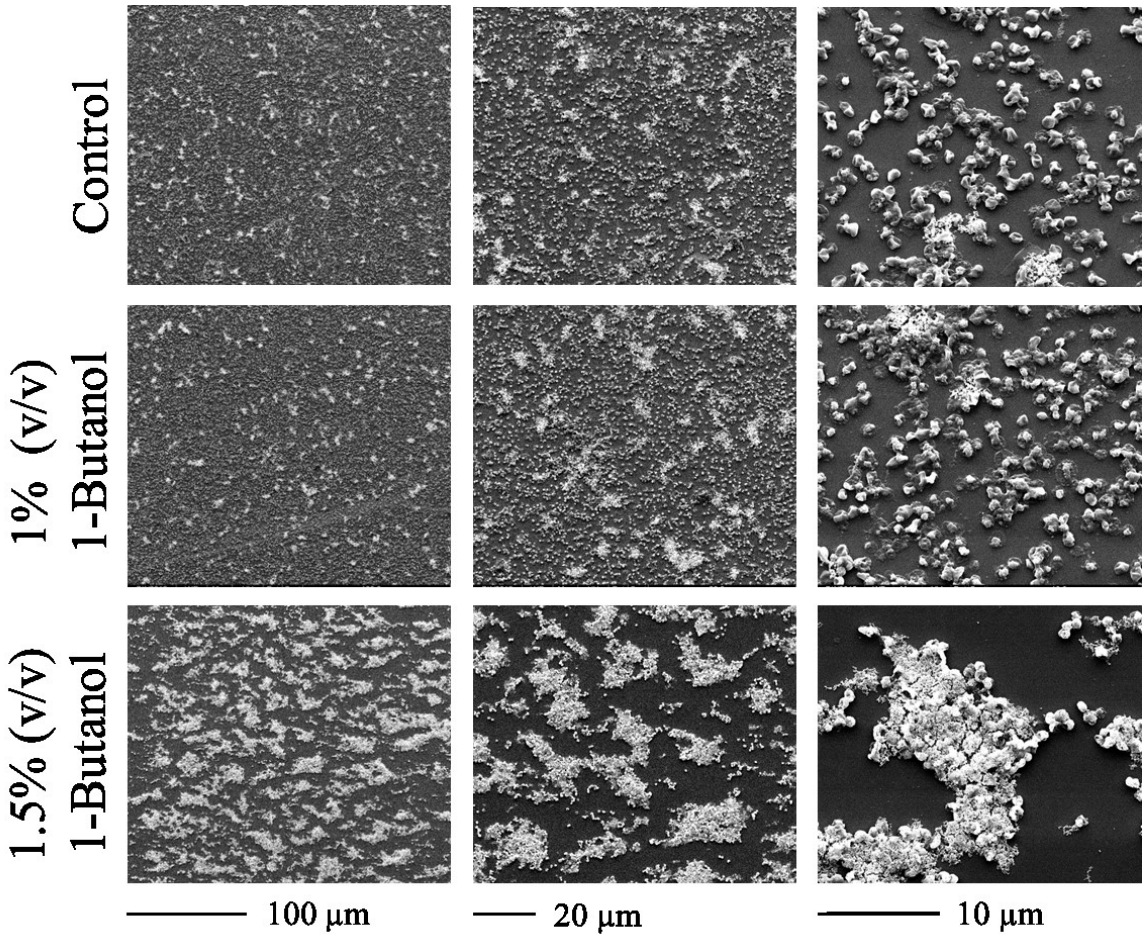


Figure 3-12: Effect of 1-butanol on *S. acidocaldarius* cell distribution and biofilm formation. *S. acidocaldarius* biofilms were grown on glass surfaces for 4d at 78°C in the absence and in presence of 1% and 1.5% (v/v) of 1- butanol. Visualization by scanning electron microscopy.

Scanning electron microscopy images (Fig. 3-12) showed a similar biofilm morphology as observed by light microscopy (Fig. 3-11). In the absence of 1-butanol *S. acidocaldarius* cells were homogeneously distributed and formed a monolayer with a carpet like structure on the glass surface. With increasing 1-butanol concentration, the cells were more heterogeneously distributed and multilayered structures and aggregates were observed. Cells located within aggregates appeared to be embedded in extracellular material, proposed to be EPS.

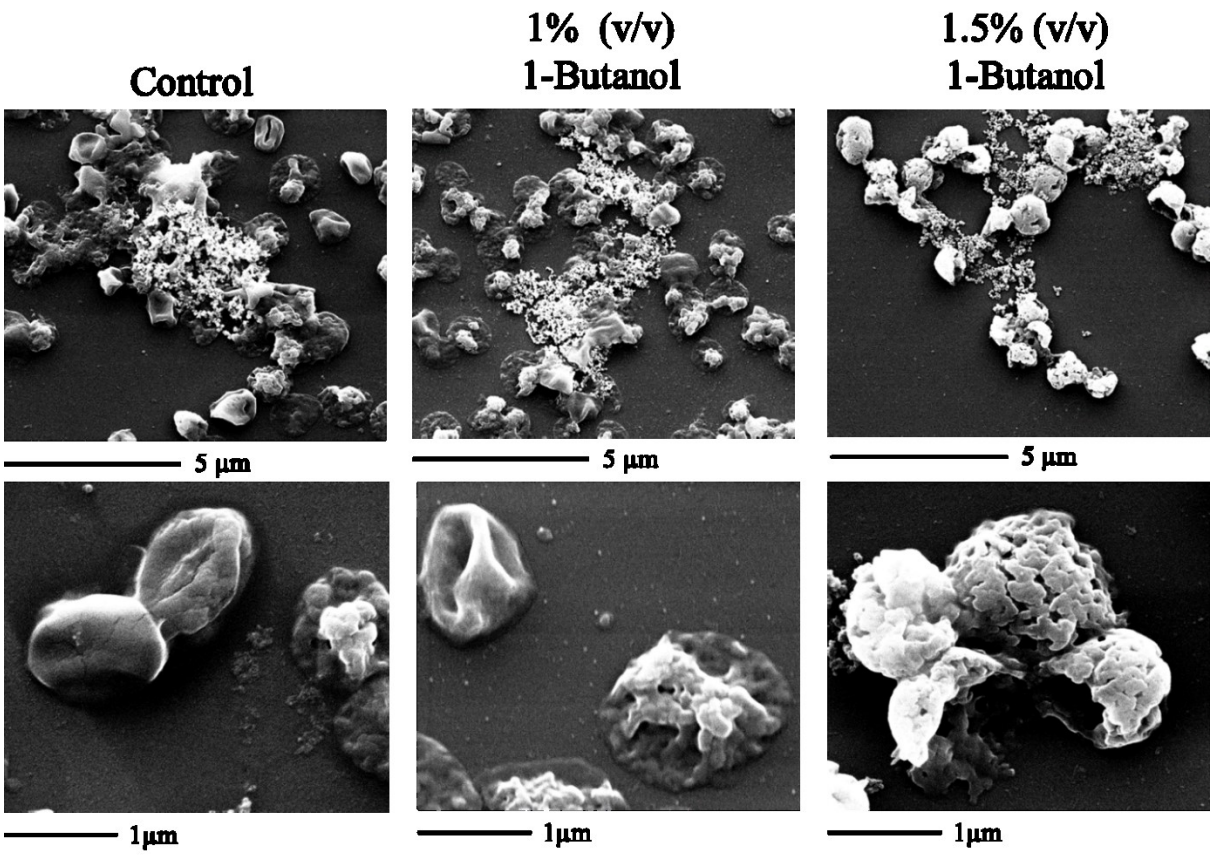


Figure 3-13: Effect of 1-butanol on *S. acidocaldarius* cell morphology. *S. acidocaldarius* biofilms were grown on glass surfaces for 4d at 78°C in the absence and in presence of 1% and 1.5% (v/v) of 1-butanol, respectively. Visualization by scanning electron microscopy.

Images of the cell aggregates with higher magnification showed a more detailed view of the cell envelope and the compounds proposed to be EPS. Under the different tested incubation conditions, the EPS appeared as filling material between the attached cells. Interestingly, the cell envelope morphology changed with increasing 1-butanol concentration. In the absence of 1-butanol and in the presence of 1% (v/v) 1-butanol the cells showed two phenotypes: (i) lobe-shaped with smooth surface structure and a size of around 1 µm as previously described by Brock *et al.* (Brock et al., 1972) and (ii) flat flat cells with a more irregular structure. The different morphologies were also confirmed by atomic force microscopy (see Fig. 3-14). In the presence of 1.5% (v/v) 1-butanol, a third phenotype was observed. The outer surface of *S. acidocaldarius* appeared more round with a perforated, brain-like structure.

3.3.3. Effect of 1-butanol on cell morphology analysed by scanning electron microscopy

Atomic force microscopy (AFM) of biofilms grown on glass slides was applied to characterize the cell morphology of *S. acidocaldarius* with and without solvent exposure (Fig. 3-14). Due to the flat surface of coverslips, microscopy of cells attached on glass is preferred for AFM. *S. acidocaldarius* cells were incubated in 6-well microtiter plates for four days in the presence of glass slides for biofilm formation as described for light microscopy and scanning electron microscopy. However, 0.2% (w/v) dextrin was used as carbon source instead of 0.2% (w/v) glucose. After incubation, the cells were carefully washed with minimal Brock-medium (pH 3) fixed with 2% (v/v) glutardialdehyde in minimal Brock medium (pH 3) and visualized by DAPI-staining for subsequent epi-fluorescence microscopy. Representative areas visualized by epi-fluorescence microscopy were further investigated by atomic force microscopy. Images show dried samples.

In the AFM two different cell morphologies were observed: Round cells and cells with irregular cell surface that seem to differ in their cell envelope. Cells grown with 1% (v/v) or without additional 1-butanol showed a comprehensive and homogeneous distribution on the glass surface. Single cell aggregates were found for both samples. However, it seemed that the size of the cells attached to the surface was larger in the presence of 1-butanol. The distribution of the two cell morphologies indicates that the increase in cell size has no influence on the cell morphology.

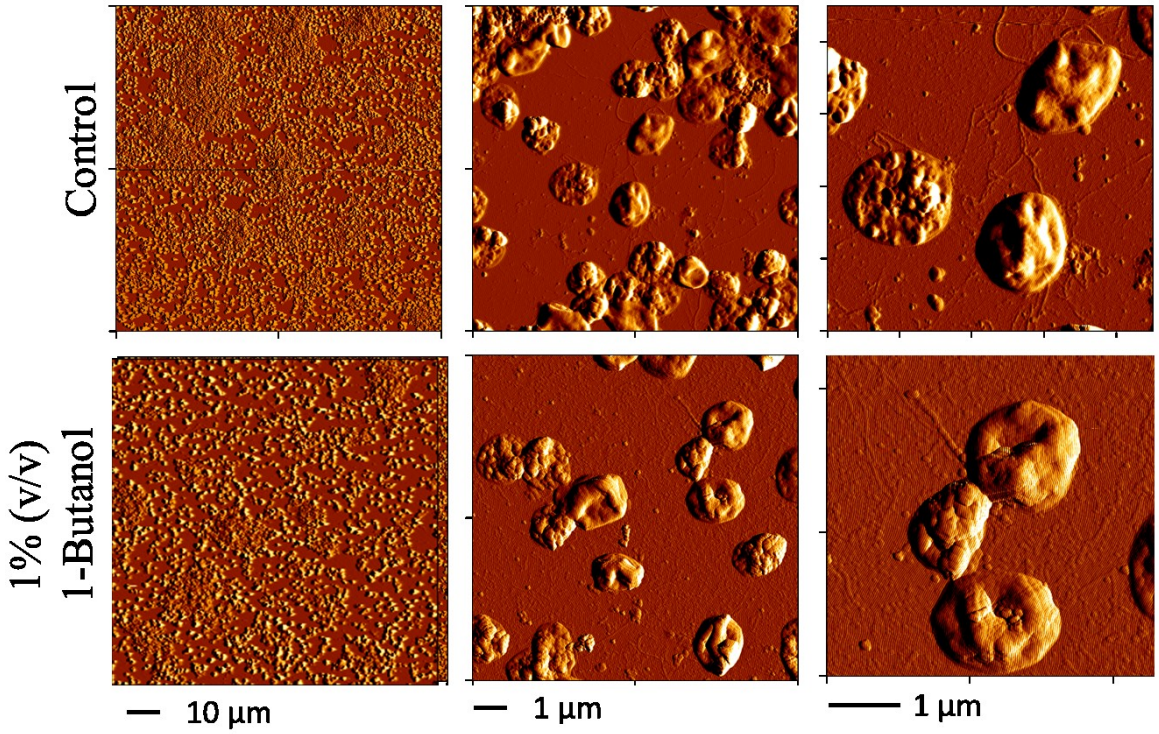


Figure 3-14: Effect of 1-butanol on *S. acidocaldarius* cell distribution and morphology analysed by AFM. *S. acidocaldarius* cells were grown statically on glass surfaces for four days at 78°C on glass slide within 6-well microtiter plates in Brock medium (pH 3) supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) dextrin as carbon sources. The cultivation was performed in absence (control) or presence of 1% (v/v) 1-butanol.

3.3.4. Effect of 1-butanol on submersed biofilms analysed by confocal laser-scanning microscopy

S. acidocaldarius cells were cultivated for four days in µ-dishes to generate submerse biofilms for subsequent visualization. For confocal laser-scanning microscopy (CLSM), cells were stained with the DNA binding fluorescence dye SYTO9 and carbohydrates within the EPS were stained by using the lectins IB4 (binding α-D-galactosyl and N-acetyl-D-galactosamine residues, blue signal) and ConA (binding α-mannopyranosy- and α-glucopyranosyl residues, red signal). Whereas the above mentioned microscopic methods like AFM and SEM were applied on dried biofilms, CLSM was applied for visualization of the three-dimensional biofilm structure and EPS, i.e. carbohydrate distribution, of hydrated biofilms. Abiotic solvent evaporation after cultivation was quantified to be 20% (v/v).

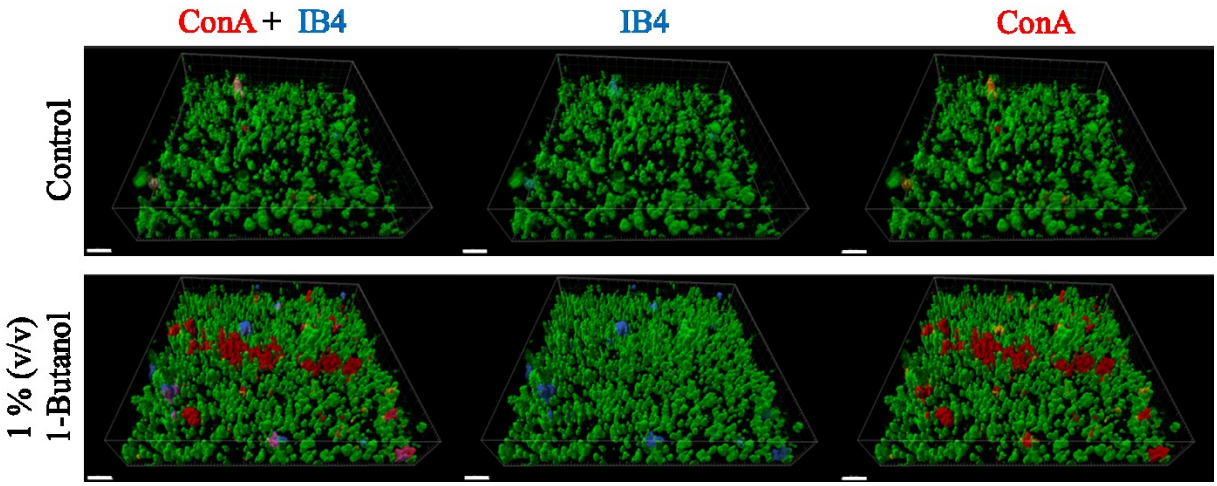


Figure 3-15: Effect of 1-butanol on biofilm formation of *S. acidocaldarius* analyzed by CLSM. Submersed biofilms of *S. acidocaldarius* cells were grown for four days under static conditions in µ-dishes (IBIDI). Cells were simultaneously stained with SYTO9, carbohydrates were stained by using the lectins IB4 (binding α-D-galactosyl and N-acetyl-D-galactosamine residues, blue signal) and ConA (binding α-mannopyranosy- and α-glucopyranosyl residues, red signal). Scale bars: 10 µm

Compared to the control without addition of 1-butanol, the presence of 1% (v/v) 1-butanol revealed an increased binding of IB4- as well as ConA-lectins (Fig. 3-15). Overall, the signals for ConA binding (red) were more dominant compared to IB4 binding (blue). Some carbohydrate structures appeared to be bound by both lectins, indicating the presence of different terminal sugar residues in the carbohydrate structure. Generally, the structures bound by lectins were not flat but tower-like structures, tending to extrude into the liquid phase.

3.4. Effect of 1-butanol on biofilm formation and established biofilms

3.4.1. Effect of 1-butanol on biofilm formation and cell viability

To analyse the effect of different 1-butanol concentrations on the growth of *Sulfolobus acidocaldarius*, the organism was cultivated under static conditions in 96-well microtiter plates for four days at 78°C in the absence and presence of different 1-butanol concentrations. Characterization of growth and biofilm quantification were performed by determination of the OD_{600} and by crystal violet (570 nm) staining, respectively. By the use of crystal violet, the biomass including cells as well as EPS components are quantified. The viability of biofilm cells was analysed by a newly established resazurin assay.

The growth of planktonic cells as well as of biofilm cells was not affected by addition of up to 1.5% (v/v) 1-butanol (Fig. 3-16 A). The absorbance of planktonic cells was quantified at 1- butanol concentrations from 0% to 1.5% (OD_{600} around 0.03). The biofilm formation quantified by crystal violet staining (570 nm) revealed no difference between 0% to 1% (v/v) 1-butanol (absorbance around 0.07) but slightly decreased at 1.5% (v/v) 1.-butanol to 0.05. The cell growth is clearly inhibited upon 1-butanol concentrations higher than 2% (v/v) as indicated by a rapid decrease of planktonic cell numbers (OD_{600}) and biofilm formation (crystal violet staining). Increasing error bars at sublethal concentrations of 1% and 1.5% (v/v) 1- butanol indicate that small changes in the concentration have significant influence in growth of *S. acidocaldarius* and the cells are very sensitive in this concentration range.

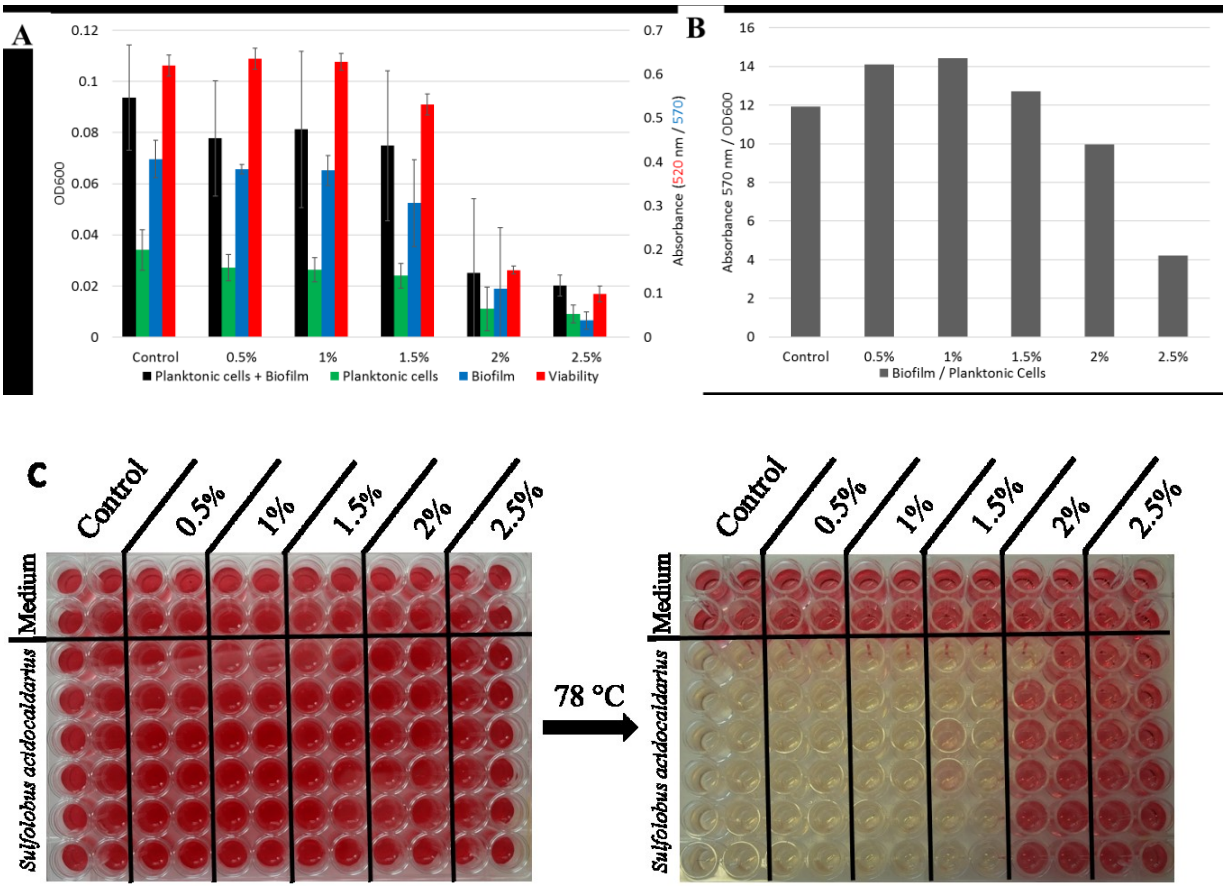


Figure 3-16: Effect of different 1-butanol concentration on biofilm formation and cell viability. *S. acidocaldarius* cells were incubated in 96-well microtiter plates in the presence of different 1-butanol concentrations for four days at 78°C. **A**, OD_{600} of planktonic cells and biofilm cells, black), OD_{600} of isolated planktonic cells transferred into a new well (green), quantification of biofilm by crystal violet staining (absorbance at 570 nm) (blue) and respiratory metabolic activity determined by the resazurin assay at 520 nm (red). **B**, Calculation of changes between planktonic and biofilm growth (Abs 570nm/ Abs 600nm). **C**, Example for the effect of 1-butanol on cell viability determined by the resazurin assay. *S. acidocaldarius* cells were grown for 4d at 78°C in 96-well microtiter plates in Brock medium (pH 3) in the presence of different 1-butanol concentrations. After addition of resazurin (0.005% (w/v) in Brock pH 3) to each cavity, plates were incubated at 78°C until colour conversion was observed (approximately 3h). Images of the 96-well microtiter plate before and after incubation at 78°C until colour conversion was observed.

Quantification of planktonic and biofilm cells as well as viability of biofilm cells indicated an increase in biofilm as well as metabolic activity in presence of 1-butanol at sublethal concentrations of 1% (v/v) and below. The quotient of the absorbance of biofilm cells and planktonic cells revealed that cells prefer biofilm growth in the presence of 0.5% - 1.5% (v/v) 1-butanol (Fig 3-16 B).

Viability of cells was determined as cellular respiratory activity by following the reduction of the red resurufin to the colourless dihydroresurufin. Resazurin as viability marker was successfully applied for other organisms but not for thermoacidophilic organisms so far (Natto et al., 2012). The resazurin assay conditions for *S. acidocaldarius* cells were modified from mesophilic Bacteria or Eukaryotes growing at neutral pH where the reduction from resazurin to resurufin is monitored (Chadha et al., 2015). Therefore, the resazurin assay was here adapted for the use in the thermoacidophilic aerobic Archaeon *S. acidocaldarius* for the first time.

Since resazurin is also a pH indicator, the absorbance strongly depends on the pH. The maximum in absorbance in Brock medium (pH 3) was determined at 520 nm. Therefore, viability analyses of *S. acidocaldarius* biofilm cells following the conversion to the colourless dihydroresurufin were performed at 520 nm and 78°C. The incubation was stopped when the cavities containing viable cells appear colourless after around 2-3h. However, control wells (incubated only with medium) and toxic 1-butanol concentrations of 2% and 2.5% (v/v) 1- butanol appeared red. No colour-change indicated no reduction to dihydroresurifin and, thus, no respiratory activity. Wells from 0% to 1.5% (v/v) 1-butanol changed to colourless dihydroresurufin, indicating respiratory activity and therefore the presence of viable cells (Fig. 3-16 C).

3.4.2. Effect of 1-butanol on established biofilms

In order to study the effect of 1-butanol on established biofilms, *S. acidocaldarius* cells were first cultivated for four days under static conditions and the developed biofilms were subsequently incubated with different concentrations of 1-butanol from 0.5% to 2.5% (v/v). In general, absorbance values below the control value indicated a reduced biofilm formation or detachment of cells. Without the addition of 1-butanol and in the presence of 0.5% (v/v) 1- butanol, the OD_{600} for quantification of planktonic cells increased from day 1 to day 2 but

kept constant during further incubation (Fig. 3-17). Concentrations of 1% and 1.5% (v/v) 1- butanol resulted in an increased OD_{600} of planktonic cells from day 1 to day 3. Without addition of 1-butanol, the formed biofilm decreased from day 1 to day 4, indicated by less crystal violet absorbance at 570nm. In the presence of 0.5% to 1% (v/v) 1-butanol an initial increase in biofilm formation from day 1 to day 2 was observed, but with ongoing incubation time the biofilm formation was reduced. In the presence of 2% and 2.5% (v/v) 1-butanol the biofilm formation was significantly reduced. Therefore, concentrations above 2% (v/v) 1- butanol induced the detachment of biofilm cells in previously established biofilms (Fig. 3-17).

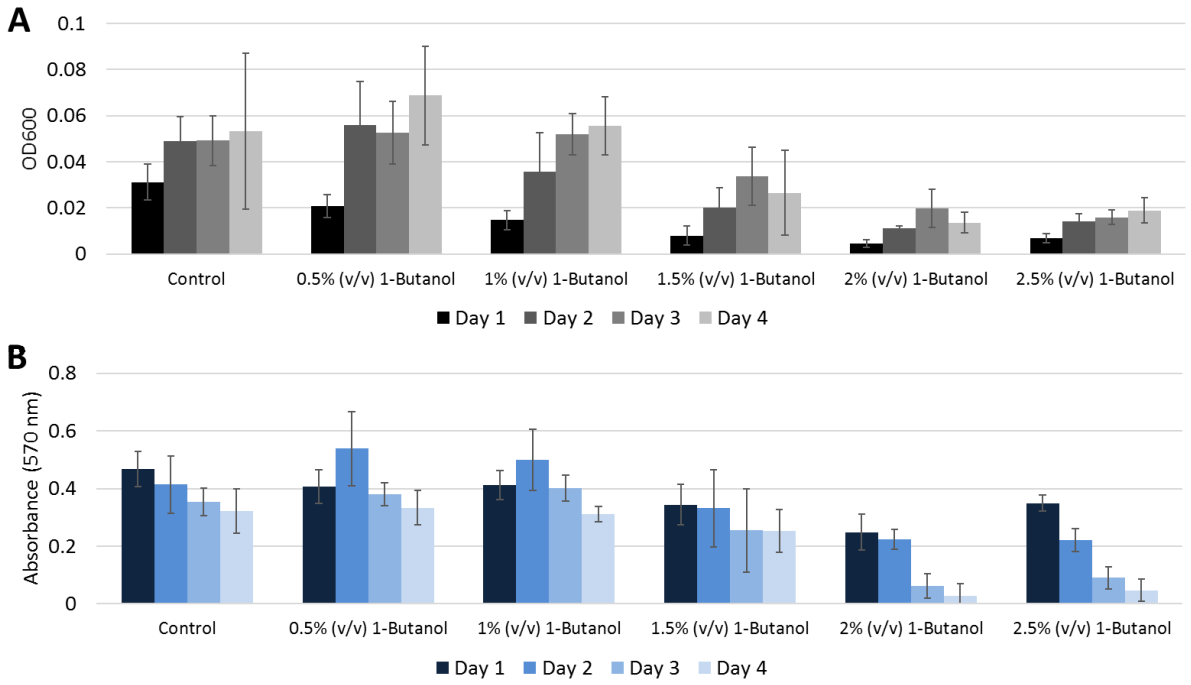


Figure 3-17: Effect of different 1-butanol concentrations on established biofilms. *S. acidocaldarius* cells were incubated for four days under static growth condition in 96-well microtiter plates for biofilm formation. Afterwards the medium was exchanged with fresh medium in presence or absence of different 1-butanol concentrations. Biofilms were further incubated for one to four days after medium exchange. The absorbance of isolated planktonic cells (OD_{600}, **A**) as well as biofilm formation determined by crystal violet staining (Absorbance 570nm, **B**) was determined.

3.5. Effect of 1-butanol on the formation and composition of extracellular polymeric substances

Since the microscopic analysis of the stress response of *S. acidocaldarius* towards 1-butanol indicated an enhanced formation of EPS, this observation was further studied by EPS isolation and quantification. Therefore, *S. acidocaldarius* biofilms were grown for four days in Petri-dishes incubated in an anaerobic jar for minimizing medium evaporation, and EPS was extracted by using the cation exchange method established previously (Jachlewski et al., 2015). Cells were inoculated in the presence of 0.5% and 1% (v/v) 1-butanol. For each cultivation condition 10 Petri-dishes were used. Biofilm formed on the bottom of the dish and planktonic cells were quantified after growth for 4d at 78°C by OD_{600} measurements. The number of planktonic cells (non-attached cells after static incubation) decreased with increasing 1-butanol concentration, especially a drop from 0.5% to 1% (v/v) 1-butanol was measured. The OD_{600} of planktonic cells grown in the presence of 1% (v/v) 1-butanol was three-fold lower compared to the control. The OD_{600} of biofilm suspension was measured as well and an at least two-fold increase in absorbance from 0.5% to 1% (v/v) 1-butanol was determined (Fig 3-18 A, B). The 1-butanol concentrations within the medium were determined using the ADH-assay revealing initial 0.51 (± 0.02) % (v/v) 1-butanol and final 0.33 (± 0.02) % (v/v) 1-butanol as well as initial 1.05 (± 0.03) and final 0.78 (± 0.03) % (v/v) 1-butanol for 1% (v/v) 1-butanol culture (Fig. 3-18 D).

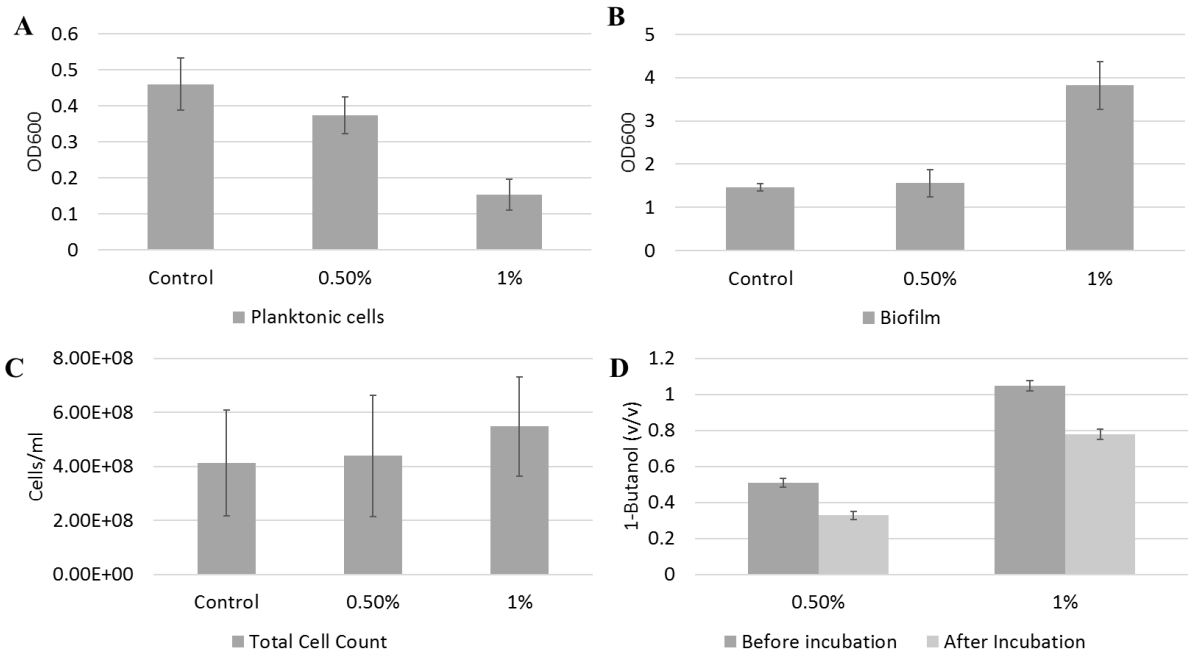


Figure 3-18: Analysis of biofilm formation of *S. acidocaldarius* in Petri-dishes in the presence of 0.5% and 1% (v/v) 1-butanol for four days at 78°C. A, Growth of planktonic cells and B, biofilm was monitored via OD_{600} measurements. C, the total cell count of resuspended biofilm solution in phosphate buffer (cells/ml) was determined by using the DAPI-method. D, the 1-butanol concentration before and after incubation determined by the ADH assay. Data represent three biological replicates, each 10 pooled technical replicates.

The number of planktonic cells (non-attached cells after static incubation) decreased with increasing 1-butanol concentration, especially from 0.5% to 1% (v/v) 1-butanol. The OD_{600} of planktonic cells grown in the presence of 1% (v/v) 1-butanol was three-fold higher compared to the control. The number of total cells in the biofilm suspension, determined by DAPI cell count, was quite similar for all three samples, indicating that the increase in biofilm formation observed is not caused by an increased cell number but probably by increased EPS formation (Fig. 3-18 C). To confirm this assumption, EPS extraction and quantification was performed.

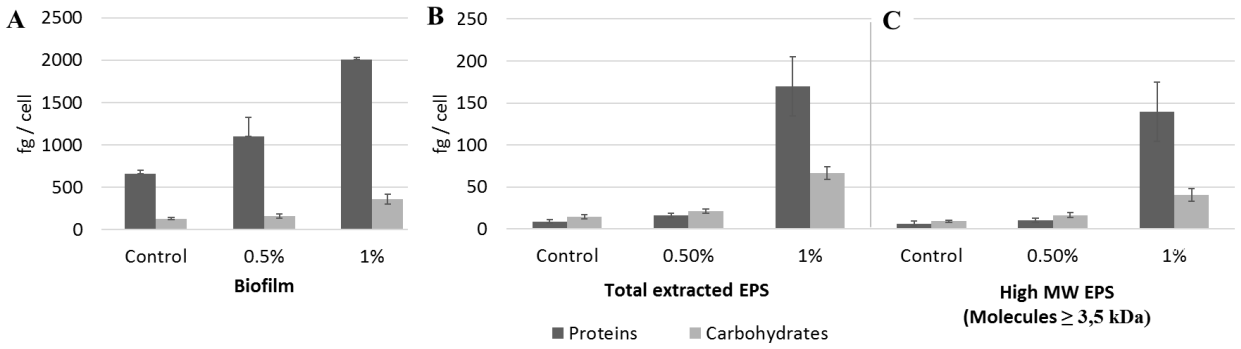


Figure 3-19: Quantification of carbohydrates and proteins of *S. acidocaldarius* biofilms grown for four days in the absence and presence of 0.5% and 1% (v/v) 1-butanol at 78°C in Petri-dishes. Quantified EPS-components were normalized to the total cell count. A, Biofilm: Isolated biofilm resuspended in phosphate buffer containing biofilm cells and total EPS. B, Total extracted EPS: Fractions after EPS extraction by the Dowex method and sterile filtration (total extracted EPS, comprising high + low MW compounds). C, High MW EPS: Molecules ≥ 3,5 kDa (High MW compounds. Fraction after dialysing of EPS sample in 3.5 kDa cut-off membrane against water). MW: Molecular weight

EPS isolation and quantification was optimized for submersed biofilms from the established protocol for unsaturated biofilms using the cation exchange resin Dowex (Jachlewski et al., 2015). Briefly, the biofilm was washed and isolated, resuspended in phosphate buffer (pH 7) and EPS was extracted by using the Dowex method. After EPS extraction, Dowex and cells were separated from EPS components by centrifugation and sterile filtration, resulting in the total extracted EPS fraction containing high and low molecular weight compounds (Fig. 3-19 B). The EPS fraction was subsequently dialysed against water using an 3,5 kDa dialysis tube. As low molecular weight compounds diffuse through the semipermeable membrane, only compounds with molecular weight above 3,5 kDa are retained (high MW EPS, Fig. 3-19 C).

The biofilm fraction consists of resuspended biofilms in phosphate buffer (pH 7) before EPS isolation, containing cells and EPS. Within the biofilm fraction, the amount of proteins as well as carbohydrates increased with increasing concentrations of 1-butanol exposure and was three-fold higher in the presence of 1% (v/v) 1-butanol compared to the control sample. The increase of carbohydrates and proteins in response to 1-butanol (1% (v/v)) was even more pronounced within the EPS fractions, including total extracted EPS. The carbohydrates increased 5-fold and the amount of proteins was even 19-fold higher. The 0.5% (v/v) 1-butanol sample, however, did not show a significant increase of carbohydrates and proteins compared to the control. Comparisons of the amount of carbohydrates and proteins within the EPS fraction before and after dialysis with 3,5 kDa cut-off membrane showed only a minor decrease, indicating the presence of mainly high molecular weight compounds in the EPS. The

main component of the EPS matrix was identified as proteins, which showed an increase in abundance in biofilms grown in the presence of 1% (v/v) 1-butanol. In comparison to the control, carbohydrate and protein amount in the 0.5% (v/v) 1-butanol sample was increased but to a much lesser extend compared to 1% (v/v) 1-butanol sample.

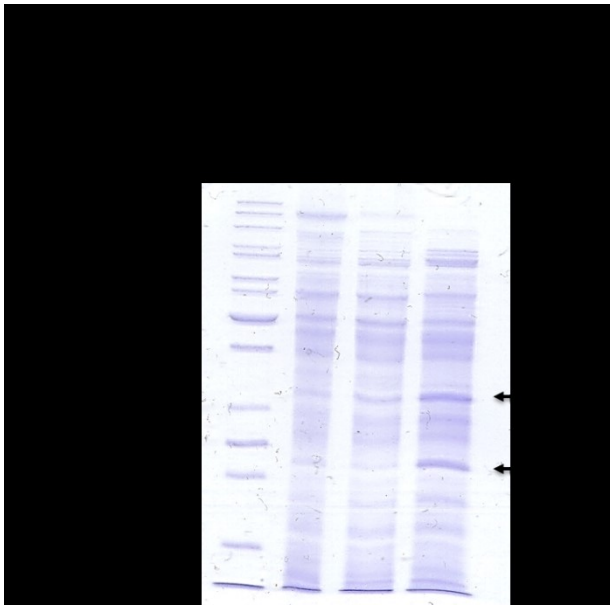


Figure 3-20: Examination of EPS proteins by 1-dimensional SDS-PAGE (12.5% PAA). Isolated and concentrated EPS fraction (proteins > 3.5 kDa) from static grown submerse *S. acidocaldarius* biofilms (4d, 78°C) are compared with membrane fraction and crude extract (soluble fraction) of planktonic grown *S. acidocaldarius* cells (shaking culture). Cultivation was performed in absence of 1-butanol. Proteins were visualised by coomassie brilliant blue staining.

Since the protein concentration of extracted EPS was too low for direct SDS-polyacrylamide gel-electrophoresis, EPS isolated from biofilms grown in the presence of 1% (v/v) 1-butanol was concentrated by medium reduction via evaporation (speed vac). Additional to the EPS fraction the membrane fraction and crude extract of *S. acidocaldarius* cells grown as shaking cultures in the absence of 1-butanol were analysed. Visual comparison of the samples showed that most of the proteins found in the concentrated EPS fraction also appear in planktonic cells of *S. acidocaldarius* membrane fraction and crude extract, indicating the presence of *S. acidocaldarius* proteins in the EPS fraction (Fig. 3-20). However, some proteins present in the EPS fraction, e. g. proteins of 25 kDa and 32 kDa size (arrows), seemed to be more dominant here than in the other fractions.

3.6. Genome-wide and cellular response of *S. acidocaldarius* towards changes in lifestyle and 1-butanol exposure

In order to study the genome-wide response of *S. acidocaldarius* towards changes in lifestyle and towards solvent exposure (i. e. 1-butanol), differences at transcriptome and proteome level were analysed. In the *S. acidocaldarius* genome a total number of 2306 genes and thereof 2224 protein encoding genes are annotated (L. Chen et al., 2005).

After static incubation of *S. acidocaldarius* in Petri-dishes for 4d at 78°C in the presence and absence of 1-butanol (0%, 0.5% and 1% (v/v)), changes in the transcriptome and proteome of isolated planktonic and biofilm cells were analysed. Briefly, Brock medium (pH 3), supplemented with 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose, was inoculated with an exponentially grown pre-culture and 25 ml were transferred into a Petri-dish. The static cultures in Petri dishes were incubated for 4d at 78°C in an anerobic jar in order to reduce evaporation of water and alcohols. Subsequently, planktonic cells were isolated by using a pipette and afterwards the attached biofilm cells were washed with minimal Brock medium (pH 3) and finally isolated. Furthermore, to address the effect of lifestyle, a shaking culture without 1-butanol addition (exponential growth phase OD_{600} 0.8) was grown and analysed. For each experiment, at least three biological replicates were pooled and further processed. Each biological replicate consisted of 10 Petri-dishes incubated in an anaerobic jar. For transcriptome studies, three biological replicates (30 Petri-dishes per 1-butanol concentration) and for proteome studies six biological replicates (60 Petri-dishes per 1- butanol concentration) were pooled, since the cell amount necessary for proteome studies is higher compared to transcriptome studies. Biofilm cells as well as planktoniccells from static and shaking cultures were labeled as shown in Tab. 3-2.

The aim of the study was to address two central questions:

i) What are the changes at transcriptome and proteome level in response to changes in lifestyle?
ii) How does *S. acidocaldarius* respond to 1-butanol exposure?

Table 3-2: Samples prepared for proteomic and transcriptomic studies. Life style samples are marked by orange shading and 1-butanol samples by blue shading.

Sample	Control	0.5% (v/v) 1-Butanol	1% (v/v) 1-Butanol
Biofilm	BF0	BF05	BF1
Planktonic cells (static culture)	PL0	PL05	PL1
Planktonic cells (shaking culture)	SC0	(-)	(-)

3.7. Genome wide response - Transcriptome studies

The changes at transcriptome level of three different samples were tested to evaluate the changes in gene expression in response to different lifestyles: (i) biofilm (BF0) and (ii) planktonic cells (PL0) from a static culture as well as (iii) planktonic grown cells from shaking cultures (SC0) of *S. acidocaldarius*.

In addition, the response of static grown biofilm and planktonic cells to different 1-butanol concentrations (0.5% and 1% (v/v) 1-butanol) was analysed. The global transcriptional responses were investigated by using Illumina sequencing.

In order to follow the effect of 1-butanol the amount of planktonic cells (OD_{600}), biofilm formation (wet weight) as well the actual 1-butanol concentration (ADH assay) was determined. The OD_{600} of statically grown planktonic cells decreased with increasing 1-butanol concentrations, whereas the biofilm wet weight is not strongly affected by 1-butanol. Notably the biofilm wet weight includes cells as well as EPS and, thus, represents the overall amount of biomass.

Table 3-3: Static cultivation of *S. acidocaldarius* in absence and presence of 0%, 0.5% and 1% (v/v) 1-butanol. Planktonic growth (OD_{600}) and biofilm wet weight were determined after 4d of incubation at 78°C. Furthermore, the initial and final 1-butanol concentration was measured. N = 3

Sample	Control	0.5% (v/v) 1-Butanol	1% (v/v) 1-Butanol
Planktonic cells (OD_{600})	0.428 ± 0.035	0.335 ± 0.079	0.170 ± 0.049
Biofilm wet weight (mg)	77.733 ± 10.300	65.567 ± 8.087	72.267 ± 13.258
1-Butanol concentration (%(v/v)) (initial)	-	0.55 ± 0.02	1.05 ± 0.00
1-Butanol concentration (%(v/v)) (final)	-	0.43 ± 0.02	0.82 ± 0.03

Sequencing yielded 7.1 – 11.4 mio reads per sample (Tab. 3-4). Of these reads, between 94.8% and 98.1% could be mapped to the reference genome and the number of reads mapping to coding sequences differed between 17.8% and 42.5%. Only the latter were used for the analysis of differential expression.

Table 3-4: Number of all sequencing reads and those mapping to the whole genome or only to coding sequences.

Sample	Total number of reads	Reads mapped to genome	Reads mapped to CDS
BF0	7.109.290	6.833.775	1.326.187
BF05	7.937.344	7.621.723	1.919.074
BF1	9.626.214	9.321.419	2.466.788
PL0	11.444.644	11.076.395	2.036.789
PL05	9.354.704	8.937.716	2.880.270
PL1	7.854.274	7.448.391	2.291.119
SC0	8.129.060	7.976.835	3.455.628

The stability indices obtained for the comparison between all RPKM values of two different conditions indicated a higher conformity with R^2-values between 0.91 and 0.93 for samples with 0 and 0.5 % Butanol (BF0 *vs* BF05 and PL0 *vs* PL05) or biofilm and planktonic culture (BF0 *vs* PL0). The low dispersion for the last example (BF0 *vs* PL1) was also visible in an MA-plot (Fig. 3-21). The comparison between 0% and 1% (v/v) 1-butanol showed a medium dispersion for both lifestyles (BF1 *vs* BF0, PL1 *vs* PL0), which resulted in R^2-values of 0.76 and 0.77. The highest differences were found for shaking sulture in comparison with biofilm (BF0 vs SC0) or planktonic lifestyle (PL0 *vs* SC0). This is confirmed by the highest number of differentially regulated genes for these comparisons (Tab. 3-5).

Table 3-5: Dataset correlation and the amount of differentially regulated genes.

Comparison		Number of regulated proteins (>4-fold)			
Samples		**R^2-value**	**Down**	**Up**	**Total**
Lifestyle	BFO *vs* PL0	0.91	13	1	14
	BFO *vs* SC0	0.58	204	118	332
	PLO *vs* SC0	0.67	54	163	217
Biofilm	BF1 *vs* BF0	0.77	74	42	116
	BF05 *vs* BF0	0.91	4	12	16
Planktonic	PL1 *vs* PL0	0.76	89	32	121
	PL05 *vs* PL0	0.93	5	3	8

The MA-plots of differentially expressed genes under different cultivation conditions are given in Fig. 3-21. The number of genes with low A-value (<2) was for all comparisons quite low. Static grown biofilm and planktonic cells showed a low number of regulated genes (A). The number of regulated genes between static grown planktonic cells and planktonic cells grown in shaking cultures was quite high (B). Biofilm (C) and planktonic cells (D) incubated in presence or absence of 1% (v/v) 1-butanol show comparable MA-plots.

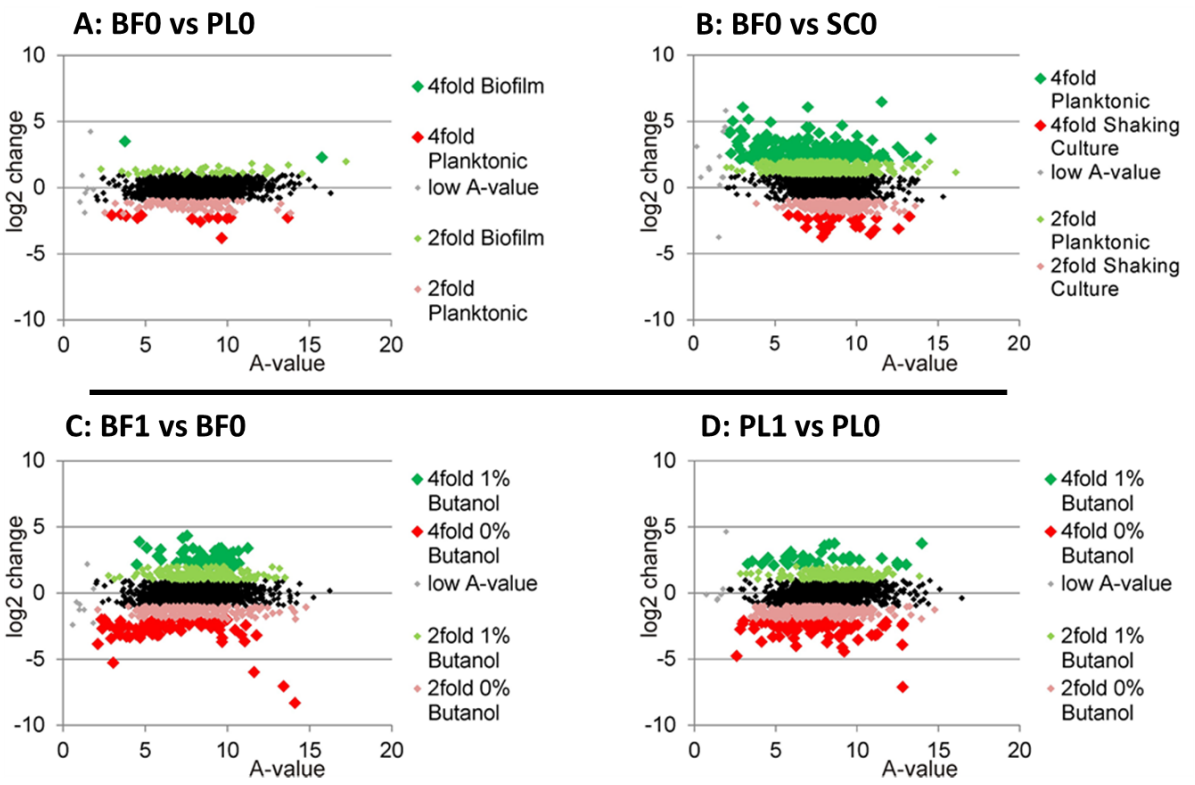


Figure 3-21: MA plots of differential regulated genes in response to lifestyle (A, B) and 1% (v/v) 1-butanol exposure (C, D). The comparison between different lifestyles Biofilm vs. Planktonic (static culture, A) (A) and Biofilm vs. Planktnic (shaking culture) (B) and the effect of 1% (v/v) 1-butanol exposure on biofilm cells (C) and planktonic cells (static culture) (D) is shown.

3.7.1. Transcriptional changes in response to lifestyle

Initially, the transcriptional changes of static incubated biofilm (BF0) and planktonic (PL0) cells were compared to investigate the transcriptional changes in response to different lifestyles. The statically incubated planktonic cells were compared with planktonic cells grown with agitation (shaking culture).

Static lifestyle: Biofilm *versus* planktonic cells

The transcriptional changes in response to static life style were compared for statically grown biofilm and planktonic cells. The comparison of biofilm cells (BF0) and planktonic cells (PL0)

grown under static cultivation conditions revealed 156 genes being more than two-fold differentially regulated: 43 genes were more than 2-fold up-regulated and 113 genes were down-regulated in biofilm cells compared to planktonic cells. Only 15 genes were regulated more than 4-fold with 2 genes being up-regulated and 13 genes down-regulated in biofilm cells (Tab. 3-6).

Table 3-6: Comparison of differentially regulated genes in *S. acidocaldarius* biofilm cells and planktonic cells grown under static cultivation conditions (Petri-dishes, 4d, 78°C). The number of up- and down-regulated genes in biofilm cells is given.

		>2-fold regulated			>4-fold regulated		
		up	down	total	up	down	total
Static lifestyle	BF0/PL0	43	113	156	2	13	15

Most of the up-regulated genes in biofilm cells belong to the arCOG categories for metabolism, cellular processes and signalling. Among other a recombinase (Saci_0677) is up-regulated 11-fold.

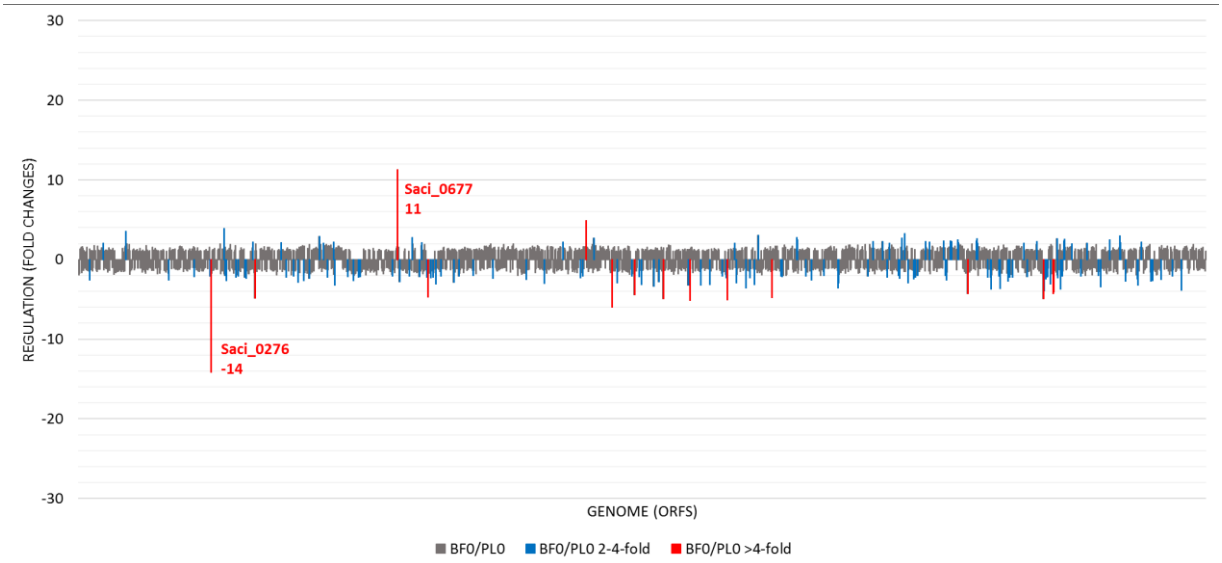


Figure 3-22: Genome-wide distribution of differentially regulated genes in *S. acidocaldarius* biofilm and planktonic cells grown under static cultivation conditions (Petri-dishes, 4d, 78°C). Genes regulated below 2-fold are depicted in grey, 2-4-fold in blue and above 4-fold in red.

The number of regulated genes according to their arCOG classification is shown in Fig. 3-23. Most of the significantly regulated genes (66%) are hypothetical or uncharacterized and the function of most genes genes is not known (arCOG category R and S). Especially the categories C (metabolism, energy production and conversion), E (metabolism, amino acid metabolism

and transport), I (metabolism, lipid metabolism), J (information storage and processing, translation), K (information storage and processing, transcription) and V (cellular processes and signalling, defence mechanisms) are down-regulated in biofilm cells. Therefore, changes in biofilm/planktonic lifestyle (BF0/PL0) under static cultivation are predominantly observed in the metabolism.

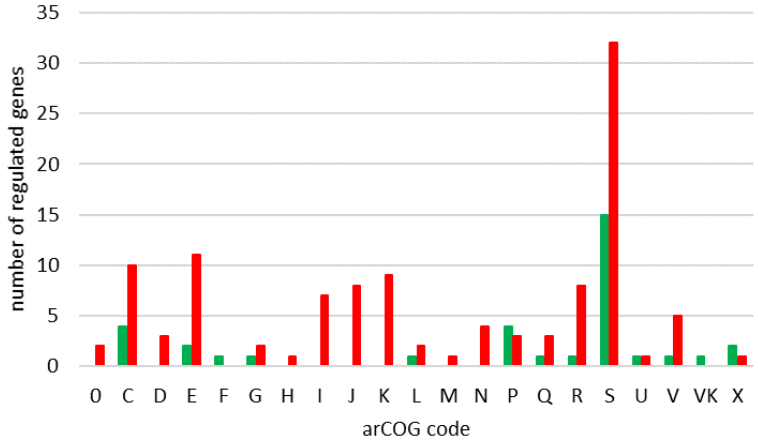


Figure 3-23: Distribution of regulated genes of *S. acidocaldarius* biofilm and planktonic cells grown under static cultivation conditions (Petri-dishes, 4d, 78°C) in arCOG categories. A cut-off of 2-fold regulated genes was used. Up-regulation in biofilm cells is depicted in green, down-regulation in red colour.

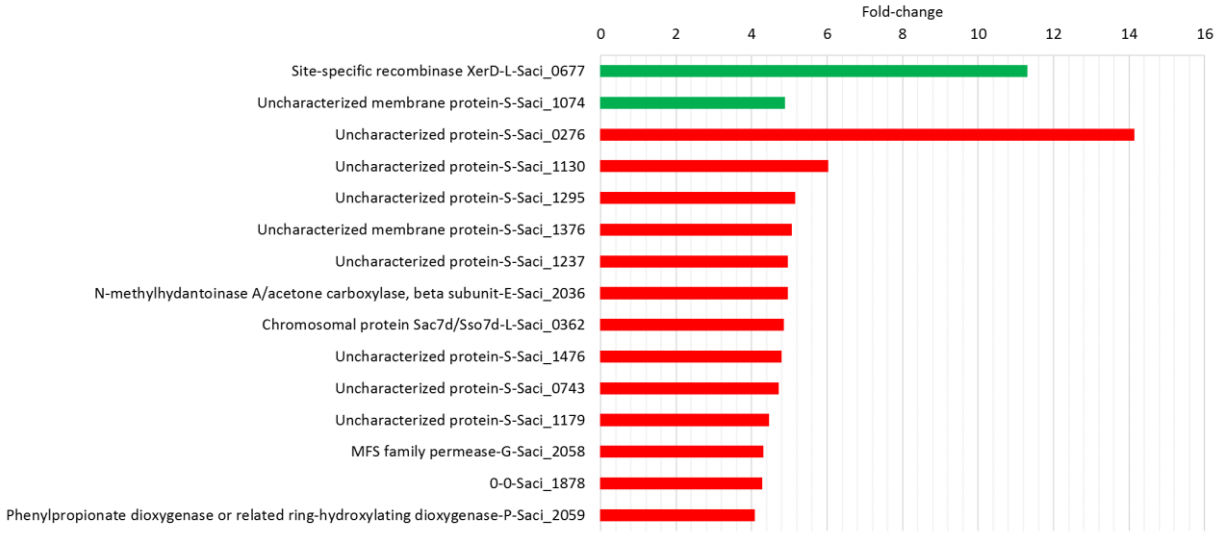


Figure 3-24: Differentially expressed genes (cut-off >4-fold) in biofilm cells and planktonic cells (BF0/PL0) grown under static cultivation conditions (Petri-dishes, 4d, 78°C). More than 4-fold up- (green colour) and down- (red colour) regulated genes in biofilm cells compared to planktonic cells (BF0/PL0) are shown. Capital letters following the respective annotation indicate the arCOG category. For genome annotation and all regulated genes see supporting information.

The more than 4-fold regulated genes above are shown in Fig. 3-24. For example, a MFS family permease (Saci_2058, 4.3-fold) and a dioxygenase (Saci_2059, 4.1-fold), which are in close vicinity, are significantly down-regulated in biofilm cells. A site-specific recombinase (Saci_0677, 11.3-fold) is highly up-regulated in biofilms and an uncharacterized protein (Saci_0276, -14.1-fold) highly down-regulated.

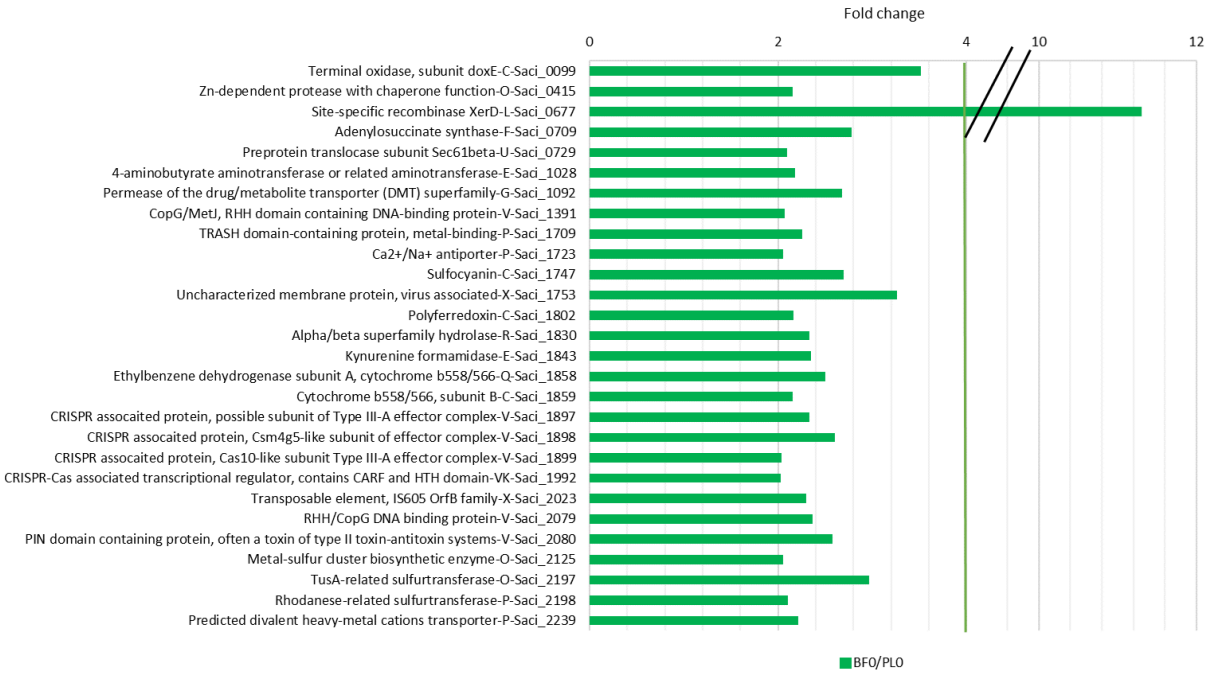


Figure 3-25: Differentially expressed genes (cut-off >2-fold) in biofilm cells and planktonic cells (BF0/PL0) grown under static cultivation conditions (Petri dishes, 4d, 78°C). Selected up-regulated genes (> 2-fold) in biofilm cells compared to planktonic cells (BF0/PL0) are shown. Capital letters following the respective annotation indicate the arCOG category. For genome annotation and all regulated genes see supporting information.

In Fig. 3-25, selected up-regulated genes with a cutoff of >2-fold are shown. The gene region from Saci_1897 to Saci_1992 is up-regulated in biofilms and is involved in the CRISPR system (CRISPR: clustered, regularly interspaced, short, palindromic repeats). In addition, genes encoding proteins of the aeroic respiratory-chain (Saci_0099, Saci_1747, Saci_1802 and Saci_1859), sulfur metabolizing genes (Saci_2125, Saci_2197 and Saci_2198) and membrane associated genes (Saci_1729, Saci_1092, Saci_1723 and Saci_2239) are up-regulated in biofilm cells. Some genes (Saci_1391, Saci_2079 and Saci_2080) belonging to the toxin-antitoxin system are up-regulated as well.

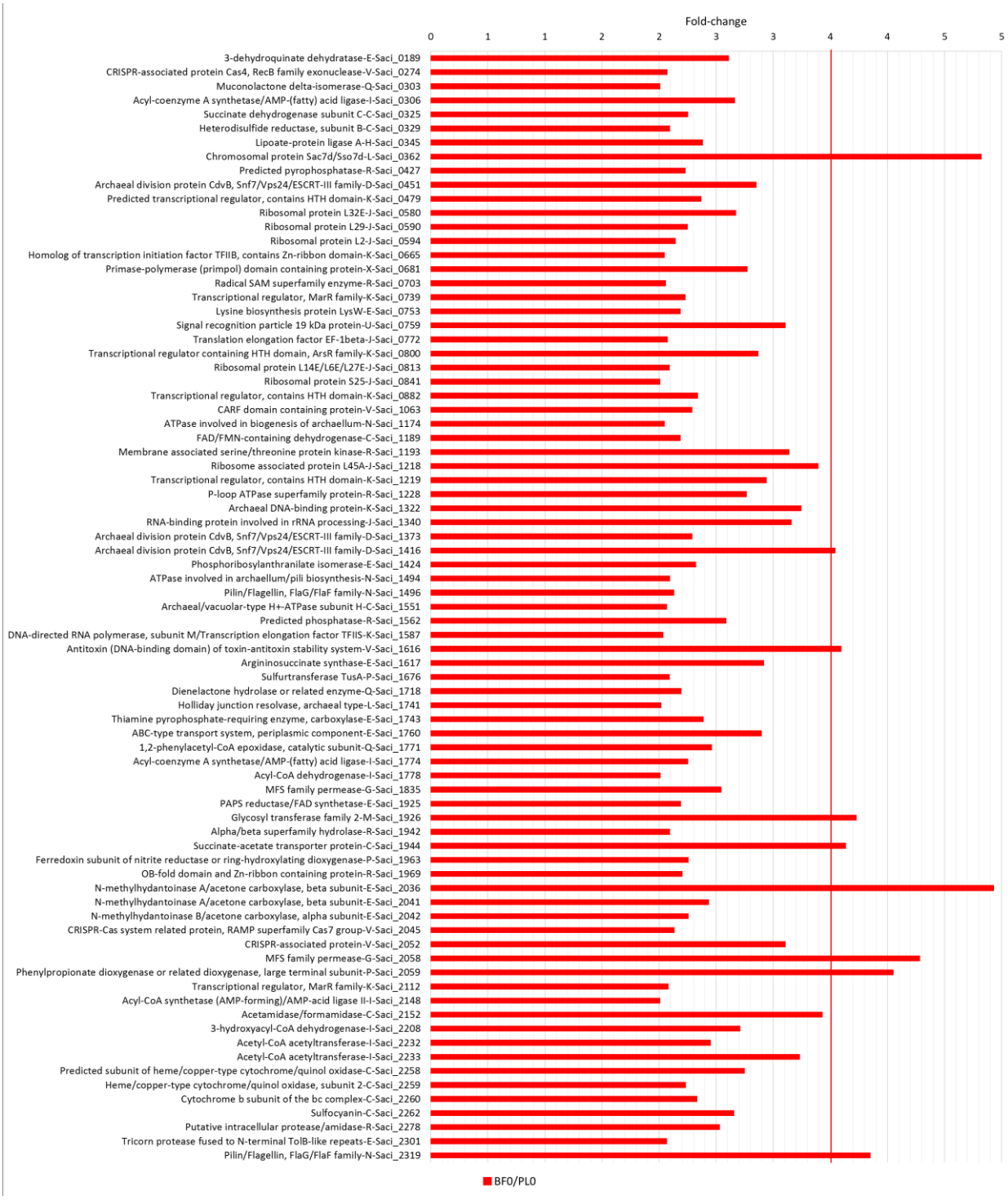


Figure 3-26: Differentially expressed genes (cut-off >2-fold) in biofilm cells and planktonic cells (BF0/PL0) grown under static cultivation conditions (Petri-dishes, 4d, 78°C). Selected down-regulated genes (> 2-fold) in biofilm cells compared to planktonic cells (BF0/PL0) are shown. Capital letters indicates the arCOG category. For genome annotation and all regulated genes see supporting information.

In Fig. 3-26, selected down-regulated genes with a cutoff of >2-fold in biofilm cells are shown. The down-regulation of a high number of metabolism-related genes involved in e.g. Acyl- (Saci_0306, Saci_1774, Saci_1778) and Acetyl-CoA metabolism (Saci_2148, Saci_2232 and

Saci_2333) was found in biofilm cells. In addition, genes encoding proteins involved in archaellum formation (Saci_1174, Saci_1494, Saci_1496 and Saci_2319) are also down-regulated. Furthermore, different transcriptional regulators (Saci_0479, Saci_0739, Saci_Saci_0800, Saci_0882, Saci_1219 and Saci_2112), genes belonging to the ESCRT-system (ESCRT: endosomal sorting complexes required for transport machinery, genes Saci_0451, Saci_1373 and Saci_1412) and the CRISPR-CAS system (Saci_0274, Saci_2045 and Saci_2052) are down-regulated in biofilm cells as well.

Planktonic cells: Static *versus* shaking lifestyle

In a next step, planktonic cells of static and shaking cultures were compared. The number of >4-fold up-regulated genes in planktonic cells of static cultures (PL0) compared to shaking cultures (SC0) is significantly higher (>4-fold). 163 genes are up- and 54-genes down-regulated compared to shaking cultures. The overall number of 217 significantly regulated genes is quite high), indicating dramatic changes in gene expression in response to the different cultivation conditions. Moreover, 863 of >2-fold regulated genes are regulated, thereof 483 up-regulated and 380 down-regulated.

Table 3-7: Comparison of differentially regulated genes of static grown cells (Petri-dishes, 4d, 78°C) and as shaking culture (Erlenmeyer flasks, 180rpm, 78°C). The number of up- and down-regulated genes in planktonic cells is given.

		>2-fold regulated			>4-fold regulated		
		up	down	total	up	down	total
Static vs shaking culture	PL0/SC0	483	380	863	163	54	217

The overall differential gene regulation in planktonic cells of static cultures (Petri-dish) and shaking cultures are shown in Fig. 3-27. In general, the number of up-regulated genes in statically grown planktonic cells is higher than of shaking cultures. The genome wide distribution of regulated genes revealed some genome regions with exceptional high expression changes.

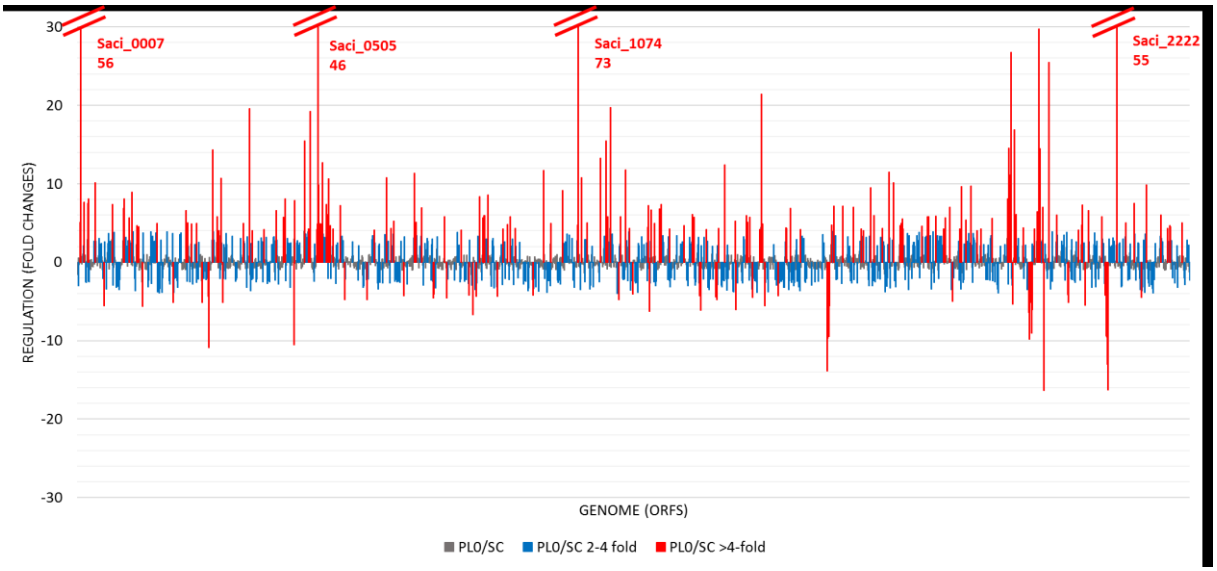


Figure 3-27: Genome-wide distribution of differentially regulated genes in *S. acidocaldarius* planktonic cells of static (Petri-dishes, 4d, 78°C) and in shaking cultures (Erlenmeyer flasks, 78°C, 180rpm). Genes regulated below 2-fold changeare depicted in grey, 2-4-fold change in blue and above 4-fold change in red.

The down-regulation of genes (> 2-fold) in statically grown planktonic cells grown under static conditions (PL0/SC0) is most abundant in the arCOG categories C (metabolism, energy production and conversion), E (metabolism, amino acid metabolism and transport) and J (information storage and processing, translation) (Fig. 3-28). Again, the most up-regulated genes were found to be not characterized (arCOG category R and S).

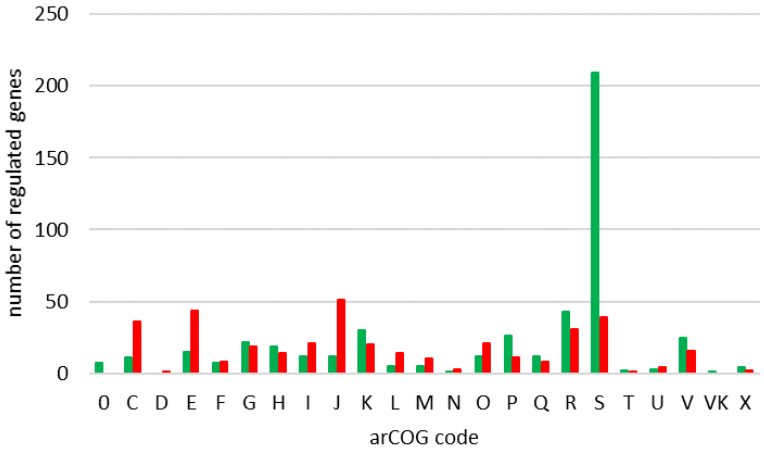


Figure 3-28: Regulated genes categorized in arCOG categories in *S. acidocaldarius* planktonic cells of static (Petri-dishes, 4d, 78°C) and shaking cultures (Erlenmeyer flasks, 78°C, 180rpm). A cut-off of 2-fold regulated genes was used. Up-regulation in statically grown planktonic cells is depicted in green, down-regulation in red colour.

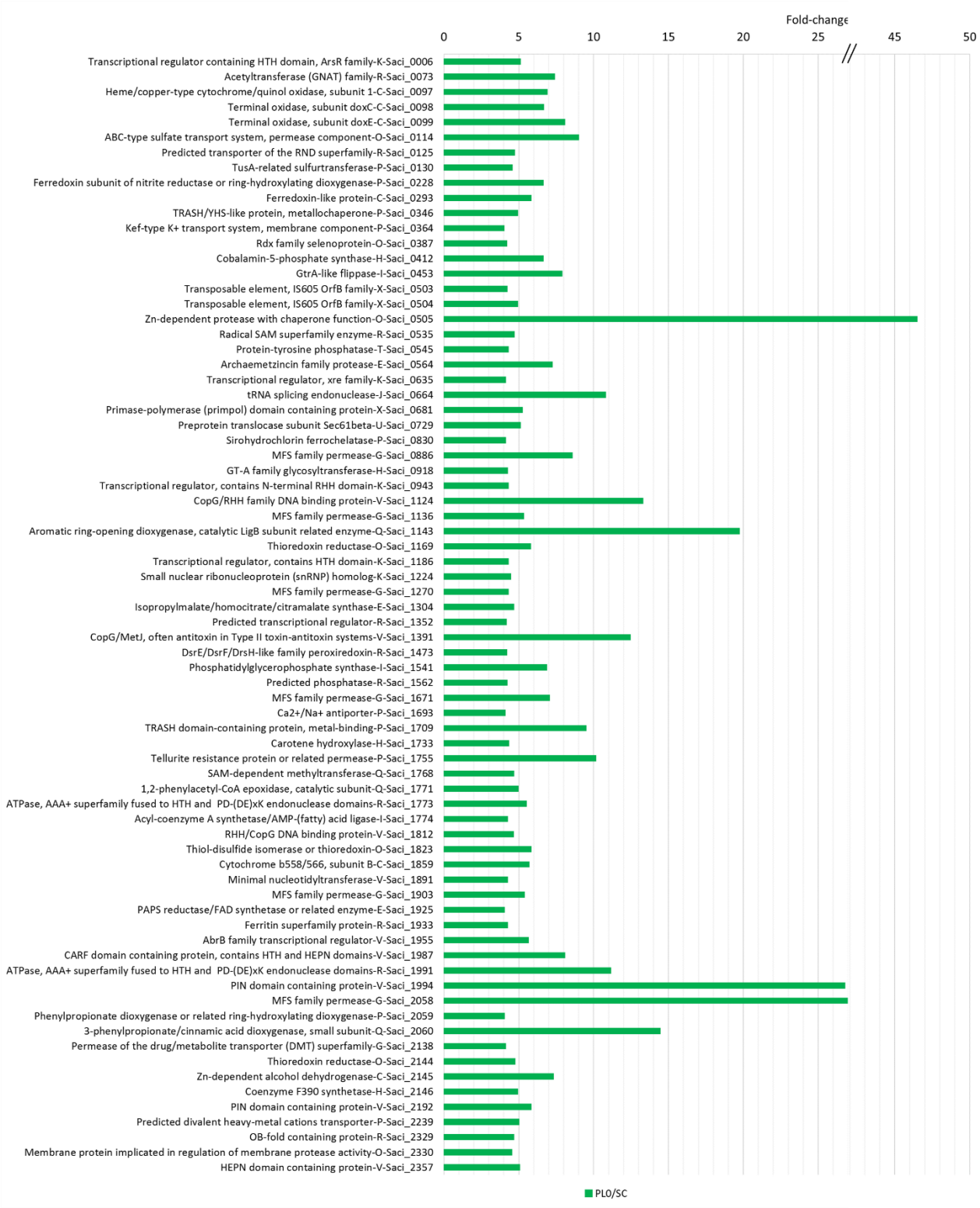


Figure 3-29: Differentially expressed genes (cut-off >4-fold) of planktonic cells (PL0/SC0) of static (Petri-dishes, 4d, 78°C) and shaking (Erlenmeyer flasks, 180rpm, 78°C) cultures. Up-regulated genes (> 4-fold) in statically grown planktonic cells compared to shaking culture. Capital letter indicates the arCOG category. For genome annotation and all regulated genes see supporting information.

The number of significantly regulated genes is quite high and various protein classes are regulated. However, Saci_0505 (Zn-dependent protease with chaperone function, 46-fold change), Saci_1994 (PIN domain containing protein, 27-fold change) and Saci_2058 (MFS family permease, 30-fold change) are the genes with highest up-regulation in planktonic cells grown under static conditions. In general, a high number of MFS (major facilitator superfamily) family permease related genes are up-regulated (Saci_0886, Saci_1136, Saci_1270, Saci_1671, Saci_1903 and Saci_2058) probably involved in the transport of small solutes across the cell membrane are up-regulated. In addition, six transcriptional regulators are up-regulated as well, suggesting major changes in transcriptional control.

Figure 3-30: Differentially expressed genes (cut-off >4-fold) of planktonic cells (PL0/SC0) of static (Petri-dishes, 4d, 78°C) and shaking (Erlenmeyer flasks, 180rpm, 78°C) cultures. Down-regulated genes (> 4-fold) in planktonic cells compared to shaking culture. Capital letter indicates the arCOG category. For genome annotation and all regulated genes see supporting information.

As already shown for up-regulated genes the number of down-regulated genes in statically grown planktonic cells compared to shaking cultures is quite high. Again, various different protein classes are regulated indicating a global transcriptional change based on the different lifestyles. In contrast to the up- regulated genes, only one gene encoding a transcriptional regulator (Saci_2116) is down-regulated in statically grown planktonic cells. Five genes encoding proteins of the ESCRT system (i. e. Saci_0451, Saci_1372, Saci_1373, Saci_1374 and Saci_1416) as well as the single subunits of a N-methylhydantoinase/acetone carboxylase (Saci_2036, Saci_2041 and Saci_2042) are down-regulated in planktonic cells of static cultures compared to shaking cultures.

3.7.2. Transcriptional changes based on 1-butanol exposure

To study the effect of 1-butanol on biofilm and planktonic cells under static growth conditions, *S. acidocaldarius* was grown in Petri-dishes for 4d at 78°C. For the identification of genes regulated only by 1-butanol exposure, genes regulated above a 4-foldchange in both lifestyles upon butanol were considered.

Table 3-8: Number of differentially regulated genes of planktonic and biofilm cells grown under static cultivation conditions in presence of 1% (v/v) 1-butanol. The number of regulated genes in presence of 1- butanol is given.

		>2-fold regulated			>4-fold regulated		
		up	down	total	up	down	total
Biofilm	BF1/BF0	206	266	272	42	74	116
	BF05/BF0	58	95	153	4	12	16
Planktonic	PL1/PL0	200	318	518	32	89	121
	PL05/PL0	67	54	121	3	5	8

The overall number of regulated genes of planktonic and biofilm cells cultivated statically with 1% (v/v) 1-butanol was comparable (121 and 116, respectively), indicating that under both growth conditions 1% (v/v) 1-butanol has a significant effect on gene expression (Tab. 3-8). However, most genes are down-regulated in biofilms (BF1, 74) and planktonic cells (PL1, 89). In contrast, the overall regulation in samples with and without 0.5% (v/v) 1-butanol was quite low. Therefore, the detailed comparative analysis of the transcriptome data was performed only for cells grown with and without 1% (v/v) 1-butanol.

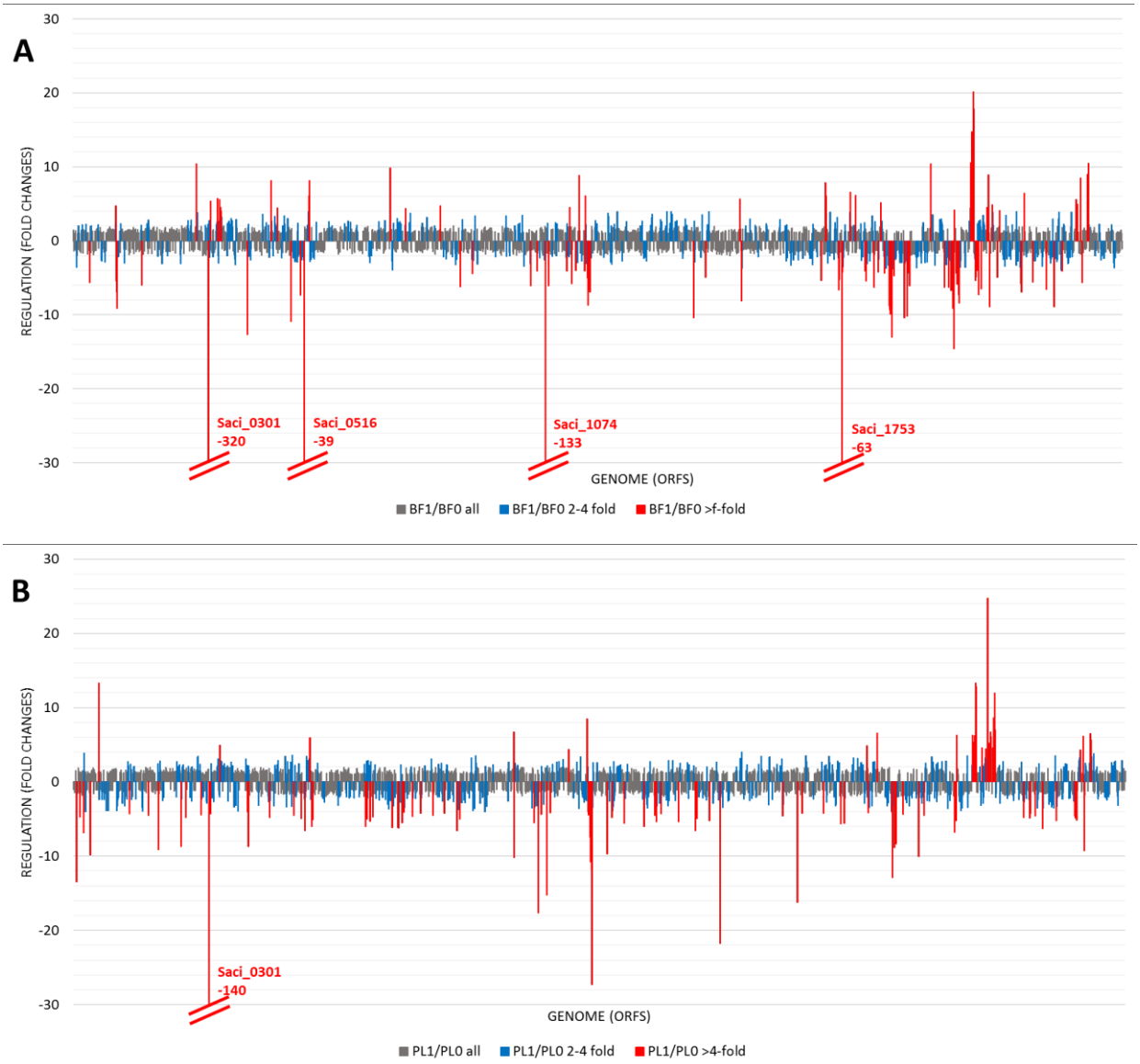


Figure 3-31: Genome-wide distribution of regulated genes of *S. acidocaldarius* biofilms (A) and planktonic cells (B) grown under static cultivation conditions in response to 1% (v/v) 1-butanol (static cultivation in Petri-dishes, 4d, 78°C). Genes regulated below 2-fold are indicated in grey, 2-4-fold in blue and above 4-fold in red.

Although some specific genome regions are strongly regulated, regulated genes are distributed over the whole chromosome, indicating general changes in gene expression (Fig. 3-31 A, B).

In biofilm cells grown in the presence of 1% (v/v) 1-butanol no gene is up-regulated more than 20-fold, whereas four genes are highly down-regulated: Saci_0301 (uncharacterized membrane protein, DUF981 family, 320-fold change), Saci_0516, (uncharacterized protein, 39-fold change) Saci_1074 (uncharacterized membrane protein, 133-fold change) and Saci_1753 (uncharacterized membrane protein, virus associated, 63-fold change). None of the proteins encoded by strongly regulated genes has been characterized so far.

As already found for biofilm cells, Saci_0301 is also strongly down-regulated in planktonic cells in response to 1-butanol exposure (140-fold change, Uncharacterized membrane protein, DUF981 family). The overall regulation pattern of genes from planktonic and biofilm cells in response to 1-butanol at transcriptome level shows significant differences dependent on the lifestyle.

The characterized proteins significantly (> 4-fold) up- and down-regulated are listed in Fig. 3- 34 and 3-35.

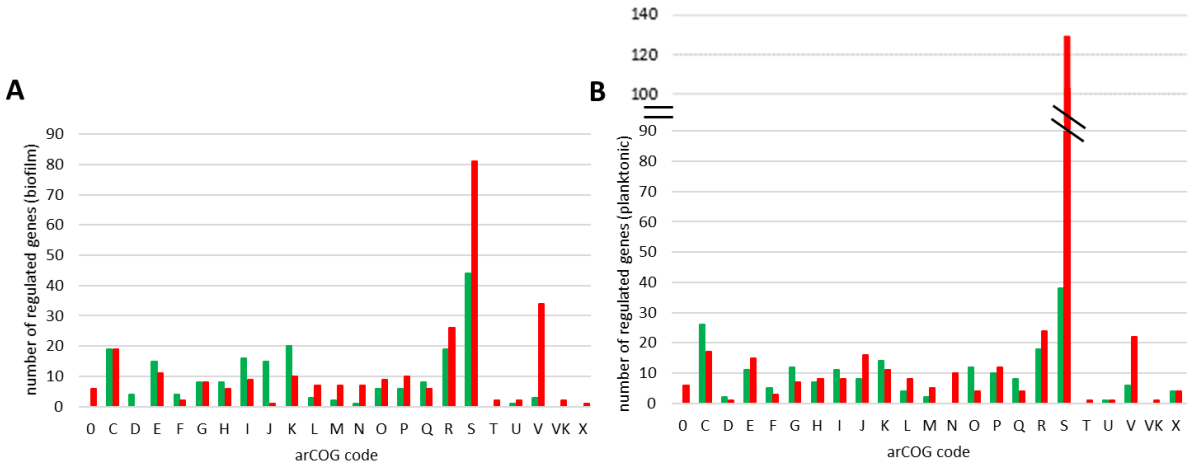


Figure 3-32: Distribution of regulated genes of *S. acidocaldarius* biofilm (A) and planktonic cells (B) grown under static cultivation conditions (Petri-dishes, 4d, 78°C) in arCOG categories. A cut-off of 2-fold regulated genes was used. Up-regulation in presence of 1-butanol is depicted in green, down-regulation in red colour.

In the presence of 1-butanol, in biofilm as well as planktonic cells, especially genes belonging to arCOG category V (cellular processes and signalling, defence mechanism) are down-regulated (Fig. 3-32). Most dominant regulated are uncharacterized genes (arCOG category S and R).

Co-regulated genes

In total 41 co-regulated genes (out of 252 regulated genes) were identified, which are regulated more than 4-fold in both biofilm and planktonic cells upon 1-butanol exposure (Fig. 3-33). 15 genes were up-regulated in the presence of 1% (v/v) 1-butanol and 26 genes were down-regulated.

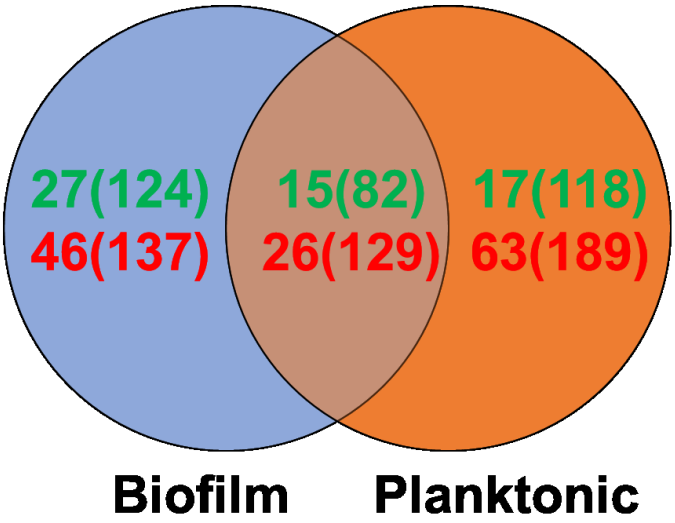


Figure 3-33: Number of differentially and co-regulated genes in static grown biofilm and planktonic cells in response to 1-butanol (1% (v/v)) exposure. The intersection gives the number of co-regulated genes. The number of 4- fold and 2-fold (in brackets) regulated genes is indicated. Green numbers indicate up-regulation upon 1-butanol exposure and red down-regulation.

The most dramatic up-regulation of genes in statical grown biofilm and planktonic cells upon 1-butanol exposure was found for Saci_2041 and Saci_2042 (N-methylhydantoinase/acetone carboxylase α and β subunit, respectively) with more than 13-fold regulation in both lifestyles. Saci_2036, encoding another β subunit homolog was up-regulated in both lifestyles as well. The up-regulation of this protein-complex is higher in biofilm cells compared to planktonic cells.

Saci_2293, Saci_2294 and Saci_2295 are located in direct gene-neighbourhood and are up-regulated significantly in both lifestyles upon 1-butanol exposure. All genes have metabolic function, Saci_2293 and Saci_2294 are categorized for secondary structure (arCOG category Q) and Saci_2295 for amino acid metabolism and transport (arCOG category E). Saci_2293 and Saci_2295 encode for proteins involved in the catechol pathway for degradation of aromatic compounds.

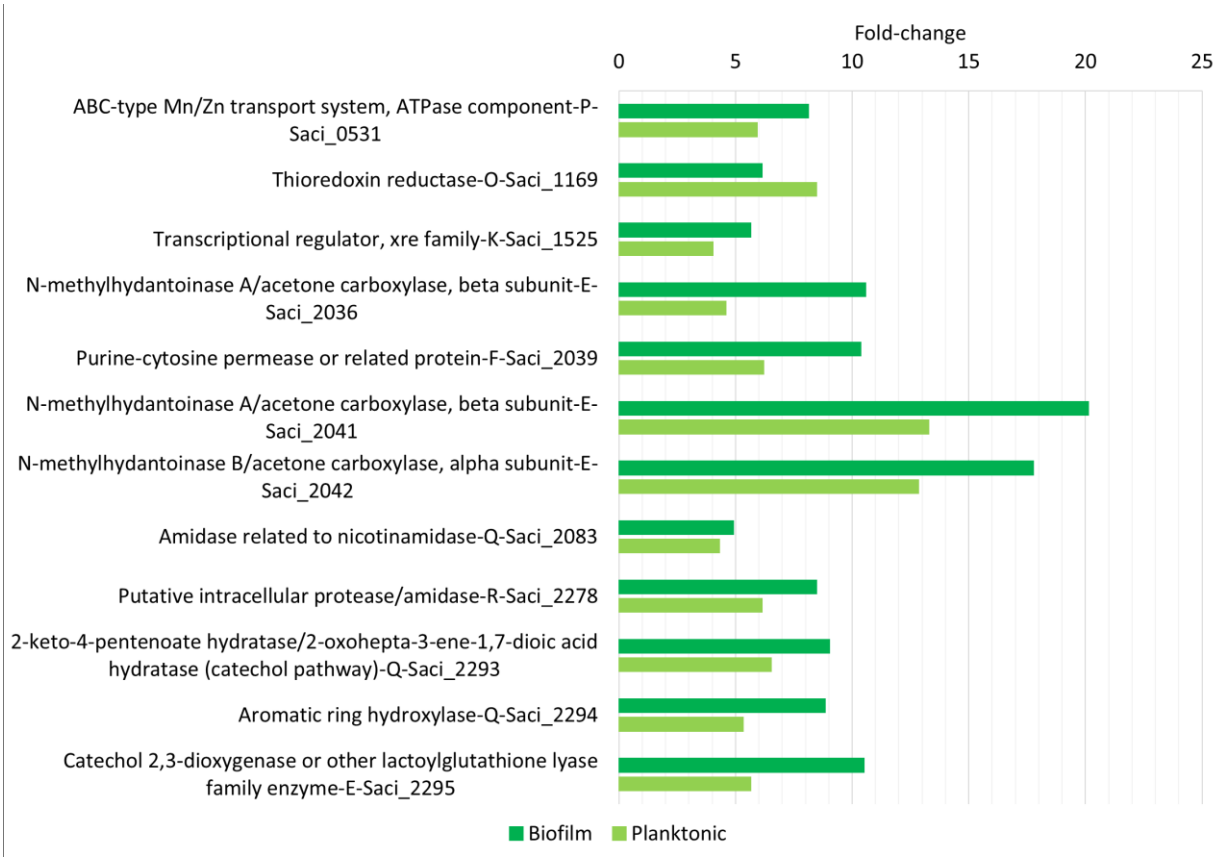


Figure 3-34: Co-regulated genes with more than 4-fold up-regulation in static grown *S. acidocaldarius* biofilm and planktonic cells in response to 1-butanol (1% (v/v) exposure (static cultivation in Petri-dishes, 4d, 78°C). Up-regulated genes show higher abundance in presence of 1-butanol. Capital letter indicates the arCOG category. For genome annotation and all regulated genes see supporting information.

Genes encoding archaellum components (i. e. Saci_1176, Saci_1177 and Saci_1178) were significantly down-regulated in biofilm and planktonic cells upon 1-butanol exposure. Furtheron, three genes belonging to arCOG category V (cellular processes and signalling: defence mechanisms): Saci_1056, Saci_1992 and Saci_1994 are significantly down-regulated. Saci 1992 and Saci_1994 are located in close neighbourhood on the chromosome.

Figure 3-35: Co-regulated genes with more than 4-fold down-regulation in static grown *S. acidocaldarius* biofilm and planktonic cells in response to 1-butanol (1% (v/v)) exposure (static cultivation in Petri-dishes, 4d, 78°C). Down-regulated genes show abundance in presence of 1-butanol. Capital letter indicates the arCOG category. For genome annotation and all regulated genes see supporting information.

Transcriptional changes based on 1-butanol exposure: Genes regulated dependent on lifestyle

Genes significantly (> 4-fold) regulated contrariwise in static grown biofilm and planktonic cells upon 1% (v/v) 1-butanol exposure (Bf1/BF0 and PL1/PL0, respectively) were compared with the regulation in opposed lifestyles (BF0/PL0). Identified genes regulated in the opposite way upon 1% (v/v) 1-butanol exposure are listed in Tab. 3-9.

Table 3-9: Genes significantly (>4-fold) regulated contrariwise in biofilm lifestyle compared to planktonic lifestyle upon 1% (v/v) 1-butanol exposure. Lifestyle-dependent regulations is depicted as control (BF0/PL0). *S. acidocaldarius* cells were incubated statically in Petri-dishes for 4d at 78°C. Biofilm (BF1/BF0) and planktonic (PL1/PL0) show up- and down-regulation of genes in the presence of 1-butanol, lifestyle shows up- and down-regulation in biofilm cells without 1-butanol exposure. Dark red and dark green colour indicate significantly (>4-fold) regulated genes, light red and light green colour indicate low regulated genes (2-4-fold regulation).

Locus	ArCOG Annotation	ArCOG Code	Biofilm BF1/BF0	Planktonic PL1/PL0	Lifestyle BF0/PL0
Saci_1897	CRISPR assocaited protein, possible subunit of Type III-A effector complex	V	-4.20	1.08	2.31
Saci_2049	CRISPR-Cas system related protein, RAMP superfamily Cas7 group	V	-4.06	1.20	1.45
Saci_2239	Predicted divalent heavy-metal cations transporter	P	-4.08	1.42	2.20
Saci_0026	Endonuclease Nob1, consists of a PIN domain and a Zn-ribbon module	J	1.42	-4.00	-1.26
Saci_0664	tRNA splicing endonuclease	J	1.75	-6.02	-1.87
Saci_0739	Transcriptional regulator, MarR family	K	3.00	-5.58	-2.22
Saci_0882	Transcriptional regulator, contains HTH domain	K	1.76	-5.03	-2.33
Saci_1212	AbrB family transcriptional regulator	V	1.12	-9.65	-1.23
Saci_2079	RHH/CopG DNA binding protein	V	-1.01	4.56	2.35
Saci_2080	PIN domain containing protein, often a toxin of type II toxin-antitoxin systems	V	-1.66	4.23	2.56

50% of the differentially regulated genes belong to arCOG category V (cellular processes and signalling, defence mechanisms). Most of the significantly down-regulated genes in planktonic cells in response to 1-butanol belong to the categories J and K for information storage and processing. Saci_0026 and Saci_0664, have a predicted function in the translation machinery and Saci_0739 and Saci_0882 in the transcription machinery. Three transcriptional regulators (Saci_0739, Saci_0882and Saci_1212) were regulated in the opposite way upon 1-butanol exposure. These regulators were strongly down-regulated in planktonic cells upon 1-butanol exposure and in biofilm cells compared to planktonic cells without solvent exposure. Two CRISPR associated genes (Saci_1897 and Saci_2049) are strongly down-regulated in biofilm cells in presence of 1% (v/v) 1-butanol and up-regulated in planktonic cells in presence of 1% (v/v) 1-butanol and in biofilm cells without 1-butanol exposure. Depending on lifestyle, these genes are up-regulated in biofilm cells and planktonic cells upon 1-butanol exposure.

3.7.3. Transcriptional changes based on 1-butanol exposure: Proteins with predicted transmembrane helices

Generally, upon 1-butanol exposure, the number of down-regulated genes is higher than the number of up-regulated genes indicating that down-regulation is a stress response towards 1-butanol exposure. Since alcohols like 1-butanol can destabilize the membrane, the number of differentially regulated genes encoding for proteins containing predicted transmembrane helices was determined. For *S. acidocaldarius*, 455 of in total 2223 annotated genes contain transmembrane helices, representing 20% of all ORFs. 16% of the up-regulated (>2-fold) and 43% of the down-regulated (>2-fold) genes in response to 1% (v/v) 1-butanol contain predicted transmembrane helices.

Table 3-10: Percentages of differentially as well as co-regulated regulated genes in static grown biofilm and planktonic cells upon 1% (v/v) 1-butanol exposure. Genes encoding for of proteins with at least one transmembrane helices and >2- and >4-fold regulation. Dark red and dark green colour indicate significantly (>4-fold) regulated genes, light red and light green colour indicate low regulated genes (2-4-fold regulation).

		>2-fold regulated		>4-fold regulated	
		up	down	up	down
Percent of genes encoding for proteins containing predicted transmembrane helices	Biofilm	20	34	21	42
	Planktonic	17	37	24	51
	Co-regulated	16	43	18	54

3.8. Cellular response - Proteome studies

The global translational response of *S. acidocaldarius* on different lifestyles and towards 1- butanol exposure was investigated by using the iTRAQ technique. This technique allows for the identification of proteins, but is limited in the quantification. Significant regulation of proteins is based on p-values. Large fold changes using iTRAQ are indicative for “on-off” regulation of protein abundance. Since the cell mass required for proteomic analysis is higher compared to transcriptome analysis, the number of samples was increased and cells from six independent experiments were pooled. The starting OD_{600} of the initial cell suspension was 0.1. Since the planktonically grown *S. acidocaldarius* cells in presence of 1% (v/v) 1-butanol did not increase in OD_{600} after incubation for 4d the cell mass was insufficient for proteome analysis. Therefore, a comparison of planktonic cells grown in the presence and absence of 1% (v/v) 1-butanol is missing.

The cell (OD_{600}) growth and 1-butanol concentrations before and after cultivation were determined and are listed in Tab. 3-11.

Table 3-11: Static cultivation of *S. acidocaldarius* in absence and presence of 0%, 0.5% and 1% (v/v) 1-butanol. Planktonic growth (OD_{600}) and biofilm wet weight were determined after 4d of incubation at 78°C. Furthermore, the initial and final 1-butanol concentration was measured. N = 6

Sample	Control	0.5% (v/v) 1-Butanol	1% (v/v) 1-Butanol
Planktonic cells (OD_{600})	0.465 ± 0.048	0.413 ± 0.092	0.100 ± 0.042
Biofilm wet weight (mg)	75.783 ± 19.367	75.350 ± 18.350	75.467 ± 23.155
1-Butanol concentration (%(v/v)) (initial)	-	0.57 ± 0.03	0.98 ± 0.03
1-Butanol concentration (%(v/v)) (final)	-	0.42 ± 0.03	0.84 ± 0.02

As already observed for the transcriptome samples the OD_{600} decreased with increasing 1-butanol concentrations, whereas the biomass is not affected by 1-butanol concentrations.

A total number of 30,113 peptides with 11,335 unique peptides were detected, corresponding to 1,374 proteins (covering up to 62% of *S. acidocaldarius* genome). Thereof 1,210 proteins were quantified with ≥ 2 unique peptides. These quantified proteins were then clustered using heat map (Dendrogram) and PCA techniques (Fig. 3-34 A and B).

T-tests analysis ($\alpha = 0.01$) were performed at the peptide level (peptides corresponding to an identified protein) to determine regulated proteins for each phenotype comparison. Proteins with p-value ≤ 0.01 from the t-tests were considered as regulated proteins, and proteins with p-value less than 8.26×10^{-6} were considered as significantly regulated proteins.

For proteomics total cells were applied, but due method limitations the identified proteins were predominantly limited to cytosolic proteins. In total 25 proteins harbouring transmembrane helices or signal peptides were identified in all samples. Therefore, the overall number of identified proteins predominantly represents cytosolic proteins.

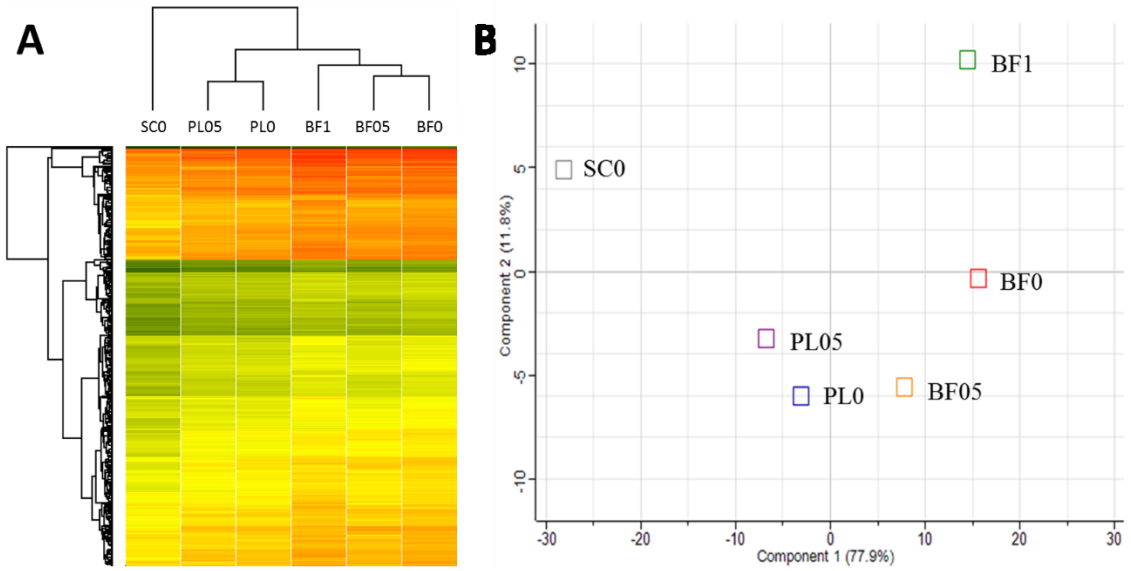


Figure 3-36: Cluster analysis of proteom data from static grown biofilm (BF) and planktonic (PL) cells as well as planktonic cells from shaking cultures (SC0, Erlenmeyer flasks, 180rpm, 78°C). Static grown *S. acidocaldarius* cells (Petri-dishes, 78°C, 4d) were incubated in the absence (BF0, PL0) and presence of different 1-butanol concentrations (0.5% (PL05, BF05) and 1% (v/v) (BF1)). **(A)**: Heatmap, **(B)**: PCA

In general, from Fig. 3-36 A and B it is obvious that proteome profiles of the cells from shaking culture (SC0) were significantly different from the other samples, reflecting the dramatic changes in lifestyle from shaking to static cultures. The proteome profiles of biofilm and statically cultivated planktonic cells with and without and with 0.5% (v/v) 1-butanol (BF0 and BF05, PL0 and PL05) did not reveal significant differences from control samples (see Tab. 3- 12). However, when the cells were grown in the presence of 1% (v/v) 1-butanol (BF1), biofilm cells showed significant differences in the proteome profiles. In the presence of 1% (v/v) 1-butanol not enough planktonic cells for proteome analysis could be isolated.

Table 3-12: Number of differentially regulated proteins of static grown biofilm (BF) and planktonic (PL) *S. acidocaldarius* cells as well as of planktonic cells from shaking cultures (SC0, Erlenmeyer flasks, 180rpm, 78°C). Static grown cells (Petri-dishes, 78°C, 4d) were incubated in the presence of different 1-butanol concentrations (0.5 and 1% (v/v)).

		Regulated proteins					
		up	significant	down	significant	total	significant
Lifestyle	BF0/SC0	153	20	141	18	294	38
	PL0/SC0	171	41	148	27	319	68
	BF0/PL0	104	9	120	8	224	17
Biofilm	BF1/BF0	93	10	114	12	207	22
	BF1/BF05	96	9	122	17	218	26
Planktonic	PL05/PL0	50	4	74	3	124	7

As indicated by the heatmap and the PCA plot, the highest number of differentially regulated proteins was identified in comparisons of cells from static cultures to the shaking culture (SC0).

3.8.1. Proteomic changes based on lifestyle

The number of differentially up- and down-regulated proteins of statically grown biofilm (BF0) and planktonic cells (PL0) is almost the same. 104 proteins were up-regulated, 120 proteins down-regulated in biofilm cells compared to planktonic cells. 17 of these regulated proteins are significantly regulated, based on their p-value (Tab. 3-12).

Most of the significantly up-regulated proteins belong to the general category metabolism: Saci_2281, Saci_ 1028 and Saci_0828 (E: amino acid metabolism and transport), Saci_1353 (F: nucleotide metabolism and transport) and Saci_1109 and Saci_0261 (I: lipid metabolism).

Most of the significantly down-regulated proteins belong to the arCOG category metabolism: Saci_ 2271, Saci_0227, Saci_1214 (C: energy production and conversion), Saci_1617 (E: amino acid metabolism and transport), Saci_0231 (P: inorganic ion transport and metabolism) and Saci_2294 (Q: secondary structure). In biofilm cells, two chaperonins (Saci_0666 and Saci_1401) are down-regulated (O: cellular processes and signalling, post-translational modification, protein turnover, chaperone functions) (Tab. 3-13).

Table 3-13: Differentially regulated proteins of static grown *S. acidocaldarius* biofilm cells and planktonic (Petri-dishes, 4d, 78°C). Significantly up- and down-regulated proteins in biofilm cells compared to planktonic cells (BF0/PL0) are shown.

Protein-ID	Locus	ArCOG annotation	arCOG functional code	Regulation (BF0/PL0)	
				Fold change	p-value
Q4J6L3	Saci_2281	Acetolactate synthase large subunit or other thiamine pyrophosphate-requiring enzyme	E	1.38	3.43E-09
Q4J941	Saci_1353	Ribonucleotide reductase, alpha subunit	F	1.18	4.73E-08
Q4J9I2	Saci_1199	N-acetylglucosamine-1-phosphate uridyltransferase	M	1.40	3.25E-07
Q4JA07	Saci_1028	4-aminobutyrate aminotransferase or related aminotransferase	E	1.44	9.67E-07
Q4J9R9	Saci_1109	3-hydroxyacyl-CoA dehydrogenase, some fused to Enoyl-CoA hydratase	I	1.31	1.17E-06
Q4JAI4	Saci_0828	Methionine synthase II (cobalamin-independent)	E	1.34	1.81E-06
P17199	Saci_0684	Ribosomal protein S10	J	1.23	7.85E-06
Q4JC01	Saci_0262	Acetyl-CoA carboxylase, carboxyltransferase component	I	1.33	8.20E-06
Q9V2T4	Saci_0666	Chaperonin GroEL, HSP60 family	O	-1.40	3.19E-17
Q4J6M3	Saci_2271	Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homolog	C	-1.33	3.87E-11
Q4J8F1	Saci_1617	Argininosuccinate synthase	E	-1.71	6.78E-11
Q4JC33	Saci_0227	Lactaldehyde dehydrogenase, Succinate semialdehyde dehydrogenase or other NAD-dependent aldehyde dehydrogenase	C	-1.45	8.26E-09
Q9V2T5	Saci_1401	Chaperonin GroEL, HSP60 family	O	-1.31	1.21E-08
Q4J9H0	Saci_1214	Aconitase A	C	-1.23	1.56E-08
P23112	Saci_0603	Translation elongation factor G, EF-G (GTPase)	J	-1.18	2.10E-08
Q4JC29	Saci_0231	Ferritin-like domain	P	-1.35	1.07E-07
Q4J6R2	Saci_2233	Acetyl-CoA acetyltransferase	I	-1.31	9.11E-06
Q4J6K2	Saci_2294	Aromatic ring hydroxylase	Q	-1.23	9.41E-06

Indicated by the proteome changes dependent on lifestyle, most changes between static planktonic and biofilm cells are visible in metabolic changes and adaptions.

As already found in the transcriptome studies for the comparison of planktonic cells from static and shaking cultures, the overall number of regulated proteins is quite high, indicating massive global changes in the proteome.

In static grown planktonic cells, 171 proteins (41 significant regulated) were up-regulated and 148 proteins (27 significant regulated) down-regulated compared to cells from shaking ultures (Fig. 3-12).

Table 3-14: Differentially regulated proteins of planktonic cells from static *S. acidocaldarius* cultures (Petri-dishes, 4d, 78°C) and as shaking cultures (Erlenmeyer flasks, 180rpm, 78°C) are shown (PL0/SC0). Significantly up-regulated proteins in planktonic cells from static cultures compared to shaking cultures.

Protein-ID	Locus	ArCOG annotation	arCOG functional code	Regulation (PL0/SC0)	
				Fold change	p-value
Q4J6M3	Saci_2271	Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homolog	C	1.95	3.32E-16
P11386	Saci_0246	Malate/lactate dehydrogenase	C	1.92	2.55E-12
Q4J6K2	Saci_2294	Aromatic ring hydroxylase	Q	1.80	2.69E-12
Q4J8F1	Saci_1617	Argininosuccinate synthase	E	2.82	3.04E-12
Q4JC33	Saci_0227	Lactaldehyde dehydrogenase, Succinate semialdehyde dehydrogenase or other NAD-dependent aldehyde dehydrogenase	C	1.67	8.16E-12
Q4JAH5	Saci_0837	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	G	1.96	1.13E-11
Q4JBU3	Saci_0320	Predicted molibdopterin-dependent oxidoreductase YjgC	R	1.42	4.73E-11
Q4JA37	Saci_0982	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit	C	1.60	3.61E-10
Q9V2T4	Saci_0666	Chaperonin GroEL, HSP60 family	O	1.25	4.29E-10
Q4JAP2	Saci_0763	Acetolactate synthase large subunit or other thiamine pyrophosphate-requiring enzyme	E	1.32	5.23E-10
Q4J6U6	Saci_2198	Rhodanese-related sulfurtransferase	P	1.88	6.77E-10
Q4J7J3	Saci_1935	Pyruvate oxidase	G	1.90	1.10E-09
Q4J6R2	Saci_2233	Acetyl-CoA acetyltransferase	I	1.64	1.98E-09
Q4JBT5	Saci_0328	Heterodisulfide reductase, subunit A, polyferredoxin	C	1.87	2.38E-09
Q4JB20	Saci_0617	Ribosomal protein L15E	J	1.52	4.83E-08
Q4JA55	Saci_0963	Acetyl-CoA acetyltransferase	I	1.64	1.06E-07
Q4J9M6	Saci_1157	Uncharacterized protein	S	1.89	1.27E-07
Q4JA93	Saci_0924	Methylmalonyl-CoA mutase	I	2.23	1.28E-07
Q4JC29	Saci_0231	Ferritin-like domain	P	1.53	2.41E-07
Q4J9H0	Saci_1214	Aconitase A	C	1.25	2.57E-07
Q4J715	Saci_2137	4-aminobutyrate aminotransferase or related aminotransferase	E	2.26	3.14E-07
Q9V2T5	Saci_1401	Chaperonin GroEL, HSP60 family	O	1.24	3.27E-07
Q4JBR8	Saci_0345	Lipoate-protein ligase A	H	1.81	4.13E-07

Protein-ID	Locus	ArCOG annotation	arCOG functional code	Regulation (PL0/SC0)	
				Fold change	p-value
Q4J8Q0	Saci_1510	Phenylalanyl-tRNA synthetase beta subunit	J	1.54	5.20E-07
Q4JAV3	Saci_0696	Nucleoside diphosphate kinase	F	1.89	7.20E-07
Q4J6L0	Saci_2285	Enoyl-CoA hydratase/carnithine racemase	I	1.59	7.81E-07
Q4JAA7	Saci_0910	Asp-tRNAAsn/Glu-tRNAGln amidotransferase A subunit or related amidase	J	1.60	1.25E-06
Q4J6K3	Saci_2293	2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway)	Q	1.93	1.26E-06
Q4JBP1	Saci_0372	Aminopeptidase N	E	1.27	1.28E-06
Q4J9S4	Saci_1104	Short-chain alcohol dehydrogenase	I	1.29	2.89E-06
Q4J6R3	Saci_2232	Acetyl-CoA acetyltransferase	I	1.49	3.90E-06
Q4J9I2	Saci_1199	N-acetylglucosamine-1-phosphate uridyltransferase	M	1.54	3.98E-06
Q4JCJ3	Saci_0057	NAD(P)H-hydrate repair enzyme Nnr, NAD(P)H-hydrate dehydratase domain	F	1.35	4.32E-06
Q4J8T9	Saci_1470	Nicotinic acid phosphoribosyltransferase	H	1.86	4.64E-06
Q4J6K0	Saci_2295	Catechol 2,3-dioxygenase or other lactoylglutathione lyase family enzyme	E	1.60	4.95E-06
Q4J8A4	Saci_1667	L-lactate utilization protein LutB, contains ferredoxin domain	C	1.96	5.08E-06
Q4J920	Saci_1377	Enolase	G	1.30	5.43E-06
Q4JAE9	Saci_0863	Arginase family enzyme	E	1.81	5.62E-06
Q4JBW4	Saci_0299	FAD/FMN-containing dehydrogenase	C	1.47	5.63E-06
Q4JCF3	Saci_0101	AbrB family transcriptional regulator	V	1.61	6.26E-06
Q4J8K7	Saci_1561	Acetolactate synthase large subunit or other thiamine pyrophosphate-requiring enzyme	E	2.06	6.31E-06

Most of the up-regulated proteins in planktonic cells belong to the arCOG category “metabolism” (arCOG categories C, E, F, G, H, I P and Q), indicating that the planktonic cultivation of *S. acidocaldarius* in different incubation systems (static *vs* shaking) induce changes in the metabolism. The up-regulated proteins belong to different enzyme classes like dehydrogeases (Saci_0246, Saci_0299 and Saci_1104), enolase (Saci_1377), kinase (Saci_0696), mutase (Saci_0924), acetyltransferase (Saci_0983 and Saci_2233) and other classes. Therefore, a wide variety of different proteins with different functionalities are up-regulated.

Table 3-15: Differentially down-regulated proteins of planktonic cells from static *S. acidocaldarius* cultures (Petri-dishes, 4d, 78°C) and shaking cultures (Erlenmeyer flasks, 180rpm, 78°C) are shown (PL0/SC). Significantly down-regulated proteins in cells from static cultures of planktonic cells compared to shaking cultures.

Protein-ID	Locus	ArCOG annotation	arCOG functional code	Regulation (PL0/SC0)	
				Fold change	p-value
Q4J9I0	Saci_1203	Chaperonin GroEL, HSP60 family	O	-1.59	4.56E-10
Q4J833	Saci_1742	Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homolog	C	-1.42	2.63E-09
Q4JC01	Saci_0262	Acetyl-CoA carboxylase, carboxyltransferase component	I	-1.69	5.59E-09
Q4JCB9	Saci_0137	Thiamine biosynthesis protein ThiC	H	-2.44	1.38E-08
Q4J6C9	Saci_2375	Isocitrate dehydrogenase	C	-1.71	1.65E-08
Q4JBU8	Saci_0315	Electron transfer flavoprotein, alpha and beta subunits	C	-1.29	4.38E-08
Q4JAU0	Saci_0710	Uncharacterized FAD-dependent dehydrogenase	R	-1.66	4.91E-08
Q4JA22	Saci_0998	Hypoxanthine phosphoribosyltransferase	H	-2.03	1.96E-07
Q4JCA3	Saci_0155	Glutamate dehydrogenase/leucine dehydrogenase	E	-1.41	2.08E-07
P39473	Saci_0085	Ribosomal protein L13	J	-1.64	2.49E-07
Q4J6S7	Saci_2218	3-hydroxyacyl-CoA dehydrogenase, some fused to Enoyl-CoA hydratase	I	-1.27	4.13E-07
P23112	Saci_0603	Translation elongation factor G, EF-G (GTPase)	J	-1.38	5.97E-07
Q4J8G0	Saci_1607	Phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR) synthase	F	-2.09	6.42E-07
Q4J9Q6	Saci_1122	Acyl-CoA synthetase (AMP-forming)/AMP-acid ligase II	I	-1.65	8.36E-07
Q4J6J5	Saci_2300	NADH:flavin oxidoreductase, Old Yellow Enzyme family	C	-1.41	1.10E-06
Q4JC84	Saci_0176	Ribosomal protein L10AE/L16	J	-1.37	1.31E-06
Q4JC46	Saci_0214	Formate-dependent phosphoribosylglycinamide formyltransferase (GAR transformylase)	F	-1.72	1.43E-06
Q4JAL1	Saci_0798	Archaeal S-adenosylmethionine synthetase	E	-1.55	2.00E-06
Q4J853	Saci_1728	Acetyl-CoA acetyltransferase	I	-2.02	2.09E-06
Q4JAI4	Saci_0828	Methionine synthase II (cobalamin-independent)	E	-1.60	3.03E-06
Q4JA33	Saci_0986	Dehydrogenase (flavoprotein)	C	-1.47	3.93E-06
Q4J9C4	Saci_1261	Threonyl-tRNA synthetase	J	-1.53	4.00E-06
O05636	Saci_0582	Ribosomal protein S8	J	-1.41	4.01E-06
P37820	Saci_0196	N-acetylglucosamine-1-phosphate uridyltransferase	M	-1.57	4.01E-06
Q9HH09	Saci_1483	Glutamine synthetase	E	-1.48	4.10E-06
Q4JAZ7	Saci_0646	S-adenosylhomocysteine hydrolase	H	-1.46	5.55E-06
Q4J8S2	Saci_1481	GTP cyclohydrolase I	H	-1.90	7.10E-06

As already found for up-regulated proteins, the dominant number of down-regulated proteins belongs to proteins involved in metabolism.

3.8.2. Proteomic changes based on 1-butanol exposure

Proteomic changes of static grown biofilm cells in Petri-dishes in absence and presence of 1% (v/v) 1-butanol were analysed. More than 2-fold significantly regulated proteins are listed in Tab. 3-19 and 3-20.

The presence of 1% (v/v) 1-butanol resulted in an up-regulation of 93 proteins (10 significant) and down-regulation of 114 proteins (12 significant) (Fig. 3-12).

The significantly up-regulated proteins in biofilm cells grown in the presence of 1-butanol are shown in Tab. 3-16.

Table 3-16: Differentially regulated proteins of static grown biofilm cells in presence and absence of 1% (v/v) 1-butanol. Significant and > 2-fold up-regulated proteins in biofilm cells are shown.

Protein-ID	Locus	ArCOG annotation	arCOG functional code	Regulation (BF1/BF0)	
				Fold change	p-value
Q9V2T4	Saci_0666	Chaperonin GroEL, HSP60 family	O	1.58	1.87E-15
Q4JBU3	Saci_0320	Predicted molibdopterin-dependent oxidoreductase YjgC	R	1.67	6.93E-11
Q9V2T5	Saci_1401	Chaperonin GroEL, HSP60 family	O	1.45	7.66E-11
Q4JCA3	Saci_0155	Glutamate dehydrogenase/leucine dehydrogenase	E	1.28	2.10E-07
P23112	Saci_0603	Translation elongation factor G, EF-G (GTPase)	J	1.26	2.44E-07
Q4JAZ9	Saci_0642	Ribosomal protein L37E	J	3.24	6.44E-04
O05635	Saci_0583	Ribosomal protein S14	J	2.18	3.59E-05

The chaperonins Saci_0666 and Saci_1401 were previously shown to be downregulated in biofilms when compared to planktonic cells in the absence of 1-butanol (Tab. 3-13). Here, these chaperonins are significantly up-regulated in biofilm cells during 1-butanol exposure. The highest up-regulation was found for the ribosomal proteins Saci_0642 and Saci_0583 (J: Information storage and processing, Translation)

Table 3-17: Differentially regulated proteins of static grown biofilm cells in presence and absence of 1% (v/v) 1-butanol. Significant and > 2-fold up-regulated proteins in biofilm cells are shown. *: Proteins with transmembrane helices.

Protein-ID	Locus	ArCOG annotation	arCOG functional code	Regulation (BF1/BF0)	
				Fold change	p-value
Q4J8Y3	Saci_1417	Phosphoenolpyruvate synthase/pyruvate phosphate dikinase	G	-1.73	8.69E-09
Q4J6V5	Saci_2190	Malate synthase	C	-1.43	1.77E-07
Q4J6L3	Saci_2281	Acetolactate synthase large subunit or other thiamine pyrophosphate-requiring enzyme	E	-1.48	2.49E-07
Q4J9I2	Saci_1199	N-acetylglucosamine-1-phosphate uridyltransferase	M	-1.40	2.75E-07
Q4JBX1	Saci_0292	Dehydrogenase (flavoprotein)	C	-1.30	3.07E-07
Q4J9E2	Saci_1242	Transcriptional regulator, contains HTH domain	K	-2.41	6.10E-04
Q4J715	Saci_2137	4-aminobutyrate aminotransferase or related aminotransferase	E	-2.35	1.40E-05
Q4JBJ9	Saci_0415*	Zn-dependent protease with chaperone function	O	-2.32	6.28E-04
Q4J7R4	Saci_1860*	Rieske Fe-S protein	C	-2.25	4.41E-03
Q4J6G5	Saci_2332*	Membrane protease subunit, stomatin/prohibitin homolog	O	-2.25	1.46E-04

Saci_1199 (N-acetylglucosamine-1-phosphate uridyltransferase) was found to be up-regulated in biofilm cells in the absence of 1-butanol when compared to static grown planktonic cells, but down-regulated in butanol-exposed biofilm cells (Tab. 3-17). Here, 3 of >2-fold down-regulated proteins contain transmembrane helices, assuming that these proteins are membrane proteins or associated to the cell membrane.

Saci_1242 (transcriptional regulator, contains HTH domain) was shown to have an regulatory role in biofilm formation of *S. acidocaldarius* (Orell et al., 2013a). However, proteome analysis showed a down-regulation in biofilm cells upon 1% (v/v) 1-butanol exposure.

4. DISCUSSION

The aim of this study was the optimization of cultivation systems for biofilm formation of *Sulfolobus acidocaldarius* and the investigation of the stress response of *S. acidocaldarius* towards organic solvents. The application of (hyper)thermoacidophilic Archaea as alternative for bacterial or eukaryal production strains for industrial processes is of great interest, e. g. for the production of biofuels like ethanol or 1-butanol or fine chemical synthesis. 1-butanol was chosen as model solvent in this study because of its important relevance as second generation biofuel.

Organisms living as biofilm are more resistant towards toxic components and therefore offer advantages to planktonic cultivation of organisms for industrial processes. This work focused on biofilm formation of S*. acidocaldarius* in the presence of organic solvents. For this, growth inhibition by organic solvents as well as their influence on biofilm formation and the response at cellular level was investigated. Beside the application and analysis of industrial potential organic solvents, this study provides more general information about stress response in the Crenarchaeon *S. acidocaldarius.* So far the effect of organic solvents has only been studied in Euryarchaeota and only the ability to metabolize organic substances was tested so far (Akolkar et al., 2008; Usami et al., 2003; Usami et al., 2005).

Since cultivation systems for analysis of biofilm formation of thermoacidophilic organisms have not been established so far, available bacterial systems were optimized in regard to biofilm growth as well as medium and solvent evaporation. In addition, a flow-through cultivation system for biofilm formation at pH 3 and 78°C was established. The challenge was the establishment of acidic and temperature stable materials and systems with reduced evaporation of medium and biocompatibility for biofilm formation. For the flow-through system, a tubing-system and a commercial µ-slide (IBIDI) was chosen for cultivation, allowing biofilm formation under constant medium flow and confocal laser-scanning microscopy. An overview of the setup is given in the materials and methods section 2-8. The applied tubing were biocompatible and could be used as substrate for biofilm formation. For all applied biofilm compartments biofilm formation could be documented.

The optimized static incubation systems were then used for analysis of biofilm formation in response to solvent exposure via different microbiological, microscopic, biochemical and polyomic methods. Focus of this work was the investigation of changes in biofilm formation, morphology and cell distribution, EPS composition and cellular changes in response to organic solvents.

Therefore, the biofilm formation in the presence of 1-butanol in submersed cultivation systems with polystyrene or glass as substrata was investigated for biofilm formation. For comparison to static systems *S. acidocaldarius* was additionally cultivated planktonically in the presence of different solvents as shaking cultures. To study the influence of solvents on planktonic and biofilm growth and the viability of biofilm cells, *S. acidocaldarius* was incubated in 96-well microtiter plates. Planktonic growth was determined via OD_{600}, biofilm growth via crystal violet staining and viability of biofilm cells via a newly adapted resazurin viability assay, monitoring respiratory activity of biofilm cells. In addition, biofilms grown in the presence of 1-butanol on glass surface were analysed using light microscopy and fluorescence microscopy as well as atomic-force microscopy and scanning electron microscopy. Microscopic images showed an aggregation of cells and enhanced EPS formation towards 1-butanol exposure. EPS extraction and quantification confirmed these observations, revealing enhanced quantification of carbohydrates and proteins.

Furthermore, the genome-wide changes at transcriptome and proteome level with major focus on changes referred to lifestyle (planktonic shaking culture (SC0), planktonic static culture (PL0 and biofilm (BF0)) and towards 1-butanol exposure were investigated. Only few changes of static incubated planktonic biofilm (BF0) and planktonic cells (PL0) were found on the transcriptome and proteome level. In contrast, planktonic cells grown as static and shaking cultures showed a high number of regulated genes and proteins. On the transcriptome level *S. acidocaldarius* responded to 1-butanol exposure predominantly with down-regulation of genes, especially of genes encoding for the motility apparatus, the archaellum, and genes encoding transmembrane proteins.

4.1. Establishment of incubation systems

Incubation systems for biofilm formation of *S. acidocaldarius* had to be optimized in order to allow for long-time cultivation (4d) at 78°C. Since changes in medium (volume and pH) and solvent concentration have an influence on biofilm formation, reproducible assay conditions are required. At the growth temperature of 78°C, medium and solvent evaporate. Evaporation of solvent is an advantage for biotechnological application by product removal via distillation, but solvent evaporation effects the aim of this study, namely the investigation of solvents towards biofilm formation. Therefore, medium and solvent evaporation had to be prevented by different strategies to enable constant medium volume and solvent concentrations. The application of gas impermeable aluminium-foil for sealing of different incubation systems (6-well and 96-well microtiter plates and µ-dishes) had no influence on biofilm growth but reduced the medium and solvent evaporation compared to the previous setups. The incubation of Petri-dishes in anaerobic jars allowed cultivation and biofilm formation of *S. acidocaldarius* with limited solvent evaporation over 4d.

For the investigation of biofilm formation towards organic solvents, cultivation systems with long-term stability over an incubation period of at least four days were developed. Dependent on the nutrients available as C-sources, *S. acidocaldarius* alkalifies the culture medium. N-Z-Amine as sole carbon source in the growth medium results in an increase in pH and therefore stresses the cells. For pH stability, glucose was added as a second carbon source next to N-Z-Amine, preventing an increase in pH. Since the use of industrial carbon sources as feedstock for biosynthesis are required for industrial processes, glucose was used (del Sol Cuenca et al., 2016). Biofilm formation strongly depends on growth medium. The applied incubation systems and optimization strategies are listed in Tab. 4-1.

Table 4-1: Overview of limitations of different incubation systems and the applied optimization strategies.

Incubation system	Limitation	Optimization
All systems	- Alkalification of medium with peptides only as C- and additional N-source	- Carbohydrate (dextrin or glucose) as a additional carbon source - No medium exchange during incubation due to medium optimization
Petri dishes	- Low amount of biofilm in Petri dishes - Evaporation of medium and solvent	- Application of Petri dishes without cams to prevent evaporation - Incubation of Petri-dishes in anaerobic jar - Increase of Petri-dish number
96-well microtiter plates	- Evaporation of medium and solvent	- Application of gas-impermeable aluminium-foil for sealing
6-well microtiter plates	- Evaporation of medium and solvent	- Application of gas-impermeable aluminium-foil for sealing
µ-dishes	- Evaporation of medium and solvent - Medium exchange during incubation	- Application of gas-impermeable aluminium-foil for sealing -

Although all incubation systems are applicable for cultivation of *S. acidocaldarius*, the solvent concentration for evaluation of stress responses had to be adapted for each single system independently, because identical solvent concentrations showed different effects depending on the incubation system. Possible reasons may be e. g. different surface materials (i. e. glass and polystyrene), culture volumes, gas phases and/or evaporation. These variations in solvent concentration over the cultivation process in different incubation systems are the reason for the different initial solvent concentrations applied. As sublethal solvent concentrations close to growth inhibition generated highest stress responses, these concentrations were chosen for further experiments.

4.1.1. Flow-through incubation system for biofilm formation

In this work, the first time an thermoacidophilic organism was incubated under flow-through conditions. For thermophilic Archaea, like the crenarchaeal *Sulfolobales*, so far no flow-through systems are published, because of the high incubation temperatures, which come along for example with medium evaporation and instability of materials. The main challenges were the establishment of a setup preventing the evaporation of medium during cultivation and the formation of air bubbles, which destroy the biofilm. Therefore, tubing with low gas diffusion were chosen. Since under the cultivation conditions most of the commonly used materials are not stable and may release toxic compounds into the media, the biocompatibility

with *S. acidocaldarius* was proved. Furthermore, the bubble trap was inserted to provide a medium reservoir and prevent the formation of bubbles.

Flow-through incubation systems are of expanding interest because knowledge of biofilm formation of Archaea under flow-through conditions is limited and especially for thermophilic species no information are available. For comparison of bacterial and archaeal biofilm formation under flow-through conditions, research is of increasing interest. For biotechnological application, flow-through systems offer advantages like constant substrate and nutrient influx as well as efflux of toxic products and, therefore, constant growth conditions and prevention of accumulation of toxic compounds. Since the production process is continuous, reduced reactor downtime for preparation, cell growth and cleaning is needed. Continuous production and reduced downtime makes the production of these systems cost-effective compared to batch or fed-batch processes.

Cultivation inside tubing and inside the commercial µ-slide (IBIDI) are the most promising biofilm compartments. Alternatively, glass capillaries are alternative compartments for cultivation but exhibit limitations for microscopy. The applied tubing system as well as the µ-slide system harbour advantages and disadvantages. Both overcome an accumulation of different metabolites and enables the cultivation of *S. acidocaldarius* over a long period of time. For evaluation and visualization of biofilm formation, in future experiments with solvent stress, µ-slides are applicable for confocal laser scanning microscopy. By using µ-slides for biofilm growth, continuous microscopy over a longer time period is possible since the biofilm does not need to be destroyed e.g. for light or fluorescence microscopy. The use of non-destructive microscopic methods is useful for visualization of biofilm development over time. Cultivation inside tubing are applicable for continuous systems and e. g. biocatalysis, because standard techniques like confocal laser-scanning microscopy for biofilm investigations cannot be used or are challenging. Biofilms can only be visualized by destructive methods, damaging the biofilm.

Viability assays for Archaea and archaeal biofilms

The amount of viable and lysed cells has a great impact on biofilm architecture as well as on EPS composition. The biomolecules from lysed cells become part of the EPS and information about the viability of biofilm cells is essential for biofilm and EPS studies. An important method to gain information about maturated biofilms and the distribution of viable and non-viable

cells are viability assays. The number of methods for viability assays of archaeal planktonic cells as well as biofilm cells is quite low. In previous studies the Live/Dead BacLight bacterial viability kit (Invitrogen Molecular Probes) was applied for archaeal species to address cell availability (Fröls et al., 2012; Koerdt et al., 2010). However, the Live/Dead kit could not be established reproducible using controls with formaldehyde treated "dead" cells as negative control. The Live/Dead assay only gives information about the viability of a small cell number of biofilm cells by fluorescence microscopy and gives no global overview about the general viability of biofilm cells. "Dead" cells are defined as cells with an permeabilized membrane, allowing the dye propidium iodide (PI) to cross the cell membrane. Since alcohols like 1-butanol influences the cell envelope, a viability assay, which addresses another viability factor other than the cell membrane had to be used. For bacterial biofilms, the redox- and pH indicator resazurin was also succesfully applied as viability indicator, addressing the respiratory activity of the cells (Pettit et al., 2005; Repp et al., 2007). Resazurin is cheap and was successfully applied for Bacteria and Eukaryotes in previous studies (Chadha et al., 2015; Z. Chen et al., 2015; Duarte et al., 2009). In this study, a viability assay based on resazurin conversion was applied for the first time for the thermoacidophilic Archaeon *S. acidocaldarius* and Archaea in general. Therefore, the applied modified resazurin assay is the first method for detection of respiratory activity of archaeal biofilms.

For mesophilic species grown at neutral pH, the intracellular conversion of resazurin (blue) to resorufin (pink) is measured. The conversion is based on reduction by $FMNH_2$, $FADH_2$, NADH, NADPH, and the cytochromes (product literature, BioRad). For the applied assay in this study, resazurin directly changed the colour from blue (neutral) to pink (acidic) due to pH 3 of the Brock medium. Therefore, the reduction of resazurin to the colourless dihydroresurufin is detected.

In previous studies, viability of planktonic cells of the extremely thermophilic *Thermococcus peptonophilus* were determined by growth using the most-probable-number method (Canganella et al., 1997). Planktonic cells of the hydrogenotrophic methanogens *Methanospirillum hungatei* and *Methanobacterium formicicum* were incubated in presence of oleate to evaluate the sensitivity of this methanogens to long-chain fatty acids. Viability was analysed by looking at the membrane activity using a Live/Dead BacLight bacterial viability kit (Sousa, 2013).

Viability assays of archaeal cells grown in biofilms are quite less published. Viability of *Archaeoglobus fulgidus* was validated by cell growth (Lapaglia et al., 1997). After removing cells from biofilms and transferring them into fresh medium for following incubation, planktonic growth of the transferred cells means the presence of viable cells in the biofilm. For different *Sulfolobus* strains, *Sulfolobus acidocaldarius*, *S. solfataricus* and *S. tokodaii*, Live/Dead stain was used to estimate the amount of viable and membrane permeable cells (Koerdt et al., 2010). For viability assays of haloarchaeal strain *Halobacterium salinarum* a modified Live/Dead assay was performed (Fröls et al., 2012). Since SYTO9 is not applicable for staining of Haloarchaea, because it is not passing the membrane, SYTO9 was substituted with acridine orange. Pearls isolated in the surface water of a spring near Regensburg (Bavaria, Germany) contain Bacteria and the SM1 euryarchaeon (Henneberger et al., 2006). Viable cells were detected by Live/Dead assay and indirect by FISH-probes, assuming that cellular rRNA indicates viable cells and serves as metabolic marker.

Therefore, the new method established for *S. acidocaldarius* in this study might represent an alternative easy approach for different extremophiles.

4.2. Phenotypic response towards different organic solvents

A wide range of primary alcohols (C2 (ethanol), C3 (1-propanol), C4 (1-butanol)) as well as secondary (2-butanol) and tertiary (isobutanol) alcohols was tested to investigate the influence of these solvents on *S. acidocaldarius* shaking and static cultures. For comparisons, also the structurally diverse solvent DMSO was applied. The alcohols are potential 2nd generation biofuels and DMSO can be applied as organic solvents for two-phase biosynthesis in biofilms.

4.2.1. Shaking cultures

S. acidocaldarius was incubated in glass shaking flasks in Brock medium (pH 3) containing 0.1% (v/v) N-Z-Amine, 0.2% (v/v) glucose and various solvents in different concentrations at 78°C. Growth of *S. acidocaldarius* was monitored via OD_{600} measurements. At the end of the experiment, the planktonic cells were discarded and the flasks were stained with crystal violet, in order to analyse for biofilm formation. Planktonic growth as shaking cultures in the

presence of different solvents gained information about the solvent tolerances, changes in growth and biofilm formation as stress response.

Biofilm formation of *S. acidocaldarius* on the glass surface of the Erlenmeyer flasks was observed for the tested alcohols ethanol, 1-propanol, 1-butanol and isobutanol. For DMSO, which was choosen as alternative organic solvent with possible application in two-phase systems, no biofilm formation was observed. Biofilm formation seems to be a stress response of *S. acidocaldarius* towards sublethal alcohol concentrations but no stress response on the organic solvent DMSO. Therefore, biofilm formation of *S. acidocaldarius* grown as shaking cultures is not a general stress response but depended on the solvent.

In shaking cultures, primary alcohols seemed to be less toxic compared to secondary alcohols. In presence of 0.25% (v/v) 2-butanol, no planktonic growth of *S. acidocaldarius* was observed. The growth inhibition of primary alcohols increased from ethanol (4-5% (v/v)), via 1-propanol (2.5-approximately 3% (v/v)) to 1-butanol (1-1.5% (v/v)). For isobutanol, growth inhibition was observed between 1-1.5% (v/v) and for DMSO between 3-4% (v/v). For Bacteria it is proposed, that alcohols with increasing carbon chain length can intercalate into the membrane and interrupt the hydrogen bonds between the lipid tails (Ly et al., 2004).

Not only differences in planktonic growth (SC) were observed in the presence of different organic solvents but also in biofilm formation. In the presence of 1-butanol, ethanol, 1-propanol and isobutanol, biofilm formation on the glass surface at the Erlenmeyer flask (solid-liquid-air interphase) were detected by crystal violet staining. Since biofilm formation was not observed in control experiments without solvents, biofilm formation seems to be a protective mechanism induced by organic solvent exposure. Cells living in the biofilm mode of life are in contrast to planktonic cells more resistant to environmental stresses (X. Z. Li et al., 2006). However, incubation with sublethal concentrations of DMSO (3% (v/v)) did not lead to biofilm formation. This indicates that biofilm formation is not a general protective mechanism, but also dependent on the environmental stress which is applied.

In response to organic solvent, extended stationary phases were observed for several alcohols like ethanol, 1-propanol and isobutanol. An extended stationary phase was not observed for DMSO. Biphasic growth was only observed in the presence of 1-butanol and with no other organic solvent tested. Since OD measurements are based on the number and size of particles (cells), the elongated stationary phase can be caused by cell lysis and increase in size of

residual cells, resulting in constant absorbance measurements. The effect of alcohols and DMSO was different regarding planktonic growth, indicating different molecular responses. Although the molecules tested are similar, regarding its chemical structure, the effect on *S. acidocaldarius* is quite different. Since e. g. for DMSO no biofilm formation was observed, the molecular mechanisms of *S. acidocaldarius* for biofilm formation seem to be quite senitive on external molecules acting as toxic compounds on the cell.

Phenotypic and molecular responses of Crenarchaeota and Euryarchaeota towards different stress-factors have been investigated focussing only on planktonic cells and only few reports dealing with stress exposure and stressresponses of Archaea have been published. Applied stress factors were change in pH, temperature, ion-concentration, UV-irradiation, metal-stress and organic solvents (Fröls et al., 2007; Götz et al., 2007; Koerdt et al., 2010; Remonsellez et al., 2006).

For example, the anaerobic marine hyperthermophile *Archaeoglobus fulgidus* was shown to form biofilms upon changes in the environment like non-physiological extremes of pH and temperature, addition of antibiotics and xenobiotics, UV-light, oxygen and high concentrations of metals (Lapaglia et al., 1997). The formed biofilms were described as heterogeneous, morphologically variable structures with extracellular polymeric substances (EPS) composed of proteins, polysaccharides and metals. In *A. fulgidus* biofilm formation was suggested to be a general protective mechanism as cells within the biofilm showed an increased tolerance compared to planktonic cells (Lapaglia et al., 1997).

Solvent tolerance of the halophilic archaeon *Halobacterium* sp. SP1(1) and its extracellular protease as well as the isolates *Haloferax* sp. SP1 and *Haloarcula* sp. SP2 were tested by Alkolkar et al. (Akolkar et al., 2008). Focus was on the protease activity because a possible application of this enzyme in antifouling coatings is desired. Growth studies in the presence of different NaCl concentrations with constant solvent concentrations (n-decane, n-undecane, n-dodecane, xylene and toluene) revealed a growth dependence on the NaCl-concentration. But no other information with respect to stress response was given (Akolkar et al., 2008).

In another study organic solvent tolerance of 16 different Haloarchaea towards n-decane, n- nonane, hexylether, n-octane, isooctane and cyclooctane on growth was tested (Usami et al., 2003). Growth curves of *Natronorubrum bangense* and *Natronobacterium gregoryi* in the presence and absence of n-decane showed two different growth pattern. The final OD_{600} of both strains was decreased in the presence of *n*-decane. Since the concentration of *n*-decane

was much higher compared to this study (10ml *n*-decane were added to 20ml medium), the effect on archaeal growth in the presence of organic solvents was not comparable. The growth of the tested organism was dependent on the logP value of the solvent as described for Bacteria and the different halophilic Archaea showed different limits for tolerance (Usami et al., 2003).

In a second study adressing solvent tolerance, the effect of different NaCl concentrations on the and polar lipid composition of haloarchaeal species was studied with different *Haloarcula* strains (Usami et al., 2005). Solvents applied in this study were n-decane, n-nonane, hexylether, isooctane and cyclooctane. In response to cyclohexane the morphology of the cells returned from triangular or irregular shape to spherical shape. After evaporation of cyclohexane, the morphology changed back to the triangular shape. For *Haloarcula argentinensis* and *Haloarcula* strains OHF-1 and OHF-2 changes in membrane lipid composition with decrease in phosphatidylglycerol and increase in phosphatidylglycerosulfate and phosphatidylglycerophosphate methyl ester in presence of *n*-decane was found. In the absence of *n*-decane the lipid composition changed back to normal. Although at higher concentration of cyclohexane no growth was observed, cells started to grow after evaporation of the solvent, suggesting that the solvent was not lethal (Usami et al., 2005).

For different kinds of planktonic grown Archaea, predominantly haloarchaeal *Haloferax* species of the Euryarchaeota, secretion of polymeric substances was documented (Anton et al., 1988; Paramonov et al., 1998; H. Parolis et al., 1996; L. A. Parolis et al., 1999; Zhao et al., 2013). The influence of nutrients on exopolysaccharide production of the hyperthermophilic archaeon *Thermococcus litoralis* and the bacterium *Thermotoga maritima* was investigated, indicating changes in attachment in the presence of different nutrients (Rinker et al., 2000). Since the secretion of polymeric substances seems to be a common mechanism of Euryarchaeota, this could be also the case for Crenarchaeota. If the secretion of polymeric substances has influences on absorbance and aggregation of cells, this leads to false determinations of cell amount and growth determination. The previously described DAPI method for total cell counts is an applicable method for cell quantification and detection of cell aggregate formation. Additionally, a counting chamber would be an alternative method for cell quantification.

Butanol tolerance was analysed in detail for different planktonic grown Bacteria, which are commonly used production strains for whole cell biocatalysis. Host strains that are commonly

used for 1-butanol production like *Escherichia coli* and *Saccharomyces cerevisiae* do not tolerate more than 2% (v/v) 1-butanol (Knoshaug et al., 2009). For *Clostridium acetobutylicum* growth inhibition was observed from 2% (v/v) 1-butanol. Furthermore, other Bacteria like *Lactobacillus* strains were screened for 1-butanol tolerance and 60% of the tested *Lactobacillus* species could grow up to 2.5% (v/v) 1-butanol (J. Li et al., 2010). An *Escherichia coli* strain was improved by metabolic engineering to tolerate 2% (v/v) 1-butanol (Bui le et al., 2015). In another study 16 aerobic and anaerobic butanol- and isobutanol tolerant Bacteria from different environmental samples, which could tolerate more than 2% (v/v) 1-butanol and isobutanol, were isolated (Kanno et al., 2013).

Studies with 24 different microorganisms (e. g. *Escherichia coli*, *Saccharomyces cerevisiae* and *Zymomonas mobilis*) was done to screen for butanol tolerant strains as potential production strains for 1-butanol production. The growth experiments showed, that the growth limitations of most organisms are between 1% and 2% (v/v) 1-butanol and only few microorganisms can tolerate 2% (v/v) 1-butanol (Knoshaug et al., 2009). 2% (v/v) 1-butanol were found to be a growth barrier for strains of *Escherichia coli*, *Zymomonas mobilis* and non-*Saccharomyces* yeast. Several *Saccharomyces cerevisiae* strains showed limited growth at 2% (v/v) 1-butanol and two strains of *Lactobacillus* grew up to 3% (v/v) 1-butanol (Knoshaug et al., 2009). It was also reported, that the decrease in incubation temperature allows for better growth at limiting concentration, however, but the upper limit was not increased (Knoshaug et al., 2009). Since *S. acidocaldarius* can grow from 65°C to 80°C, reduction of the incubation temperature may have a positive effect on growth

In this study, *S. acidocaldarius* grows in the presence of 1% (v/v) 1-butanol. These results indicate a comparable tolerance of *S. acidocaldarius* towards 1-butanol as observed for different bacterial strains. However, *S. acidocaldarius* was neither adapted to 1-butanol nor genetically manipulated and in the previously mentioned studies no biofilm formation in presence of sublethal 1-butanol concentrations was reported.

4.2.2. Biofilm formation of static cultures

For analysis of biofilms grown on glass surfaces, *S. acidocaldarius* was incubated for four days at 78°C in cavities of 6-well microtiter plates with glass coverslip as substrate for biofilm formation on the bottom of the well. 1-Butanol, ethanol, isobutanol, 1-propanol and DMSO

were added to the culture medium in sublethal concentration. After incubation, the biofilms were stained by crystal violet and further investigated by light microscopy.

For the control experiments and all applied solvents, predominantly mono layered biofilms were visualized. Without addition of solvents, *S. acidocaldarius* cells are homogeneously distributed on the glass surface. For all applied solvents, aggregation of cells with sublethal concentrations were observed. Therefore, the aggregation of cells can be a stress response independent of the structure of the tested solvent. DMSO was shown to induce no biofilm formation in shaking cultures incubated with sublethal DMSO concentrations in contrast to the applied alcohols. However, under static incubation conditions *S. acidocaldarius* responds with aggregation on DMSO exposure. The aggregates are surrounded by weak crystal violet signals. Since crystal violet not only stains cells but also unselective EPS components, the aggregates are potentially cells embedded in EPS.

The aggregation of *S. acidocaldarius* and *S. solfataricus* by pilins was strongly investigated (Fröls et al., 2008; Henche et al., 2012a; Henche et al., 2012b; Zolghadr et al., 2010). Since some of the observations of aggregate formation are caused by stresses, e. g. UV-irradiation, solvent stress also enhances the formation of pili and therefore aggregation as protective mechanism. For *Haloferax volcanii*, mutations in Type IV pili (like the archaellum) lead to formation of microcolonies (Esquivel et al., 2013). Additionally, glycosylation on pilus assembly and function has an effect on microcolony formation of *Haloferax volcanii* (Esquivel et al., 2016). Beside the mentioned studies, no altered biofilm formation on glass slides was published so far.

4.3. Phenotypic response towards 1-butanol exposure

In previous experiments *S. acidocaldarius* showed biofilm formation in shaking cultures and aggregation of cells in static incubation in the presence of 1-butanol. These effects were also found for the majority of the solvents tested in this study. Therefore, 1-butanol was chosen as model solvent for additional analysis and was investigated more detailed. 1-Butanol is a 2nd generation biofuel and has a higher energy density and boiling point e. g. compared to ethanol. The characterization of the stress response of *S. acidocaldarius* towards 1-butanol exposure is of important relevance to investigate the industrial potential of *S. acidocaldarius*.

The influence of 1-butanol on biofilm formation and established biofilms of *S. acidocaldarius* was investigated in different incubation systems with varying methods to achieve extensive information. Stress response on biofilm formation was the focus in this study, therefore *S. acidocaldarius* was grown in most of the experiments directly in the presence of 1-butanol. Biofilms were grown on polystyrene (96-well microtiter plates, µ-dishes and Petri-dishes) or glass surface (cover slips) in submersed incubation systems for 4d at 78°C.

The application of glass coverslips has some advantages compared to other substrates, e. g. they are small and transferable, thin, harbour a quite smooth surface and allow biofilm formation. Biofilm formation on glass surfaces enables the application of different microscopic methods like light- and epifluorescence microscopy, atomic force microscopy and laser scanning microscopy. Disadvantage of this method is the circumstance, that only microscopy of dried biofilms was successful. Atomic force as well as scanning electron microscopy was not successfully applied for hydrated samples. Therefore, during preparation for microscopy, cells as well as EPS components were lost and not visualized by the applied microscopic techniques. Detected cells as well as EPS are therefore tightly bound to the glass surface as well as to the attached cells.

4.3.1. Influence of 1-butanol on biofilm formation and established biofilms

The investigation of 1-butanol on biofilm formation and established biofilms was done by using 96-well microtiter plates as incubation system, since the growth of planktonic and biofilm cells as well as cell viabilitycan be measured in parallel.

Influence of 1-butanol on biofilm formation

For initial characterization of biofilm formation in the presence of different solvent concentrations, 96-well microtiter plate assays in polystyrene plates were applied. In this cultivation system growth inhibition occurred at a solvent exposure of $\geq$2% (v/v) 1-butanol and biofilm growth is slightly preferred over planktonic growth at lower 1-butanol concentrations but decreased at $\geq$ 1.5% (v/v) 1-butanol. The resazurin viability assays showed respiratory activity of biofilm cells grown in the presence of up to 1.5% (v/v) 1-butanol. No respiratory activity at 1-butanol concentrations in the range of 2 to 2.5% (v/v) 1-butanol was detected. Resazurin, resolved in acidic minimal Brock medium, was used and the conversion to the colourless dihydroresurufin was measured. Resazurin directly changed its colour while

diluted in Brock medium (pH 3), and the reduction to dihydroresurufin is an irreversible reaction. The detection of dihydroresurufin formation has the advantage that no reverse oxidation of dihydroresurufin to coloured resurufin can occur.

The reduction of respiratory activity is accompanied by inhibition of biofilm formation. The amount of planktonic cells and biofilm droped significantly at 1.5 to 2% (v/v) 1-butanol. With the reduction of biofilm, the conversion of resazurin was stopped as well. From 0.5 to 1.5% (v/v) 1-butanol biofilm growth is favoured compared to planktonic growth, potentially as protective mechanism. The sublethal concentration of 1.5% (v/v) 1-butanol led to increasing standard errors for biofilm formation, indicating a critical concentration range when small changes e. g. due to differences in evaporation, led to growth inhibition.

Influence of 1-butanol on established biofilms

For investigating the influence of 1-butanol on already established biofilms*, S. acidocaldarius* cells were first cultivated for four days to allow for biofilm formation and subsequently for one to four days in the presence of different 1-butanol concentrations. The effect of 1-butanol on planktonic and biofilm cells was found to be different. With longer incubation time, the absorbance of planktonic cells increased, indicating planktonic growth in the presence of 1-butanol. Without 1-butanol exposure, the biofilm amount remained constant. In the presence of sublethal 1-butanol concentrations (above 2% (v/v)), the biofilm increased within the first two days but decreased with ongoing cultivation time. This decrease in biofilm might explain the observed increased amount of planktonic cells. Thus, a high 1-butanol concentration seems to lead to a detachment of biofilm cells resulting in a decrease of biofilm cells and an increase of planktonic cells. The solvent concentration which causes biofilm detachment is important for industrial applications of biofilms. The solvent concentration must be below the detachment-concentration because otherwise the process is negatively affected by detachment of biofilm cells. Since sublethal solvent concentration promote biofilm growth, concentrations below the detachment limit might positively influence the biofilm formation.

The 96-well microtiter plate assay is a rapid method for first insights of planktonic as well as biofilm growth under different conditions. Since growth is only quantified via absorbance measurements, (at 600 nm for planktonic cells and 570 nm for biofilm via resuspended crystal violet), this method is not very sensitive. Crystal violet does not only bind to cells but also to

EPS components (negatively charged molecules), thus, an increase in absorbance not only indicate an increase of biofilm cells but also biomass, including EPS components (Peeters et al., 2008). The resazurin assays showed respiratory cell activity of biofilm cells. The velocity or kinetic of resurufin conversion may also indicate the viability status of the cell as well as the cell quantity since the reduction depends on cell number as well as respiratory cell activity. The velocity of conversion was strongly dependent on incubation temperature, being highest at 78 °C. Kinetic measurements at mesophilic temperatures, necessary for reducing medium evaporation and condensation as well as temperature limitations of common microtiter plate readers, are not ideal

96-well microtiter plate experiments with *S. acidocaldarius* grown for two days under different incubation conditions were performed previously by Koerdt et al. (Koerdt et al., 2010). The influence of temperature, pH, iron concentration and different combinations of pH and iron concentration were determined as ratio of biofilm and planktonic grown cells. The results indicated that high (85°C) and low (60°C) temperatures, both representing temperature stress, as well as an increase in pH from pH 2 to pH 6 caused increased biofilm formation compared to planktonic growth. Changes of iron concentration from 0.015 g/L to 0.065 g/L revealed a constant growth behaviour of *S. acidocaldarius*, maybe indicating that the applied iron concentrations were no stress factors for the cells (Koerdt et al., 2010).

4.3.2. Analysis of phenotypic changes by different microscopic methods

Biofilm for visualization by atomic force and scanning electron microscopy were grown as previously described for light microscopy. In contrast to light microscopy, only 1-butanol was applied as solvent.

Atomic force microscopy

As light microscopy only gives insights on cell distribution on the glass surface, atomic force microscopy was applied for visualization of single cells. Although atomic force microscopy was successfully applied, no information on cell envelope could be gained. For atomic force microscopy, only concentrations of up to 1% (v/v) 1-butanol could be applied. Higher concentration of 1-butanol lead to enhanced EPS formation and since atomic force microscopy is based on a thin oscillating cantilever scanning the surface, high amounts of EPS molecules stick to the cantilever and interfere with the scanning. By atomic force microscopy, two

different cell types were detected: Lobe-shaped cells and flat cells. Since the atomic force microscopy is an invasive method, the flat cells may appear by cell lysis caused by the force of the cantilever. Therefore, also the non-invasive scanning electron microscopy was applied.

Scanning electron microscopy

For electron microscopy, biofilm formation was conducted as described for light- and atomic force microscopy. For visualization, the biofilms were dried and sputtered with heavy metals for electron microscopy. The overall morphology of biofilms visualized by electron microscopy revealed the formation of aggregates in presence of sublethal concentration of 1-butanol as already visualized by light microscopy. The slight shadows of crystal violet, observed with light microscopy, which are speculated to be EPS, were visualized by electron microscopy in more detailed. Although the identity of the observed structures is still unknown, the observed structures resemble EPS components. Since these observed components were not removed by the washing steps and during sample preparation, the molecules seem to be tightly bound to the cells and the biofilm aggregates. Images of single cells without and with 1% (v/v) 1-butanol revealed the same phenotype as already detected by atomic force microscopy, lobe-shaped and flat *S. acidocaldarius* cells, indicating that the flat cells observed in atomic force microscopy are not caused due to the pressure of the cantilever. Therefore, *S. acidocaldarius* has two different morphologies under standard growth conditions. However, the cell morphology changed with growth in presence of 1.5% (v/v) 1-butanol and a third phenotype appeared. Lobe-shaped *S. acidocaldarius* cells harbour a rather smooth cell surface, whereas the surface of flat cells is rough. The third phenotype detected after incubation with 1.5% (v/v) 1-butanol are round-like lobe shaped cells with a rough surface. Potentially they produce large amounts of EPS in which the cell aggregates are embedded. Alternatively, the 1-butanol exposure leads to structural changes of the cell envelope forming a novel phenotype. Information about the viability of these cells is missing.

By scanning electron microscopy an altered cell morphology in response to 1% (v/v) 1-butanol was found, which might be related to the enhanced EPS production.

Scanning electron microscopy images of stationary-phase grown *Bacillus subtilis* cells showed an almost two-fold increase in length in the presence of 1.4% (v/v) 1-butanol compared to control cells (Vinayavekhin et al., 2015). Changes in size were not considered in this study and in contrast to the rod-shaped *Bacillus* strain the Archaeon *Sulfolobus acidocaldarius* used in

this study is round. In future experiments, changes in size during 1-butanol exposure can be investigated by either microscopic methods or dynamic light scattering (DLS). Changes in size are a commonly detected stress response of Bacteria on solvents and might be a stress response in Archaea as well.

In previous studies on biofilm formation and cell appendages of *S. acidocaldarius* as well as studies about cell appendages scanning electron microscopy images were performed (Henche et al., 2012b; Koerdt et al., 2010). Glass slides were completely covered with *S. acidocaldarius* cells and microcolonies were observed 48h after incubation (Koerdt et al., 2010). Linke in this study, the glass surface was after 4d of incubation homogeneously covered with cells with heterogeneous aggregates of cells. With elongated incubation time, a network of filamentous structures and EPS formation was observed, which increased with prolonged incubation time.

Confocal laser scanning microscopy

The effect of 1-butanol on submersed biofilms was investigated by *S. acidocaldarius* cultivation for four days in µ-dishes. Subsequently, cells were stained via the DNA-binding fluorophore SYTO9 and two different lectins conjugated with Alexa-fluorophores, respectively. Visualization was performed by confocal laser scanning microscopy. The lectins Concanavalin A (ConA, preferred binding of α-mannopyranosy- and α-glucopyranosyl residues) and Isolectin GS-IB4 (IB4, favoured binding of α-D-galactosyl and N-acetyl-D-galactosamine residues) were applied for carbohydrate binding. Since autofluorescence by the cells was observed in the green wavelength range, which overlays with the autofluorescence of SYTO9, SYTO9 was chosen for cell staining. As the pH strongly affects lectins and the conjugated fluorophores, staining and detection procedure were performed at pH 7. Changes in pH can alter the favoured binding structures of the lectins because they affect their tertiary structure and therefore also the binding site of the lectins. The applied Alexa-fluorophores, conjugated to the lectins, are described as one of the most thermostable fluorophores and are also applicable at more acidic pH.

Staining of biofilm components and visualization by CLSM revealed the same effect on submerse biofilms as observed previously for to the other microscopic methods: In the presence of 1%(v/v) 1-butanol the biofilm is more densely packed and the amount of carbohydrates is increased. Signals of IB4-stained residues appear predominantly in 1-butanol exposed biofilms and not in the control biofilms and also the amount of ConA-signals increases

dramatically in the presence of 1-butanol. Therefore, enhanced carbohydrate formation could be shown as well as changes in carbohydrate composition since IB4 binds predominantely to α-D-galactosyl and N-acetyl-D-galactosamine residues and ConA to α-mannopyranosy- and α-glucopyranosyl residues. The predominant staining by ConA indicates that most of the detected carbohydrates harbour terminal α-mannopyranosy- and α-glucopyranosyl residues. Interestingly, D-glucose and D-mannose were also identified as major components of the glycosylated S-layer proteins (Koerdt et al., 2010).

Lectin staining and confocal laser scanning microscopy were applied previously for *S. acidocaldarius* and *S. solfataricus* wild-type and knock-out mutant cells (Henche et al., 2012a; Henche et al., 2012b; Koerdt et al., 2010; Orell et al., 2013b). It was shown that molecular changes via gene deletions have influences on biofilm architecture and carbohydrate amount and distribution (Koerdt et al., 2012; Orell et al., 2013b). For example, by application of the *S. solfataricus* mutant strain PBL2025 and compelementation of missing genes encoding a α-mannosidase (SSO3006) and a β-galactosidase (SSO3019) it was shown, that the α-mannosidase might be involved in the modulation of the EPS composition (Koerdt et al., 2012).

In the present study it is shown, that also extrinsic factors like solvent exposure have an influence on carbohydrate amount and distribution. 1-Butanol at sublethal concentrations of 1% (v/v) led to higher carbohydrate amounts and changes in carbohydrate composition as visualized by both lectins, ConA and IB4. Carbohydrates, loosely bound, are released from the biofilm into the liquid phase. Further studies may be performed with other lectins to get more detailed information about carbohydrate identities and quantities. Furthermore, protein binding dyes might be applied in future experiments. A screening of different dyes for EPS visualization of biofilms formed by Crenarchaea has been published recently (R. Zhang et al., 2015; R. Y. Zhang et al., 2015). Biofilms of the meso- and thermoacidophilic metal-oxidizing Archaea *Ferroplasma acidiphilum* DSM 28986, *Sulfolobus metallicus* DSM 6482T and a novel isolate *Acidianus* sp. DSM 29099 were screened with 75 commercial available lectins by fluorescence lectin-binding analysis (R. Y. Zhang et al., 2015). Analogous experiments with various lectins for visualization of EPS carbohydrates of *S. acidocaldarius* biofilms after 1-butanol exposure can give more detailed information of carbohydrate amount, identity and distribution within the EPS.

For Bacteria, changes of biofilm structure and alginate production of *Pseudomonas aeruginosa* towards subinhibitory concentrations of the antibiotic imipenem were observed by confocal laser scanning microscopy (Bagge et al., 2004). With imipenem exposure, biofilms showed an increased thickness and more biomass per substratum area. Staining with lectin ConA showed more signals in biofilms formed with antibiotic exposure compared to the control experiment, indicating alginate formation (Bagge et al., 2004). Also *Pseudomonas* sp. grown in a modified flow-cell reactor exposed to the solvent styrene showed increased ConA binding compared to the control, indicating enhanced carbohydrates bound by ConA (Halan et al., 2011).

The visualization of submerse biofilms by lectin staining and confocal-laser scanning microscopy confirmed the enhanced EPS-formation in the presence of 1-butanol as already shown for dried biofilms by light- and electron microscopy. By lectin staining it was confirmed that an enhanced carbohydrate formation, predominantly consisting of α-mannopyranosy- and α-glucopyranosyl residues, occurs. Since microscopy only reveales qualitative information about EPS composition, the quantification of EPS components is not possible.

4.3.3. Influence of 1-butanol on EPS amount and composition

The by light microscopy and scanning electron microscopy observed enhanced EPS-formation was further analysed by EPS isolation and quantification. Therefore, *S. acidocaldarius* cells were grown as submerged biofilms for four days in Petri dishes with Brock medium (pH 3) containing 0.1% (v/v) N-Z-amine, 0.2% (v/v) glucose. To investigate the response towards different 1-butanol concentration (0, 0.5, 1% (v/v)) were added. The biofilms formed at the bottom of the Petri dish were isolated and used for subsequent EPS extraction with the cation exchange resin DOWEX. The EPS fraction (biomolecules ≥ 3,5 kDa) increased in biofilms formed in presence of 1% (v/v) 1-butanol: The amount of carbohydrates per cell increased 4.5-fold and the amount of proteins 21-fold compared to the control without 1-butanol exposure. The increase of EPS components was not found in presence of 0.5% (v/v) 1-butanol. Therefore, concentrations above 0.5% and 1% (v/v) 1-butanol result in stress and enhanced EPS formation. EPS are important to increase the robustness of biofilms and facilitate the attachment of e. g. *Pseudomonas aeruginosa* (Colvin et al., 2011; Hatch, 1998).

The extraction method used in this study was previously established for *S. acidocaldarius* grown as unsaturated biofilms on semisolid agar (Jachlewski et al., 2015). This metod was also

applied for different *Sulfolobus* strains in previous studies (Koerdt et al., 2012; Orell et al., 2013b). In the published as well as in this study, the generation of sufficient amount of biomass from submerse biofilms was quite challenging and an upscaling of single experiments had to be performed for providing a sufficient amount of EPS for colorimetric assays for quantification of carbohydrate and protein concentration. The increased amount of proteins leads to the speculation, that the increase in carbohydrates is caused by cell lysis. As already described by Jachlewski *et al*., analysis of extracted EPS proteins by SDS-PAGE and MS analysis revealed the presence of extracellular active enzymes but also enzymes of cytoplasmatic origin suggesting release of embrane vesicles or biofilm-inherent cell lysis (Jachlewski et al., 2015). However, catalytic active enzymes with various function were identified, indicating the occurrence of extracellular enzymes as part of the EPS matrix. Since *S. acidocaldarius* grew as biofilm with concentration of 1% (v/v) 1-butanol and the microscopic images indicate EPS formation as stress response, the extracted proteins are potentially a combination of secreted proteins of viable cells and proteins from lysed cells. As the EPS can serve as digestion compartment, secretion of e. g. alcohol dehydrogenases into the EPS-matrix can be performed as protective mechanism to convert the toxic 1-butanol to less toxic compounds before affecting the cells (Flemming et al., 2016).

The identification of formed carbohydrates within the EPS was so far only performed by lectin binding and confocal laser microscopy. Within the established EPS isolation method, further analytical methods can be performed in future studies for the identification of the different carbohydrates within the EPS matrix (Ruhmann et al., 2015). For *S. acidocaldarius*, thin-layer chromatography (TLC) after hydrolysis of EPS polysaccharides was applied and glucose was found to be the most abundant sugar monomer (Jachlewski, 2013). Previous studies with cultures of *S. solfataricus* (MT3 and MT4) grown in fermenter or as batch culture produced an extracellular sulfated heteropolysaccharide with glucose, mannose, glucosamine and galactose as major components (Nicolaus, 1993). The production of exopolysaccharides of the *S. solfataricus* strains MT4 and MT3 was dependent on the incubation system. Both strains showed 2.6-fold increased exopolysaccharide amount in fermenter compared to batch cultures. The protein content was determined as 0-0.1%.

In numerous studies the EPS of planktonically grown Archaea was investigated with focus on carbohydrates, however, predominantly haloarchaea and planktonically grown cells were

analysed and the analytical methods were out of date (Anton et al., 1988; Paramonov et al., 1998; H. Parolis et al., 1996; L. A. Parolis et al., 1999; Rinker et al., 2000; Zhao et al., 2013). Therefore, studies with up-to-date methods like HPLC, MS or NMR need to be applied for further detailed analysis of the xopolysaccharides produced by Archaea.

4.4. Transcriptomic and proteomic studies of *S. acidocaldarius* cells grown under different lifestyles and with solvent exposure

Poly-omic studies enable to analyse for genome-wide changes within an organism or cells and are commonly applied for example to compare different strains (e. g. wild type and deletion mutant), different lifestyles (planktonic and biofilm) or the response to environmental changes (e.g. stress).

So far, only a couple of studies dealing with proteomic and transcriptomic changes of biofilm and planktonic cells of *Sulfolobus spp* and Archaea in general has been published (Koerdt et al., 2011; Losensky et al., 2016). In the study performed by Koerdt and coworkers, statically incubated biofilm and planktonic cells of three *Sulfolobales* (*S. acidocaldarius*, *S. solfataricus* and *S. tokodaii*) were compared and investigated by proteomic and transcriptomic approaches (Koerdt et al., 2011). Losensky and coworkers investigated the proteomic changes of *Halobacterium salinarum* R1 cells grown planktonically or as and biofilms (Losensky et al., 2016). Additional studies addressing molecular changes in Archaea based on the lifestyle are lacking since research is more focussed on other experiments than changes in different lifestyles. Available transcriptomic studies of Archaea were reviewed by Walther and colleagues in 2010 revealing that mainly different environmental stressors (e. g. heat and cold shock stress, oxygen stress and UV irradiation) or growth on different carbon sources were analysed so far (Walther et al., 2011). The response of extremophilic Archaea towards organic solvents has not been addressed so far.

In this study, *S. acidocaldarius* was incubated statically in Petri-dishes in the presence and absence of 1-butanol for 4d at 78°C and attached (biofilm, BF) and suspended cells (planktonic, PL) were analysed separately. Additionally, planktonic cells of shaking cultures (SC0) were compared with statically grown planktonic cells to unravel changes in response to the choosen microaerophilic lifestyle.

The identification of genes and proteins which are co-regulated under different growth conditions turned out to be difficult because the proteomics technique identified predominantly soluble, cytoplasmic proteins in this study. The number of genes encoding proteins with predicted transmembrane helices identified in *S. acidocaldarius* is 20%, and can therefore not be compared in both approaches. Thus, only a part of parallel regulated genes and proteins can be considered in this study.

4.4.1. Regulation based on different lifestyles

To address the regulation of genes and proteins in response to the applied static incubation systems (i.e. statically grown biofilm and planktonic cells) cells were compared to planktonic cells from shaking cultures. Generally, in statically grown biofilm and planktonic cells only few genes and proteins are differentially regulated (e.g. 156 genes >2-fold and 15 genes >4-fold regulated). In contrast, the differences between planktonic cells from static and shaking cultures are much more dramatic (e.g. 863 genes >4-fold, 217 genes >4-fold). Under both planktonic lifestyles in static and shaking cultures, especially the arCOG classes C (metabolism, energy production and conversion), I (metabolism, lipid metabolism) and J (information storage and processing, translation) are regulated.

Static incubation of biofilm cells *versus* planktonic cells

The transcriptional changes of statically incubated planktonic cells and biofilm cells (BF0/PL0, Tab 3.6) are quite low. Only two genes are more pronounced regulated; Saci_0276 is -14-fold down-regulated and Saci_0677 is 11-fold up-regulated in biofilm cells. Saci_0677 is annotated as site-specific recombinase XerD (arCOG annotation) whereas Saci_0276 is an uncharacterized protein of unknown function. However, Saci_0677 shows no similarity to the previously characterized single Xer homologue of *S. solfataricus* (SSO0375, (Duggin et al., 2011)).

Also in the proteome analysis, no strongly regulated proteins were identified. Since the cells were incubated for 4d under microaerophilic conditions the differentiation between biofilm and planktonic cells is maybe at a late stage. Maybe the biofilm is already maturated and biofilm dispersal starts with the release of cells. Generally, in biofilm cells more genes and proteins are down-regulated compared to planktonic cells.

In the previous study by Koerdt and colleagues three different *Sulfolobus spp.*, *S. acidocaldarius*, *S. solfataricus* and *S. tokodaii,* were compared with respect to transcriptomic and proteomic changes based on sessile (biofilm) and planktonic lifestyle (Koerdt et al., 2011). In contrast to this study the cells were incubated for 2d in large Petri-dish (ø 150mm, 50mm height with ventilation cams) inside a metal box instead of 4d in regular Petri-dish (ø 92mm, 16mm height without ventilation cams) in an anerobic jar. In addition, Koerdt and co-workers used 0.1% (w/v) tryptone for growth instead of 0.1% (w/v) N-Z-Amine and 0.2% (w/v) glucose.

Furthermore, in the study published by Koerdt et al. the transcriptome was analysed by microarray technique whereas in this study RNAseq via Illumina sequencing was used. Proteomic data were generated in both studies by iTRAQ analysis, however, with improvements in hardware and software in this later study. Theses significant differences between both studies seem to explain why the overlap between both studies is rather low (Tab. 4-2).

In both studies, the transcriptional regulators Saci_0882 and Saci_1223 were found to be regulated. The deletion strain of the Lrs14 transcriptional regulator ΔSaci_1223 is known to be impaired in biofilm formation (Orell et al., 2013b). In addition, several chaperons are up-regulated in biofilm cells in both studies, Saci_0666, Saci_1401 and Saci_1665. Additionally, several ATPases (Saci_0838, Saci_1174 and Saci_1548) are regulated that are either part of the respiratory chain complex V (ATPase/ATP synthase, subunit A, Saci_1548) or are involved in various secretion and motility systems (Makarova et al., 2016).

Table 4-2: Comparison of proteome and transcriptome data of statically grown biofilm and planktonic cells of *S. acidocaldarius* from this study with Koerdt et al. (Koerdt et al., 2011). In both studies, cells were grown grown under static cultivation conditions (Petri-dishes, 2d (Koerdt et al.), 4d (this study), 78°C) with different medium compositions and oxygen supply. In this study, cut-offs by p-value were set for proteomics and 2-fold changes for transcriptomics. Positive values (green) indicate up-regulation and negative values (red) down-regulation in biofilm cells compared to planktonic cells. (-): not (significantly) regulated.

		This study			Koerdt et al		
Locus	ArCOG annotation	Proteome		Trans-criptome	Prote-ome	Transcriptome	
		Fold change	p-value	Fold change	Fold change	Log_2 fold change	p-value
Saci_0246	Malate/lactate dehydrogenase	1.35	1.94E-03	(-)	-1.03	(-)	(-)
Saci_0292	Dehydrogenase (flavoprotein)	-1.18	3.79E-03	(-)	0.61	(-)	(-)
Saci_0362	Chromosomal protein Sac7d/Sso7d	(-)	(-)	-4.82	0.73	(-)	(-)
Saci_0666	Chaperonin GroEL, HSP60 family	1.16	3.14E-03	(-)	-0.4	(-)	(-)
Saci_0838	ATPase of the AAA+ class, CDC48 family	1.19	4.57E-03	(-)	0.54	(-)	(-)
Saci_0882	Transcriptional regulator, contains HTH domain	(-)	(-)	-2.33	1.11	-0.94	Not declared
Saci_1109	3-hydroxyacyl-CoA dehydrogenase, some fused to Enoyl-CoA hydratase	-1.25	2.98E-03	(-)	0.5	(-)	(-)
Saci_1174	ATPase involved in biogenesis of archaellum	(-)	(-)	-2.04	(-)	1.42	0.003
Saci_1223	Transcriptional regulator, contains HTH domain	1.60	4.74E-04	(-)	0.85	(-)	(-)
Saci_1322	Archaeal DNA-binding protein	(-)	(-)	-3.24	0.74	(-)	(-)
Saci_1401	Chaperonin GroEL, HSP60 family	-1.14	8.81E-03	(-)	-0.24	(-)	(-)
Saci_1417	Phosphoenolpyruvate synthase/pyruvate phosphate dikinase	1.26	5.39E-05	(-)	0.56	(-)	(-)
Saci_1548	Archaeal/vacuolar-type H+-ATPase subunit A	1.20	1.36E-03	(-)	-0.55	(-)	(-)
Saci_1665	Molecular chaperone (HSP20 family)	1.43	3.70E-03	(-)	0.97	(-)	(-)
Saci_1860	Rieske Fe-S protein	1.57	1.46E-05	(-)	(-)	2.57	0,001
Saci_2117	Aerobic-type carbon monoxide dehydrogenase, large subunit CoxL/CutL homolog	1.20	6.19E-03	(-)	(-)	-0.51	0.002

For *Clostridium acetobutylicum*, used for biobutanol production, changes between biofilm and static planktonic cells on transcript level were recently published (Liu et al., 2016). For *C. acetobutylicum*, in the biofilm mode of life, 16.2% of total genes are differentially expressed, most of them up-regulated in biofilm cells. Most of the up-regulated genes belong to cell metabolism (i.e. amino acid biosynthesis, sulfur uptake and cystein biosynthesis), whereas histidine biosynthesis was down-regulated (Liu et al., 2016). In this study, the majority of regulated genes and proteins of *S. acidocaldarius* cells was down-regulated in biofilms compared to statically grown planktonic cells.

Proteomic studies of colonies (cells grown on solid agar plates), biofilms (grown in silicone tubes under constant medium flow) and planktonic cells of *Pseudomonas aeruginosa* showed that expression pattern of biofilms cells and exponentially grown planktonic cells were comparable (Mikkelsen et al., 2007). Biofilms (grown in tubes with medium flow) were found to be much more metabolically active then planktonic cells in the exponential phase. Therefore, although shaking cultures and static biofilms were compared in both studies, differences in the experimental setups have a major effect on the number of regulated genes in static biofilms and planktonic cells from shaking cultures.

Planctonic cells from static cultures *versus* shaking cultures

Transcriptomic analysis of the gene expression of planktonic cells from static cultures and shaking cultures revealed a high number of differentially regulated genes (>2-fold: 863, >4-fold: 217) with a significant higher up-regulation in planktonic cells from static cultures. Between static and shaking cultures the cells seem to envision major physiological changes. In transcriptomic studies four genes were identified, which are highly up-regulated in static cultures compared to shaking cultures: Saci_0007 (56-fold), Saci_0505 (46-fold), Saci_1074 (73-fold) and Saci_2222 (55-fold). Saci_0505 is annotated as Zn-dependent protease with chaperone function whereas the other genes encode proteins of unknown function. Saci_0007, Saci_1074 and Saci_2222 contain transmembrane helices but are not annotated.

For the comparison of planktonic cells from static cultures and shaking cultures one important difference resulting probably in a high number of regulated genes and proteins is the different oxygen supply. The static incubated cultures in this study were incubated in a closed system, resulting in limited loss of medium and solvent but minimizing oxygen availability. For *S. solfataricus* the influence of different O_2 concentration on growth and gene expression in

shaking cultures has been investigated (G. Simon et al., 2009). *S. solfataricus* is able to grow in the presence of oxygen concentration from 1.5 – 24% and responds by physiological and transcriptional changes to different O_2 concentration. DNA microarray experiments showed altered expression of different genes, especially genes encoding for the three terminal oxidases of the respiratory chain (Tab. 4.7). This indicates that *S. solfataricus* responds with an adaptation of the respiratory system to fluctuating oxygen concentrations.

Just recently differences in proteome of planktonic and biofilm cells *of Halobacterium salinarum* E1 were published (Losensky et al., 2016). Around 60% of the predicted *Hbt. salinarum* proteome could be detected and quantified. 68% of these proteins showed significant abundance changes between planktonic and sessile states, indicating a dramatic change on the molecular level in response to different lifestyles. The identified regulated proteins predominantly belong to the arCOG categories information storage and processing, cellular processes and signalling and to metabolismus (Losensky et al., 2016).

4.4.2. Regulation based on 1-butanol exposure

Major changes were observed at the transcriptome and proteome level in response to 1-butanol exposure. From the observations of *S. acidocaldarius* cells and biofilms using microscopic techniques (e.g. light microscopy. SEM, AFM and CLSM) it was obvious that these changes target the morphology of the cell as well as the biofilm structure and EPS composition. In accordance with this observations, major changes in the expression of membrane proteins, S-layer and cell appendages as well as enzymes involved in the synthesis of extracellular polysaccharides, one component of the EPS, as well as vesicle formation was observed.

In response to 1-butanol exposure, 18% (up) and 54% (down) of the genes encoding for proteins with one or more predicted transmembrane helices were significantly (>4-fold) up- and down-regulated, respectively, suggesting a major change in the composition of the membrane.

Actually, most of the highly regulated genes in response to 1-butanol are predicted membrane proteins. For example, in both, planktonic cells and biofilm cells, Saci_0301 with six predicted transmembrane helices is strongly down-regulated (320-fold for biofilm and 140-fold for static

planktonic cells) in the presence of 1-butanol at the transcriptional level. Saci_1074 (-133-fold) and Saci_1753 (-63-fold), both with three predicted transmembrane helices, are highly down-regulated in biofilm cells in the presence of 1% (v/v) 1-butanol. Unfortunately, the function of the encoded proteins is unknown and could also not be predicted using bioinformatic approaches e. g. HHPred. Knock-out experiments may help in the future to access the possible function of these proteins.

Cell surface structures

In *Sulfolobus* spp., a proteinaceous S-layer forms the sole cell envelope, which is composed of the two S-layer proteins SlaA and SlaB (Albers et al., 2011). SlaA is the larger outer protein and SlaB the smaller inner protein, which anchors the S-layer to the mebrane (Veith et al., 2009). The S-layer proteins SlaA (Saci_2354, -2.99) and SlaB and (Saci_2355, -3.66) are down-regulated in biofilm cells in response to 1-butanol. Changes in the S-layer may also result in different cell morphologies which were observed in the scanning electron microscopy (Fig. 3- 11). Since the S-layer is anchored by SlaB to the cell membrane, the down-regulation may stabilize the cell envelope.

In *S. acidocaldarius*, three different types of surface structures have been identified: the archaeallum (Saci_1171-Saci_1180, Fig. 4-4), the UV-induced pili (ups, Saci_1493 - Saci_1495) and the adhesive pili (aap, Saci_2317 - 2319) (Henche et al., 2012b). In our studies in response to lifestyle or butanol exposure, the genes encoding the adhesive pili were not found to be differentially regulated. Although pili are involved in the attachment of cells, the down-regulation of pili is consistent with the observation of the down-regulation of membrane proteins in the presence of 1-butanol. However, without 1-butanol exposure in different lifestyles, the attachment of cells is potentially facilitated by other mechanisms, like physical adsoption. The two genes involved in formation of the UV induced pili, Saci_1493 (-2.57 fold, predicted component of type IV pili like system) and Saci_1495 (-2.48, Pilus assembly protein TadC) are down-regulated in planktonic cells in presence of 1% (v/v) 1-butanol. UV-inducible pili have been shown to play a major role in cell aggregation and DNA exchange, which allows for DNA repair via homologous recombination in response to UV damage (van Wolferen et al., 2013). Since UV stress is not applied in this experiment and 1-butanol exposure induces biofilm formation and might interfere with proteinaceous surface structures, the reduced formation of UV-induced pili might be expected under this conditions. The parallel down-regulation of

surface structures like the archaellum and UV-induced pili are potentially a protective mechanism to stabilize the cell envelope by reduction of the protein number bound to the membrane.

Further on, statically incubated planktonic and biofilm cells respond with significant down-regulation of genes involved in the archaellum biosynthesis in the presence of 1% (v/v) 1-butanol. The archaellum is well-characterized and the proteins involved in archaellum formation are known (Albers et al., 2015; Jarrell et al., 2012). The archaeal motility structure, the archaellum, is related to the type IV pilus and has no similarity to the bacterial flagella, only the function as motility structure is identical. The motor complex consists of the integral membrane protein FlaJ, which is surrounded by the ring-forming scaffold protein FlaX. FlaH and FlaI are close to FlaJ and probably interact with this membrane protein. FlaB is the rotor protein (Albers et al., 2015). The regulation and a model of the archaellum is shown in Fig. 4- 1.

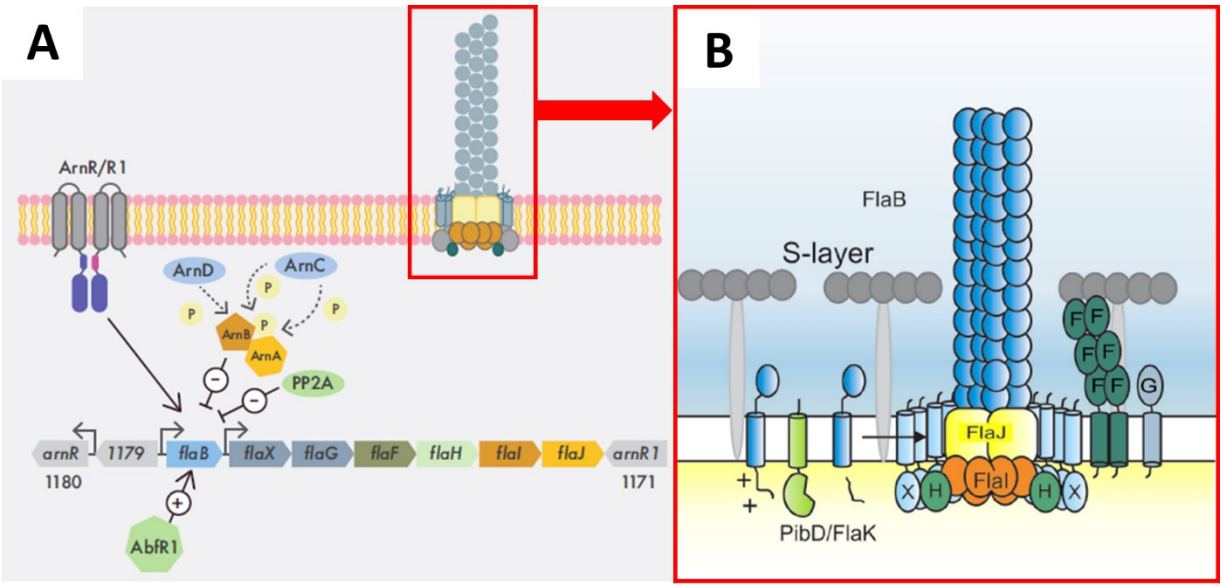


Figure 4-1: Overview of the archaellum regulatory network and model of the archaellum. The archaellum is a rotating type IV pilus-like motility structure encoded by the locus flaBXGFHIJ (Esser et al., 2016). (A), The archaellum operon (Saci_1171-Saci_1180) is under control of two promotors (flab and flax promotor). ArnR (Saci_1180) and ArnR1 (Saci_1171) are flanking the operon and are activators of archaellum expression. The expression by the the two transcriptional he repressors ArnA (Saci_1210) and ArnB (Saci_1211) xx. AbfR1 (Saci_0446) is a positive regulator of the *flab* gene (Lassak et al., 2013; Orell et al., 2013b). The regulation of transcription is performed at protein level by reversible protein phosphorylation of the repressors by the protein kinases ArnD and ArnC. The protein phosphatase PP2A controls the motility operon negatively (Esser et al., 2016). (B), After the proteins PibD/FlaK form the pre-archaellin, the motor complex is formed by a ring-complex of FlaX. Inside the scaffold protein FlaX is the membrane protein FlaJ which interacts with FlaH and FlaI. FlaG interacts with the S-layer (Albers et al., 2015).
Figure modified from Esser et. al. (A) and Albers & Jarrell (B) (Albers et al., 2015; Esser et al., 2016)

ArnR (Saci_1180) and ArnR1 (Saci_1171) are known to be positive regulators of FlaB expression, whereas ArnA (Saci_1210) and ArnB (Saci_1211) are transcriptional repressors of the *flaX-flaJ* gene cluster (Lassak et al., 2013; Reimann et al., 2012). The schematic genome organization of the archaeallum operon and its gene neighbourhood is shown in Fig. 4-2.

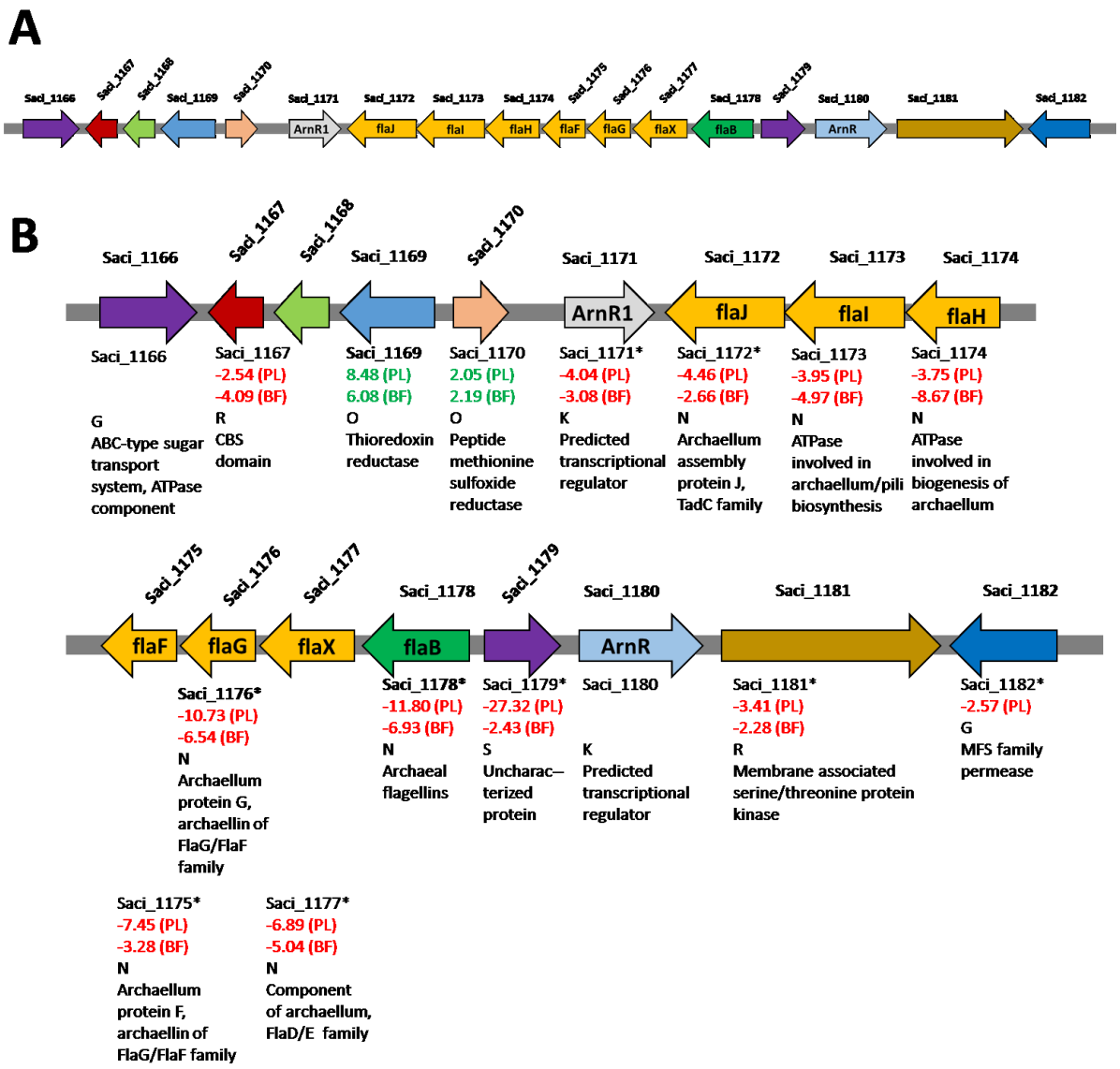


Figure 4-2: Schematic overview of the gene region comprising the archaeallum-operon for cell motility in *S. acidocaldarius*. A general overview of the gene cluster (A) as well as an enlargement with additional information (B) is shown. The arCOG category and annotation for each gene is given and the down-regulation (red) and up-regulation (green) of genes in response to 1% (v/v) 1-butanol in planktonic (PL) and biofilm (BF) cells of statically grown cultures is shown.

The gene region encoding the proteins necessary for archaellum synthesis were predominantly down-regulated, except for Saci_1169 (Thioredoxin reductase) and Saci_1170 (Peptide methionine sulfoxide reductase). Both proteins are categorized as proteins

responsible for cellular processes and signalling in general and post-translational modification, protein turnover, chaperone functions in detail (arCOG category O). Saci_1171 (ArnR1), one of the positive transcriptional regulators for the archaellum biosynthesis was also significantly down-regulated in response to 1-butanol exposure, whereas Saci_1180 (ArnR,) showed no differential regulation (Reimann et al., 2013). Both repressors (ArnA and ArnB) are less than 2-fold regulated in statically grown biofilm and planktonic cells in the presence of 1-butanol. Since the archaellum is involved in motility, the down-regulation of genes involved in archaellum synthesis is an important adaptation of *S. acidocaldarius* to facilitate biofilm formation (Henche et al., 2012b). In addition, surface structures like the archaellum potentially destabilize the membrane in the presence of 1-butanol because incorporations of proteins into the membrane may destabilize the cell envelope. Archaella assembly as well as surface structure-driven motility are highly energy-consuming/demanding processes and energy is probably required for other adaptive or protective processes under this growth conditions (Albers et al., 2015). Therefore, the observed down-regulation of genes involved in archaellum formation and motility is in line with the increased biofilm formation in response to 1-butanol exposure, which might protect the cell against harmful organic solvents.

Exopolysaccharide synthesis and transport (Glycosyltransferase gene cluster)

In addition to changes of the cell morphology also significant changes in biofilm structure and EPS composition were observed in response to 1-butanol exposure using EPS extraction methods as well as CLSM. In response to 1-butanol an increase in the amount of protein and exopolysaccharides in the EPS as well as a change in exopolysaccharide composition was observed. In previous work a gene cluster in *S. acidocaldarius* was identified, which encodes several membrane proteins, a predicted flippase (Saci_1905) and Glycosyltransferases (GTs) of different enzyme families, called here the GT cluster. It was shown that Saci_1908 is under the control of the Lrs-like transcriptional regulator (abfR1, repressor of biofilm formation) and deletion of Saci_1908 and of the GT family 2 (Saci_1909) resulted in an obvious change in biofilm structure and EPS production (Orell et al., 2013b). Therefore, it is assumed that the GT gene cluster plays a major role in EPS formation.

As shown in Fig 4-3 for several of the genes in the GT gene cluster a differential expression in response to 1-butanol was observed. For the membrane proteins Saci_1908, Saci_1919 and Saci 1920 a down-regulation in planktonic cells in response to 1-butanol is observed, whereas

the membrane protein Saci_1917 and the GT Saci_1923 are down-regulated in biofilm cells. Two uncharacterized proteins (Saci_1913 and Saci_1924) and two glycosyltransferases (Saci_1914 and Saci_1915) were differentially regulated more than 2-fold in both lifestyles upon 1-butanol exposure. The observed changes in the GT gene cluster seem to support the proposed function in exopolysaccharide synthesis and the observed morphological changes at cellular and biofilm level, however, for a final evaluation additional enzymatic and functional information for the encoded proteins has to been awaited.

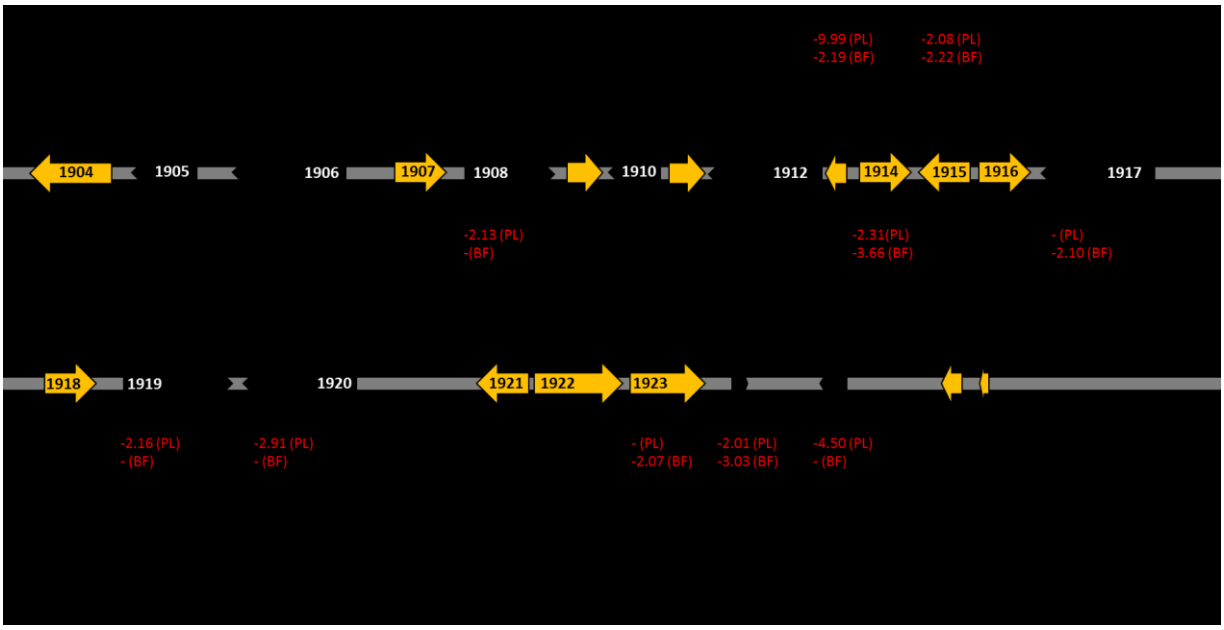


Figure 4-3: Schematic overview of the EPS/glycosyltransferase gene cluster of *S. acidocaldarius*. Orange arrows indicate proteins annotate as glycosyltransferase. The arCOG category and annotation for each gene is given and the down-regulation of genes in response to 1% (v/v) 1-butanol in planktonic (PL) and biofilm (BF) cells of statically grown cells is shown.

Vesicle/ESCRT

Quantification of EPS of biofilms indicated enhanced carbohydrate and protein concentrations in the presence of 1-butanol. In addition to the increase in exopolysaccharides in response to 1-butanol exposure also significant increase in the amount of proteins in the EPS was observed. Membrane vesicles are extracellular, membrane bound organelles of prokaryotic cells, which have several different functions from binding and delivery of DNA, removal of toxic cellular proteins or protecting reason (Manning et al., 2013). Membrane vesicles are part of the EPS matrix but less investigated because the common used techniques for EPS isolation inherently select for water soluble EPS (Flemming, 2011). Membrane vesicle formation was observed in many Bacteria and Archaea, although the archaeal cell wall differs fundamentally

from the bacterial one (Deatherage et al., 2012; Ellen et al., 2009; Manning et al., 2013). Studies of *Sulfolobus* and *Thermococcales* showed, that both Archaea are able to produce membrane vesicles (Ellen et al., 2009; Soler et al., 2008). Since both organisms are known to be able to form biofilms, vesicles are a potential constituent of the EPS. The presence of cytosolic proteins within the EPS was reported in a previous study dealing with EPS isolation and characterisation of *S. acidocaldarius* (Jachlewski et al., 2015). It was suggested that the proteins of cytoplasmic origin are either released via membrane vescicle or biofilm inherent cell lysis during biofilm maturation. *Sulfolobus* cells were shown to form membrane vesicles involving an endosomal sorting complex required for transport (ESCRT) III-dependent budding process, which is similar to the endosomal sorting pathway of exosomes in eukaryotes (Ellen et al., 2010).

The ESCRT-system is important for cell division and vesicle formation. Whereas Eukaryotes rely on an FtsZ-dependent cell devision mechanism, all Crenarchaota possess the archaeal *Cdv* (for cell division) genes (*Cdv*A, B and C), which typically form an operon (Ettema et al., 2009). CdvB and CdvC are related to the eukaryal ESCRT-III protein sorting machinery. In all Archaea that possess the Cdv machinery multiple copies (paralogs) of the *cdvB* gene have been identified. Saci_1373 is organized in the *cdv* operon, involved in cell-cycle regulation and was shown to be repressed upon UV exposure (Ettema et al., 2009). Saci_1601 was shown to be cell-cycle regulated. Saci_1416 and Saci_0451 have been previously shown to be important in membrane vesicle formation (Ellen et al., 2009; Ettema et al., 2009). The ESCRT-III proteins assemble at the site of membrane vesicle formation and induce the outward protrusion of the membrane including the S-layer. Afterwards the ATPase (Saci_1372) catalyses the dissassembly of ESCRT-III proteins and enables membrane vesicle release. Saci_1372 is regulated in the presence of 1-butanol and in different lifestyles according to the other genes involved in the ESCRT-system. All *cdvB* homologues, except Saci_1601 and the ATPase, show a significant down-reglation in response to lifestyle, suggesting that vesicle formation is reduced under static growth conditions compared to shaking cultures. The highest regulation was observed for the *cdvB* genes involved in vesicle formation, Saci_0451 and Saci_1416, which were 10.5- and 6.1-fold down-regulated, respectively. In statically grown cells in response to 1-butanol, Saci_0451 was shown to be up-regulated in planktonic cells (2.9-fold) and more significantly in biofilm cells (4.4-fold). The other *cdvB* homologues, except Saci_1601 and the ATPase, were up-regulated only in biofilm cells (2.4- and 2.7-fold). Therefore, vesicle

formation seems to be a stress response of biofilm cells towards 1-butanol exposure. The ESCRT-III system has been previously investigated in *S. acidocaldarius*, but the influence of solvent stress on biofilm cells was not analysed so far (Härtel et al., 2014; Samson et al., 2008).

Table 4-3: Differentially regulated genes of the ESCRT-III system in *S. acidocaldarius* biofilm and planktonic cells grown statically in the presence and absence of 1% (v/v) 1-butanol (Petri-dishes, 4d, 78°C) and cells grown under different lifestyles (i.e. statically grown biofilm and planktonic cells and planktonic cells from shaking cultures). Dark red and dark green colour indicate significantly (>4-fold) regulated genes, light red and light green colour indicate low regulated genes (2-4-fold regulation). (-): not regulated

Locus	arCOG annotation	arCOG code	Biofilm (BF1/BF0)	Plank-tonic (PL1/PL0)	Lifestyle Static (BF0/PL0)	Lifestyle (PL0/SC0)
Saci_0451	Archaeal division protein CdvB, Snf7/Vps24/ESCRT-III family	D	4.41	2.85	-2.85	-10.5
Saci_1372	Cell division ATPase of the AAA+ class, ESCRT system component	D	2.57	(-)	(-)	-4.4
Saci_1373	Archaeal division protein CdvB, Snf7/Vps24/ESCRT-III family	D	2.39	(-)	-2.29	-4.1
Saci_1416	Archaeal division protein CdvB, Snf7/Vps24/ESCRT-III family	D	2.66	(-)	-3.54	-6.1
Saci_1601	Archaeal division protein CdvB, Snf7/Vps24/ESCRT-III family	D	(-)	(-)	(-)	(-)

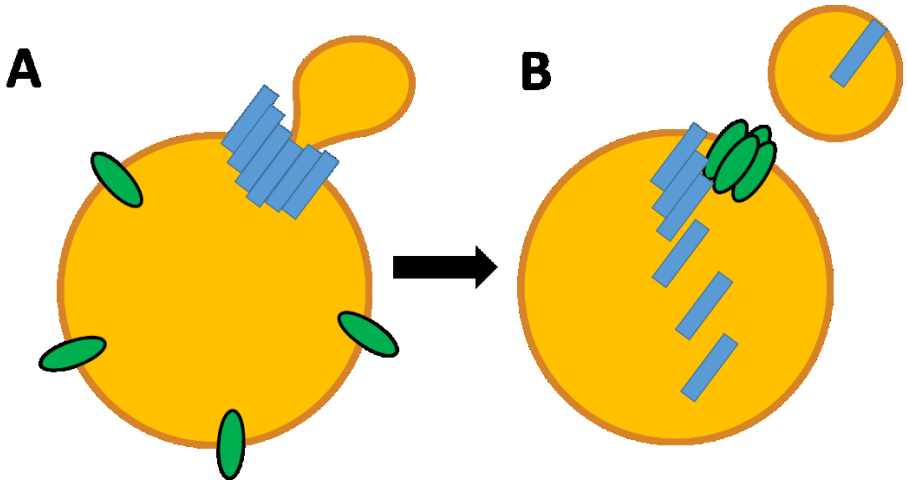


Figure 4-4: Membrane vesicle formation in Archaea. A, The ESCRT-III homologous protein (blue) is conserved in Archaea and Eukaryotes and facilitate the outward membrane budding. ESCRT proteins are located at the site of membrane vesicle formation and induce the outward protrusion of the membrane (including the S-layer). B, For membrane vesicle release, Vps4 homologue ATPases catalyse the disassembly of ESCRT-III homologous. Figure modified from Deatherage et al. (Deatherage et al., 2012)

For *Pseudomonas putida* the formation of membrane vesicles was shown to be a protecting mechanism against stress, because cells become more hydrophobic and enhance their ability

to form biofilms (Baumgarten et al., 2012). It is likely that *S. acidocaldarius* has comparable mechanisms to promote biofilm formation as *Pseudomonas*. Biofilms of the hyperthermophilic Archaeon *Thermococcus litoralis* were characterized, but membrane vesicles were not considered (Rinker et al., 1996). For *Sulfolobus* it was shown that the membrane vesicles contain antimicrobial proteins named sulfolobicins, which act as growth inhibitors for other *Sulfolobus* species (Ellen et al., 2009; Prangishvili et al., 2000). However, vesicle formation was so far only investigated for planktonic grown *Sulfolobus spp.* and vesicle formation in biofilms has not been reported so far.

Stress response (CRISPR-Cas, toxin-antitoxin system)

Besides changes in the S-layer, surface structures and EPS also significant changes in the CRISPR-Cas as well the toxin-antitoxin system were observed.

The CRISPR-Cas (CRISPR: clustered, regularly interspaced, short, palindromic repeats; Cas: CRISPR-associated) encode adaptive immune systems for protection against foreign DNA in Archaea and Bacteria by re-arrangement of DNA (Makarova et al., 2015). Immunity is established by insertion of foreign DNA into CRISPR loci, transcription and processing of these loci, and finally by hybridization of CRISPR RNAs to the complementary sequence of the invading nucleic acid guided by Cas proteins. The hybridization of the invading nucleic acid by compementery sequences mediated by the CRISPR-Cas system leads to target interference and inhibition of gene expression (van der Oost et al., 2014). Furthermore, the target is degradated by Cas3, Csm6 and/or non-Cas nucleases (van der Oost et al., 2014). The CRISPR-Cas system is grouped into three main subtypes: types I, II and III (Makarova et al., 2011). CRISPR-Cas systems are more prevalent in Archaea than in Bacteria and Archaea possess significantly more type III systems than Bacteria. The chromosome of *S. acidocaldarius* carries five repeat-clusters, which fall into CRISPR families II and III with 133, 78, 11, 5 and 2 repeats (Lillestol et al., 2009). The CRISPR system in *S. acidocaldarius* is devided into type I-D system (Saci_1864-Saci_1881), *Sulfolobus* specific Type III system (Saci_1893-Saci_1899), adaptation/processing module (Saci_2008-Saci_2012) and Type III-D system (Saci_2053-Saci_2052) according to Makarova (unpublished).

Table 4-4: Differentially regulated genes of the CRISPR-Cas system in *S. acidocaldarius* biofilm and planktonic cells grown statically in the presence and absence of 1% (v/v) 1-butanol (Petri-dishes, 4d, 78°C) and cells grown under different lifestyles (i.e. statically grown biofilm and planktonic cells and planktonic cells from shaking cultures). Dark red and dark green colour indicate significantly (>4-fold) regulated genes, light red and light green colour indicate low regulated genes (2-4-fold regulation). (-): not regulated, n.a.: not available. Classification of genes to corresponding CRISPR-Cas systems according to Makarova (unpublished).

Locus	arCOG annotation	CRISPR-Cas system	arCOG code	Biofilm (BF1/BF0)	Plank-tonic (PL1/PL0)	Lifestyle Static (BF0/PL0)	Lifestyle (PL0/SC0)
Type I-D system							
Saci_1867	RecB-nuclease fused to coiled-coil domain	n.a.	R	-2.53	(-)	(-)	(-)
Saci_1868	Uncharacterized protein	n.a.	S	-2.31	(-)	(-)	7.04
Saci_1872	CRISPR-Cas system related heleicase, Cas3 (C-terminal HD nuclease domain)	cas3	V	(-)	(-)	(-)	-3.52
Saci_1873	CRISPR associated protein, RAMP family Cas5 group	csc1gr5	V	(-)	(-)	(-)	-2.74
Saci_1874	CRISPR-Cas system related protein, RAMP superfamily Cas7 group	csc2gr7	V	(-)	(-)	(-)	-5.01
Saci_1876	CRISPR-Cas associated transcriptional regulator, contains CARF and HTH domain	casR	VK	-2.35	(-)	(-)	(-)
Saci_1877	CRISPR-Cas system related protein, RAMP superfamily Cas6 group	cas6	V	-2.95	(-)	(-)	-2.00
Saci_1881	CRISPR-associated protein Cas1	cas1	V	-2.91	(-)	(-)	(-)
Sulfolobus specific Type III system							
Saci_1896	CRISPR-Cas system related protein, RAMP superfamily Cas7 group	csm3gr7	V	-2.20	(-)	(-)	(-)
Saci_1897	CRISPR assocaited protein, possible subunit of Type III-A effector complex	csx26	V	-4.20	(-)	2.31	-2.61
Saci_1898	CRISPR associated protein, Csm4g5-like subunit of effector complex	csm4gr5	V	-4.35	(-)	2.58	-2.24
Saci_1899	CRISPR associated protein, Cas10-like subunit Type III-A effector complex	cas10	V	-6.06	-2.20	2.02	(-)
Saci_1928	CRISPR-Cas system associated transcriptional regulator, containing Rossmann-like and HTH domain	n.a.	K	2.43	2.50	(-)	(-)
Saci_1992	CRISPR-Cas associated transcriptional regulator, contains CARF and HTH domain	n.a.	VK	-9.13	-5.10	2.01	3.94

Locus	arCOG annotation	CRISPR-Cas system	arCOG code	Biofilm (BF1/BF0)	Plank-tonic (PL1/PL0)	Lifestyle Static (BF0/PL0)	Lifestyle (PL0/SC0)
Adaptation/processing module							
Saci_2008	CRISPR-Cas system related protein, RAMP superfamily Cas6 group	cas6	V	-8.40	-2.71	(-)	(-)
Saci_2010	CRISPR-associated protein Cas2	cas2	V	-3.56	(-)	(-)	(-)
Saci_2011	CRISPR-associated protein Cas1	cas1	V	-2.64	(-)	(-)	(-)
Type III-D system							
Saci_2045	CRISPR-Cas system related protein, RAMP superfamily Cas7 group	csm3gr7	V	(-)	(-)	-2.13	(-)
Saci_2049	CRISPR-Cas system related protein, RAMP superfamily Cas7 group	csm3gr7	V	-4.06	(-)	(-)	(-)
Saci_2052	CRISPR-associated protein	csm2gr11	V	2.33	2.04	-3.10	(-)

More recent studies indicate that the CRISPR-Cas system is not only used as defence mechanism against foreign DNA, but is also involved in stress response and regulation of envelope structures (Ratner et al., 2015). *Sulfolobus solfataricus* P2, a close relative to *S. acidocaldarius* used in this study, has six CRISPR-loci (Zhang et al., 2013). Cas5, belonging to the type I CRISPR-Cas system, was experimentally found to be toxic for *S. solfataricus* (He et al., 2014). However, CRISPR-Cas system in *Sulfolobales* is under further research (Manica et al., 2013; Zebec et al., 2014).

For *S. acidocaldarius* cells grown in the presence of 1% (v/v) 1-butanol, genes encoding for CRISPR components are predominantly down-regulated. One of the up-regulated genes encodes for a transcriptional regulator (Saci_1928). Interestingly, Saci_1897 – Saci_1899 and Saci_1992 are differentially regulated in different lifestyles without 1-butanol exposure. Saci_1868 (uncharacterized protein) is strongly up-regulated (7-fold) in static planktonic cells compared to planktonic shaking culture. The regulation of these CRISPR-genes is, therefore, lifestyle and 1-butanol dependent.

For *Pseudomonas aeruginosa* it was shown that biofilm formation is regulated by the type I CRISPR-Cas system (Cady et al., 2011; Zegans et al., 2009). In the study, the Bacteria are infected with phages and the infection of cells leads to inhibition of biofilm formation, potentially as quarantine from the biofilm community (Zegans et al., 2009). In *S. acidocaldarius* the gene region from Saci_1864 to Saci_1881 is annotated as type I CRISPR-

Cas sytem and is not regulated in response to 1-butanol exposure in planktonic cells but in biofilm cells (K. Makarova, unpublished data). Depending on lifestyle of static cells the genes are not regulated as well. In static planktonic cells, only Saci_1868 (uncharacterized protein) is up-regulated (7.04-fold), but the other genes encoding proteins of the gene region are down- regulated. Saci_1872, Saci_1873 and Saci_1874 are only down-regulated in statically grown planktonic cells compared to planktonic shaking cultures, indicating a regulation based on lifestyle.

Toxin/antitoxin

Two major types of toxin-antitoxin systems are well established: In Type I systems the antitoxin is an mRNA molecule that prevent the translation of the toxin mRNA by binding. In type II systems the antitoxin binds to the toxin and inactivates the toxin by protein-protein interaction (Makarova et al., 2009). In total, three types are described (Van Melderen, 2010). Toxin-antitoxin systems are ubiquitous in prokaryotes and in Bacteria it is proposed that toxin-antitoxin (TA) loci play a major role in microbial stress response elements (Cooper et al., 2009). In response to environmental stress, toxin-antitoxin systems are triggered (Chan et al., 2015). Stress conditions inducing toxin-antitoxin systems are e. g. amino acid starvation, oxidative stress (H_2O_2) and antibiotics (Wang et al., 2011). Toxin-antitoxin systems are important for biofilm formation (Wen et al., 2014). Toxin-antitoxin modules were found to be regulated in *Bacillus subtilis* biofilms, showing an influence of the toxins TxpA and YqcG (Bloom-Ackermann et al., 2016). In *E. coli* biofilms, the toxi-antitoxin systems MqsRA and YocB/YefM are induced (Ren et al., 2004). The detailed mechanisms of toxin-antitoxin systems on biofilm formation are not well understood so far (Wang et al., 2011). However, recent studies investigate the influence of toxin-antitoxin systems in bacterial biofilms (Bloom-Ackermann et al., 2016).
In response to 1% (v/v) 1-butanol, four genes related to the antitoxin system were down-regulated and one gene was up-regulated (Tab. 4-5).

Table 4-5: Differentially regulated genes of the toxin-antitoxin system in *S. acidocaldarius* biofilm and planktonic cells grown statically in the presence and absence of 1% (v/v) 1-butanol (Petri-dishes, 4d, 78°C) and cells grown under different lifestyles (i.e. statically grown biofilm and planktonic cells and planktonic cells from shaking cultures). Dark red and dark green colour indicate significantly (>4-fold) regulated genes, light red and light green colour indicate low regulated genes (2-4-fold regulation). (-): not regulated

Locus	arCOG annotation	arCOG code	Biofilm (BF1/BF0)	Plank-tonic (PL1/PL0)	Lifestyle Static (BF0/PL0)	Lifestyle (PL0/SC0)
Saci_0264	Transcriptional regulator, CopG/Arc/MetJ family (DNA-binding and a metal-binding domains)	V	2.03	-2.57	(-)	(-)
Saci_0322	CopG/RHH family DNA binding protein, antitoxin	V	-2.03		(-)	(-)
Saci_0942	CopG/MetJ, RHH domain containing DNA-binding protein, often an antitoxin in Type II toxin-antitoxin systems	V	(-)	-3.27	(-)	(-)
Saci_1056	Antitoxin, CopJ/RHH family	V	-4.08	-17.63	(-)	3.46
Saci_1124	CopG/RHH family DNA binding protein	V	-4.08	(-)	(-)	13.27
Saci_1812	RHH/CopG DNA binding protein	V	-2.71	-3.23	(-)	4.62
Saci_1932	RHH/copG family antitoxin	V	-2.97	-2.57	(-)	2.10
Saci_1936	RHH/CopG DNA binding protein	V	-2.89	-2.60	(-)	(-)
Saci_1947	RHH/CopG DNA binding protein	V	(-)	-2.16	(-)	2.16
Saci_1952	CopG/RHH family DNA binding protein, antitoxin	V	(-)	-2.64	(-)	3.02
Saci_1980	RHH/CopG DNA binding protein	V	(-)	-2.53	(-)	(-)
Saci_2003	CopG/RHH family DNA binding protein, antitoxin	V	(-)	(-)	(-)	(-)
Saci_2079	RHH/CopG DNA binding protein	V	(-)	4.56	2.35	(-)

The genes Saci_1056 (Antitoxin, CopJ/RHH family, BF: -4.08-fold, PL -17.63-fold) and Saci_1932 (RHH/copG family antitoxin, BF: -2.97-fold, PL: -2.57-fold) were regulated in biofilm and planktonic cells. CopG is an antitoxin and a member of a type II toxin-antitoxin system family found in Bacteria and Archaea. It binds DNA through N-terminal ribbon-helix-helix (RHH) motifs (Gerdes et al., 2005). Comparative genomics of defence systems in Archaea and Bacteria are summarized by Makarova et al. (Makarova et al., 2013). In static grown planktonic and biofilm cells, no toxin-antitoxin genes are regulated, except Saci_2079 (2.35-fold). In contrast to 1-butanol exposed cells, however, in statically grown planktonic cells the toxin-antitoxin genes are only up-regulated compared to planktonic cells grown under shaking conditions.

A previous study on heat shock response of *S. solfataricus* by temperature shift from 80 to 90°C revealed that 5 min after temperature shift around one third of the open reading frames

were differentially expressed (Tachdjian et al., 2006). In the early stages of stress response many toxin-antitoxin loci and insertion elements were found to be regulated and it was suggested that genome plasticity and metabolic regulation are connected. The number of the overall up- and down-regulated genes was comparable, indicating that up- or down-regulation are not preferred (Tachdjian et al., 2006). This was also confirmed by a later study on heat shock response in *S. solfataricus*, which showed that several vapBC genes were triggered by the thermal shift from 80°C to 90°C (Cooper et al., 2009). In the bacterial toxin-antitoxin gene pair vapB is the antitoxin of the cognate toxin vapC (Pandey et al., 2005; Ramage et al., 2009).

Regulation at gene and protein level

Transcription is the synthesis of RNA molecules by a RNA-polymerase from a DNA template. Transcription factors, e. g. proteins, contribute to the generation of RNA from a DNA template and, therefore, play a major role in gene expression. Transcriptional regulators control the transcription by influencing the RNA polymerase in a positive (facilitation of DNA binding) or negative (prevention of DNA binding) way during transcription. Notabely, in Archaea bacterial-like transcription regulators interact with an eukaryal-type transcription machinery that is characterized by a multi-subunit RNA polymerase and homologues of the TATA binding proteins (TBP), transcription factor IIB (TFB). The thermoacidophilic Crenarchaeon *S. acidocaldarius* possesses three TFBs (TFB1, TFB2 and TFB3) and one TBP. TFB1 is the most commonly expressed TFIIB homologue under standard growth conditions and supports transcription initiation *in vitro*, whereas TFB3 is up-regulated following UV-exposure and acts as a co-activator in the presence of TFB1 (Bell et al., 2001; Götz et al., 2007; Paytubi et al., 2009).

So far, only a limited number of transcriptional regulators has been characterized and the function of most transcriptional regulators has to be elucidated in the future. Functional poly-omic studies are promising tools to unravel the possible function of regulators, since they allow to address genome-wide changes in response to different lifestyles or stress conditions. In a previous study addressing biofilm formation of three *Sulfolobus* species, six putative Lrs14-like transcriptional regulators were identified as possible regulatory factors during biofilm development (Orell et al., 2013b). Lrs14-regulators have been studied in *S. solfataricus* previously (Napoli et al., 1999), however, their role on biofilm and EPS formation as well as motility was only studied later on (Orell et al., 2013b). The genes encoding the Lrs14

transcriptional regulators Saci_1223, Saci_1242 and Saci_0446 were shown to be involved in regulation of different aspects of biofilm development.

Table 4-6: Differentially regulated transcriptional regulators and general transcription factors in *S. acidocaldarius* biofilm and planktonic cells grown statically in the presence and absence of 1% (v/v) 1-butanol (Petri-dishes, 4d, 78°C) and cells grown under different lifestyles (i.e. statically grown biofilm and planktonic cells and planktonic cells from shaking cultures). Dark red and dark green colour indicate significantly (>4-fold) regulated genes, light red and light green colour indicate low regulated genes (2-4-fold regulation). (-): not regulated

Locus	arCOG annotation	arCOG code	Biofilm (BF1/BF0)	Planktonic (PL1/PL0)	Lifestyle Static (BF0/PL0)	Lifestyle (PL0/SC0)
Saci_0102	Transcriptional regulator, contains HTH domain	K	-2.07	(-)	(-)	3.20
Saci_0466	Transcriptional regulator, contains HTH domain	K	3.37	(-)	(-)	(-)
Saci_0665	Homolog of trans-cription initiation factor TFIIB, contains Zn-ribbon domain	K	(-)	-2.66	-2.04	2.40
Saci_1171	Predicted trans-criptional regulator	K	-3.07	-4.03	(-)	(-)
Saci_1209	Transcriptional regulator, contains HTH domain	K	3.27	(-)	(-)	(-)
Saci_1223	Transcriptional regulator, contains HTH domain	K	-2.91	(-)	(-)	-2.38
Saci_1588	DNA-binding transcriptional regulator, Lrp family	K	(-)	3.32	(-)	(-)

Saci_0446, (*abfR1*, for Archaeal Biofilm Regulator 1) was up-regulated in biofilm cells upon 1-butanol exposure. AbfR1 is suggested to activate cell motility and to repress EPS formation (Orell et al., 2013b). ArnR1 (Saci_1171) is the activator for the archaellum operon expression and its down-regulation in static biofilm and planktonic cells in response to 1-butanol is in line with the induced biofilm formation (Lassak et al., 2013). Saci_1588 was found to be the only transcriptional regulator down-regulated in static planktonic and biofilm cells grown in the presence of 1% (v/v) 1-butanol. The other transcriptional regulators were regulated only in biofilm cells.

The gene encoding the transcription factor *tfb3*, is down-regulated in static planktonic cells grown in the presence of 1-butanol (-2.66-fold), up-regulated (+2.4-fold) in planktonic cells from static cultures compared to shaking cultures and down-regulated (-2.04-fold) in biofilm

cells compared to statically grown planktonic cells. Tfb3 has been previously shown to be highly up-regulated upon UV-irradiation and acts as a transcriptional co-activator (Götz et al., 2007; Paytubi et al., 2009). Therefore, the differential expression of *tfb3* might allow to adapt transcription to different environmental stress conditions. Depending on lifestyle, especially Saci_0665 is regulated: down in biofilm cells (-0.24-fold) compared to planktonic cells and up (2.40-fold) in planktonic cells grown under shaking conditions compared to static planktonic cells. Other regulated transcriptional regulators are Saci_0102 (3.20-fold up) in static planktonic cells compared to shaking culture and Saci_1223 (-2.38-fold down) under the same conditions.

In general, a wide variety of transcriptional regulators is regulated depending on 1-butanol exposure and lifestyle. Since the regulator Saci_1171 is only down-regulated in the presence of 1-butanol and not in different lifestyles, further investigations are necessary to confirm its importance under solvent stress. Like already performed for Lrs14-like genes, knock-out studies are potential experiments for further studies (Orell et al., 2013b).

Protein kinases and phosphatases

In addition to the regulation at gene level the regulation at protein level by post-translational modification plays a major role. Protein kinases as well as protein phosphatases are important for signal transduction via reversible protein phosphorylation, i.e. phosphorylation and dephosphorylation, in all three domains of life (Esser et al., 2016). Reversible phosphorylation of proteins enables organisms to respond directly to environmental changes by modifying the functional properties of enzymes. *S. acidocaldarius* comprises six Hanks-type protein kinases, five atypical protein kinases and two protein phosphatases (PP2A, PTP) (Esser et al., 2016). Protein kinases and phosphatases were regulated in this study mostly to a low amount and were predominantly down-regulated in the presence of 1-butanol (Fig. 4-7). The highly regulated archaellum operon is regulated by phosphorylation of the repressors ArnA (Saci_1210) and ArnB (Saci_1211) by the protein kinases ArnC (Saci_1193) and ArnD (Saci_1694) (Esser et al., 2016). The gene encoding ArnC (Saci_1193) is up-regulated in biofilm cells and not in planktonic cells in the presence of 1-butanol, but is significantly down-regulated in planktonic cells from static cultures compared to shaking cultures and in biofilm cells compared to planktonic cells (both from static cultures). Since ArnC phosphorylates the negative regulators of the archaeallum biosynthesis, ArnA and ArnB, the general down-

regulation of the archaellum operon is conducted by higher expression of the negative regulator. ArnD (Saci_1694) is not regulated either in reponse to 1-butanol solvent stress nor to different lifestyles. The membrane associated kinase Saci_1181 is down-regulated in both biofilm and planktonic cells and the RIO2 atypical protein kinase (Saci_0796) only in planktonic cells in the presence of 1% (v/v) 1-butanol. Saci_1181 was recently named ArnS and identified as starvation-induced Ser/Thr protein kinase (Haurat et al., 2016). The bifunctional protein tyrosine phosphatase (PTP, Saci_0545) is highly up-regulated (4.3-fold) in static planktonic cells compared to planktonic cells from shaking cultures (Reimann et al., 2013).

Table 4-7: Selected differentially regulated kinases and phosphatases in *S. acidocaldarius* biofilm and planktonic cells grown statically in the presence and absence of 1% (v/v) 1-butanol (Petri-dishes, 4d, 78°C) and cells grown under different lifestyles (i.e. statically grown biofilm and planktonic cells and planktonic cells from shaking cultures). Dark red and dark green colour indicate significantly (>4-fold) regulated genes, light red and light green colour indicate low regulated genes (2-4-fold regulation). (-): not regulated

Locus	arCOG annotation	arCOG functional code	Biofilm (BF1/BF0)	Planktonic (PL1/PL0)	Lifestyle Static (BF0/PL0)	Lifestyle (PL0/SC0
Saci_0796	RIO-like serine/threonine protein kinase fused to N-terminal HTH domain (RIO2)	T	(-)	-2.13	(-)	(-)
Saci_1181	Membrane associated serine/threonine protein kinase	R	-2.28	-3.41	(-)	2.77
Saci_1193	Membrane associated serine/threonine protein kinase	R	2.33	(-)	-3.13	-4.08
Saci_0545	Protein-tyrosine phosphatase	T	-2.46	(-)	(-)	4.27

Metabolism

In addition to morphology, stress response and changes in regulation at gene and protein level, also major changes were observed in metabolism. Major changes were observed in the branched respiratory chain, which consists of reduced quinones, cytochromes and three terminal oxidase complexes (Schäfer et al., 1990). Electrons from oxidation processes (e. g. NADH) enter the respiratory chain via oxidoreductases. Afterwards, electrons are transported via reduced quinones to the cytochrome oxidase complex SoxLN-CbsAB-OdsN (Hiller et al., 2003). Electrons are then transferred to one of the terminal oxidase complexes (SoxABCDL, SoxEFGHIM and DoxBCE), which reduce O_2 to H_2O and transfer H^+ across the membrane (Gleissner et al., 1997; Komorowski et al., 2002; Lübben et al., 1994). The proteins involved in

the respiratory chain are membrane bound and predominantly down-regulated upon 1-butanol exposure (Tab. 4-8).

Table 4-8: Differentially regulated genes encoding for proteins involved in the respiratory chain in *S. acidocaldarius* biofilm and planktonic cells grown statically in the presence and absence of 1% (v/v) 1-butanol (Petri-dishes, 4d, 78°C) and cells grown under different lifestyles (i.e. statically grown biofilm and planktonic cells and planktonic cells from shaking cultures). Dark red and dark green colour indicate significantly (>4-fold) regulated genes, light red and light green colour indicate low regulated genes (2-4-fold regulation). (-): not regulated

Locus	arCOG annotation	arCOG code	Biofilm (BF1/BF0)	Planktonic (PL1/PL0)	Lifestyle Static (BF0/PL0)	Lifestyle (PL0/SC0)
SoxNL-CsAV-OdsN						
Saci_1859	Cytochrome b558/566, subunit B	C	-13.00	-8.82	2.14	5.67
Saci_1860	Rieske Fe-S protein	C	-3.16	(-)	(-)	(-)
Saci_1861	Cytochrome b subunit of the bc complex	C	-3.84	-2.38	(-)	(-)
Saci_1862	Heme-degrading monooxygenase HmoA and related ABM domain proteins	H	-2.83	(-)	(-)	(-)
SoxABCDL (Saci_2086-2089): Not regulated						
SoxEFGHIM						
Saci_2258	Predicted subunit of heme/copper-type cytochrome/quinol oxidase	C	(-)	-2.57	(-)	(-)
Saci_2259	Heme/copper-type cytochrome/quinol oxidase, subunit 2	C	3.86	(-)	-2.23	(-)
Saci_2260	Cytochrome b subunit of the bc complex	C	(-)	-4.59	-2.33	-2.27
Saci_2261	Rieske Fe-S protein	C	-2.19	-4.82	(-)	(-)
Saci_2262	Sulfocyanin	C	(-)	-3.94	-2.65	(-)
Saci_2263	Heme/copper-type cytochrome/quinol oxidase, subunit 1 and 3	C	(-)	-2.89	(-)	(-)
DoxBCE						
Saci_0097	Heme/copper-type cytochrome/quinol oxidase, subunit 1	C	-5.46	-3.84	(-)	6.87
Saci_0098	Terminal oxidase, subunit doxC	C	-6.92	-3.23	(-)	6.62
Saci_0099	Terminal oxidase, subunit doxE	C	-9.13	-2.36	(-)	8.05

Most of the genes encoding for proteins involved in the respiratory chain are down-regulated in planktonic as well as biofilm cells in the presence of 1-butanol. The terminal SoxABCDL complex (Saci_2086-2089), generating a proton motive force, is not regulated (Gleissner et al., 1997; Lübben et al., 1994). Transcriptomic analysis of *S. solfataricus* P2 incubated in the presence of different oxygen concentrations showed that the SoxABCD quinol oxidase complex is up-regulated under low oxygen concentrations, which are possible stress conditions (G. Simon et al., 2009). The terminal oxidase complex DoxBCE, consisting of Saci_0097, Saci_0098 and Saci_0099 and responsible as primary energy converter, is down-regulated (Purschke et al., 1997). Saci_1859, Saci_1860, Saci_1861 and Saci_1862 are mono-heme cytochromes and down-regulated as response upon 1-butanol exposure (Hiller et al., 2003).

Although the generation of energy is essential for *S. acidocaldarius*, the membrane bound complexes of the respiratory chain are predominantly down-regulated in response to 1-butanol exposure. As found also for other membrane-bound proteins, the down-regulation of the complexes may lead to a stabilization of the cell envelope in accordance to the static lifestyle with low oxygen supply. In this study, Saci_1821 (protein distantly related to bacterial ferritins) was down-regulated in biofilm cells upon 1-butanol exposure (-6.28-fold).

During oxygen respiration partially reduced and highly reactive oxygen (ROS) intermediates are produced, which represent an intracellular stress for the cells (G. Simon et al., 2009). The thioredoxin reductase Saci_1169, catalysing the reduction of thioredoxin and encoded in a gene region closed to the archaellum genes, is up-regulated. Since thioredoxin systems reduce the intracellular redox state by oxidation of reactive oxygen species in mammalian cells, the thioredoxin reductase may also act as antioxidance defence in *S. acidocaldarius* (Lillig et al., 2007). Since additional genes like thioredoxin (Saci_1823, BF: 2.58-fold, PL: 6.54-fold) and peroxiredoxin (Saci_1125, BF: 11.20-fold, PL: 4.38-fold) were also up-regulated, these protein class is in general regulated in the presence of 1-butanol.

The up-regulation of thioredoxin proteins were also found in a study with *S. solfataricus* as an oxidative stress response (Maaty et al., 2009). The up-regulation of thioredoxin proteins towards oxidative stress was found in all three domains of life (Reimann et al., 2013). In a previous study in response to UV irradiation and formation of reactive oxygen species, *S. acidocaldarius* responded with an up-regulation of genes Saci_1820 - Saci_1823, which

probable play a role in the detoxification of reactive oxygen species (Götz et al., 2007). In *E. coli*, the regulation of genes normally expressed under oxidative stress were regulated with 1-butanol exposure as well (Rutherford et al., 2010).

Depending on different lifestyles, a reduced number of genes encoding for the respiratory chain are regulated compared to 1-butanol exposed cells. In static planktonic cells, the DoxBCE complex is highly up-regulated compared to planktonic cells grown in shaking cultures. Especially Saci_1859 (Cytochrome b558/566, subunit B) is strongly down-regulated in cells with 1-butanol exposition, but also in different lifestyles without 1-butanol. Therefore, the gene expression is effected by 1-butanol as well as different lifestyles. Single genes (Saci_2259, Saci_2260 and Saci_2262) encoding for proteins of the SoxEFGHIM complex are down-regulated in biofilm cells compared to static planktonic cells.

Further genes involved in the metabolism are down-regulated dependent on 1-butanol exposure and lifestyle. In biofilm and planktonic cells different subunits of a N-methylhydantoinase/acetone carboxylase, involved in the amino acid metabolism, are up-regulated in the presence of 1% (v/v) 1-butanol. Additionally, genes of enzymes involved in the catechol pathway are up-regulated (Saci_2293 and Saci_2295). Catechol is the ortho isomer of the benzenediol and since Saci_2294, an aromatic ring hydroxylase, is up-regulated as well, enzymes for metabolization of aromatic compounds seem to be up-regulated. These genes are not regulated in static grown cells without 1-butanol exposure but down-regulated in static planktonic cells compared to planktonic cells from shaking cultures.

Proteome and transcriptional analysis of *S. solfataricus* P2 grown in the presence of ethanol and propanol were performed to address the metabolism of alcohols (Chong et al., 2007a, 2007b). For *S. solfataricus* P2 it was proposed that ethanol was channeled via acetyl-CoA into the central metabolism and carbon flux in ethanol grown cultures shifted toward the glyoxylate cycle (Chong et al., 2007b). Propanol degradation in *S. solfataricus* P2 was proposed to proceed via the citrate cycle involving succinyl-CoA intermediates (Chong et al., 2007a). Since 1-butanol has a longer alkyl chain compared to ethanol and propanol and *S. solfataricus* and *S. acidocaldarius* show some differences in metabolic versatility the possible conversion of 1-butanol in *S. acidocaldarius* is unclear. However, in this study various coenzyme A dependent proteins are up-regulated upon 1-butanol exposure.

Especially in the industrial relevant *Pseudomonas putida* the 1-butanol degradation was investigated via omics to investigate the 1-butanol metabolism. In *Pseudomonas putida* genes encoding for key enzymes in 1-butanol response were identified via omics-based analysis. Various 1-butanol assimilation pathways channeling 1-butanol through the glyoxylate shunt pathway into the central carbohydrate metabolism were identified (del Sol Cuenca et al., 2016). Analysis by different proteomic approaches of the molecular response of *Pseudomonas putida* KT2440 to 1-butanol was done recently (O. Simon et al., 2015). Enzymes involved in 1-butanol degradation were identified and a pathway for the metabolism of 1-butanol was constructed (O. Simon et al., 2015). Systems biology tools (amongst others transcriptome and proteome studies) of *Pseudomonas putida* KT2440 were focussed on 1-butanol degradation and the degradation pathway could be unravelled (Vallon et al., 2015).

Changes in gene expression upon 1-butanol exposure was also investigated or additional industrial relevant species like *Clostridium*. The transcriptional analysis of 1-butanol stress and tolerance of modified *Clostridium acetobutylicum* was tested for 0.25% and 0.75% (v/v) 1-butanol (Tomas et al., 2004). The increased expression of stress protein genes, like encoding chaperons, was found to be 1-butanol dose-dependent.

Cell-wide studies on 1-butanol stress were performed for *Escherichia coli* to obtain a global view on 1-butanol stress response (Rutherford et al., 2010). For this, transcript, protein and metabolite levels were investigated. The effect of 1-butanol stress on *E. coli* had comparable responses on components to other stresses, like changes of respiratory function, heat-shock and envelope stress, oxidative stress and metabolite transport and biosynthesis. Since major changes in the respective fields were also found to be regulated in *S. acidocaldarius* upon 1-butanol exposure, the general stress response of both organisms seem to be comparable. Application of different methods indicated a large increase of reactive oxygen species of 1-butanol-exposed cells (Rutherford et al., 2010). In the present study no information about the formation of reactive oxygen species was conducted.

4.5. Outlook

In this thesis, unadapted *S. acidocaldarius* DSM639 was used for the experiments on stress response. Adaptation of bacterial as well as archaeal strains can enhance the tolerance for toxic compounds like organic solvents because the cells can adapt by changes in membrane and metabolism. However, growth inhibitions of unadapted *S. acidocaldarius* give first insights about intrinsic solvent tolerances. Biofilm formation of *S. acidocaldarius* could be observed on a wide range of substrates with industrial relevance, e. g. glass and polystyrene. Also, other substrates like PDMS, Nylon- and PVDF membranes (data not shown) are capable substrates for biofilm formation. Biofilm formation of *S. acidocaldarius* can potentially be further improved by the application of substrata enabling a better attachment and surface for biofilm formation. For separation of 1-butanol from medium and other compounds by the pervaporation method, different membranes can be applied (Abdehagh et al., 2014). Since PDMS membranes are substrates for biofilm formation, theses membranes offer potentially also the ability for parallel separation of 1-butanol.

The enhanced biofilm formation and production of extracellular material under stress conditions offers the possibility of industrial application. Since the formation of EPS, especially protein and carbohydrate compounds are increased, biofilm formation under stress conditions can be used as cultivation system for generation of EPS because secreted archaeal carbohydrates and proteins have a possible industrial relevance (Poli et al., 2011).

Especially the production of second generation biofuels from renewable resources as alternative to petroleum-based fuels has an increasing importance nowadays (Nigam et al., 2011). Since some production methods require high temperature and acidic conditions, which are the optimal growth conditions of *S. acidocaldarius*, there are potential applications in biofuel production. For biotechnological production of biobutanol from lignocellulosic biomass not only the tolerance of the production strain against toxic compounds is important. Also production of biobutanol in high yields as well as tolerance of extreme pH and high temperatures are necessary (Fischer et al., 2008).

Problems with microbial production of 1-butanol are diverse, but some limitations can be overcome by the use of *S. acidocaldarius* (Zheng et al., 2009). Main problems are e. g. for commonly used mesophilic *Clostridia* srains the tolerance level of C*lostridia* towards ABE-fermentation products (acetate, 1-butanol, ethanol), sporulation and low 1-butanol titer

(Zheng et al., 2009). Production strains like *Clostridium acetobutylicum* are under constant metabolic optimization to enhance solvent production, sporulation, process stability and resistance (Zheng et al., 2009). Genetic tools for manipulation and investigations of central carbohydrate metabolism and, therefore, potential routes for 1-butanol production are under research (Bräsen et al., 2014). Ongoing research and the application of transcriptomics and proteomics may give further insights about metabolic pathways for 1-butanol metabolism.

Low 1-butanol titer, which makes the recovery quite expensive and the removal of toxic 1-butanol can be overcome by distillation when using a thermophilic organism like *S. acidocaldarius*. Separation of 1-butanol and other volatile toxic compounds could be done during long-term production using distillation. Initial experiments in this study showed, that 1-butanol with a boiling point of 118°C evaporates during incubation. This indicates, that distillation is applicable for 1-butanol and even better for e. g. ethanol with a boiling pint of 78 °C. Application of *Clostridium acetobutylicum* grown as biofilms for 1-butanol production was successfully applied and discussed (Huang et al., 2004; Liu et al., 2014; Liu et al., 2013). Compared to planktonic cells, the 1-butanol tolerance and production rate were improved. As the biobutanol formation of *Clostridium acetobutylicum* grown in biofilms has been shown to be a possible application, also *S. acidocaldarius* grown as biofilm may be applicable (Liu et al., 2014).

In summary, cultivation systems for static as well as flow-through cultivation of *S. acidocaldarius* were optimized or established. For different incubation systems further methods for investigation of biofilms were developed. Biofilm formation, aggregation of cells and enhanced production of EPS are phenotypic stress responses of *S. acidocaldarius* cultivated in the presence of different organic solvents.

4. REFERENCES

Abdehagh, N., Tezel, F. H., & Thibault, J. (2014). Separation techniques in butanol production: Challenges and developments. *Biomass and Bioenergy, 60*, 222-246.

Akita, H., Nakashima, N., & Hoshino, T. (2015). Bacterial production of isobutanol without expensive reagents. *Appl Microbiol Biotechnol, 99*(2), 991-999.

Akolkar, A. V., Deshpande, G. M., Raval, K. N., Durai, D., Nerurkar, A. S., & Desai, A. J. (2008). Organic solvent tolerance of *Halobacterium* sp. SP1(1) and its extracellular protease. *J Basic Microbiol, 48*(5), 421-425.

Albers, S. V., & Jarrell, K. F. (2015). The archaellum: how Archaea swim. *Front Microbiol, 6*, 23.

Albers, S. V., & Meyer, B. H. (2011). The archaeal cell envelope. *Nat Rev Microbiol, 9*(6), 414-426.

Anton, J., Meseguer, I., & Rodriguez-Valera, F. (1988). Production of an Extracellular Polysaccharide by *Haloferax mediterranei*. *Appl Environ Microbiol, 54*(10), 2381-2386.

Antoni, D., Zverlov, V. V., & Schwarz, W. H. (2007). Biofuels from microbes. *Appl Microbiol Biotechnol, 77*(1), 23-35.

Atomi, H., Imanaka, T., & Fukui, T. (2012). Overview of the genetic tools in the Archaea. *Front Microbiol, 3*, 337.

Atomi, H., Sato, T., & Kanai, T. (2011). Application of hyperthermophiles and their enzymes. *Curr Opin Biotechnol, 22*(5), 618-626.

Auchtung, T. A., Takacs-Vesbach, C. D., & Cavanaugh, C. M. (2006). 16S rRNA phylogenetic investigation of the candidate division "Korarchaeota". *Appl Environ Microbiol, 72*(7), 5077-5082.

Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E. P., et al. (2004). *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. *Antimicrob Agents Chemother, 48*(4), 1175-1187.

Basen, M., Schut, G. J., Nguyen, D. M., Lipscomb, G. L., Benn, R. A., Prybol, C. J., et al. (2014). Single gene insertion drives bioalcohol production by a thermophilic archaeon. *Proc Natl Acad Sci U S A, 111*(49), 17618-17623.

Baumgarten, T., Sperling, S., Seifert, J., von Bergen, M., Steiniger, F., Wick, L. Y., et al. (2012). Membrane vesicle formation as a multiple-stress response mechanism enhances *Pseudomonas putida* DOT-T1E cell surface hydrophobicity and biofilm formation. *Appl Environ Microbiol, 78*(17), 6217-6224.

Bell, S. D., & Jackson, S. P. (2001). Mechanism and regulation of transcription in archaea. *Curr Opin Microbiol, 4*(2), 208-213.

Berkner, S., & Lipps, G. (2008). Genetic tools for *Sulfolobus spp*.: vectors and first applications. *Arch Microbiol, 190*(3), 217-230.

Berkner, S., Wlodkowski, A., Albers, S. V., & Lipps, G. (2010). Inducible and constitutive promoters for genetic systems in *Sulfolobus acidocaldarius*. *Extremophiles, 14*(3), 249-259.

Bewley, M. A., Pham, T. K., Marriott, H. M., Noirel, J., Chu, H. P., Ow, S. Y., et al. (2011). Proteomic evaluation and validation of cathepsin D regulated proteins in macrophages exposed to *Streptococcus pneumoniae*. *Mol Cell Proteomics, 10*(6), M111 008193.

Bloom-Ackermann, Z., Steinberg, N., Rosenberg, G., Oppenheimer-Shaanan, Y., Pollack, D., Ely, S., et al. (2016). Toxin-Antitoxin systems eliminate defective cells and preserve symmetry in Bacillus subtilis biofilms. *Environ Microbiol*.

Borden, J. R., & Papoutsakis, E. T. (2007). Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. *Appl Environ Microbiol, 73*(9), 3061-3068.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, 72*, 248-254.

Bräsen, C., Esser, D., Rauch, B., & Siebers, B. (2014). Carbohydrate metabolism in Archaea: current insights into unusual enzymes and pathways and their regulation. *Microbiol Mol Biol Rev, 78*(1), 89-175.

Brock, T. D., Brock, K. M., Belly, R. T., & Weiss, R. L. (1972). *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch Mikrobiol, 84*(1), 54-68.

Bui le, M., Lee, J. Y., Geraldi, A., Rahman, Z., Lee, J. H., & Kim, S. C. (2015). Improved n-butanol tolerance in *Escherichia coli* by controlling membrane related functions. *J Biotechnol, 204*, 33-44.

Cady, K. C., & O'Toole, G. A. (2011). Non-identity-mediated CRISPR-bacteriophage interaction mediated via the Csy and Cas3 proteins. *J Bacteriol, 193*(14), 3433-3445.

Calderon, K., Gonzalez-Martinez, A., Gomez-Silvan, C., Osorio, F., Rodelas, B., & Gonzalez-Lopez, J. (2013). Archaeal diversity in biofilm technologies applied to treat urban and industrial wastewater: recent advances and future prospects. *Int J Mol Sci, 14*(9), 18572-18598.

Camilli, A., & Bassler, B. L. (2006). Bacterial small-molecule signaling pathways. *Science, 311*(5764), 1113-1116.

Canganella, F., Gonzalez, J. M., Yanagibayashi, M., Kato, C., & Horikoshi, K. (1997). Pressure and temperature effects on growth and viability of the hyperthermophilic archaeon *Thermococcus peptonophilus*. *Arch Microbiol, 168*(1), 1-7.

Castelle, C. J., Wrighton, K. C., Thomas, B. C., Hug, L. A., Brown, C. T., Wilkins, M. J., et al. (2015). Genomic expansion of domain archaea highlights roles for organisms from new phyla in anaerobic carbon cycling. *Curr Biol, 25*(6), 690-701.

Castro, C., Zhang, R., Liu, J., Bellenberg, S., Neu, T. R., Donati, E., et al. (2016). Biofilm formation and interspecies interactions in mixed cultures of thermo-acidophilic archaea Acidianus spp. and Sulfolobus metallicus. *Res Microbiol*.

Cavicchioli, R. (2011). Archaea--timeline of the third domain. *Nat Rev Microbiol, 9*(1), 51-61.

Chadha, S., & Kale, S. P. (2015). Simple fluorescence-based high throughput cell viability assay for filamentous fungi. *Lett Appl Microbiol, 61*(3), 238-244.

Chan, W. T., Balsa, D., & Espinosa, M. (2015). One cannot rule them all: Are bacterial toxins-antitoxins druggable? *FEMS Microbiol Rev, 39*(4), 522-540.

Chen, L., Brugger, K., Skovgaard, M., Redder, P., She, Q., Torarinsson, E., et al. (2005). The genome of *Sulfolobus acidocaldarius*, a model organism of the Crenarchaeota. *J Bacteriol, 187*(14), 4992-4999.

Chen, Z., Wu, Y., Huang, J., & Liu, D. (2015). Metabolic engineering of *Klebsiella pneumoniae* for the de novo production of 2-butanol as a potential biofuel. *Bioresour Technol, 197*, 260-265.

Cheng, K. C., Demirci, A., & Catchmark, J. M. (2010). Advances in biofilm reactors for production of value-added products. *Appl Microbiol Biotechnol, 87*(2), 445-456.

Chimileski, S., Franklin, M. J., & Papke, R. T. (2014). Biofilms formed by the archaeon *Haloferax volcanii* exhibit cellular differentiation and social motility, and facilitate horizontal gene transfer. *BMC Biol, 12*, 65.

Choi, K. H., Hwang, S., Yoon, N., & Cha, J. (2014). A novel integrative expression vector for *Sulfolobus* species. *J Microbiol Biotechnol, 24*(11), 1503-1509.

Chong, P. K., Burja, A. M., Radianingtyas, H., Fazeli, A., & Wright, P. C. (2007a). Proteome analysis of *Sulfolobus solfataricus* P2 propanol metabolism. *J Proteome Res, 6*(4), 1430-1439.

Chong, P. K., Burja, A. M., Radianingtyas, H., Fazeli, A., & Wright, P. C. (2007b). Proteome and transcriptional analysis of ethanol-grown *Sulfolobus solfataricus* P2 reveals ADH2, a potential alcohol dehydrogenase. *J Proteome Res, 6*(10), 3985-3994.

Colvin, K. M., Gordon, V. D., Murakami, K., Borlee, B. R., Wozniak, D. J., Wong, G. C., et al. (2011). The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathog, 7*(1), e1001264.

Cooper, C. R., Daugherty, A. J., Tachdjian, S., Blum, P. H., & Kelly, R. M. (2009). Role of vapBC toxin-antitoxin loci in the thermal stress response of *Sulfolobus solfataricus*. *Biochem Soc Trans, 37*(Pt 1), 123-126.

Cornillot, E., Nair, R. V., Papoutsakis, E. T., & Soucaille, P. (1997). The genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. *J Bacteriol, 179*(17), 5442-5447.

Crueger, W. a. C., A. (1990). Acetic acids *Biotechnology: A Textbook of Industrial Microbiology*: Science Tech.

Crusz, S. A., Popat, R., Rybtke, M. T., Camara, M., Givskov, M., Tolker-Nielsen, T., et al. (2012). Bursting the bubble on bacterial biofilms: a flow cell methodology. *Biofouling, 28*(8), 835-842.

Davies, D. (2003). Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov, 2*(2), 114-122.

Deatherage, B. L., & Cookson, B. T. (2012). Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. *Infect Immun, 80*(6), 1948-1957.

del Sol Cuenca, M., Roca, A., Molina-Santiago, C., Duque, E., Armengaud, J., Gomez-Garcia, M. R., et al. (2016). Understanding butanol tolerance and assimilation in Pseudomonas putida BIRD-1: an integrated omics approach. *Microb Biotechnol, 9*(1), 100-115.

DeLong, E. F. (1998). Everything in moderation: archaea as 'non-extremophiles'. *Curr Opin Genet Dev, 8*(6), 649-654.

Demirci, A., Pometto, A. L., 3rd, & Ho, K. L. (1997). Ethanol production by Saccharomyces cerevisiae in biofilm reactors. *J Ind Microbiol Biotechnol, 19*(4), 299-304.

Di Meglio, L., Busalmen, J. P., Pastore, J. I., Ballarin, V. L., & Nercessian, D. (2014). Hyperhalophilic archaeal biofilms: growth kinetics, structure, and antagonistic interaction in continuous culture. *Biofouling, 30*(2), 237-245.

Dickschat, J. S. (2010). Quorum sensing and bacterial biofilms. *Nat Prod Rep, 27*(3), 343-369.

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev, 15*(2), 167-193.

Duarte, M., Giordani, R. B., De Carli, G. A., Zuanazzi, J. A., Macedo, A. J., & Tasca, T. (2009). A quantitative resazurin assay to determinate the viability of *Trichomonas vaginalis* and the cytotoxicity of organic solvents and surfactant agents. *Exp Parasitol, 123*(2), 195-198.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry, 28*(3), 350-356.

Duggin, I. G., Dubarry, N., & Bell, S. D. (2011). Replication termination and chromosome dimer resolution in the archaeon Sulfolobus solfataricus. *EMBO J, 30*(1), 145-153.

Durre, P. (2007). Biobutanol: an attractive biofuel. *Biotechnol J, 2*(12), 1525-1534.

Egorova, K., & Antranikian, G. (2005). Industrial relevance of thermophilic Archaea. *Curr Opin Microbiol, 8*(6), 649-655.

Elias, S., & Banin, E. (2012). Multi-species biofilms: living with friendly neighbors. *FEMS Microbiol Rev, 36*(5), 990-1004.

Ellen, A. F., Albers, S. V., & Driessen, A. J. (2010). Comparative study of the extracellular proteome of Sulfolobus species reveals limited secretion. *Extremophiles, 14*(1), 87-98.

Ellen, A. F., Albers, S. V., Huibers, W., Pitcher, A., Hobel, C. F., Schwarz, H., et al. (2009). Proteomic analysis of secreted membrane vesicles of archaeal *Sulfolobus* species

reveals the presence of endosome sorting complex components. *Extremophiles, 13*(1), 67-79.

Embley, T. M., & Williams, T. A. (2015). Evolution: Steps on the road to eukaryotes. *Nature, 521*(7551), 169-170.

Esquivel, R. N., Schulze, S., Xu, R., Hippler, M., & Pohlschroder, M. (2016). Identification of Haloferax volcanii Pilin N-Glycans with Diverse Roles in Pilus Biosynthesis, Adhesion, and Microcolony Formation. *J Biol Chem, 291*(20), 10602-10614.

Esquivel, R. N., Xu, R., & Pohlschroder, M. (2013). Novel archaeal adhesion pilins with a conserved N terminus. *J Bacteriol, 195*(17), 3808-3818.

Esser, D., Hoffmann, L., Pham, T. K., Brasen, C., Qiu, W., Wright, P. C., et al. (2016). Protein phosphorylation and its role in archaeal signal transduction. *FEMS Microbiol Rev, 40*(5), 625-647.

Ettema, T. J., & Bernander, R. (2009). Cell division and the ESCRT complex: A surprise from the archaea. *Commun Integr Biol, 2*(2), 86-88.

Fischer, C. R., Klein-Marcuschamer, D., & Stephanopoulos, G. (2008). Selection and optimization of microbial hosts for biofuels production. *Metab Eng, 10*(6), 295-304.

Flemming, H. C. (2011). The perfect slime. *Colloids Surf B Biointerfaces, 86*(2), 251-259.

Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nat Rev Microbiol, 8*(9), 623-633.

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol, 14*(9), 563-575.

Frock, A. D., & Kelly, R. M. (2012). Extreme Thermophiles: Moving beyond single-enzyme biocatalysis. *Curr Opin Chem Eng, 1*(4), 363-372.

Fröls, S. (2013). Archaeal biofilms: widespread and complex. *Biochem Soc Trans, 41*(1), 393-398.

Fröls, S., Ajon, M., Wagner, M., Teichmann, D., Zolghadr, B., Folea, M., et al. (2008). UV-inducible cellular aggregation of the hyperthermophilic archaeon *Sulfolobus solfataricus* is mediated by pili formation. *Mol Microbiol, 70*(4), 938-952.

Fröls, S., Dyall-Smith, M., & Pfeifer, F. (2012). Biofilm formation by haloarchaea. *Environ Microbiol, 14*(12), 3159-3174.

Fröls, S., Gordon, P. M., Panlilio, M. A., Duggin, I. G., Bell, S. D., Sensen, C. W., et al. (2007). Response of the hyperthermophilic archaeon *Sulfolobus solfataricus* to UV damage. *J Bacteriol, 189*(23), 8708-8718.

Fuqua, W. C., Winans, S. C., & Greenberg, E. P. (1994). Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol, 176*(2), 269-275.

Gerdes, K., Christensen, S. K., & Lobner-Olesen, A. (2005). Prokaryotic toxin-antitoxin stress response loci. *Nat Rev Microbiol, 3*(5), 371-382.

Gleissner, M., Kaiser, U., Antonopoulos, E., & Schafer, G. (1997). The archaeal SoxABCD complex is a proton pump in *Sulfolobus acidocaldarius*. *J Biol Chem, 272*(13), 8417-8426.

Götz, D., Paytubi, S., Munro, S., Lundgren, M., Bernander, R., & White, M. F. (2007). Responses of hyperthermophilic crenarchaea to UV irradiation. *Genome Biol, 8*(10), R220.

Green, E. M. (2011). Fermentative production of butanol--the industrial perspective. *Curr Opin Biotechnol, 22*(3), 337-343.

Gronenberg, L. S., Marcheschi, R. J., & Liao, J. C. (2013). Next generation biofuel engineering in prokaryotes. *Curr Opin Chem Biol, 17*(3), 462-471.

Gross, R., Hauer, B., Otto, K., & Schmid, A. (2007). Microbial biofilms: new catalysts for maximizing productivity of long-term biotransformations. *Biotechnol Bioeng, 98*(6), 1123-1134.

Gross, R., Lang, K., Buhler, K., & Schmid, A. (2010). Characterization of a biofilm membrane reactor and its prospects for fine chemical synthesis. *Biotechnol Bioeng, 105*(4), 705-717.

Guy, L., & Ettema, T. J. (2011). The archaeal 'TACK' superphylum and the origin of eukaryotes. *Trends Microbiol, 19*(12), 580-587.

Halan, B., Buehler, K., & Schmid, A. (2012). Biofilms as living catalysts in continuous chemical syntheses. *Trends Biotechnol, 30*(9), 453-465.

Halan, B., Letzel, T., Schmid, A., & Buehler, K. (2014). Solid support membrane-aerated catalytic biofilm reactor for the continuous synthesis of (S)-styrene oxide at gram scale. *Biotechnol J, 9*(10), 1339-1349.

Halan, B., Schmid, A., & Buehler, K. (2011). Real-time solvent tolerance analysis of *Pseudomonas* sp. strain VLB120{Delta}C catalytic biofilms. *Appl Environ Microbiol, 77*(5), 1563-1571.

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol, 2*(2), 95-108.

Härtel, T., & Schwille, P. (2014). ESCRT-III mediated cell division in *Sulfolobus acidocaldarius* - a reconstitution perspective. *Front Microbiol, 5*, 257.

Hatch, R. A. a. S., N. J. (1998). Alginate Lyase Promotes Diffusion of Aminoglycosides through the Extracellular Polysaccharide of Mucoid *Pseudomonas aeruginosa*. *Antimicrobial Agents and CHhemotherapy, 42*(4), 974–977.

Hatzinikolaou, D. G., Kalogeris, E., Christakopoulos, P., Kekos, D. and Macris, B.J. . (2001). Comparative growth studies of the extreme thermophilic *Sulfolobus acidocaldarius* in submerged and solidified substrate cultures. *World Journal of Microbiology & Biotechnology, 17*, 229-234.

Haurat, M. F., Figueiredo, A. S., Hoffmann, L., Li, L., Herr, K., Wilson, A., et al. (2016). ArnS, a kinase involved in starvation-induced archaellum expression. *Mol Microbiol*.

He, F., Chen, L., & Peng, X. (2014). First experimental evidence for the presence of a CRISPR toxin in *Sulfolobus*. *J Mol Biol, 426*(22), 3683-3688.

Hekmat, D., Bauer, R., & Fricke, J. (2003). Optimization of the microbial synthesis of dihydroxyacetone from glycerol with Gluconobacter oxydans. *Bioprocess Biosyst Eng, 26*(2), 109-116.

Henche, A. L., Ghosh, A., Yu, X., Jeske, T., Egelman, E., & Albers, S. V. (2012a). Structure and function of the adhesive type IV pilus of *Sulfolobus acidocaldarius*. *Environ Microbiol, 14*(12), 3188-3202.

Henche, A. L., Koerdt, A., Ghosh, A., & Albers, S. V. (2012b). Influence of cell surface structures on crenarchaeal biofilm formation using a thermostable green fluorescent protein. *Environ Microbiol, 14*(3), 779-793.

Henneberger, R., Moissl, C., Amann, T., Rudolph, C., & Huber, R. (2006). New insights into the lifestyle of the cold-loving SM1 euryarchaeon: natural growth as a monospecies biofilm in the subsurface. *Appl Environ Microbiol, 72*(1), 192-199.

Hilker, R., Stadermann, K. B., Doppmeier, D., Kalinowski, J., Stoye, J., Straube, J., et al. (2014). ReadXplorer--visualization and analysis of mapped sequences. *Bioinformatics, 30*(16), 2247-2254.

Hiller, A., Henninger, T., Schafer, G., & Schmidt, C. L. (2003). New genes encoding subunits of a cytochrome bc1-analogous complex in the respiratory chain of the hyperthermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*. *J Bioenerg Biomembr, 35*(2), 121-131.

Hoffman, L. R., D'Argenio, D. A., MacCoss, M. J., Zhang, Z., Jones, R. A., & Miller, S. I. (2005). Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature, 436*(7054), 1171-1175.

Hottes, A. K., Meewan, M., Yang, D., Arana, N., Romero, P., McAdams, H. H., et al. (2004). Transcriptional profiling of *Caulobacter crescentus* during growth on complex and minimal media. *J Bacteriol, 186*(5), 1448-1461.

Huang, W. C., Ramey, D. E., & Yang, S. T. (2004). Continuous production of butanol by *Clostridium acetobutylicum* immobilized in a fibrous bed bioreactor. *Appl Biochem Biotechnol, 113-116*, 887-898.

Hwang, S., Choi, K. H., Yoon, N., & Cha, J. (2015). Improvement of a *Sulfolobus-E. coli* shuttle vector for heterologous gene expression in *Sulfolobus acidocaldarius*. *J Microbiol Biotechnol, 25*(2), 196-205.

Inoue. A; Horikoshi, K. (1991). Estimation of Solvent-Tolerance of Bacteria by the Solvent Parameter Log P. *Journal of Fermentation and Bioengineering, 71*(3), 194-196.

Isken, S., & de Bont, J. A. (1998). Bacteria tolerant to organic solvents. *Extremophiles : life under extreme conditions, 2*(3), 229-238.

Jachlewski, S. (2013). *Biofilm formation and EPS analysis of the thermoacidophilic Archaeon Sulfolobus acidocaldarius.* (Dissertation), University of Duisburg-Essen.

Jachlewski, S., Jachlewski, W. D., Linne, U., Bräsen, C., Wingender, J., & Siebers, B. (2015). Isolation of Extracellular Polymeric Substances from Biofilms of the Thermoacidophilic Archaeon *Sulfolobus acidocaldarius*. *Front Bioeng Biotechnol, 3*, 123.

Jarrell, K. F., & Albers, S. V. (2012). The archaellum: an old motility structure with a new name. *Trends Microbiol, 20*(7), 307-312.

Jarrell, K. F., Ding, Y., Nair, D. B., & Siu, S. (2013). Surface appendages of archaea: structure, function, genetics and assembly. *Life (Basel), 3*(1), 86-117.

Jarrell, K. F., Stark, M., Nair, D. B., & Chong, J. P. (2011). Flagella and pili are both necessary for efficient attachment of *Methanococcus maripaludis* to surfaces. *FEMS Microbiol Lett, 319*(1), 44-50.

Jiang, Y., Liu, J., Jiang, W., Yang, Y., & Yang, S. (2014). Current status and prospects of industrial bio-production of n-butanol in China. *Biotechnol Adv*.

Kandiba, L., & Eichler, J. (2014). Archaeal S-layer glycoproteins: post-translational modification in the face of extremes. *Front Microbiol, 5*, 661.

Kandler, O., & Konig, H. (1978). Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. *Arch Microbiol, 118*(2), 141-152.

Kang, Q., Appels, L., Tan, T., & Dewil, R. (2014). Bioethanol from lignocellulosic biomass: current findings determine research priorities. *ScientificWorldJournal, 2014*, 298153.

Kanno, M., Katayama, T., Tamaki, H., Mitani, Y., Meng, X. Y., Hori, T., et al. (2013). Isolation of butanol- and isobutanol-tolerant bacteria and physiological characterization of their butanol tolerance. *Appl Environ Microbiol, 79*(22), 6998-7005.

Kelman, Z., & White, M. F. (2005). Archaeal DNA replication and repair. *Curr Opin Microbiol, 8*(6), 669-676.

Knoshaug, E. P., & Zhang, M. (2009). Butanol tolerance in a selection of microorganisms. *Appl Biochem Biotechnol, 153*(1-3), 13-20.

Koerdt, A., Godeke, J., Berger, J., Thormann, K. M., & Albers, S. V. (2010). Crenarchaeal biofilm formation under extreme conditions. *PLoS One, 5*(11), e14104.

Koerdt, A., Jachlewski, S., Ghosh, A., Wingender, J., Siebers, B., & Albers, S. V. (2012). Complementation of *Sulfolobus solfataricus* PBL2025 with an alpha-mannosidase: effects on surface attachment and biofilm formation. *Extremophiles, 16*(1), 115-125.

Koerdt, A., Orell, A., Pham, T. K., Mukherjee, J., Wlodkowski, A., Karunakaran, E., et al. (2011). Macromolecular fingerprinting of *Sulfolobus* species in biofilm: a transcriptomic and proteomic approach combined with spectroscopic analysis. *J Proteome Res, 10*(9), 4105-4119.

Komorowski, L., Verheyen, W., & Schafer, G. (2002). The archaeal respiratory supercomplex SoxM from S. acidocaldarius combines features of quinole and cytochrome c oxidases. *Biol Chem, 383*(11), 1791-1799.

Koonin, E. V. (2015). Archaeal ancestors of eukaryotes: not so elusive any more. *BMC Biol, 13*, 84.

Kozubal, M. A., Romine, M., Jennings, R., Jay, Z. J., Tringe, S. G., Rusch, D. B., et al. (2013). Geoarchaeota: a new candidate phylum in the Archaea from high-temperature acidic iron mats in Yellowstone National Park. *ISME J, 7*(3), 622-634.

Kunduru, M. R., & Pometto, A. L., 3rd. (1996). Continuous ethanol production by Zymomonas mobilis and Saccharomyces cerevisiae in biofilm reactors. *J Ind Microbiol, 16*(4), 249-256.

Lan, E. I., & Liao, J. C. (2013). Microbial synthesis of n-butanol, isobutanol, and other higher alcohols from diverse resources. *Bioresour Technol, 135*, 339-349.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat Methods, 9*(4), 357-359.

Lapaglia, C., & Hartzell, P. L. (1997). Stress-Induced Production of Biofilm in the Hyperthermophile *Archaeoglobus fulgidus*. *Appl Environ Microbiol, 63*(8), 3158-3163.

Lassak, K., Peeters, E., Wrobel, S., & Albers, S. V. (2013). The one-component system ArnR: a membrane-bound activator of the crenarchaeal archaellum. *Mol Microbiol, 88*(1), 125-139.

Lewis, V. P., & Yang, S. T. (1992). Continuous propionic acid fermentation by immobilized Propionibacterium acidipropionici in a novel packed-bed bioreactor. *Biotechnol Bioeng, 40*(4), 465-474.

Li, J., Zhao, J. B., Zhao, M., Yang, Y. L., Jiang, W. H., & Yang, S. (2010). Screening and characterization of butanol-tolerant micro-organisms. *Lett Appl Microbiol, 50*(4), 373-379.

Li, X. Z., Webb, J. S., Kjelleberg, S., & Rosche, B. (2006). Enhanced benzaldehyde tolerance in *Zymomonas mobilis* biofilms and the potential of biofilm applications in fine-chemical production. *Appl Environ Microbiol, 72*(2), 1639-1644.

Lillestol, R. K., Shah, S. A., Brugger, K., Redder, P., Phan, H., Christiansen, J., et al. (2009). CRISPR families of the crenarchaeal genus Sulfolobus: bidirectional transcription and dynamic properties. *Mol Microbiol, 72*(1), 259-272.

Lillig, C. H., & Holmgren, A. (2007). Thioredoxin and related molecules--from biology to health and disease. *Antioxid Redox Signal, 9*(1), 25-47.

Liu, D., Chen, Y., Ding, F. Y., Zhao, T., Wu, J. L., Guo, T., et al. (2014). Biobutanol production in a *Clostridium acetobutylicum* biofilm reactor integrated with simultaneous product recovery by adsorption. *Biotechnol Biofuels, 7*(1), 5.

Liu, D., Chen, Y., Li, A., Ding, F., Zhou, T., He, Y., et al. (2013). Enhanced butanol production by modulation of electron flow in *Clostridium acetobutylicum* B3 immobilized by surface adsorption. *Bioresour Technol, 129*, 321-328.

Liu, D., Xu, J., Wang, Y., Chen, Y., Shen, X., Niu, H., et al. (2016). Comparative transcriptomic analysis of *Clostridium acetobutylicum* biofilm and planktonic cells. *J Biotechnol, 218*, 1-12.

Lopez, D., Vlamakis, H., & Kolter, R. (2010). Biofilms. *Cold Spring Harb Perspect Biol, 2*(7), a000398.

Losensky, G., Jung, K., Urlaub, H., Pfeifer, F., Fröls, S., & Lenz, C. (2016). Shedding light on biofilm formation of *Halobacterium salinarum* R1 by SWATH-LC/MS/MS analysis of planktonic and sessile cells. *Proteomics*.

Lübben, M., Warne, A., Albracht, S. P., & Saraste, M. (1994). The purified SoxABCD quinol oxidase complex of *Sulfolobus acidocaldarius* contains a novel haem. *Mol Microbiol, 13*(2), 327-335.

Ly, H. V., & Longo, M. L. (2004). The influence of short-chain alcohols on interfacial tension, mechanical properties, area/molecule, and permeability of fluid lipid bilayers. *Biophys J, 87*(2), 1013-1033.

Maaty, W. S., Wiedenheft, B., Tarlykov, P., Schaff, N., Heinemann, J., Robison-Cox, J., et al. (2009). Something old, something new, something borrowed; how the thermoacidophilic archaeon *Sulfolobus solfataricus* responds to oxidative stress. *PLoS One, 4*(9), e6964.

Mah, T. F., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol, 9*(1), 34-39.

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J., Charpentier, E., Horvath, P., et al. (2011). Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol, 9*(6), 467-477.

Makarova, K. S., & Koonin, E. V. (2015). Annotation and Classification of CRISPR-Cas Systems. *Methods Mol Biol, 1311*, 47-75.

Makarova, K. S., Koonin, E. V., & Albers, S. V. (2016). Diversity and Evolution of Type IV pili Systems in Archaea. *Front Microbiol, 7*, 667.

Makarova, K. S., Wolf, Y. I., & Koonin, E. V. (2009). Comprehensive comparative-genomic analysis of type 2 toxin-antitoxin systems and related mobile stress response systems in prokaryotes. *Biol Direct, 4*, 19.

Makarova, K. S., Wolf, Y. I., & Koonin, E. V. (2013). Comparative genomics of defense systems in archaea and bacteria. *Nucleic Acids Res, 41*(8), 4360-4377.

Manica, A., Zebec, Z., Steinkellner, J., & Schleper, C. (2013). Unexpectedly broad target recognition of the CRISPR-mediated virus defence system in the archaeon Sulfolobus solfataricus. *Nucleic Acids Res, 41*(22), 10509-10517.

Manning, A. J., & Kuehn, M. J. (2013). Functional advantages conferred by extracellular prokaryotic membrane vesicles. *J Mol Microbiol Biotechnol, 23*(1-2), 131-141.

Meleigy, S. A., & Khalaf, M. A. (2009). Biosynthesis of gibberellic acid from milk permeate in repeated batch operation by a mutant Fusarium moniliforme cells immobilized on loofa sponge. *Bioresour Technol, 100*(1), 374-379.

Merritt, J. H., Kadouri, D. E., & O'Toole, G. A. (2005). Growing and analyzing static biofilms. *Curr Protoc Microbiol, Chapter 1*, Unit 1B 1.

Mikkelsen, H., Duck, Z., Lilley, K. S., & Welch, M. (2007). Interrelationships between colonies, biofilms, and planktonic cells of *Pseudomonas aeruginosa*. *J Bacteriol, 189*(6), 2411-2416.

Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L., & Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods, 5*(7), 621-628.

Müller, V. (2014). Bioalcohol production by a new synthetic route in a hyperthermophilic archaeon. *Proc Natl Acad Sci U S A, 111*(49), 17352-17353.

Najafpour, G., Younesi, H., & Syahidah Ku Ismail, K. (2004). Ethanol fermentation in an immobilized cell reactor using Saccharomyces cerevisiae. *Bioresour Technol, 92*(3), 251-260.

Napoli, A., van der Oost, J., Sensen, C. W., Charlebois, R. L., Rossi, M., & Ciaramella, M. (1999). An Lrp-like protein of the hyperthermophilic archaeon *Sulfolobus solfataricus* which binds to its own promoter. *J Bacteriol, 181*(5), 1474-1480.

Natto, M. J., Savioli, F., Quashie, N. B., Dardonville, C., Rodenko, B., & de Koning, H. P. (2012). Validation of novel fluorescence assays for the routine screening of drug susceptibilities of *Trichomonas vaginalis*. *J Antimicrob Chemother, 67*(4), 933-943.

Ng, S. Y., Zolghadr, B., Driessen, A. J., Albers, S. V., & Jarrell, K. F. (2008). Cell surface structures of archaea. *J Bacteriol, 190*(18), 6039-6047.

Nicolaus, B., Manca, M. C., Romano, I. and Lama, L. (1993). Production of an exopolysaccharide from two thermophilic archaea belonging to the genus *Sulfolobus*. *FEMS Microbiology Letters, 103*, 203-206.

Nigam, P. S., & Singh, A. (2011). Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science, 37*(1), 52-68.

Oger, P. M., & Cario, A. (2013). Adaptation of the membrane in Archaea. *Biophys Chem, 183*, 42-56.

Orell, A., Fröls, S., & Albers, S. V. (2013a). Archaeal biofilms: the great unexplored. *Annu Rev Microbiol, 67*, 337-354.

Orell, A., Peeters, E., Vassen, V., Jachlewski, S., Schalles, S., Siebers, B., et al. (2013b). Lrs14 transcriptional regulators influence biofilm formation and cell motility of Crenarchaea. *ISME J, 7*(10), 1886-1898.

Osborne, S. J., Leaver, J., Turner, M. K., & Dunnill, P. (1990). Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. *Enzyme Microb Technol, 12*(4), 281-291.

Pandey, D. P., & Gerdes, K. (2005). Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Res, 33*(3), 966-976.

Paramonov, N. A., Parolis, L. A., Parolis, H., Boan, I. F., Anton, J., & Rodriguez-Valera, F. (1998). The structure of the exocellular polysaccharide produced by the Archaeon *Haloferax gibbonsii* (ATCC 33959). *Carbohydr Res, 309*(1), 89-94.

Parolis, H., Parolis, L. A., Boan, I. F., Rodriguez-Valera, F., Widmalm, G., Manca, M. C., et al. (1996). The structure of the exopolysaccharide produced by the halophilic Archaeon *Haloferax mediterranei* strain R4 (ATCC 33500). *Carbohydr Res, 295*, 147-156.

Parolis, L. A., Parolis, H., Paramonov, N. A., Boan, I. F., Anton, J., & Rodriguez-Valera, F. (1999). Structural studies on the acidic exopolysaccharide from *Haloferax denitrificans* ATCC 35960. *Carbohydr Res, 319*(1-4), 133-140.

Paytubi, S., & White, M. F. (2009). The crenarchaeal DNA damage-inducible transcription factor B paralogue TFB3 is a general activator of transcription. *Mol Microbiol, 72*(6), 1487-1499.

Peeters, E., Nelis, H. J., & Coenye, T. (2008). Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J Microbiol Methods, 72*(2), 157-165.

Pester, M., Schleper, C., & Wagner, M. (2011). The Thaumarchaeota: an emerging view of their phylogeny and ecophysiology. *Curr Opin Microbiol, 14*(3), 300-306.

Pettit, R. K., Weber, C. A., Kean, M. J., Hoffmann, H., Pettit, G. R., Tan, R., et al. (2005). Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. *Antimicrob Agents Chemother, 49*(7), 2612-2617.

Peyfoon, E., Meyer, B., Hitchen, P. G., Panico, M., Morris, H. R., Haslam, S. M., et al. (2010). The S-layer glycoprotein of the crenarchaeote *Sulfolobus acidocaldarius* is glycosylated at multiple sites with chitobiose-linked N-glycans. *Archaea, 2010*.

Pham, T. K., Roy, S., Noirel, J., Douglas, I., Wright, P. C., & Stafford, G. P. (2010). A quantitative proteomic analysis of biofilm adaptation by the periodontal pathogen *Tannerella forsythia*. *Proteomics, 10*(17), 3130-3141.

Pohlschröder, M., & Esquivel, R. N. (2015). Archaeal type IV pili and their involvement in biofilm formation. *Front Microbiol, 6*, 190.

Poli, A., Di Donato, P., Abbamondi, G. R., & Nicolaus, B. (2011). Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by archaea. *Archaea, 2011*, 693253.

Prangishvili, D., Holz, I., Stieger, E., Nickell, S., Kristjansson, J. K., & Zillig, W. (2000). Sulfolobicins, specific proteinaceous toxins produced by strains of the extremely thermophilic archaeal genus *Sulfolobus*. *J Bacteriol, 182*(10), 2985-2988.

Prangishvili, D., Stedman, K., & Zillig, W. (2001). Viruses of the extremely thermophilic archaeon Sulfolobus. *Trends Microbiol, 9*(1), 39-43.

Probst, A. J., & Moissl-Eichinger, C. (2015). "Altiarchaeales": uncultivated archaea from the subsurface. *Life (Basel), 5*(2), 1381-1395.

Purschke, W. G., Schmidt, C. L., Petersen, A., & Schafer, G. (1997). The terminal quinol oxidase of the hyperthermophilic archaeon *Acidianus ambivalens* exhibits a novel subunit structure and gene organization. *J Bacteriol, 179*(4), 1344-1353.

Qureshi, N., Annous, B. A., Ezeji, T. C., Karcher, P., & Maddox, I. S. (2005). Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. *Microb Cell Fact, 4*, 24.

Ramage, H. R., Connolly, L. E., & Cox, J. S. (2009). Comprehensive functional analysis of Mycobacterium tuberculosis toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. *PLoS Genet, 5*(12), e1000767.

Ratner, H. K., Sampson, T. R., & Weiss, D. S. (2015). I can see CRISPR now, even when phage are gone: a view on alternative CRISPR-Cas functions from the prokaryotic envelope. *Curr Opin Infect Dis, 28*(3), 267-274.

Reimann, J., Esser, D., Orell, A., Amman, F., Pham, T. K., Noirel, J., et al. (2013). Archaeal signal transduction: impact of protein phosphatase deletions on cell size, motility, and energy metabolism in *Sulfolobus acidocaldarius*. *Mol Cell Proteomics, 12*(12), 3908-3923.

Reimann, J., Lassak, K., Khadouma, S., Ettema, T. J., Yang, N., Driessen, A. J., et al. (2012). Regulation of archaella expression by the FHA and von Willebrand domain-containing proteins ArnA and ArnB in *Sulfolobus acidocaldarius*. *Mol Microbiol, 86*(1), 24-36.

Remonsellez, F., Orell, A., & Jerez, C. A. (2006). Copper tolerance of the thermoacidophilic archaeon *Sulfolobus metallicus:* possible role of polyphosphate metabolism. *Microbiology, 152*(Pt 1), 59-66.

Ren, D., Bedzyk, L. A., Thomas, S. M., Ye, R. W., & Wood, T. K. (2004). Gene expression in Escherichia coli biofilms. *Appl Microbiol Biotechnol, 64*(4), 515-524.

Repp, K. K., Menor, S. A., & Pettit, R. K. (2007). Microplate Alamar blue assay for susceptibility testing of *Candida albicans* biofilms. *Med Mycol, 45*(7), 603-607.

Rinker, K. D., & Kelly, R. M. (1996). Growth Physiology of the Hyperthermophilic Archaeon *Thermococcus litoralis:* Development of a Sulfur-Free Defined Medium, Characterization of an Exopolysaccharide, and Evidence of Biofilm Formation. *Appl Environ Microbiol, 62*(12), 4478-4485.

Rinker, K. D., & Kelly, R. M. (2000). Effect of carbon and nitrogen sources on growth dynamics and exopolysaccharide production for the hyperthermophilic archaeon *Thermococcus litoralis* and bacterium *Thermotoga maritima*. *Biotechnol Bioeng, 69*(5), 537-547.

Rosche, B., Li, X. Z., Hauer, B., Schmid, A., & Buehler, K. (2009). Microbial biofilms: a concept for industrial catalysis? *Trends Biotechnol, 27*(11), 636-643.

Ruhmann, B., Schmid, J., & Sieber, V. (2015). Methods to identify the unexplored diversity of microbial exopolysaccharides. *Front Microbiol, 6*, 565.

Rutherford, B. J., Dahl, R. H., Price, R. E., Szmidt, H. L., Benke, P. I., Mukhopadhyay, A., et al. (2010). Functional genomic study of exogenous n-butanol stress in *Escherichia coli*. *Appl Environ Microbiol, 76*(6), 1935-1945.

Samson, R. Y., Obita, T., Freund, S. M., Williams, R. L., & Bell, S. D. (2008). A role for the ESCRT system in cell division in archaea. *Science, 322*(5908), 1710-1713.

Sardessai, Y., & Bhosle, S. (2002). Tolerance of bacteria to organic solvents. *Res Microbiol, 153*(5), 263-268.

Sauer, K. (2003). The genomics and proteomics of biofilm formation. *Genome Biol, 4*(6), 219.

Schäfer, G., Anemüller, S., Moll, W., Meyer, W., and , & Lübben, M. (1990). Electron transport and energy conservation in the archaebacterium *Sulfolobus acidocaldarius*. *FEMS Microbiology Reviews, 75*, 335-348.

Schiraldi, C., Giuliano, M., & De Rosa, M. (2002). Perspectives on biotechnological applications of archaea. *Archaea, 1*(2), 75-86.

Schopf, S., Wanner, G., Rachel, R., & Wirth, R. (2008). An archaeal bi-species biofilm formed by *Pyrococcus furiosus* and *Methanopyrus kandleri*. *Arch Microbiol, 190*(3), 371-377.

Sikkema, J., de Bont, J. A., & Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev, 59*(2), 201-222.

Simon, G., Walther, J., Zabeti, N., Combet-Blanc, Y., Auria, R., van der Oost, J., et al. (2009). Effect of O2 concentrations on *Sulfolobus solfataricus* P2. *FEMS Microbiol Lett, 299*(2), 255-260.

Simon, O., Klebensberger, J., Mukschel, B., Klaiber, I., Graf, N., Altenbuchner, J., et al. (2015). Analysis of the molecular response of *Pseudomonas putida* KT2440 to the next-generation biofuel n-butanol. *J Proteomics, 122*, 11-25.

Soler, N., Marguet, E., Verbavatz, J. M., & Forterre, P. (2008). Virus-like vesicles and extracellular DNA produced by hyperthermophilic archaea of the order Thermococcales. *Res Microbiol, 159*(5), 390-399.

Sousa, D. Z., Salvador, A. F. , Ramos, J., Guedes, A. P., Barbosa, S., Stams, A. J. M., Alves, M. M., Pereira M. A. (2013). Activity and Viability of Methanogens in Anaerobic Digestion of Unsaturated and Saturated Long-Chain Fatty Acids. *Applied and Environmental Microbiology, 79*(14), 4239–4245.

Spang, A., Saw, J. H., Jorgensen, S. L., Zaremba-Niedzwiedzka, K., Martijn, J., Lind, A. E., et al. (2015). Complex archaea that bridge the gap between prokaryotes and eukaryotes. *Nature, 521*(7551), 173-179.

Spormann, A. M. (2008). Physiology of microbes in biofilms. *Curr Top Microbiol Immunol, 322*, 17-36.

Stewart, P. S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol, 292*(2), 107-113.

Stoodley, P., Sauer, K., Davies, D. G., & Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Annu Rev Microbiol, 56*, 187-209.

Tachdjian, S., & Kelly, R. M. (2006). Dynamic metabolic adjustments and genome plasticity are implicated in the heat shock response of the extremely thermoacidophilic archaeon *Sulfolobus solfataricus*. *J Bacteriol, 188*(12), 4553-4559.

Tay, A., & Yang, S. T. (2002). Production of L(+)-lactic acid from glucose and starch by immobilized cells of Rhizopus oryzae in a rotating fibrous bed bioreactor. *Biotechnol Bioeng, 80*(1), 1-12.

Tomas, C. A., Beamish, J., & Papoutsakis, E. T. (2004). Transcriptional Analysis of Butanol Stress and Tolerance in *Clostridium acetobutylicum*. *Journal of Bacteriology, 186*(7), 2006-2018.

Usami, R., Fukushima, T., Mizuki, T., Inoue, A., Yoshida, Y., & Horikoshi, K. (2003). Organic solvent tolerance of halophilic archaea. *Biosci Biotechnol Biochem, 67*(8), 1809-1812.

Usami, R., Fukushima, T., Mizuki, T., Yoshida, Y., Inoue, A., & Horikoshi, K. (2005). Organic solvent tolerance of halophilic archaea, Haloarcula strains: effects of NaCl concentration on the tolerance and polar lipid composition. *J Biosci Bioeng, 99*(2), 169-174.

Vallon, T., Simon, O., Rendgen-Heugle, B., Frana, S., Mückschel, B., Broicher, A., et al. (2015). Applying systems biology tools to studyn-butanol degradation in *Pseudomonas putida* KT2440. *Engineering in Life Sciences, 15*(8), 760-771.

van der Oost, J., Westra, E. R., Jackson, R. N., & Wiedenheft, B. (2014). Unravelling the structural and mechanistic basis of CRISPR-Cas systems. *Nat Rev Microbiol, 12*(7), 479-492.

Van Melderen, L. (2010). Toxin-antitoxin systems: why so many, what for? *Curr Opin Microbiol, 13*(6), 781-785.

van Wolferen, M., Ajon, M., Driessen, A. J., & Albers, S. V. (2013). Molecular analysis of the UV-inducible pili operon from Sulfolobus acidocaldarius. *Microbiologyopen, 2*(6), 928-937.

Veith, A., Klingl, A., Zolghadr, B., Lauber, K., Mentele, R., Lottspeich, F., et al. (2009). *Acidianus*, *Sulfolobus* and *Metallosphaera* surface layers: structure, composition and gene expression. *Mol Microbiol, 73*(1), 58-72.

Venkatesan, N., Perumal, G., & Doble, M. (2015). Bacterial resistance in biofilm-associated bacteria. *Future Microbiol, 10*, 1743-1750.

Vinayavekhin, N., Mahipant, G., Vangnai, A. S., & Sangvanich, P. (2015). Untargeted metabolomics analysis revealed changes in the composition of glycerolipids and phospholipids in Bacillus subtilis under 1-butanol stress. *Appl Microbiol Biotechnol, 99*(14), 5971-5983.

Wagner, M., Berkner, S., Ajon, M., Driessen, A. J., Lipps, G., & Albers, S. V. (2009). Expanding and understanding the genetic toolbox of the hyperthermophilic genus Sulfolobus. *Biochem Soc Trans, 37*(Pt 1), 97-101.

Wagner, M., van Wolferen, M., Wagner, A., Lassak, K., Meyer, B. H., Reimann, J., et al. (2012). Versatile Genetic Tool Box for the Crenarchaeote *Sulfolobus acidocaldarius*. *Front Microbiol, 3*, 214.

Wagner, M., Wagner, A., Ma, X., Kort, J. C., Ghosh, A., Rauch, B., et al. (2014). Investigation of the malE promoter and MalR, a positive regulator of the maltose regulon, for an improved expression system in Sulfolobus acidocaldarius. *Appl Environ Microbiol, 80*(3), 1072-1081.

Walther, J., Sierocinski, P., & van der Oost, J. (2011). Hot transcriptomics. *Archaea, 2010*, 897585.

Wang, X., & Wood, T. K. (2011). Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. *Appl Environ Microbiol, 77*(16), 5577-5583.

Wen, Y., Behiels, E., & Devreese, B. (2014). Toxin-Antitoxin systems: their role in persistence, biofilm formation, and pathogenicity. *Pathog Dis, 70*(3), 240-249.

Westman, J. O., & Franzen, C. J. (2015). Current progress in high cell density yeast bioprocesses for bioethanol production. *Biotechnol J, 10*(8), 1185-1195.

Woese, C. R., Kandler, O., & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci U S A, 87*(12), 4576-4579.

Wolf, J., Stark, H., Fafenrot, K., Albersmeier, A., Pham, T. K., Müller, K. B., et al. (2016). A systems biology approach reveals major metabolic changes in the thermoacidophilic archaeon *Sulfolobus solfataricus* in response to the carbon source L-fucose versus D-glucose. *Mol Microbiol*.

Zebec, Z., Manica, A., Zhang, J., White, M. F., & Schleper, C. (2014). CRISPR-mediated targeted mRNA degradation in the archaeon Sulfolobus solfataricus. *Nucleic Acids Res, 42*(8), 5280-5288.

Zegans, M. E., Wagner, J. C., Cady, K. C., Murphy, D. M., Hammond, J. H., & O'Toole, G. A. (2009). Interaction between bacteriophage DMS3 and host CRISPR region inhibits group behaviors of *Pseudomonas aeruginosa*. *J Bacteriol, 191*(1), 210-219.

Zeldes, B. M., Keller, M. W., Loder, A. J., Straub, C. T., Adams, M. W. W., & Kelly, R. M. (2015). Extremely thermophilic microorganisms as metabolic engineering platforms for production of fuels and industrial chemicals. *Frontiers in Microbiology, 6*.

Zhang, J., & White, M. F. (2013). Hot and crispy: CRISPR-Cas systems in the hyperthermophile *Sulfolobus solfataricus*. *Biochem Soc Trans, 41*(6), 1422-1426.

Zhang, R., Neu, T. R., Zhang, Y., Bellenberg, S., Kuhlicke, U., Li, Q., et al. (2015). Visualization and analysis of EPS glycoconjugates of the thermoacidophilic archaeon *Sulfolobus metallicus*. *Appl Microbiol Biotechnol, 99*(17), 7343-7356.

Zhang, R. Y., Neu, T. R., Bellenberg, S., Kuhlicke, U., Sand, W., & Vera, M. (2015). Use of lectins to in situ visualize glycoconjugates of extracellular polymeric substances in acidophilic archaeal biofilms. *Microb Biotechnol, 8*(3), 448-461.

Zhao, D., Cai, L., Wu, J., Li, M., Liu, H., Han, J., et al. (2013). Improving polyhydroxyalkanoate production by knocking out the genes involved in exopolysaccharide biosynthesis in *Haloferax mediterranei*. *Appl Microbiol Biotechnol, 97*(7), 3027-3036.

Zheng, Y. N., Li, L. Z., Xian, M., Ma, Y. J., Yang, J. M., Xu, X., et al. (2009). Problems with the microbial production of butanol. *J Ind Microbiol Biotechnol, 36*(9), 1127-1138.

Zhou, B., Martin, G. J., & Pamment, N. B. (2008). Increased phenotypic stability and ethanol tolerance of recombinant Escherichia coli KO11 when immobilized in continuous fluidized bed culture. *Biotechnol Bioeng, 100*(4), 627-633.

Zolghadr, B., Klingl, A., Koerdt, A., Driessen, A. J., Rachel, R., & Albers, S. V. (2010). Appendage-mediated surface adherence of *Sulfolobus solfataricus*. *J Bacteriol, 192*(1), 104-110.

5. APPENDIX

5.1. Glossary

ACN	acetonitrile
ADH	alcohol dehydrogenase
AFM	atomic force microscopy
AO	acridine orange
arCOG	archaeal clusters of orthologous groups
BF0	Static incubated biofilm cells (Petri-dishes, 4d, 78°C)
BF1	Static incubated biofilm cells in presence of 1% (v/v) 1-butanol (Petri-dishes, 4d, 78°C)
BSA	bovine serum albumin
°C	degree centigrade
CBB	coomassie brilliant blue
CE	crude extract
CER	cation exchange resin
cfu	colony forming units
CLSM	confocal laser scanning microscopy
ConA	concanavalin A
d	day
Da	Dalton
DAPI	4’,6-diamidino-2-phenylindole
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eDNA	extracellular deoxyribonucleic acid

EDTA	ethylene diamine tetra-acetic acid
EPDM	ethylene propylene diene monomer
EPS	extracellular polymeric substances
FA	formic acid
FDR	false discovery rate
FISH	fluorescence in situ hybridization
G	gravity
h	hour
HILIC	hydrophobic interaction chomatography
HPLC	high pressure liquid chromatographie
IB4	lectin from Griffonia simplicifolia
kDa	kilo Dalton
l	liter
LEW buffer	lysis-equilibration-wash buffer
ml	milliliter
MF	membrane fraction
MMTS	methyl methanethiosulfonate
MS	mass spectrometer
MW	molecular weight
MWCO	molecular weight cut-off
NAD+	nicotinamide-adenine-dinucleotide (oxidized)
NADH	nicotinamide-adenine-dinucleotide (reduced)
NCBI	national center for biotechnology information
Nt	nucleotides
OD	optical density
ORF	open reading frame

PAA	Polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PCA	principal component analysis
PDMS	polydimethylsiloxane
PI	propidium iodide
PL0	Static incubated planktonic cells (Petri-dishes, 4d, 78°C)
PL1	Static incubated biofilm cells in presence of 1% (v/v) 1-butanol (Petri-dishes, 4d, 78°C)
PMMA	polymethylmethacrylate
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RPKM	reads per kilobase million
Rpm	rotations per minute
RT	room temperature
SC0	Shaking culture (180rpm, 78°C)
SDS	sodium dodecyl sulfate
TCC	total cell count
TCEP	tris- (2-carboxyethyl) phosphine
µl	mikroliter
TEAB	triethylammonium bicarbonate
uHPLC	ultra high performance liquid chromatography
v/v	volume per volume
w/v	weight per volume
XTT	(2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide)

5.2. Supporting information

Table 5-1: Conversion of solvent concentration in % (v/v) in mM.

Solvent	Concentration [% (v/v)]	Concentration [mM]
Ethanol	0	0
	1	171
	2	342
	3	514
	4	685
	5	857
1-Propanol	0	0
	0.25	33
	0.5	67
	1	133
	1.5	200
	2	266
	2.5	333
1-Butanol	0	0
	0.5	55
	1	109
	1.5	164
2-Butanol	0	0
	0.25	27
	0.5	55
Isobutanol	0	0
	0.5	54
	1	108
	1.5	162
DMSO	0	0
	1	141
	2	282
	3	422
	4	563

Table 5-2: Overview of the arCOG codes and categories of *S. acidocaldarius* according to Makarova (unpublished data). The number and percentage for all arCOG categories were calculated for the whole *S. acidocaldarius* genome. Identical general categories are highlighted in different colours.

ArCOG Code	Number	Percent	General category	Category
C	168	7.6	Metabolism	Energy production and conversion
D	9	0.4	Cellular processes and signalling	Cell cycle control and mitosis
E	159	7.1	Metabolism	Amino Acid metabolis and transport
EF	2	0.1		
F	68	3.1	Metabolism	Nucleotide metabolism and transport
G	99	4.5	Metabolism	Carbohydrate metabolism and transport
H	105	4.7	Metabolism	Coenzyme metabolism
I	91	4.1	Metabolism	Lipid metabolism
J	201	9.0	Information storage and processing	Translation
K	135	6.1	Information storage and processing	Transcription
L	84	3.8	Information storage and processing	Replication and repair
M	64	2.9	Cellular processes and signalling	Cell wall/membrane/envelop biogenesis
N	18	0.8	Cellular processes and signalling	Cell motility
O	78	3.5	Cellular processes and signalling	Post-translational modification, protein turnover, chaperone functions
P	74	3.3	Metabolism	Inorganic ion transport and metabolism
Q	38	1.7	Metabolism	Secondary Structure
R	219	9.8	Poorly characterized	General Functional Prediction only
S	437	19.6	Poorly characterized	Function Unknown
T	13	0.6	Cellular processes and signalling	Signal Transduction
U	13	0.6	Cellular processes and signalling	Intracellular trafficing and secretion
V	122	5.5	Cellular processes and signalling	Defense mechanisms
VK	2	0.1		
X	16	0.7	Mobilome	Phage-derived proteins, transposases and other mobilome components
-	9	0.4		
Sum:	2224	100.0		

Liste der Veröffentlichungen

Publikationen

2017	**Benninghoff, J. C,** Albersmeier, A., Pham, T. K., Wright, P., Kalinowski, J., Wingender, J., & Siebers, B. (2017). The effect of 1-butanol on biofilm formation of the thermoacidophilic Archaeon *Sulfolobus acidocaldarius.* (in Vorbereitung)

Buchkapitel

2016	**Jens C. Benninghoff**, Jost Wingender, Hans-Curt Flemming and Bettina Siebers (2016). Biofilms X-treme: composition of extracellular polymeric substances in Archaea. In: Hans-Curt Flemming, Thomas R. Neu, and Jost Wingender (Eds.): The Perfect Slime: Microbial Extracellular Polymeric Substances (EPS). IWA Publishing, London, (in press)

Vorträge

07/2013	Genome function and gene regulation in Archaea, 16.7.-17.7.2013, Schmitten (Frankfurt) **Jens Benninghoff**, Jost Wingender, Bettina Siebers, „Stress response towards organic solvents in *Sulfolobus acidocaldarius* biofilms"

Poster

03/2013	VAAM Konferenz, 10.3.-13.3.2013, Bremen **Jens Benninghoff**, Jost Wingender, Bettina Siebers, "Stress response in biofilms of the thermoacidophilic Archaeon *Sulfolobus acidocaldarius*"
05/2014	Biofilms 6 Konferenz, 11.5.-13.5.2014, Wien (Österreich) **Jens Benninghoff**, Jost Wingender, Bettina Siebers, "Extremofilms" - Biofilm formation of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* and response towards 1-butanol exposure"
09/2014	The Perfect Slime Konferenz, 10.9.-12.9.2014, Essen **Jens Benninghoff**, Jost Wingender, Bettina Siebers, „Change of EPS composition of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* in response to 1-butanol exposure"
06/2015	FEMS Konferenz, 7.6.-11.6.2015, Maastricht (Niederlande) **Jens Benninghoff**, Jost Wingender, Bettina Siebers, "Biofilms X-treme: Tolerance of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* to 1-butanol exposure"
09/2015	Summer School in Bioinformatics, 20.9.-26.9.2015, Gießen **Jens Benninghoff**, Jost Wingender, Bettina Siebers, „Physiological and molecular responses of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* to 1-butanol exposure"

Lebenslauf

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Erklärung

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

The effect of organic solvents on biofilm formation 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 Oktober 2016

Jens Carlo Benninghoff

ACKNOWLEDGEMENTS

Besonders dankbar bin ich Frau Prof. Dr. Bettina Siebers für die Ratschläge und Diskussionen, die uneingeschränkten Möglichkeiten und das Vertrauen, welches mir während meiner Arbeit entgegengebracht wurde. Danke!

Herrn Prof. Dr. Hans-Curt Flemming danke ich für die Übernahme des Zweitgutachtens.

Herrn Dr. Christopher Bräsen danke ich für viele konstruktive und hilfreiche Diskussionen.

Ein großes Dankeschön geht an Herrn Dr. Jost Wingender für die langen und informativen Diskussionen über Biofilme. Kein Paper oder Buch konnte mir mehr Informationen über Biofilme vermitteln.

Prof. Dr. Andreas Schmid, Prof. Dr. Katja Bühler und Dr. Babu Halan vom UFZ Leipzig gilt mein aufrichtiger Dank für die gute Zusammenarbeit in diesem Projekt. Für die Durchführung der Transkriptomuntersuchungen danke ich Andreas Albersmeier und Prof. Dr. Jörn Kalinowski von der Universität Bielefeld. Für die Durchführung der Proteomuntersuchungen danke ich Trong Khoa Pham und Prof. Dr. Philip Wright von der Universität Sheffield (UK). Smail Boukercha danke ich für die Durchführung der Elektronenmikroskopie. Andrzej Kuklinski und Christian Thyssen möchte ich für die Unterstützung bei den Rasterkraftmikroskopie-Aufnahmen danken. Dr. Witold Jachlewski danke ich für Einführung am Konfokalen Laser-Raster Mikroskop. Prof. Dr. Sonja-Verena Albers und Dr. Alvaro Orell danke ich für das „Biofilm-Praktikum“ am MPI Marburg. Meinen Studenten Nikolas Ditz, Joel Sanchez und Claudia Bruhn danke ich für die hervorragende Arbeit im Labor.

Danke an das „Damenlabor“: Dr. Anna Hagemann, Dr. Julia Verheyen, Dr. Silke Jachlewski und Dr. Bernadette Rauch! Es war toll, wie ihr mich in eurem Labor aufgenommen habt! Vielen Dank für das gute Essen beim „Perfekten Dinner“! Besonders möchte ich mich bei Dr. Julia Verheyen für die Korrekturen meiner Dissertation bedanken! Dr. Theresa Kouril, Dr. Dominik Esser und Dr. Verena Kallnik danke ich für die unterhaltsamen Kaffeepausen! Agathe Materla danke ich für die Unterstützung außerhalb des Labors. Den „Neuen“ Christina Stracke, Claus-Rüdiger Wallis, Katharina Fafenroth, Marcel Blum, Frank Schult, Lu Shen, Xiaoxiao Zhou sowie Sabine Dietl und Thomas Knura danke ich für die tolle Arbeitsatmosphäre. Larissa Schocke und Astrid Neu danke ich für die vielen unterhaltsamen Stunden.

Dem gesamten Biofilm Centre danke ich für die tolle und freundliche Arbeitsatmosphäre.

Dem Mercator Research Center Ruhr (MERCUR) möchte ich herzlich für die Finanzierung danken.

Mein größter Dank für die jahrelange Unterstützung in jeglicher Hinsicht gilt meiner Familie.