Grappling extremes: Molecular methods combined with cultivation reveal the composition and biology of space-relevant microbial communities

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Dissertation

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by

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from Munich

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List of publications

Publications listed here have appeared or will appear in peer-reviewed journals. All publications appeared in the frame of the PhD program. Copies of all accepted and submitted publications are available on the supporting CD (./All publications/Published manuscripts and ./All publications/Submitted manuscripts)

Published manuscripts (sorted by year)

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13 Wolf, A; Moissl-Eichinger, C; Perras, A; Koskinen, K; Tomazic, PT; and Thurnher D (2016) The salivary microbiome mirrors tumor stage and preconditions of patients with oropharyngeal squamous cell carcinoma.

14 Monaghan, E; Cockell, C; Schwendner, P; Perras, A; Rettberg, P; Beblo-Vranesevic, K; Bohmeier, M; Rabbow, E; Moissl-Eichinger, C; Wink, L; Marteinsson, V; Vannier, P; Gomez, F; Garcia-Descalzo, L; Ehrenfreund, P; Westall F; Gaboyer, F; Amils, R; Malki, M; Pukall, R; Cabezas, C; and Walter, N (2016): Mars on Earth? Even the best analogs bear the imprint of 2.5 billion years of oxygenated, carbon-rich world.

Other contributions:

Patent

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Conference Proceedings

Contributions at international conferences included >20 posters and talks presented as first author (date: January 2016). A full list of all contributions can be requested from the author (akperras@gmail.com).

Proposals (as principal investigator)

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I. Abstract

Extreme environments are characterised by the presence of various physical and chemical stressors and offer the opportunity to understand the terrestrial and extraterrestrial borders of life (e.g. onboard the International Space Station and on Mars). Some terrestrial extreme environments resemble Mars-like conditions and enable us to monitor potential Martian habitability. A key factor here is the limitation of oxygen on Mars. Anoxic environments are the most defiant samples in sample collection and cultivation and thus vastly unexplored. However, the assessment of anaerobic microorganisms enables researcher to draw conclusions on possible extinct and extant extraterrestrial life. Also, the logistics of travel to Mars has to be considered. Long lasting, manned space missions to Mars require more knowledge on the diversity and behaviour of microorganisms in space-environments to maintain a secure working place. This thesis encompasses the assessment of the microbial diversity in space-relevant, extreme environments and analysis of members thereof by combining cultivation-based and cultivation-independent methods.

One extreme environment is represented by the confined indoor environment of the **International Space Station** (ISS). While the presence of numerous human-associated bacteria onboard the ISS was already reported, the occurrence of Archaea remained unclear. Archaea are omnipresent on Earth and were frequently detected in cleanrooms, where the spacecraft hardware is assembled. The molecular investigation of long-term stored dust samples of the ISS confirmed the presence of human-associated microbiota and uncovered also archaeal signatures, which were detected for the first time in an extraterrestrial environment. Moreover, obtained bacterial cultures and subsequent stress-tests uncovered an exceptional resistance against desiccation and antibiotics. These findings strongly enlighten insights into the extraterrestrial microbiome and the archaeome and have to be considered for future space missions.

Terrestrial Mars analogues are in the scope of interest, because they offer the opportunity to study microbial model organisms and model communities, which might be able to withstand Mars-analogue conditions. Several Mars-analogue settings have been investigated before; however, a substantial lack of knowledge is given on oxygen-depleted settings. Several extreme, anoxic settings, which resemble abiotic Early and Present Martian conditions, were investigated in the frame of the **MASE project** (Mars Analogues for Space Exploration). Extensive cultivation efforts delivered numerous valuable, novel anaerobic model organisms, which were unconsidered for astrobiology so far. Findings of cultivation-independent methods uncovered an overall environment-specific diversity including highly adapted microorganisms. Deeper analysis revealed a cosmopolitan group of bacterial signatures present in all Early Mars-analogue settings. A small fraction of these signatures were also present in the Present Mars-analogue environment. These results emphasise a high microbial diversity in anoxic, extreme environments including versatile core genera with significant implications for astrobiology.

Subsurface environments, such as the MASE sulphidic springs, were already known to harbour an exceptional archaeon. In the nutrient-poor aquifers, uncultivated **SM1 euryarchaeal biofilm** (now: "*Candidatus* Altiarchaeum hamiconnexum") flocks are washed up and have been investigated thoroughly upon their phylogeny and life-style throughout the last decade. The work carried out along in this thesis helped to decipher novel findings on the ultrastructure. *Candidatus* Altiarchaeum hamiconnexum possesses an Archaea-atypical double membrane and cell surface appendages ("hami"), which are anchored and assembled (most likely) similarly to bacterial type 4 pili. The hami are formed by one major protein species, namely hamin protein, which showed high N-terminal similarity to S-layers. These findings propose the divergent evolution of a highly-ordered proteinaceous sheath into exceptionally organized filaments. The full-length hamin-encoding gene was identified and served as foundation for heterologous expression attempts. The hami filaments pose exceptional tools in nanobiotechnology.

In summary, the findings presented in this thesis remarkably contribute to understand life in extreme extraterrestrial and terrestrial settings with high impacts in astrobiology and industry.

II. General Introduction

1. Tackling the dogma of microbial diversity

"Microorganisms will give you anything you want if you know how to ask them"[1]

1.3 billion years after the formation of the Earth (~4.5 billion years ago), after the Hadean Eon, the planet cooled down, solid crusts and oceans formed and established the major prerequisite for the origin of life. The oldest biosignatures of life found so far are "stromatolites", dome-shaped clumps formed by mats of microorganisms, and are dated back to around 3.4 billion years ago in the Archean Eon (Allwood et al., 2007; Schopf et al., 2007). The world back then was very different from the Earth today. It was characterised by extreme conditions including high temperatures, and an oxygen-depleted atmosphere comprised of large amounts of ammonia, methane, water vapour, and carbon dioxide. One hypothesis speculates that first lifeforms were extremophiles (meaning "loving the extreme"; Brock, 1978; Fredrickson and Onstott, 1996), which had adapted to anaerobic and hyperthermophilic conditions. Many of these microorganisms are evolutionary relics and belong to the domain Archaea. The existence of Archaea was first recognized in 1977, when Carl Woese and George Fox rearranged the tree of life based on universal molecular markers (small ribosomal subunits; Woese and Fox, 1977; Woese et al., 1990; Figure II.1-1). Archaea are morphological similar to Bacteria at first sight and are comparable in size and shape. Nonetheless, closer investigations on genetic and ultrastructural levels disclosed high dissimilarities of Archaea to Bacteria as well as to Eukaryota. Hence, Archaea were grouped in a separate branch in the tree of life. For a long time, archaeal existence was thought to be restricted to extreme environments on Earth: high/low temperatures, high acidic or alkaline conditions, high amounts of salts, very low water activity and low nutrient availability (Rothschild and Mancinelli, 2001). This may be due to the fact that the majority of Archaea were isolated from specialized and extreme niches. However, archaeal as well as bacterial signatures are detected in almost every environment on Earth. Their omnipresence demonstrates that microorganisms are equipped with physiological versatility and are consequently assigned as "survival specialists".

[1] Kinichiro Sakaguchi

The detailed assessment of the microbial diversity has challenged microbiologists for a long time. Thus far, ~11.000 microbial species have been cultivated and validly described and ~600 are added each year (Rosselló-Móra, 2012). Traditional cultivation techniques in the laboratory involve plating and inoculation in broth medium including or excluding the presence of oxygen. Anaerobic cultivation demands much more effort due to the strict avoidance of oxygen. The preparation of anoxic media requires the expulsion of all bound oxygen molecules and has been standardized over years (Hungate, 1969; Miller and Wolin, 1974). The air in the atmosphere is replaced by gas mixtures of H_2, CO_2, N_2 depending on the metabolic demand. This technique is carried out using suitable equipment such as gas stations and anaerobic glove boxes (Aranki and Freter, 1972). Growth of microorganisms takes place in gas-tight vessels, which do not allow atmospheric oxygen to enter the medium.

When looking at the estimated microbial richness of 10^7-10^{12} species, the amount of cultivated microorganisms count a very small proportion (Dykhuizen, 1998; Pedrós-Alió, 2006). One explanation is that the majority of microorganisms are considered to be uncultivable (Amann et al., 1995; Joseph et al., 2003). There are many reasons for this: (**i**) cells are known to enter a non-culturable state when exposed to unsuitable growth conditions (Roszak et al., 1984, Xu et al., 1982), (**ii**) lack of knowledge on growth conditions for unknown species (Dewi Puspita et al., 2012), (iii) time-demanding slow growth (especially cold-loving psychrophiles; Franzmann et al., 1997) and (**iv**) dependence on symbioses (one or more), which does not allow pure cultures of single species (Jahn et al., 2007; Morris et al., 2013).

A second explanation is that obtaining pure cultures is in many cases time-consuming as well as labour intensive. In particular, cultivation becomes increasingly challenging while working with microorganisms that require complex metabolic demands.

Despite the obstacles, cultivated microorganisms are valuable and indispensable for microbiologists. A valid description of a microbial species requires a cultivated representative, which are subjected to various techniques to guarantee a clear classification (e.g. DNA-DNA hybridization and spectrometric techniques, such as MALDI-TOF spectrometry (Welker and Moore, 2011).

In addition, cultivation enables to uncover features behind the gene sequences (e.g. metabolomics, ultrastructure, interaction with the environment). Although cultivation is ambitious, it is essential to obtain a comprehensive insight into an organism's lifestyle.

Researchers have tackled some shortcomings of cultivation in very creative ways and have tried to find a trade-off between standard laboratory cultivation and growing microorganisms in their natural environments. *In situ* cultivation techniques were developed by promoting the microorganisms` growth directly within their natural biotope (Gavrish et al., 2008; Nichols et al., 2010). In the best case, one microorganism is trapped within a cell-device and required growth factors are delivered throughout a semipermeable membrane, which keeps other microorganisms out of the trapping system and prevents overgrowth. This system has improved over the years and nowadays even high-throughput chip-systems are available (isolation chip; iChip), allowing the simultaneous *in situ* isolation and cultivation of thousands of microorganisms in the same biotope (Ling et al., 2015).

As promising as the *in situ* cultivation method is described, it still has drawbacks. The first and most obvious limitation is restricted access to some extraordinary biotopes such as the hydrothermal vents in the Deep Sea. Hence, sampling and monitoring is limited by a long preparation time and very cost-intensive. A second limitation is represented by very slow growers which do not show up in a researcher`s lifetime. Lastly, the purification of interdependent communities is still not feasible with this method.

Yet, it is possible to analyse certain traits of uncultivated microorganisms by culture-independent methods such as stable isotope probing (SIP; Radajewski et al., 2000; Radajewski et al., 2003), microscopy techniques (including scanning, transmission and fluorescence-based microscopy such as fluorescence *in situ* hybridisation; Wagner et al., 1994) and molecular methods (e.g. sequencing techniques). The latter attracted increasing and justified attention, since the methods are rapidly improving. Sequencing methods are enabling the assessment of entire microbial communities, which are not sufficiently covered by cultivation.

Sanger and colleagues established the first sequencing method which enabled us to decipher complete gene sequences and entire genomes (Sanger et al., 1977). The Sanger technique lead to the ground-breaking novel tree of life suggested by Woese and Fox (Woese and Fox, 1977; Woese et al., 1990) and eventually facilitated the sequencing of the first human

genome (Consortium, 2004). The diversity of microbial communities can be estimated by community fingerprinting, whereas PhyloChip analyses enables the quantification and identification thereof (Muyzer and Smalla, 1998; DeSantis et al., 2007). However, the aforementioned methods are more and more replaced by high-throughput sequencing methods.

The trend nowadays is to focus on Next Generation Sequencing (NGS) methods, which cover the small subunit ribosomal RNA (SSU rRNA) genes of a microbial population. Different sequencing platforms are available, ranging from Roche 454 pyrosequencing (Quince et al., 2009), Pacific Biosciences (PacBio RS II; Hoefler et al., 2013) to Illumina sequencing (MiSeq and HiSeq; Caporaso et al., 2012). The sequencing technique has evolved so rapidly that nowadays sequencing is even possible on the International Space Station. In an experiment referred to as the Biomolecule Sequencer, the MinION device demonstrated successfully sequencing of DNA in microgravity (Castro-Wallace et al., 2016). All technologies provide high speed, massively parallel sequencing, but inherently introduce inherent error rates (Quail et al., 2012). The least biased method is represented by Illumina MiSeq sequencing, which is currently the method of choice.

NGS allows a deep characterisation of the composition of the biological world. However, there are also limitations, which falsify the “real picture”. For instance, organisms from novel lineages remain hidden due to an inappropriate primer matching (Bergmann et al., 2011; Brown et al., 2015). Other biases are introduced by (**i**) DNA extraction (Feinstein et al., 2009), (**ii**) PCR amplification (Pinto and Raskin, 2012), (**iii**) sequencing artefacts (Lee et al., 2012), (**iv**) DNA copy number (Kembel et al., 2012), and (**v**) sampling depth (Lagier et al., 2012). Some errors can be tackled by performing replicates, suitable DNA extraction methods (Feinstein et al., 2009), multiple combinations of primer sets, reducing the number of PCR cycles to avoid chimera formation (Ahn et al., 2012) and negative controls (meaning no template controls), which are carried along each step. Although researchers are aware of these problems and try to eliminate them, microbiome projects are still lacking a standard operation procedure (SOP), which guides each step of sample preparation and data processing (Brooks et al., 2015).

A microbial community is more than a list of types of organisms. 16S rRNA gene surveys do not provide information on microbial properties and their function. A more sophisticated

sequencing method is represented by “whole-genome shotgun sequencing” (Venter et al., 2004). Here, in contrast to sequencing a single gene or genome (Rinke et al., 2013), the DNA amount of an entire community is sequenced and provides valuable insights into encoded functional capacities and the genetic repertoire. Currently, more than 30.000 draft genomes (i.e. reconstructed genome fragments) are available, obtained from all domains of life. Recently, the draft genomes have served as a basis to recalculate the tree of life. The reconstructed tree contains a vast number of uncultivated representatives, in particular, of organisms deriving from extreme, yet unexplored environments (Hug et al., 2016; Figure II.1-1). When compared to the tree of Woese and collegues, which was calculates almost 30 years ago, there has been tremendous progress in capturing the uncultivated microbial diversity by NGS as is illustrated in Figure II-1.1.

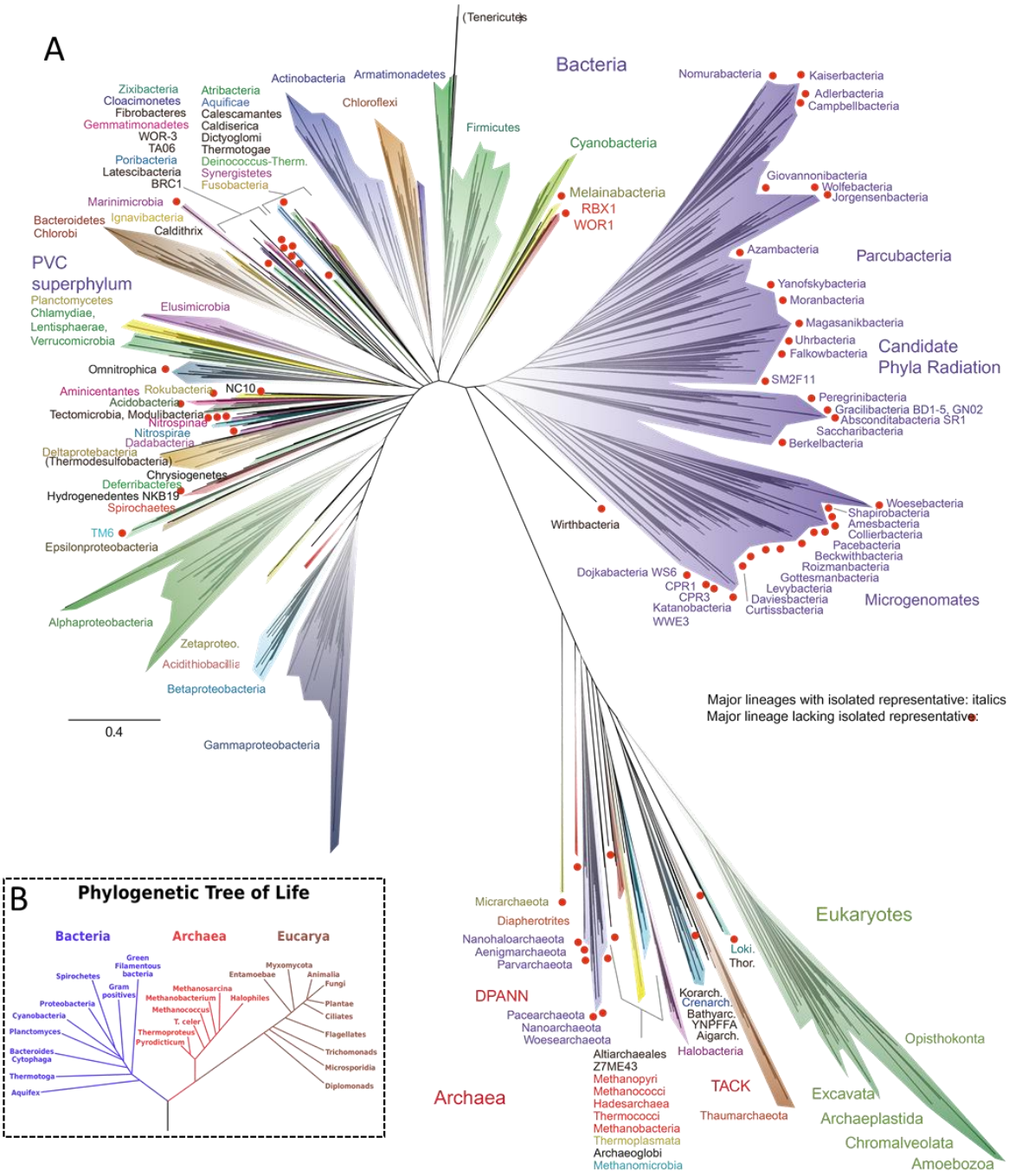

Figure II.1-1 The new tree of life (Hug et al., 2016) (A) is facing the old tree (B, Woese et al., 1990). The old tree was calculated on single sequences (16S/18S rRNA gene of Prokaryotes and Eukaryotes, respectively), while the new tree is based on entire (draft) genomes. While comparing their dimensions, it becomes clear that the knowledge on the diversity is rapidly growing. The major part of organisms remains uncultivated (for instance in the Candidate Phyla Radiation, coloured in purple). In (A) red dots and non-italicized names highlight lineages, which lack an isolated representative.

However, as described above, culture-dependent and culture-independent methods are facing the same problem: On a standalone basis, each method does not create a true illustration of a whole microbial community. On the hand, NGS allows capturing the diversity, but phenotype characteristics remain hidden behind the sequences. On the other

hand, cultivation of the entire microbial community is not possible. An obvious solution is the combination of cultivation and cultivation-independent methods, which delivers the best result (Donachie et al., 2007; Lagier et al., 2012; Dubourg et al., 2013).

Within the frame of this thesis, both methods are combined in order to uncover the "microbial dark matter" of terrestrial, extreme environments. Results of Illumina MiSeq sequencing will provide enlightenment on the microbial diversity of various extreme environments, namely the International Space Station and Mars-analogue sites. An extensive cultivation approach allows drawing conclusions on stress resistances of isolated microbes. Cultivation is not always possible in the laboratory and the following chapter shows how *in situ* cultivation combined with cultivation-independent methods sheds insights into the lifestyle of an archaeon, which is hosted in one of the investigated extreme, environments. These sulphidic springs are also in the scope of astrobiological research as Mars-analogue aquifers. In the subsequent chapter, extreme environments on Earth and in space are described with respect to their relevance for the search of extraterrestrial life.

2. A grappling archaeon: Traits of the SM1 Euryarchaeon

"I have never been disappointed upon asking microorganisms for whatever I wanted"[2]

2.1. Profiling the SM1 Euryarchaeon

More than 15 years ago, the discovery of an archaeon was reported, which exhibited a very unusual lifestyle (Rudolph et al., 2001). In the Sippenauer Moor, a sulphide-rich anoxic spring in close vicinity to Regensburg, Bavaria (Germany), whitish pearl-like assemblages were found to be attached to solid surfaces and float on the subsurface. Their eye-catching and macroscopically visible appearance drew interest on their composition. Different microscopy techniques such as scanning electron or phase contrast microscopy and fluorescence *in situ* hybridization (FISH) revealed that the pearls and the connected threads were formed by two main morphologically as well as phylogenetically different microorganisms (Rudolph et al., 2001; Moissl et al., 2002; Moissl et al., 2003; Rudolph et al., 2004b): Filamentous, sulfur-oxidizing bacteria (*Thiothrix* sp. SipK4, Gammaproteobacteria; Rudolph et al., 2001; Moissl et al., 2002) connect whitish pearls, which mainly consist of anaerobic coccoid archaea (referred to as "SM1 Euryarchaeon", based on the location Sippenauer Moor).

[2] Kinichiro Sakaguchi

It was already hypothesised at this point that this unusual partnership relies on an atypical form of cohabitation based on a syntrophic or even symbiotic relationship (Moissl et al., 2002). The partnership was proposed to rely on a sulfur cycling process, where the anaerobic SM1 Euryarchaeon potentially reduces sulphate to H_2S, which is metabolized by their surrounding sulfur-oxidizing bacterial partners and *vice versa*. Cultivation experiments on the archaeal enrichment failed and the authors speculated on a partner-dependent lifestyle (Rudolph et al., 2001; Moissl et al., 2002).

The unusual lifestyle in form of the “strings-of-pearls community” (SOPC) was not restricted to the Sippenauer Moor. Another sulphidic spring in Bavaria, the Islinger Mühlbach, shows the presence of similar structures. The spring was artificially formed by a drill hole and is characterised by a similar water chemistry and temperature compared to the Sippenauer Moor (~10°C throughout the year; Probst et al., 2013b). Near the outflow, coccoid archaeal cells accumulate to macroscopically visible pearls, surrounded and connected by filamentous bacteria, which, however, belonged to a different bacterial class (IMB1; Epsilonproteobacterium; Rudolph et al., 2004b; later classified as *Sulfuricurvum* sp.; Kodama and Watanabe, 2004). The phylogenetic different cohabitation was explained by the lower oxygen concentration in the Islinger Mühlbach compared to the Sippenauer Moor, which is enhancing the growth of IMB1 and outcompetes the growth of *Thiothrix* sp. (Probst et al., 2013b). The macroscopic and microscopic view on the SOPC is shown in Figure II.2-1.

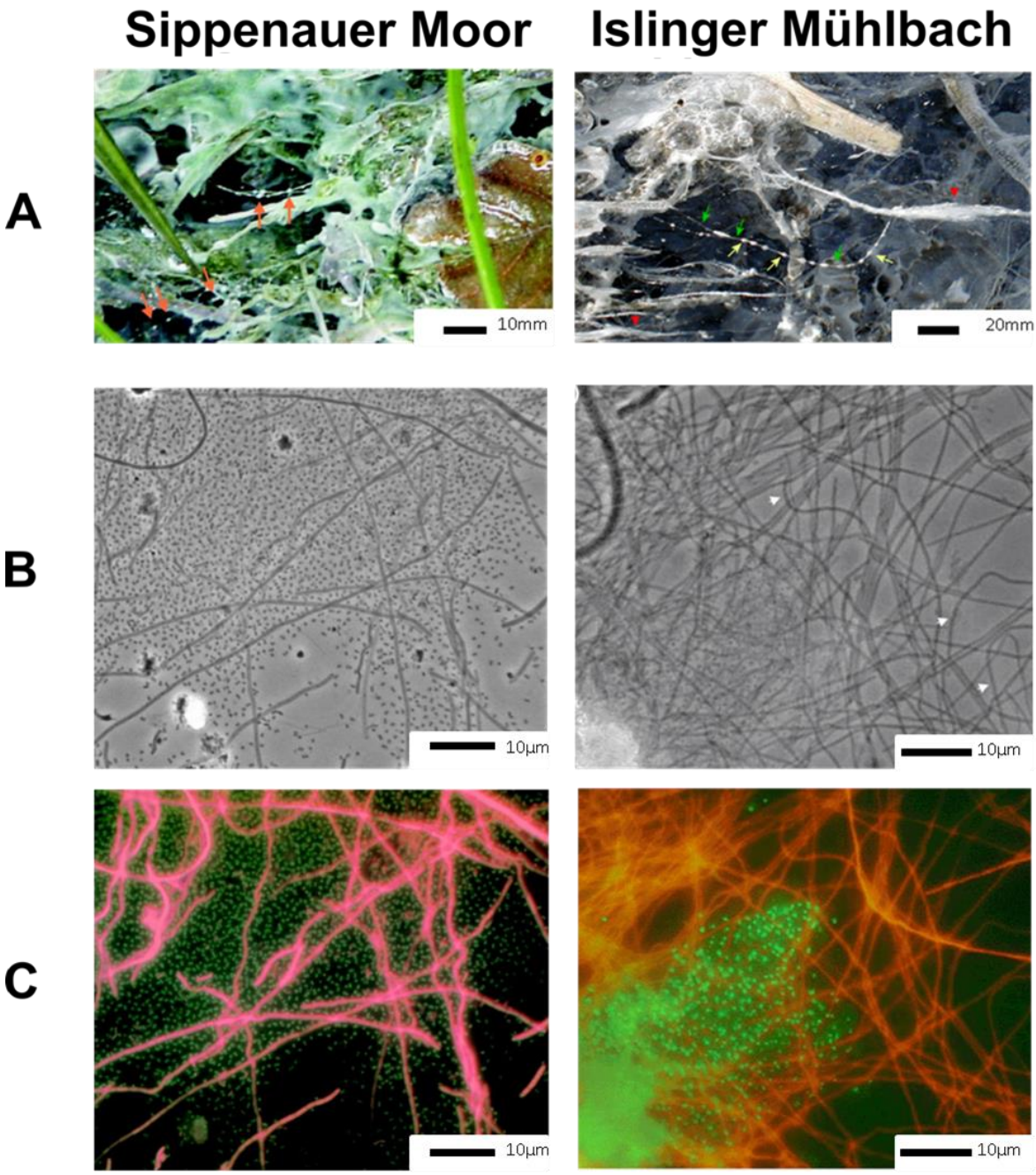

Figure II.2-1 String-of-pearl community differently imaged. The structure can already be observed by naked eye (A). It is formed by coccoid and filamentous microorganisms (B, phase contrast microscopy), which were identified as Archaea and Bacteria, respectively (Fluorescence *in situ* hybridisation imaging (C)). Images were taken from Rudolph et al. 2001 and 2004**.**

Further studies focused on the microcolony-forming archaea encapsulated within the pearls. The cocci with a diameter of 0.5 µm were analysed by clone libraries and taxonomic analyses classified the archaeal strains from both springs. They belong to a single phylotype and branch separately

within the Euryarchaeota (Rudolph et al., 2001; Moissl et al., 2002; Moissl et al., 2003; Rudolph et al., 2004b). Signatures of the SM1 Euryarchaeon were also detected in environmentally remote locations (e.g. in Turkey) and proved them to be widely distributed (Rudolph et al., 2004b, Bird et al., 2016; Figure II.2-2).

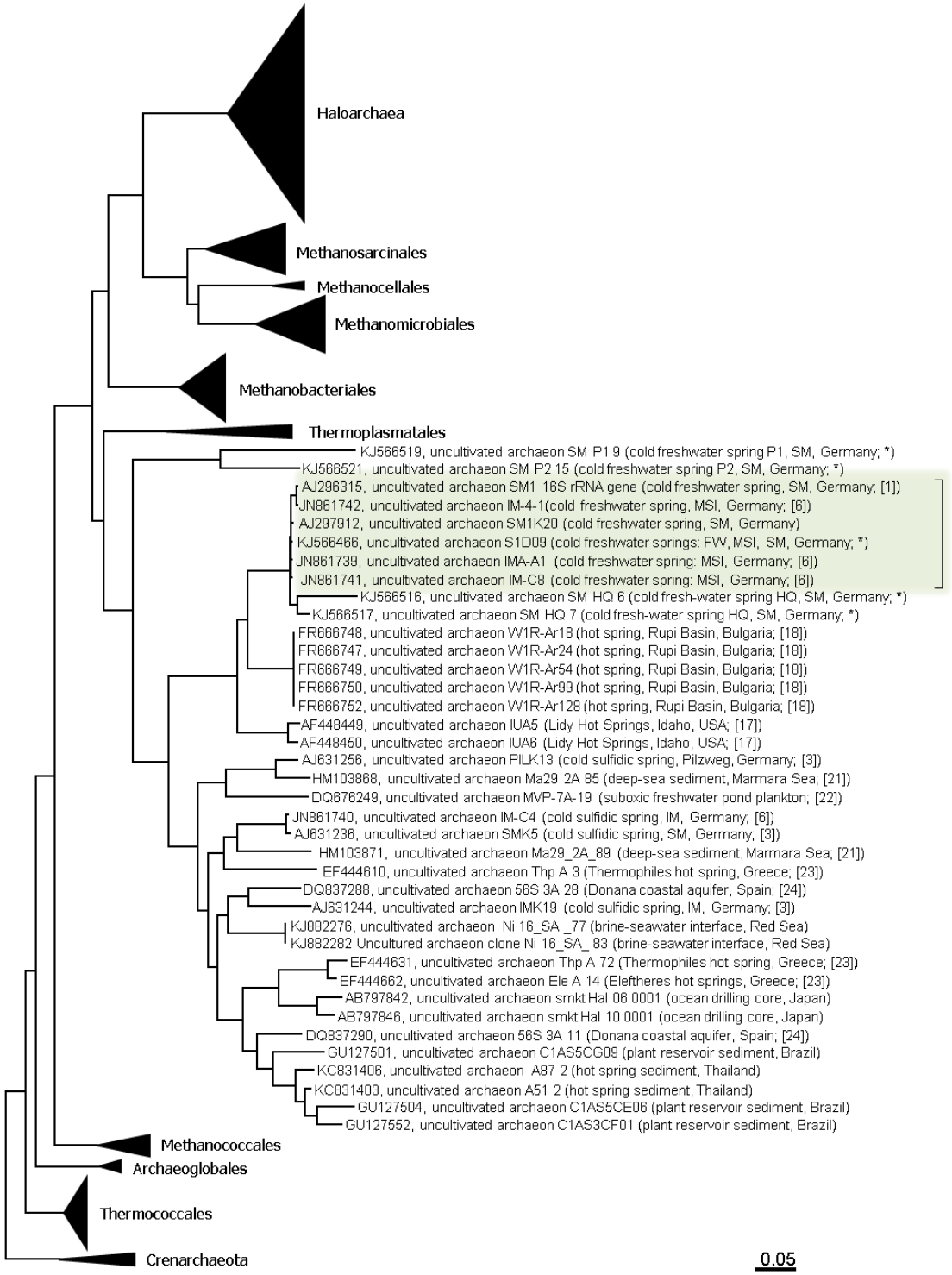

Figure II.2-2 The SM1 phylotype within the Euryarchaeota tree (highlighted in green). Closely related signatures were detected in many Bavarian freshwater settings. More information can be found in Probst and Moissl-Eichinger, 2015.

Interestingly, their lifestyle was not restricted to the SOPC morphology, but could also be detected in the form of a mono-archaeal, partner-independent biofilm appearance. In both springs, an *in situ* trapping system overcame the unfeasible cultivation in the laboratory (Henneberger et al., 2006).

Polyethylene nets were exposed in anoxic areas of the springs and after minutes, slimy, whitish flocks were randomly attaching to the trapping system. Phylogeny and FISH studies revealed the flocks to be dominated by the same archaeal species, with only a minor proportion (5%) of their respective bacterial partners and thus forming an almost pure archaeal biofilm (Henneberger et al., 2006; Probst et al., 2013b; Figure II.2-3). The composition of the core microbiome of the biofilm was assessed using 16S rRNA gene microarray (PhyloChip G3) analysis, and was further confirmed by *dsrB* gene targeting FISH. Quantitative PCR assays revealed representatives of the Deltaproteobacteria (sulphate-reducing group) as significantly enriched (Probst et al., 2013b). It was speculated that these bacteria provided a valuable function within the biofilm, as they were consistently abundant over a long period of time (Henneberger et al., 2006; Probst et al., 2013b). In addition, the sensitive PhyloChip technique detected additional archaeal species embedded in the biofilm, however, in a small proportion (Probst et al., 2013b). This finding was confirmed by FISH and clone library experiments.

The SM1 euryarchaeal cells, as previously seen in SOPCs, were surrounded by a thick matrix of extracellular polymeric substance (EPS; Costerton et al., 1995). The EPS layer consisted of polysaccharides and proteins and was most likely also produced by the SM1 euryarchaeal cells (Henneberger et al., 2006). The EPS of archaeal biofilms is, similar to bacterial EPS matrices, the key mediator for structure and function of biofilms (Flemming and Wingender, 2010). Only a few archaea-containing biofilms are known (Edwards et al., 2000; Battin et al., 2001; Couradeau et al., 2011) and most information is based on static incubation systems conducted under laboratory conditions (LaPaglia and Hartzell, 1997; Schopf et al., 2008; Baker-Austin et al., 2010; Fröls et al., 2012; Bang et al., 2014; Di Meglio et al., 2014). Naturally formed archaeal mono-species biofilms, as formed by the SM1 Euryarchaeon, are rare. The best knowledge available is on the ARMAN consortium which dominates one environment (Orphan et al., 2001). Hence, archaeal mono-species biofilms pose a special feature within natural archaeal lifestyles.

The biofilm formation of the SM1 Euryarchaeon was observed in deeper, anoxic subsurfaces of two biotopes (Islinger Mühlbach and Sippenauer Moor). Interestingly, they exhibit macroscopically different appearances, presumably caused by different strains (Probst et al., 2013b).

Strikingly, as observed in all SM1 euryarchaeal lifestyles (SOPC and biofilms), the cells were arranged in a regular, three-dimensional pattern and each cell kept a certain distance from the other (Figure II.2-3).

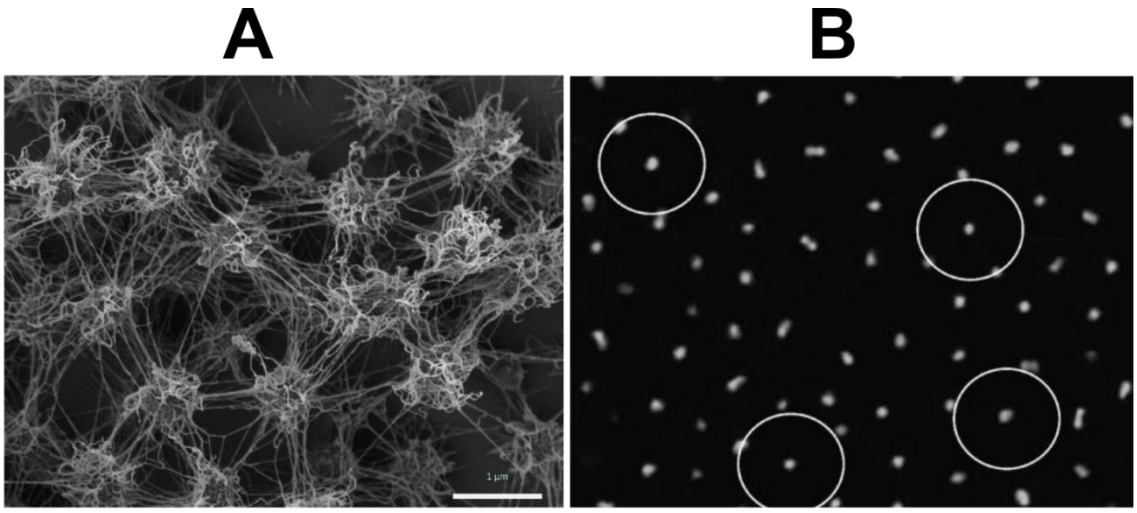

Figure II.2-3 Scanning electron micrograph depicting the dense, well-ordered biofilm (A). Each cell is kept in a certain distance by spacers (Bar: 1 µm; Probst and Moissl-Eichinger, 2015). This phenomenon is also observed in confocal scanning micrographs (B, each circle indicates for a diameter of 8 µm; Henneberger et al., 2006).

The spacers were observed as proteinaceous cell surface appendages exhibiting an extraordinary appearance and architecture never seen before.

2.2. Archaeal ninjas

Each SM1 euryarchaeal cell analysed from the Sippenauer Moor and Islinger Mühlbach was observed to be covered by more than a hundred surface appendages, which exhibit an extraordinary architecture (Moissl et al., 2005). The barbed-wire filaments had a length of ~1-3 µm and carried so-called prickles (length: 30 nm) sticking out in regular intervals (46 nm) with a tripartite, grappling hook (diameter: 60 nm) at its distal end. The hook region was responsible for the name assignment of the unique filaments: hami (sing. hamus; Latin for anchor, hook). Tomographic reconstructions suggested a helical, twisted structure, which was confirmed by Fourier filtering. Further observation pictured a detailed view on the composition of one filament: the helical filament was thicker than the prickle region in

diameter (7-8 nm vs. 4.6 nm, respectively). Each filament is divided into three proto-filaments. The architecture of the hami is shown in Figure II.2-4.

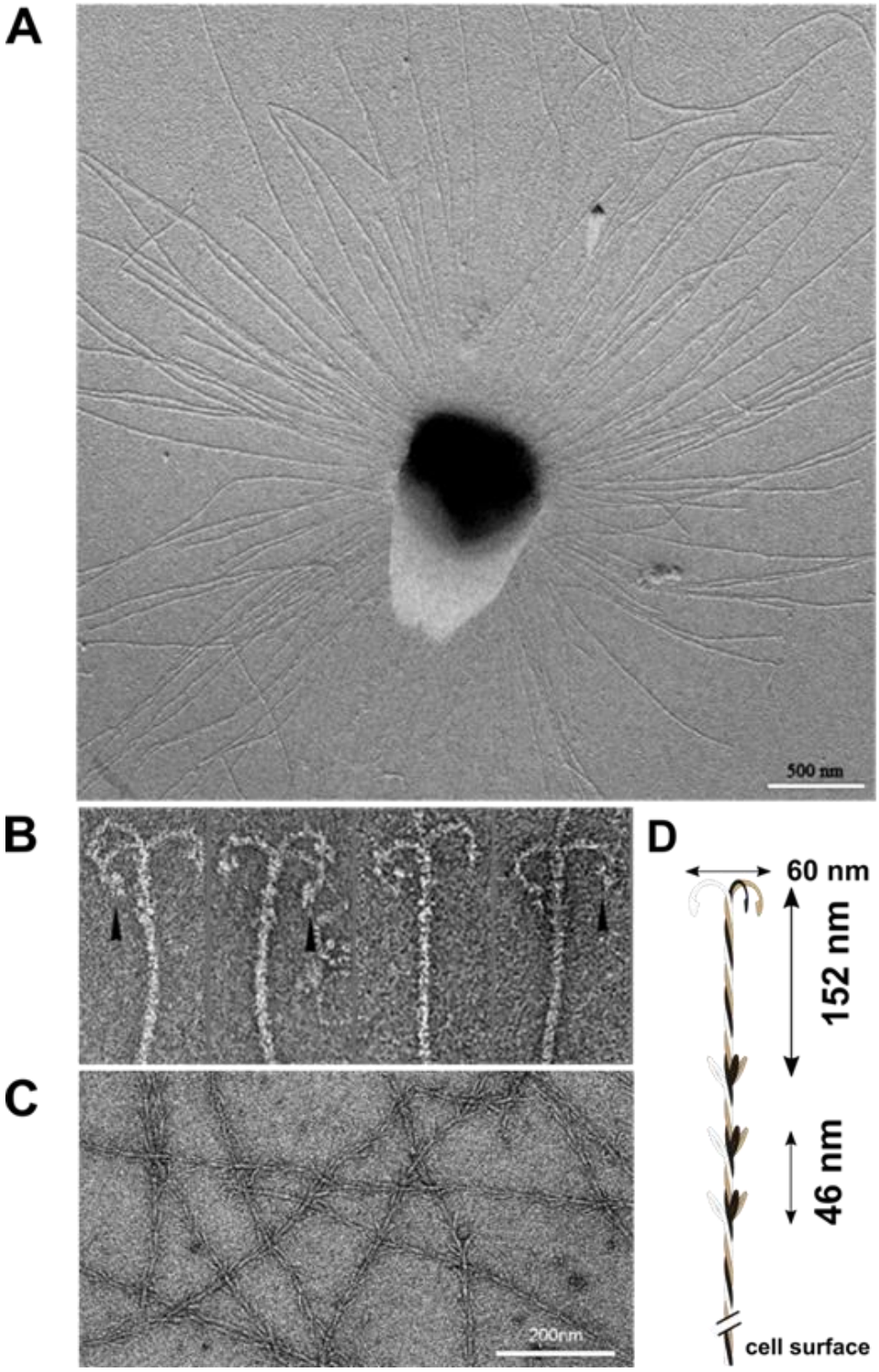

Figure II.2-4 Each SM1 Euryarchaeal cell is surrounded by numerous cell surface appendages, referred to as "hami" (Moissl et al., 2005; A). The hami carry a tripartite, grappling hook at the distal end (Moissl et al., 2005; B), the filaments are reminiscent of barbed-wires with prickles sticking out in regular intervals (Moissl et al., 2005; Probst and Moissl-Eichinger, 2015; C). Based on microscopic investigations, a hamus model was created (Probst and Moissl-Eichinger, 2015; D).

The hamus structure was described to be unique in nature; however, marginal structural similarities were drawn with actin filaments (Milligan et al., 1990; Holmes et al., 2003; Moissl et al., 2005). The hami filaments exhibit a well-defined top-to-bottom organisation, which speculates a complex assembly involving a vast variety of proteins similar to eukaryotic actin filaments (Pollard, 2007).

Interestingly, denaturing SDS-PAGE and immunological experiments revealed that the proto-filaments were composed of one major protein subunit with a molecular weight of 120 kDa without sequence homologies to any deposited proteins sequences in databases (Moissl et al., 2005).

These experiments revealed a rather simple assembly design, which applies to findings on archaeal flagella filaments. Archaeal flagella systems are composed of less than 13 different proteins (Thomas et al., 2001). The archaeal flagella filament is composed of one to several proteins with no homologues to eukaryotic or bacterial flagellar proteins, suggesting a convergent evolution (Cohen-Krausz and Trachtenberg, 2002). In fact, the archaeal flagella synthesis is similar to synthesis of bacterial type IV pili (Albers et al., 2006; Trachtenberg and Cohen-Krausz, 2006; Jarrell and McBride, 2008; Ng et al., 2008).

Up to this day, nothing was known about the hami assembly process and anchoring system, which was speculated to be complex (Moissl et al., 2005), but requires further investigation.

The stability of hami filaments was remarkable in both a wide pH and temperature range: The filaments kept their three dimensional characteristic structure up to 70°C and in a pH range of 0.5 to 11.5 (Moissl et al., 2005). This finding was surprising, since the host thrives in a pH neutral and cold environment.

The stable filaments most likely function as a spacer and mediate the highly ordered cell-pattern within the biofilm. The hooks serve as attachments with strong adhesive forces between the cells and might even trap various organic and inorganic materials in their natural biotope (Moissl et al., 2005; Moissl-Eichinger et al., 2012a).

In summary, the extraordinary hami filaments show unusual traits. They are (**i**) complex in structure maintaining a barbed-wire region and a hook region, (**ii**) composed of one major, high molecular weight protein species (**iii**) highly stable within a wide pH and temperature range, and (**iv**) acting as spacer between the cells in the biofilm. The mentioned properties of

hami filaments suggest that they are excellent tools for nanobiotechnology (Moissl et al., 2005; Moissl-Eichinger et al., 2012a). As the natural host is resisting any cultivation efforts so far, a heterologous hami host expression system was considered. However, the hamus encoding gene was merely partly elucidated and its full length needed to be uncovered (Moissl, 2005).

The SM1 Euryarchaeon possesses unusual cell surface appendages. Its ultrastructure might even exhibit more extraordinary traits, which remained hidden so far and awaits further examination.

3. The era of astrobiology

"Everything is everywhere, but the environment selects" [3]

Astrobiology is the study of the origin, evolution, and distribution of life in the entire Universe. It addresses the question whether life exists/ existed beyond Earth and how it can be detected. In recent years, tremendous technical progress enabled mankind to explore and study extra-terrestrial settings such as the moon (in manned explorations) and Mars (in unmanned explorations using numerous orbiters and rovers). Mars is supposed to be the most promising planet for extant and extinct life in our Solar System, and thus attracts the interest for future missions (next to icy moons). Mars rovers like Mars Science Laboratory (better known as "Curiosity rover") and orbiters detected frequent outbursts of methane gas on the Martian surface (Formisano et al., 2004; Geminale et al., 2008; Fonti and Marzo, 2010; Geminale et al., 2011; Webster et al., 2015). These outbreaks arise spontaneously and are speculated to emerge from subsurface aquifers, where methane has previously been locked, or where basalt is serpentinised. However, the methane might also have a biogenic source. On Earth, the atmospheric methane is primarily produced by methanogenic microorganisms and for this reason there is hope to detect life on Mars (Atreya et al., 2007).

Several rovers and orbiters deliver valuable information on the Mars, but future, long-term missions to Mars aspire to search for life by human beings. The travel to Mars itself will take place in confined space shuttles and lasts for several months. The mission requires extensive preparation and a microbiological-safe work place has to be provided. This scenario is already trained using the International Space station (ISS).

[3] Lourens Baas Beckings

In 1998, the first component of the ISS launched into the low Earth orbit and set another milestone for human space flight. At the present time, it consists of several modules organized by five space agencies, namely National Aeronautics and Space Administration (NASA; USA), European Space Agency (ESA; Europe), Russian Federal Space Agency (Roscosmos; Russia), Canadian Space Agency (CSA; Canada), and Japanese Aerospace Exploration Agency (JAXA; Japan). The spacecraft provides the platform for several scientific experiments on astrobiology, astronomy, physical science, material science, space weather meteorology and human research including space life and medicine.

The ISS represents a completely sealed, confined (meaning restricted to a defined number of parameters) man-made environment. It is characterised by extreme parameters such as higher radiation levels compared with Earth settings, low nutrient levels due to reduced exchange with the environment and microgravity. In addition, the indoor-environment is characterised by a stable, constant temperature (~22°C) and humidity (~60%; Coil et al., 2016). Since 2000, the ISS has been inhabited by humans – being accompanied by millions of microorganisms (the human body carries up to $4x10^{13}$ microbial cells; Sender et al., 2016). The monitoring and control of the ISS microbiome is of uttermost interest as some species may impose a life-threatening hazard to the inhabiting crew. It has been shown that the increased microgravity and (presumably) starvation conditions about 400 km above ground as experienced onboard the ISS lead to an enhanced pathogenicity of *Salmonella typhimurium (*Wilson et al., 2007; Zea et al., 2016). Not only may the crew be affected, but the spacecraft is also endangered. Technophilic microorganisms (in particular fungi) were already responsible for major problems in the Russian space station (Mir) corroding alloys and polymers (Novikova et al., 2001; Novikova, 2004; Alekhova et al., 2005). Consequently, the cleaning procedure onboard the ISS is very strict including periodical disinfection procedures and air/subsurface controls, which check for microbial and fungal contaminations. The vast investigation on the bacterial community onboard the ISS revealed that they were human-associated (Venkateswaran et al., 2014).

Respective ground controls on Earth, cleanrooms, where the spacecraft hardware is assembled, are reflecting the same image. Cleanrooms are highly controlled, and defined environments and are used to build hardware designated to leave Earth. In the course of the planetary protection regulations, the microbial contamination of hardware has to be

controlled and kept to a minimum (Kminek and Rummel, 2015). Assays include the assessment of viable cells (Vaishampayan et al., 2013), spores and the total microbial bioburden by combining standard cultivation techniques (Stieglmeier et al., 2009) with molecular methods (e.g. qPCR, cloning and Next Generation Sequencing; La Duc et al., 2003; La Duc et al., 2004; Bashir et al., 2016). The assessment of viable cells is conducted by the use of the chemical compound propidium monoazide (PMA). PMA masks free DNA, which is most likely derived from dead cells in subsequent PCR reactions (Nocker et al., 2007), while DNA originating from viable cells is protected by the intact cell wall. The main proportion of detected microorganisms was uncovered to be human-associated (Moissl et al., 2007; Bashir et al., 2016), which is in accordance with the microbial population detected on the ISS (Checinska et al., 2015). However, these microorganisms are considered to be very hardy and adapt very well to the harsh conditions prevalent in cleanrooms and the ISS.

Possible adaptation of microorganisms towards space stresses are addressed within an flight project originally named “ARBEX” (Archaeal and Bacterial Extremophiles on board the ISS; now “EXTREMOPHILES”; Moissl-Eichinger et al., 2016a) and focuses on the investigation of adaptation processes of moderate and extremotolerant microorganisms onboard the ISS.

Studies on the ISS provide important knowledge to future crewed space missions to Mars. So far, no human being has reached Mars, and it still requires rovers and orbiters equipped with technologies to search for Martian life signatures. The most recent project was the ExoMars mission a program of ESA and Roscosmos (Montroni et al., 2016). The Trace Gas Orbiter (TGO) already reached the Martian orbit and collects atmospheric data. The landing demonstrator Schiaparelli reached the Martian surface, however, unfortunately could not demonstrate a successful landing and crashed on the Martian surface at 300 km/h (Clery, 2016). In 2020, the next ExoMars mission will be equipped with hardware enabling the search for signatures of life on the Martian surface and in its subsurface by drilling experiments. In the course of planning this project, two main questions arise:

- **Do we expect Earth-derived life (contamination), which is brought along with the hardware?**

Forward contamination is a main issue in planetary protection. Forward contamination means the transfer of microbes to other planets (e.g. the Mars). The microbial hitchhikers would interfere with life-detection procedures and cause false positive signals in life-detection measurements. For this reason, possible contamination, in particular on life-detection systems must be kept to a minimum.

- **How does possible Martian life look like?**

No one knows what lifeforms we are facing on Mars. Consequently, we have to sustain and draw our knowledge on lifeforms we are also facing in Mars-like settings on Earth. Based on this, it is possible to develop life-detection systems, which can search purposefully for Martian life.

The first question can be addressed by assessing the microbial communities, which are in contact with any hardware designated to be sent to Mars. In addition, an uttermost care of decontamination is carried out to minimize this undesired scenario.

The second question is more challenging. All speculations are based on the assumption that Mars provides or rather has provided prerequisites for the origin and persisting of life.

The appearance of Mars underwent a dramatic change throughout the last billions of years and it has to be emphasised that the conditions of Early Mars and Present Mars are highly dissimilar and have to be regarded individually.

Today, Mars is a cold, desert world. The globally averaged atmospheric pressure at the surface was measured to be about 7 mBar and is consequently extreme thin. Hence, the temperature is only slightly above the freezing point of water near the equator during the warmest part of the day and does not allow water to stay permanently in its liquid form (Liu et al., 2003). In fact, a high amount of water is supposed to be present on Mars, however, due to the inappropriate conditions, water remains in ice-rich permafrost and is not accessible (Séjourné et al., 2012).

Yet, the climate on Mars may not always have been as extreme as it is today and might once have supported the origin of life. In the Noachian period (~4.1–3.7 Ga ago; Bibring et al., 2006; Carr and Head, 2010) the atmosphere of Mars was expected to be significantly denser and consisted primarily of carbon dioxide, admixed with methane, sulphur gases, and only traces of oxygen. The dense atmosphere might have resulted in increased Greenhouse warming leading to stable, warm temperatures above the freezing point (Jakosky and Phillips, 2001). This may have permitted the common and stable occurrence of liquid water at the surface such as large oceans in the Northern hemisphere and lakes within crater

regions (Ruiz et al., 2004; Perron et al., 2007; Hiesinger and Head Iii, 2002; Carr and Head, 2010). Liquid water is an essential prerequisite for the emerging of any lifeforms and habitability (Grotzinger et al., 2014; Brack, 2002). Other criteria for habitable conditions are: Access to energy sources (Nisbet et al., 2007; Southam et al., 2015), organic carbon and biologically essential elements (Cockell et al., 2016a). Energy sources are commonly delivered in the form of thermodynamic disequilibria between chemical species in close proximity (Westall et al., 2011). Carbon on Mars is likely to originate from meteorites and comets (Duprat et al., 2010), which impacted the planet in a regular manner and in hydrothermal systems such as Martian volcanic environments (Martin and Russell, 2007; Hofmann and Bolhar, 2007). Volcanic rocks simultaneously provide the source for essential elements (H,N,O,P,S) and trace minerals (Nisbet et al., 2007). This scenario has already been observed for subaqueous volcanic sediments of similar shaped Early Earth (Westall et al., 2011). The sediments contained essential elements and energy sources and represented appropriate biotopes for heterotrophic microorganisms (Furnes et al., 2004; Nisbet et al., 2007; Westall, 2009; Southam et al., 2015). Thus, the Early Mars fulfilled all criteria necessary for a habitable planet. The geochemical settings on Early Mars are described to range from (**i**) acidic conditions (for instance indicated by the presence of jarosite in Burns formation at Meridiani Planum; Squyres and Knoll, 2005; Bibring et al., 2006; Knoll and Grotzinger, 2006), to (**ii**) pH neutral aquifers with low salinity and variable redox stages of iron, phosphor and sulphur (for instance the Fluvio-Lacustrine Environment at Yellowknife Bay, Gale Crater; Bristow and Milliken, 2011; McLennan et al., 2014; Grotzinger et al., 2014).

These data show that Early Mars appears to have hosted localized environments that would have been compatible with the requirements of primitive terrestrial life (Grotzinger et al., 2014). However, life may not have been persisted throughout the change into a hygroscopic planet. In this scenario, we expect to find preserved fossilised microorganisms on present Mars (Friedmann, 1986; Hofmann et al., 1997). Another scenario represents life, which has adapted to a very low water activity and three different possibilities are supposed:

a) It is speculated that the evolution of life might have been formed in hygroscopic surfaces by obtaining water solely from the atmosphere (Davila and Schulze-Makuch, 2016). The speculations are based on a similar setting on Present Earth: The Atacama Desert, which is considered to be the driest region on Earth. Within the desert an

amazing diversity of microorganisms adapted to desiccation, high radiation and high salinity reside on the surface (e.g. the Terrabacteria, which include Cyanobacteria, Chloroflexi and Deinoccus-Thermus members). Considering the capacity of terrestrial microorganisms to adapt to arid conditions, Early Martian life could have adapted and evolved throughout the transformation of Mars.

b) Recently delivered data describe occasionally occurring last remnants of narrow, up to ~5 meter in diameter, liquid water streamlets (recurring slope lineae, RSL; Chevrier and Rivera-Valentin, 2012; Ojha et al., 2014; Ojha et al., 2015). The RSLs are restricted to small scale flow features and appear mostly during the Martian spring and summer, which suggests a melting result of frozen liquid solution. The large amounts of sedimentary salts lead to high salinity conditions within the RSL and thus, they are reflected as brines (Wang et al., 2006; Chevrier and Melchiorri, 2016). Similar brines on Earth host halophilic microorganisms, which demonstrates their habitability (Leuko et al., 2010).
c) Permafrost settings on Mars might enclose life in a metabolically very slow state as in similar settings, which have been investigated on Earth (Soina and Vorobyova, 1996).

Life might still exist on Mars in a cryptobiotic state and bloom whenever water becomes available. Eventually, settings on Early and Present Mars are considered as potentially habitable and thus, there is a possibility of extant/extinct life. The search for Martian life is supported by model organisms, which thrive in terrestrial Mars-analogue environments. Terrestrial extreme, harsh environments host model organisms, which are widely adapted to withstand the challenging circumstances. A range of Mars-like environments were studied from the aforementioned Atacama Desert to permafrost settings and caves (Fernández-Remolar et al., 2008). However, in the body of literature, one main Martian feature is widely ignored: The atmospheric oxygen abundance is ~10^{-5} times less than the present atmospheric levels on Earth (Franchi et al., 1999; Mahaffy et al., 2013). Earth is not exposed to comparable harsh physical and chemical conditions (i.e. high radiation dose, high amount of perchlorates) as experienced on Mars. Still, terrestrial model conditions must be at least very close to Martian conditions. Anaerobic analogue settings must include at least a few of physical extremes such as high acidity, high salinity, low temperature, and low nutrient availability, however, not necessarily all at once. Studies on anoxic, extreme Mars-like

settings are rare, and there is no systematic attempt to cultivate and study microorganisms from a diverse set of anaerobic Mars-analogue environments. In particular, the examination of cultivated microorganisms on physiological capabilities and reactions to simulated Mars-similar stresses (e.g. high radiation and desiccation) delivers valuable information on their resistances. Moreover, the microbial diversity has to be assessed with molecular techniques such as NGS to obtain a broad picture of the overall community and eventually identify novel model organisms for astrobiology. This will advance the knowledge of possible life in Mars-analogue conditions.

The MASE (Mars Analogues for Space Exploration; http://mase-eu.org/[4]) project is a collaborative research project with more than 20 members from 7 different European countries. It was kicked-off in the beginning of 2014 with the major aim to fill in knowledge gaps upon anaerobic, Mars similar microorganisms. The work plan aimed to cover the following main points:

a) Sampling of anoxic, Mars-like settings and development of a standard operation protocol for anoxic sampling
b) Anaerobic cultivation of microorganisms using a designated standard medium and providing the resulting anaerobic culture collection to the scientific community
c) Assessing and describing the overall microbial community using molecular methods
d) Study the response of cultivated isolates to combined environmental stressors also experienced on Mars
e) Artificial fossilisation of selected microorganisms (stressed and unstressed counterparts) and assess the potential of their preservation
f) Development of biosignature detection techniques based on fossilised and unfossilised microorganisms

The tasks of the MASE project are divided and every member is allocated certain responsibilities. This dissertation covers the microbiological part (points a-c, see above), however, everyone in the team contributed to all work packages. The outcome of this

[4] also social outreach projects such as facebook and twitter are available (https://twitter.com/MarsAnalogues, https://www.facebook.com/MASE-371726352989521)

dissertation will help to provide knowledge on extreme, Mars-like life – an essential prerequisite for detecting extraterrestrial life.

4. Scope and publication guide

The scope of this thesis was to investigate extreme environment, namely the International Space Station (in the frame of the ESA flight EXTREMOPHILES project) and Mars-like environments within the frame of the MASE project upon their microbial community using cultivation-independent and cultivation-based techniques. One extreme setting, also involved in the MASE project, namely the sulphidic springs, hosts an extraordinary archaeon with exceptional traits (e.g. unusual lifestyles, and ultrastructure) – the SM1 Euryarchaeon. The uncultivated archaeal cells were thoroughly investigated upon ultrastructure. The following paragraphs provide the guidance for the respective publications, where major findings are presented and discussed in detail.

Elucidating the grappling ultrastructure of the SM1 Euryarchaeon

The SM1 Euryarchaeon has attracted the interest of researchers for more than a decade. Although thoroughly studied, not all knowledge gaps on the ultrastructure are filled. The publication "Grappling Archaea: ultrastructural analyses of an uncultivated, cold-loving archaeon and its biofilm" reports on the microbial interaction within the biofilm and ultrastructure using electronic microscopy. The results confirmed previous findings (Probst et al., 2014a) which evidenced a divergence in the biofilms of the two biotopes. Moreover, it was shown that the SM1 Euryarchaeon exhibits a double membrane, a rarity in the archaeal domain. This observation was confirmed in another study carried out in the frame of this thesis (Probst et al., 2014b).

Notwithstanding this archaeon being uncultivable in the laboratory, a thorough study based on the ultrastructure and genetic features of the "hami" structures was feasible and the results are described in detail in "S-layers at a second glance? Altiarchaeal grappling hooks (hami) resemble archaeal S-layer proteins in structure and sequence". The full length of the hami-encoding gene was obtained and the N-terminus region revealed a high similarity to S-layer proteins. The results expand the knowledge on archaeal cell surface appendages and their evolution.

The outlasting microbiome of the International Space Station

Another extreme environment is represented onboard the International Space Stations. The microbial investigation is important as well for future space missions. Long-term stored dust samples deriving from the Russian modules were investigated upon their microbial diversity using cultivation combined with NGS. For the first time, signatures of Archaea were detected; however their viability remains be confirmed. Cultivation revealed an outlasting microbiome equipped with various, unexpected resistances. A comparison with the microbial community of the US-module manifest a different composition, however, both harbour a substantial part of human-associated signatures. The results are presented in the publication “Resilient microorganisms in dust samples of the International Space Station – Survival of the adaptation specialists.”

The MASE project: Expanding the knowledge on extraterrestrial life

The body of literature contains solid knowledge on terrestrial Mars-like life, however lacks substantial information on the anaerobic proportion of microorganisms thereof. In the frame of the MASE project, we assessed the microbial diversity of (Present and/or Early) Mars-similar environments. Again, cultivation and cultivation-independent methods were combined and delivered impacts for extraterrestrial life. The findings are presented in “Mars exploration begins on Earth: Systematic comparison of the anaerobic, intact and cultivable microbiome of extreme, anoxic Mars-analogue, environments”. Studies, which were carried out along the MASE project contained stress tests upon selected MASE organisms, fossilisation thereof, physiochemical description of sampling sites, and a detailed description of the MASE sampling procedure (Beblo-Vranesevic et al., 2016; Cockell et al., 2016b; Gaboyer et al., 2016). Taken together, they merge to a detailed picture on potential Mars-similar life.

III. Manuscripts and publications

Overview

This cumulative dissertation contains four articles. Three manuscripts were already published, and one has been submitted to Nature communications. In any case, the PhD candidate, Alexandra K. Perras, has authored every manuscript printed in the following as a first author.

Concerning the data structure on the supporting CD the reader is referred to Chapter VII.

The PhD student's contributions to the manuscripts are as follows:

III-1. Perras AK, Wanner G et al., (2014): Alexandra performed sample processing, contributed to transmission electron microscope imaging and wrote the paper. The manuscript and supporting information can be found on the supporting CD (./Selected publications/*03*).

III-2. Perras AK et al., (2015): Alexandra performed experiments, analysed data and wrote the paper. The manuscript and supporting information can be found on the supporting CD (./Selected publications/*04*).

III-3. Mora M, Perras AK et al., (2016): Alexandra performed bioinformatics, biostatistics and wrote the paper. The manuscript and supporting information can be found on the supporting CD (./Selected publications/*06*).

III-4. Perras et al., submitted (2016): Alexandra planned the study, participated during sampling trips, performed experiments, bioinformatics and wrote the manuscript. The manuscript and supporting information can be found on the supporting CD (./Selected publications/*08*).

A full list of publications, authored by the PhD candidate, is listed on pages 8 and 9. The articles (published and submitted) are individually provided on the supplementary CD.

III.1. Grappling archaea: ultrastructural analyses of an uncultivated, cold-loving archaeon, and its biofilm

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Abstract

Similarly to Bacteria, Archaea are microorganisms that interact with their surrounding environment in a versatile manner. To date, interactions based on cellular structure and surface appendages have mainly been documented using model systems of cultivable archaea under laboratory conditions. Here, we report on the microbial interactions and ultrastructural features of the uncultivated SM1 Euryarchaeon, which is highly dominant in its biotope. Therefore, biofilm samples taken from the Sippenauer Moor, Germany, were investigated via transmission electron microscopy (TEM; negative staining, thin-sectioning) and scanning electron microscopy (SEM) in order to elucidate the fine structures of the microbial cells and the biofilm itself. The biofilm consisted of small archaeal cocci (0.6 µm diameter), arranged in a regular pattern (1.0–2.0 µm distance from cell to cell), whereas each archaeon was connected to 6 other archaea on average. Extracellular polymeric substances (EPS) were limited to the close vicinity of the archaeal cells, and specific cell surface appendages (hami; Moissl et al., 2005) protruded beyond the EPS matrix enabling microbial interaction by cell-cell contacts among the archaea and between archaea and bacteria. All analysed hami revealed their previously described architecture of nano-grappling hooks and barb-wire basal structures. Considering the archaeal cell walls, the SM1 Euryarchaea exhibited a double-membrane, which has rarely been reported for members of this phylogenetic domain. Based on these findings, the current generalized picture on archaeal cell walls needs to be revisited, as archaeal cell structures are more complex and sophisticated than previously assumed, particularly when looking into the uncultivated majority.

Keywords: Archaea, Biofilm, Ultrastructure, Hami, EPS, SEM, TEM, Microbial interaction

Introduction

Understanding the microbial “dark matter” has become one of the driving desires of the scientific community (Rinke et al., 2013). In particular, deep-branching, uncultivated archaea have attracted the interest, being largely unexplored but widespread and likely major drivers of the nutrient cycles in various ecosystems (Cavicchioli et al., 2007). Systems that allow unbiased and direct analyses of uncultivated microorganisms on microscopic and macroscopic levels due to one organism's predominance are extremely rare. However, such systems are of utmost importance to understand the functioning of microorganisms in the environment, their natural cellular composition, their actual metabolic activity and their interactions with the abiotic and biotic environment (Morris et al., 2013).

The majority of microorganisms remain uncultivable using standard methods (Amann et al., 1995; Joseph et al., 2003). The unsatisfying success in this regard might be rooted in the interwoven interactivity of microorganisms in their natural biotope, such as natural ecosystems, or macrobes, such as plants or the human body. The human body itself is colonized by 10–100 times more microbial cells than own cells (Schleifer, 2004). Analyzing the (human) microbiome has become a major scientific focus, benefitting from state-of-the-art, cultivation-independent methods which include next generation sequencing of 16S rRNA genes and –OMICS technologies (Zhang et al., 2010). Altogether, these methods allow first glances at the diversity and function of an entire microbial community, which interacts

closely with its host, forming a "superorganism": the holobiont (Margulis and Margulis, 1993; Rohwer et al., 2002). It is assumed, that the cooperation of host and microbes represents a unit of selection in evolution and changes in composition and function have severe impact on further development or even next host generations (Zilber-Rosenberg and Rosenberg, 2008). As a consequence, evolution appears to be a coordinated process of entire (microbial) communities, which need to be scientifically addressed as a whole.

The effects of microbial interactions for the different partners can vary. In symbiotic relationships all partners benefit, whereas commensal interaction is beneficial for one partner and not harmful for the other. Parasites, however, strongly affect the fitness of one partner (Moissl-Eichinger and Huber, 2011). A well-documented model system of a bacterial symbiotic interaction is "*Chlorochromatium aggregatum*", a clearly structured consortium of immobile green sulfur bacteria epibionts and a motile beta-proteobacterium (Müller and Overmann, 2011). This association provides mobility to the epibionts and, in exchange, amino acids and 2-oxoglutarate to the inner partner. Detailed ultrastructural analyses revealed that hair-like filaments protrude from the epibionts and directly interconnect with the central bacterium. The latter connects with the epibionts via periplasmic tubes, which attach to the epibiont's outer membrane (Wanner et al., 2008).

In general, structural analyses of syntrophic and interactive consortia and communities that include an archaeal partner have rarely been reported, and information on the structure of natural archaeal populations in the literature is scarce. A likely syntrophic interaction between two hyperthermophilic archaea was artificially established under laboratory conditions: during co-culture conditions, *Pyrococcus furiosus* attaches to *Methanopyrus kandleri* forming an unusual bi-species biofilm on provided surfaces ("fried-egg colonies"; Schopf et al., 2008). The contact between the two types of archaeal cells is mediated by flagella and possibly by extracellular polymeric substances (EPS). One example for a natural and uncultivated archaeal-archaeal interactive community is the ARMAN (archaeal Richmond Mine acidophilic nanoorganisms) system, where the ARMAN cells interact closely with *Thermoplasmatales* cells leading to a potential nutrient or molecule exchange (Comolli et al., 2009; Baker et al., 2010; see also article in this issue).

A model system for archaeal interspecies relationships is represented by the "intimate association" of *Ignicoccus hospitalis* and its partner *Nanoarchaeum equitans* (Huber et al., 2002; Jahn et al., 2008). The relationship is based on the attachment of *N. equitans* to the outer cellular membrane (OCM) of *I. hospitalis* (Jahn et al., 2004). It has been shown that this obligate dependence on *I. hospitalis* is a consequence of the transfer of membrane lipids, amino acids and probably even ATP from *I. hospitalis* to *N. equitans* (Huber et al., 2012). Other investigations gave evidence for the lateral transfer of genetic material in both directions, during the co-evolution of these two archaeal cells (Podar et al., 2008). While *I. hospitalis* is able to grow in pure culture, *N. equitans* still resists cultivation without its host. This system can be maintained in the laboratory, and since one of the microorganisms is strictly dependent on the other, it actually reflects the interaction of two archaea in the natural biotope, where both species thrive.

Moreover, interactive microbial communities of Bacteria and Archaea are known, such as the anaerobic methane oxidizing (AMO) consortia, consisting of anaerobic, methanotrophic archaea (ANME) in loose association with sulfate reducing bacteria (SRB) of the

Desulfococcus/Desulfosarcina group (Hoehler et al., 1994; Elvert et al., 1999; Hinrichs et al., 1999; Thiel et al., 1999).

Another bacterial/archaeal consortium was detected in the sulphidic springs of the Sippenauer Moor (SM), a cold (~10°C) swamp area, located in the southeast of Germany. Coccoid archaea, designated as "SM1 Euryarchaeon", were found to be the major constituents of macroscopically visible whitish pearls, floating in the surface waters of the springs. The outer sheath of these pearls is formed by a sulfur-oxidizing, filamentous bacterial partner (*Thiothrix* sp.; Rudolph et al., 2001; Moissl et al., 2002). The pearls are connected by thin threads, exclusively formed by *Thiothrix* sp. (Moissl et al., 2002), giving the microbial community a "string-of-pearls" like appearance. The SM1 Euryarchaeon was also detected in another, distinct sulphidic setting, the Mühlbacher Schwefelquelle (MSI; nearby Regensburg, Germany), where the string-of-pearls community (SOPC) can be found in a similar microbial composition (Rudolph et al., 2004b).

Interestingly, subsequent studies revealed that the MSI-SM1 Euryarchaeon seeks the vicinity to sulphide-oxidisers only in (oxygenated) surface waters, whereas in the deeper, anaerobic subsurface it grows as an almost pure biofilm (Henneberger et al., 2006). Within the biofilm, the MSI-SM1 Euryarchaeon predominates a minor bacterial community, which is mostly composed of sulfate-reducing bacteria (Henneberger et al., 2006; Probst et al., 2013b). Since the SM1 Euryarchaeon remains uncultured under laboratory conditions, many features, including its metabolic capability, are yet to be fully understood. The archaeal biofilms are transported with the water flow from the subsurface to the spring outflow, where biomass can be harvested in sufficient quantities for further analyses (Henneberger et al., 2006; Probst et al., 2013b). Similar biofilms, mainly consisting of coccoid SM1 Euryarchaeota and a minor fraction of bacteria, were also observed in upwelling, anoxic waters of the SM (Henneberger et al., 2006).

The SM1 Euryarchaeon has revealed extraordinary properties, clearly distinguishing it from the archaeal strains characterised in the literature. Firstly, the SM1 Euryarchaeon is one of a few reported archaea capable of biofilm formation in its natural biotope. Additionally, it is the only archaeon known to clearly dominate a low-temperature biotope: the literature suggests that ecosystems are either dominated by bacteria or mixtures of diverse archaea (Schrenk et al., 2003; Schrenk et al., 2004; Koch et al., 2006; Webster and Negri, 2006; Weidler et al., 2008; Briggs et al., 2011; Couradeau et al., 2011; Ionescu et al., 2012). The appearance of the SM1 Euryarchaeon in a variety of ecosystems (Rudolph et al., 2004b) and in extremely high density (as almost pure biofilms, "hot spots") suggests an important role in the subsurface with a vast impact on local biogeochemistry. Thirdly, the SM1 Euryarchaeon carries a novel type of cell surface appendages. Being as thin as pili, these appendages (up to 4 µm long) exhibit barb-wire like prickles (which might function as distance holders in the biofilm) and small nano-hooks at their distal end. These structures were described as "hami" (latin for anchors, hooks; Moissl et al., 2005). So far no comparable microbial or artificial similar structures of similar size have been described. These unique properties of the SM1 Euryarchaeon biofilm have made the ecosystems, the microbial assemblages, and the archaeon itself a model system for studying cold-loving archaea in a natural biotope.

The SM1 euryarchaeal biofilms from the two biotopes SM and MSI were compared in a very recent study via genetic and chemical microbiome profiling, which revealed that both

biofilms are different in their bacterial composition and are thus unlikely to originate from one single biotope in the subsurface. The archaea of both biofilms were initially judged to be identical - based on an identical 16S rRNA gene of both populations. However, the SM and MSI cells were different in size, showed strong variations in membrane lipid composition and in their genomic information, and revealed also minor differences in ultrastructure (EPS and hami). Thus, we concluded that the two biofilms are dominated by the same archaeal species, but by two different strains thereof (Probst et al., 2014a).

Based on this finding, a deeper ultrastructural investigation of the SM population became warranted, which was conducted in this study. Here, we provide novel insights into the multifarious aspects of the SM1 Euryarchaeon lifestyle from structural biofilm organization and the interactions with the bacterial and archaeal neighbors via its unique cell surface appendages to cell wall architecture.

Material and methods

***Sampling and sample processing*.** Samples for ultrastructural analyses were taken in a cold sulphidic spring in close vicinity to Regensburg, Germany (SM; Rudolph et al., 2001; Rudolph et al., 2004b). Archaeal biofilms were harvested from raw-meshed nets, placed right within the spring outflow (Henneberger et al., 2006). The samples were collected using sterile syringes and transported on ice to the laboratory.

***Ultrastructural analyis*.** Freshly taken biofilms were fixed in original spring water including 0.1% glutardialdehyde (w/v). Scanning electron microscopy was carried out as described elsewhere (Probst et al., 2014a). Samples were examined using a Zeiss Auriga scanning electron microscope operated at 1–2 kV. For TEM, the sample preparation and procedure is described in Probst et al., 2014b. Samples were examined using a CM12 transmission electron microscope (FEI Co., Eindhoven, The Netherlands) operated at 120 kV. All images were digitally recorded using a slow-scan charge-coupled device camera that was connected to a computer with TVIPS software (TVIPS GmbH, Gauting, Germany).

Results

***The SM1 euryarchaeon forms a biofilm with EPS and cell surface appendages*.** The SM SM1 Euryarchaeon forms a biofilm, which is dominated by a single species. Macroscopically, the biofilm droplets (diameter up to 2 cm) appear milky and viscous, and show strong attachment to various types of surfaces. Using different microscopy techniques, a homogenous cell-population was observed (e.g., Figure III.1-1A). The rare (less than 5%; Probst et al., 2014a), mostly unflagellated and unpiliated bacterial cells were embedded within the biofilms and morphologies ranged from short rods, spirilla and cocci to several µm-long filaments (Figures III.1-1B, C).

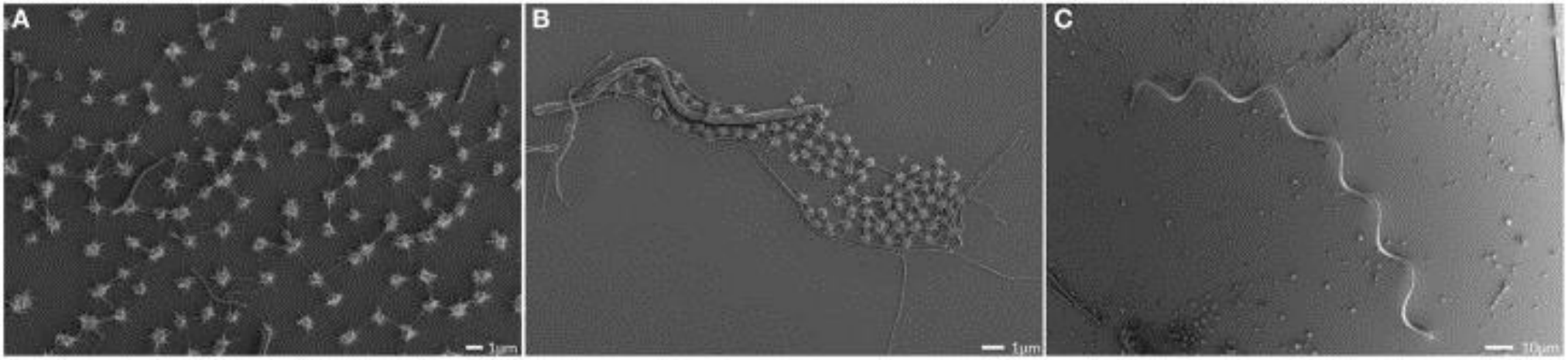

Figure III.1-1 Scanning electron micrographs of the SM biofilm. Overview, showing the homogenous archaeal population (small coccoid shaped cells; A,B) and large, spiral-shaped bacterium (C).

Viruses were not detected in any of the preparations. The archaeal cells were visible as regular cocci, although many cells appeared to be actively dividing at the time point of sampling, with an oval morphology and a clear, central contraction (Figure III.1-2). The average cell diameter of non-dividing cells was determined to be about 0.6 µm (±0.1 µm), corresponding to a cell volume of 0.11 μm^3 on average (Probst et al., 2014a).

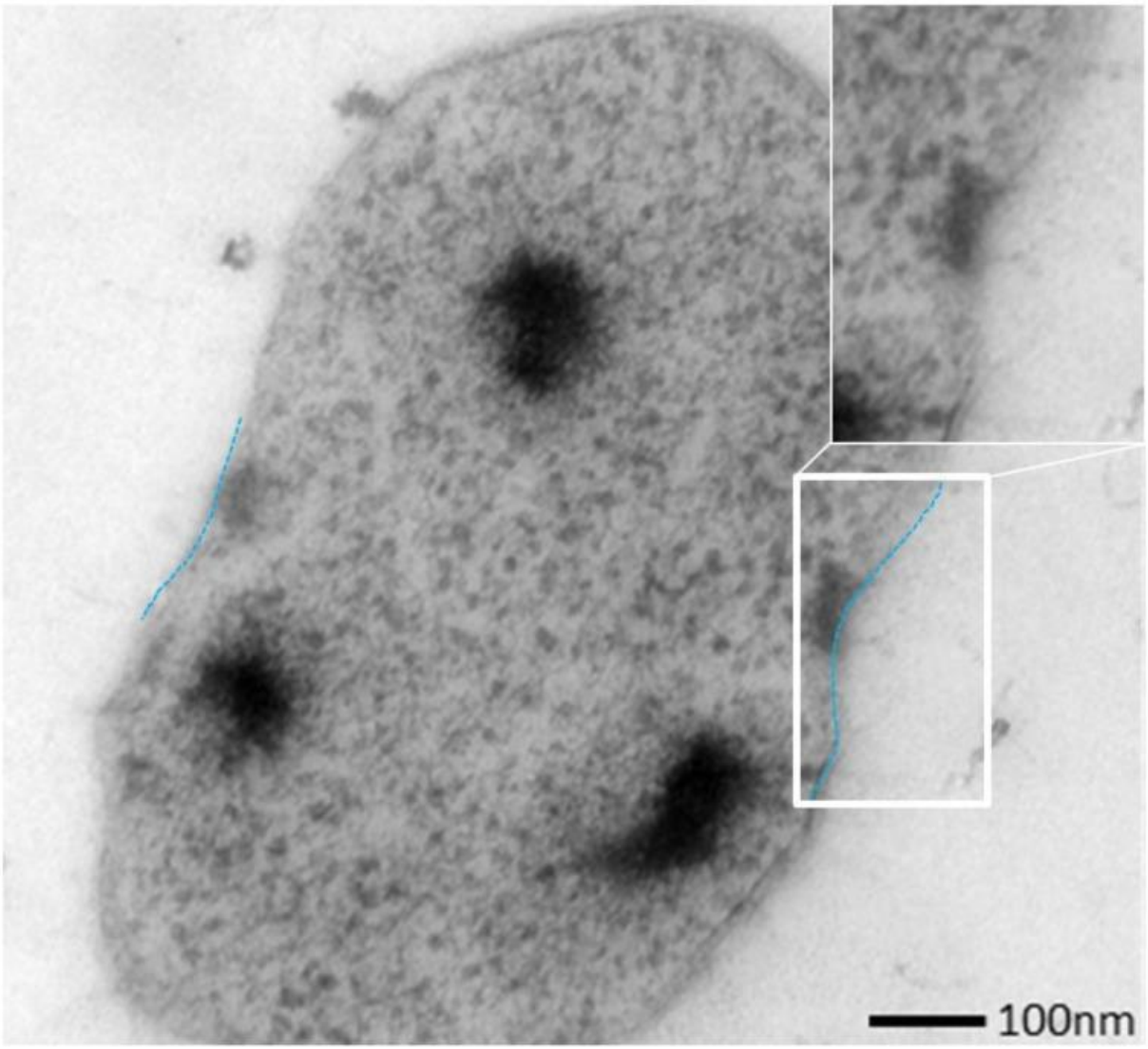

Figure III.1-2 Ultrathin section of one dividing SM1 coccus with a visible invagination.

The archaeal cells were arranged in mostly regular distances [~1.0–2.0 µm, mean: 1.26 µm, standard deviation (*SD*): 0.5 µm], forming a spacious, penetrable, but strongly connected cell-to-cell network (Figures III.1-3A, B). Each cell within the biofilm was linked to 1–7 (mostly 6) cells by a dense web of cell-cell contact threads (Figures III.1-3B and Figures III.1-1A). These connections occasionally appeared like tubes or bars (not shown), caused by drying artifacts due to a high amount of EPS, often covering the fine structures. This EPS layer resulted in the smooth appearance of cell surfaces and their surface appendages (Figure III.1-3C).

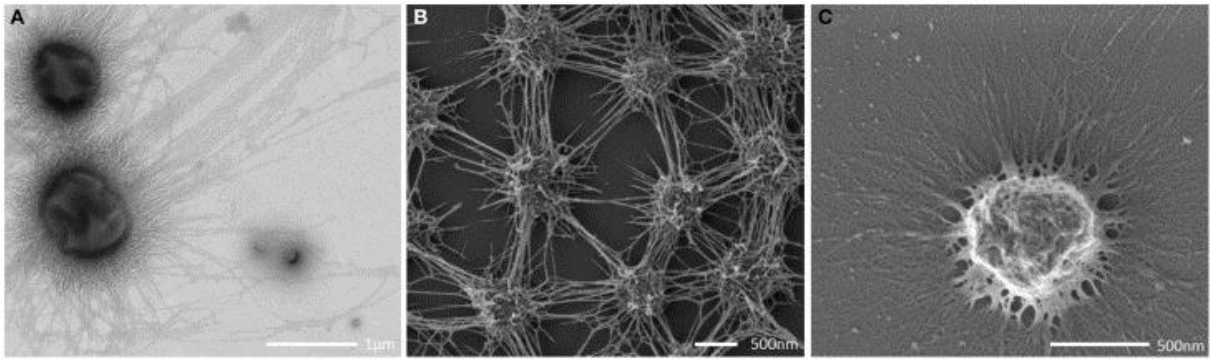

Figure III.1-3 Transmission electron (A) and scanning electron (B,C) micrographs. (A,B) show intraspecies contact via the cell appendages (bars: A: 500 nm; B: 400 nm). (C) shows a single coccus embedded in a thick EPS layer.

However, in different areas of the biofilm, where the EPS was thinner or absent, the fine-structures of cell-cell connections (the hami Moissl et al., 2005) could be visualized in more detail (Figure III.1-4).

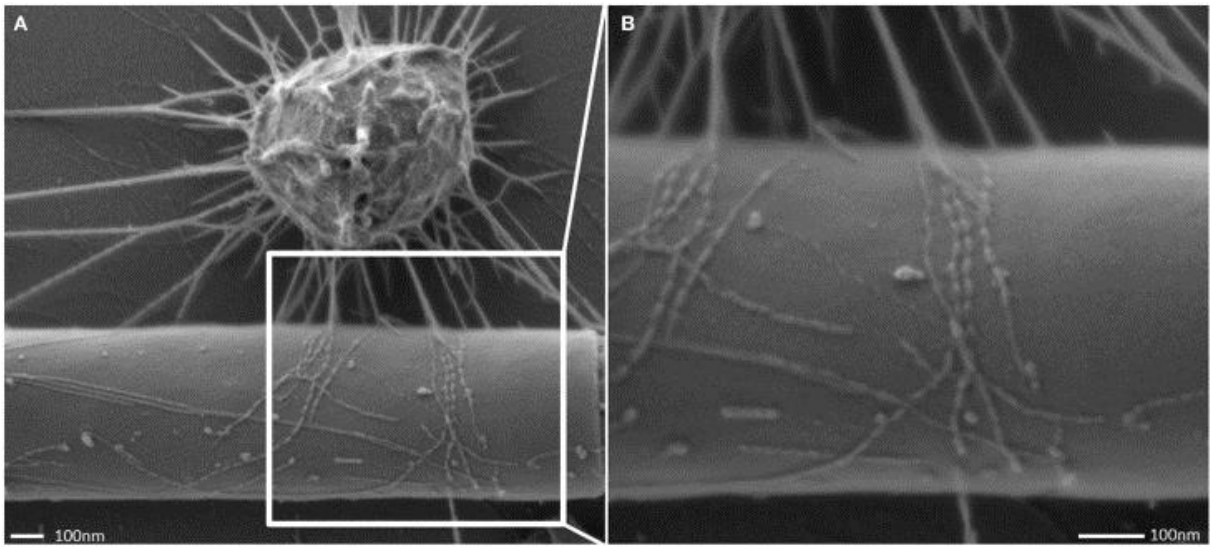

Figure **III.1-4** Scanning electron micrograph of the cell appendages: ‘‘hami” . Hami attaching to a filamentous bacterium (A) and close up view (B)**.**

The EPS was shown to form a ~400 nm wide matrix around the cells (Figure III.1-5).

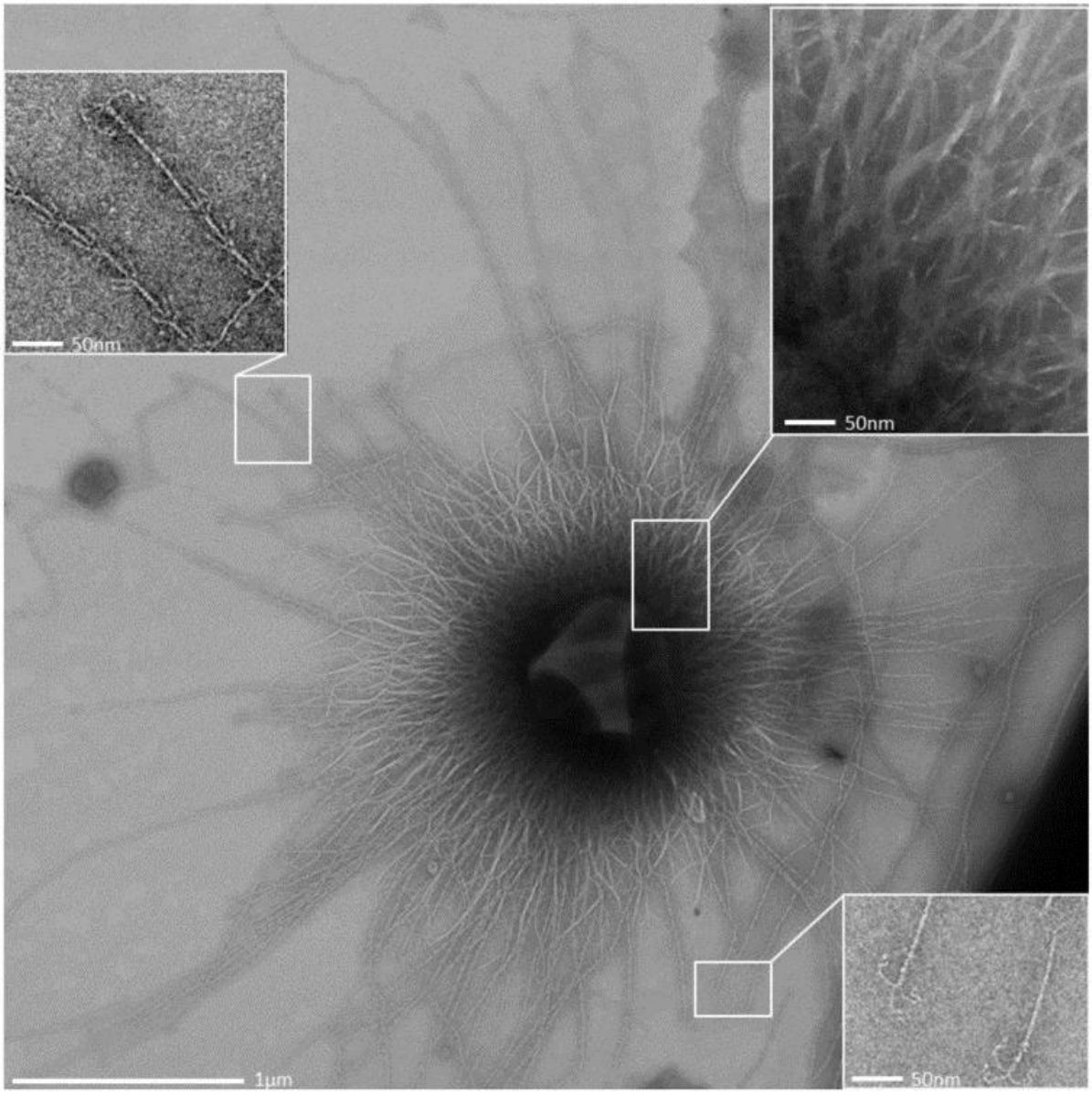

Figure III.1-5 Overview transmission electron micrograph (negative staining) of a SM1 euryarchaeal cell embedded in the EPS layer. The architecture of the hami is shown in the close up views.

The hami protruded beyond the EPS, still allowing the cells to contact other cells or abiotic surfaces (Figure III.1-6).

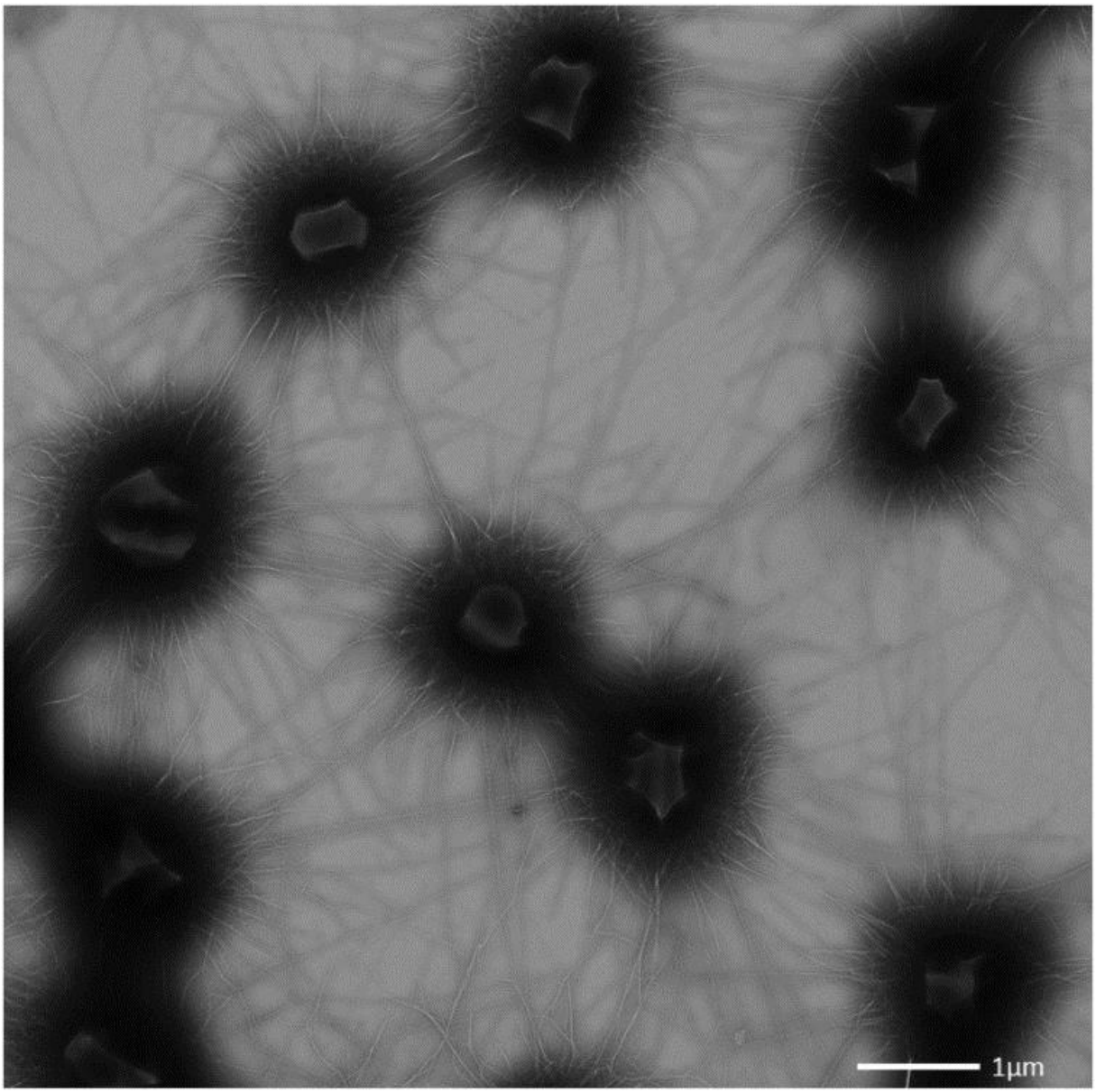

Figure III.1-6 Transmission electron micrograph (negative staining) of the SM1 euryarchaeal biofilm**.**

In contrast to the regularly organized pattern between the archaeal cocci, bacteria did not have a certain distance to the archaea but were embedded in an irregular manner - they were either directly attached to an archaeal cell, located between several archaeal cells, or not attached to other cells at all (Figure III-1), leading to the assumption that the interacting hami, and not the EPS, are the driving force to maintain the archaeal biofilm structure with defined cell-cell distances.The interconnected coccoid archaea seemed to seek additional contact to bacterial cells (Figures III.1-7) via their hami. Noteworthy, some bacterial morphotypes (filament-forming rods) within the biofilm appeared to be cocooned by hami (Probst et al., 2014a; Figure III.1-7), whereas other bacteria (such as spirilla, Figure III.1-7B) were only sparsely contacted.

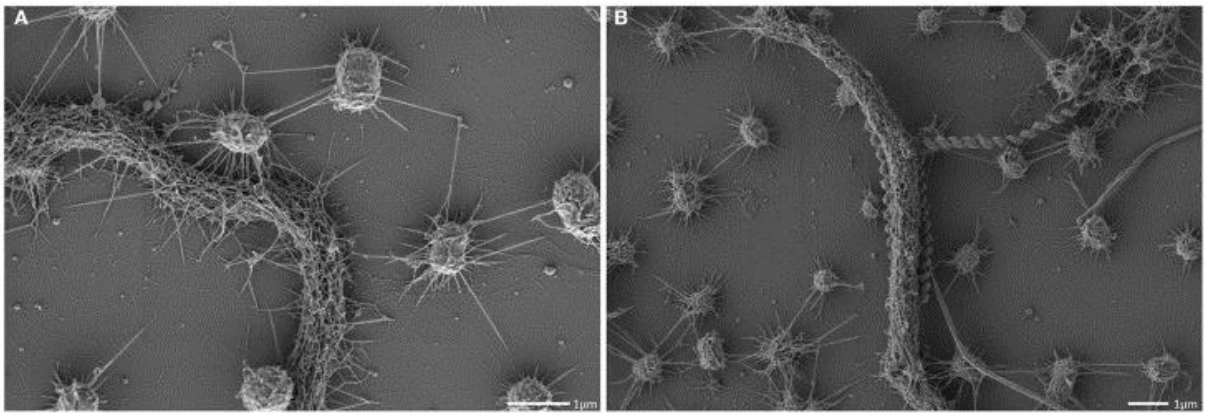

Figure III.1-7 Scanning electron micrograph. Archaeal cocci of the SM1 biofilm with numerous hami, cocooning bacterial filaments of varying diameter (A,B)**.**

***The SM1 euryarchaeal cell appendages: the hami*.** All archaeal cells revealed the presence of hundreds of hami that protrude from their cell surfaces (Figures III.1-5, III.1-6, III.1-8A). All hami analysed (incl. TEM following negative-staining and unstained by cryo-TEM; Moissl et al., 2005) showed nano-grappling hooks at their distal ends (Figures III.1-5, III.1-8B). The hami architecture was clearly distinguishable in hook- and prickle-regions, where three prickles were formed in regular distances by the major filament (Figure III.1-8B). These prickles are shaped by local bending of the three basic proteinaceous fibers (Moissl et al., 2005). The hooks were on average 60 nm in diameter (Figures III.1-5, III.1-8B) and were found to attach to the surfaces of other cells and to the prickle-regions or hooks of hami belonging to neighboring cells. The length of single hami was determined to be in the range of 0.4–3.7 µm, with an average length of 1.3 µm (*SD*: 0.6 µm).

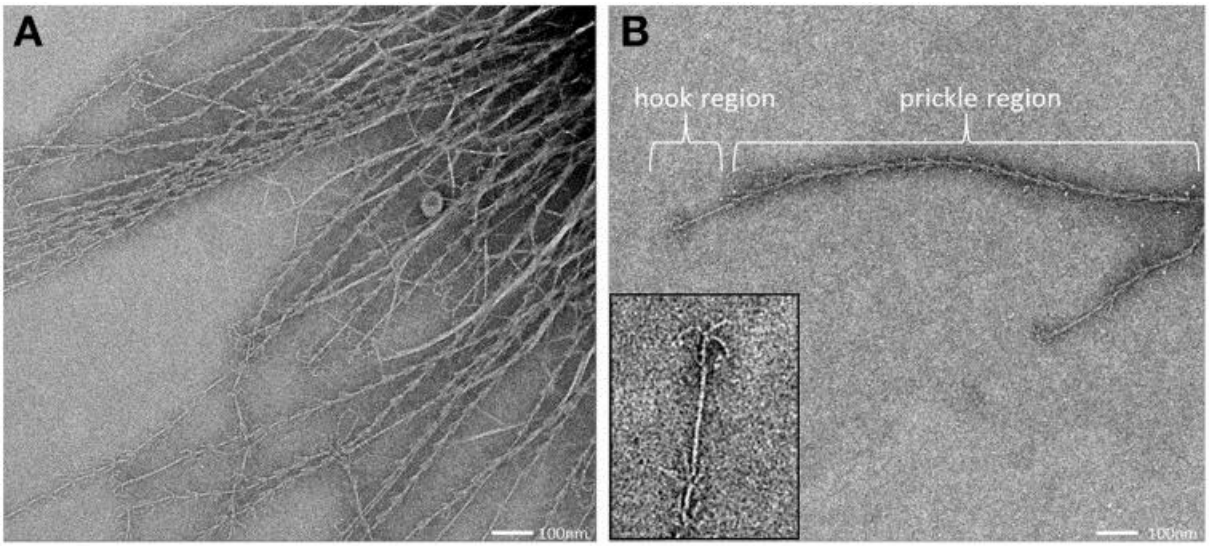

Figure III.1-8 Transmission electron micrograph (negative staining) of cell appendages (hami) protruding from a cell (A). The hamus architecture is distinguishable into a prickle region and a hook region (B) (Moissl et al., 2005).

***The SM1 euryarchaeal cell wall is composed of an inner and outer membrane*.** SM biofilm samples were subjected to thin sectioning in order to analyse their ultrastructure in more detail. The outer sheath was identified as an additional membrane (Figure III.1-9) and not, as often seen within the Archaea, as an S-layer. The SM1 euryarchaeal cell wall thus is composed of an inner membrane, periplasm, and an outer membrane. The inner and outer membranes revealed a typical structure (electron-dense, electron-lucent, electron-dense) and each showed an average thickness of 5–6 nm. The periplasm was determined to span 25 nm on average. The periplasm did not include any particles or other larger conglomerates or vesicles, as analysed so far. Thin sections of cells further confirmed the presence of an EPS-layer and the hami forming a dense network around the cells (Figure III.1-9).

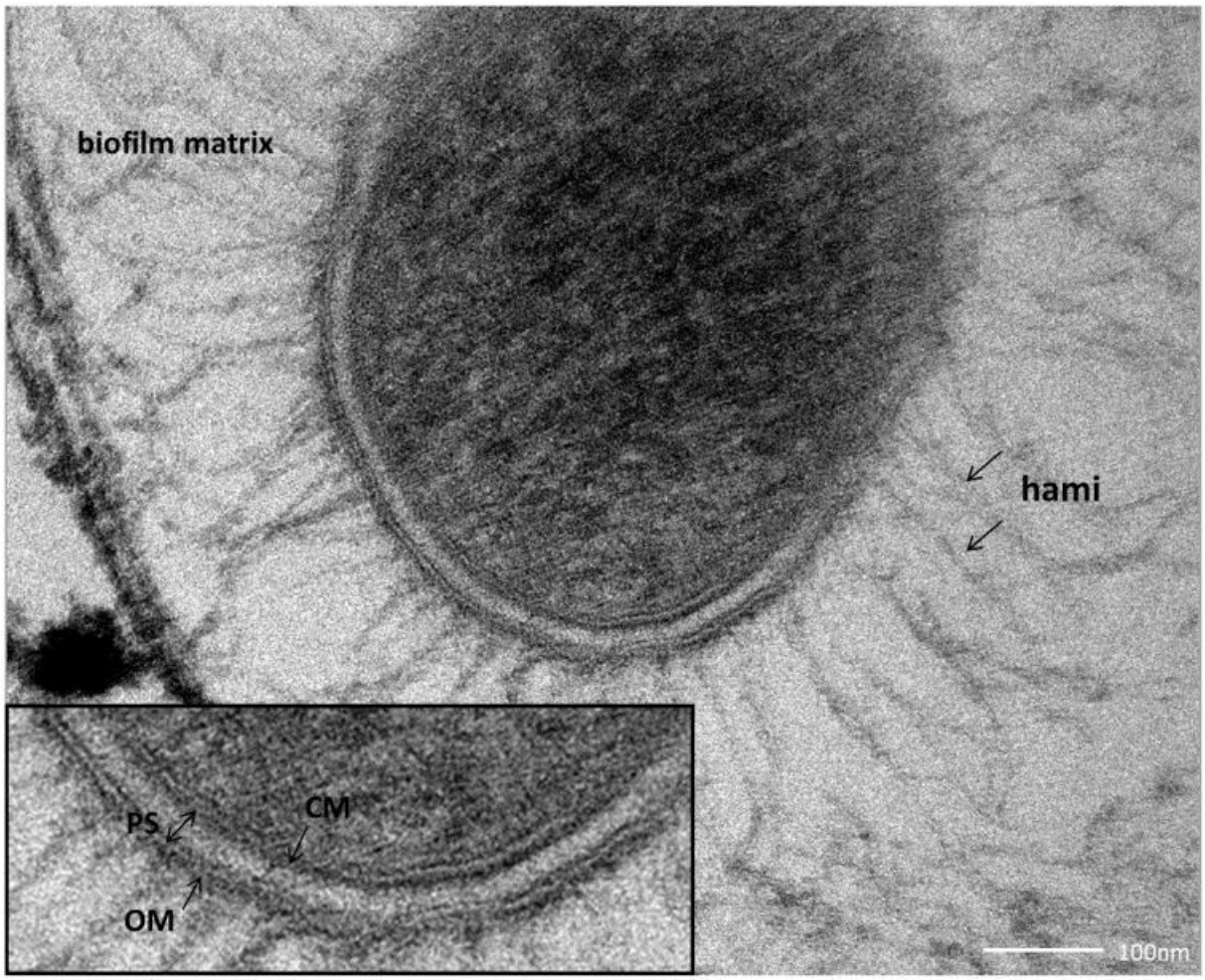

Figure III.1-9 Thin section of a single coccus, embedded in the biofilm matrix. The close-up view reveals the clearly visible cellular membrane (CM), the periplasmic space (PS) and the outer membrane (OM).

Although the anchorage of the hami could not be resolved so far, these filaments seemed to span both membranes (Figure III.1-10).

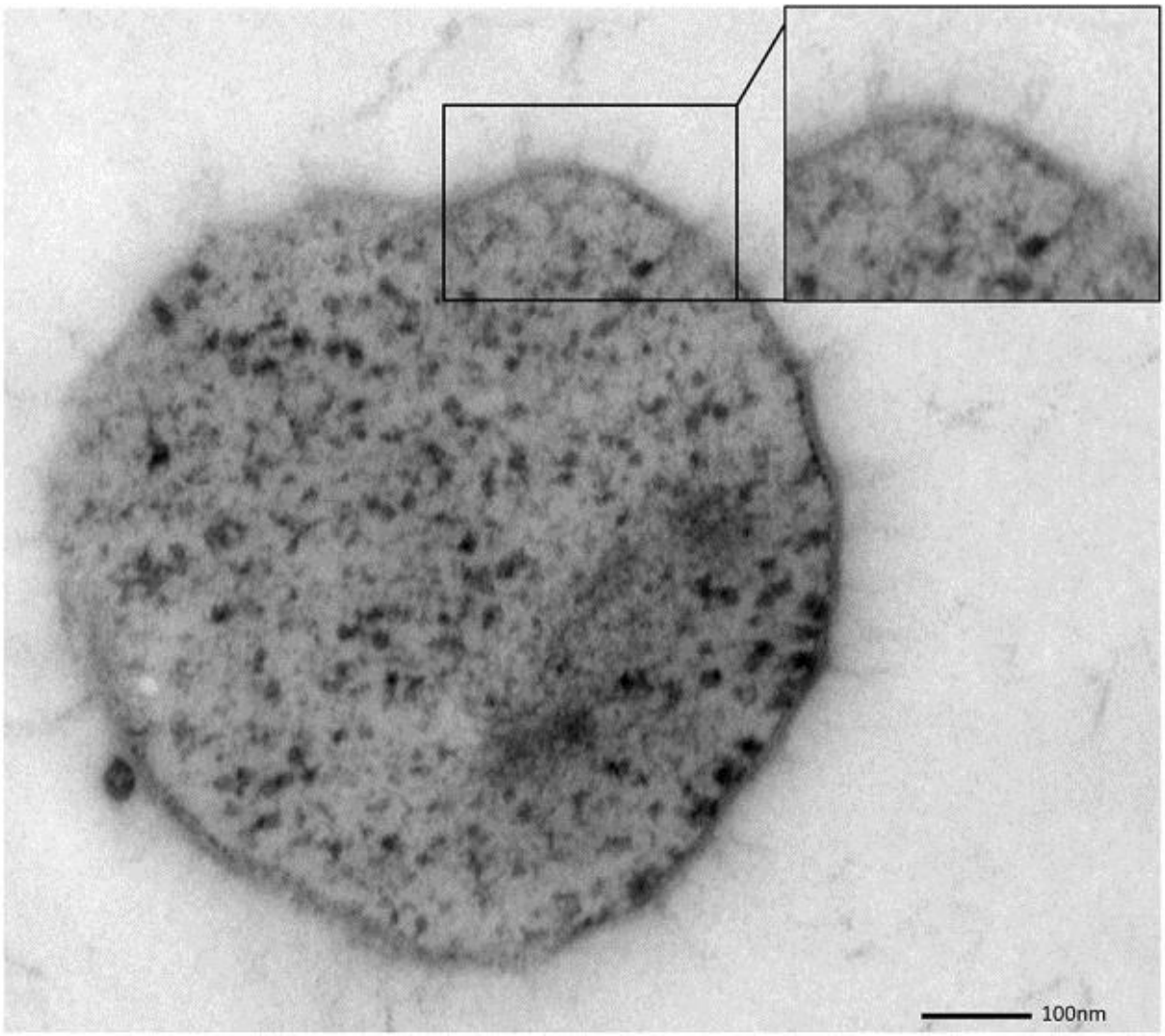

Figure III.1-10 Thin section of a single, coccoid, SM1 euryarchaeal cell. The close-up view highlights several structures, possibly hami, which might cross both membranes.

Within dividing cells, right at the central contraction site, belt-like structures were visible, suggesting protein aggregations involved in cell division machineries, such as FtsZ (Figure III.1-2). The cytoplasm appeared packed with ribosomes and dark regions, which could display the chromosome or the location of storage substances (Figure III.1-2).

Discussion

The SM1 Euryarchaeon is a unique organism that shows many features not observed in other microorganisms. Its distinct position within the phylogenetic tree (Rudolph et al., 2004b), the ability for biofilm-formation, and its predominance over associated bacteria, as well as the biofilms' origin in the subsurface of sulphidic springs warranted a detailed analysis of the ultrastructure. In this communication, we focused on the biofilms found in upwelling waters of sulphidic springs in the SM. Besides the discovery of the hami (Moissl et al., 2005), this current study provides the first detailed ultrastructural analyses of the SM biofilm population. Most of the knowledge about the SM1 euryarchaeal biofilms, however, was so far retrieved from the MSI environment (Henneberger et al., 2006; Probst et al., 2013b; Probst et al., 2014a), including preliminary ultrastructural insights (Henneberger et al., 2006).

The archaeal biofilm fine-structure appeared to be similar to described bacterial biofilm architecture (Sutherland, 2001), where the microbial cells are typically enclosed in a matrix of EPS (Costerton et al., 1995). Generally, this matrix is composed of DNA, proteins and polysaccharides and forms a slimy layer around the cells (Wingender et al., 1999). Data on the EPS composition of the SM SM1 biofilm are not available yet. DNA, however, was not detected in the highly hydrated MSI biofilm EPS, and the protein component was attributed to the presence of hami (Henneberger et al., 2006). Noteworthy, the amount of EPS was found to be variable: some cells were completely covered by EPS, whereas others were without detectable matrix.

In the bacterial domain, biofilm-formation is highly common and can cause severe problems in, e.g., medical environments (Donlan, 2001) or industrial facilities (Mattila-Sandholm and Wirtanen, 1992). On the other hand, biofilms are highly beneficial for food production or wastewater treatment (Park et al., 1990; Nicolella et al., 2000). EPS generally mediates the surface attachment, and forms a protection-shield against harmful chemical compounds (Bridier et al., 2011). Besides other important advantages, the biofilm matrix entraps excreted enzymes in close proximity to the cell (“external digestion system”; Flemming and Wingender, 2010). Water channels have been observed frequently in bacterial biofilms, which can support the distribution of nutrients and signal molecules, as well as the removal of inhibitory metabolic products (Costerton et al., 1994). The cells within the SM biofilms are organized in a strikingly regular pattern, in a spacious but strong and very sticky network, hinting at (1) a rapid flowing stream in its natural biotope in the subsurface, (2) the necessity of being attached to a surface, and (3) a requirement for a permanent water flow through the biofilm. Strikingly, compared to natural, non-medical bacterial biofilms, the purity and predominance of one species is extraordinary and was observed in both biofilms studied so far (Probst et al., 2014a).

During the course of this analysis, numerous samples were taken from the sulphidic spring environment, transported under cool conditions and prepared for ultrastructural analyses as soon as possible. Due to the close vicinity of the two sampling sites to the Regensburg laboratory, transportation time was minimal (<1 h). However, due to the origin of the biofilms in the deeper subsurface of the sulphidic springs, which cannot be assessed at the moment, we have no information on the age or status of the biofilm pieces welled up with the spring water. In a previous study, the viability of the cells was found to be extraordinarily

high (up to 90%), and cells exhibited excellent FISH (fluorescence *in situ* hybridization) signals due to the high content of ribosomes (Moissl et al., 2003), which are indications for a physiologically healthy status of the archaeal cells. Although precautions were taken in order to avoid preparation artifacts, caused by sampling or subsequent preparation for electron microscopy, alterations and damages cannot completely be avoided. This could be overcome by an immediate, on-site freezing of the samples for, e.g., cryo-electron tomographical analyses. This technique would allow for the detailed study of the cell division machinery, the hami anchorage, and the two-membrane system itself and thus is a desirable goal for subsequent studies.

All of the cells analysed by electron microscopy carried about 150 hami on their surface, with an average length of 1.3 µm. This is within to the reported length-range of pili found on the surface of *Escherichia coli* (1.0–2.0 µm; Russell and Orndorff, 1992), which usually carries 100–300 pili (Neidhardt et al., 1990). Obviously the unique hami are well suited for the formation of such a biofilm, being responsible for cell-cell and cell-surface attachment. In addition, the hami, and in particular the prickle region, seem to facilitate the regular distance pattern, forming spacers between the cells (Moissl et al., 2005). Noteworthy, the SM biofilm cells were found to be significantly smaller than those of the MSI biofilms (0.60 µm vs. 0.72 µm; Probst et al., 2014a). Based on SEM. the distances of SM cells to each other were 1.3 µm (on average), which is in strong contrast to confocal laser scanning microscopy data from the MSI population (4 µm distance). Currently it is unknown, whether this difference is based on strain-specific properties, or on method-specific preparation.

At this point of research, additional function(s) of the hami, besides attachment to surfaces, remain speculative. The energetic cost of hami synthesis appears higher than the production of simple, filamentous pili (which could also mediate surface adhesion), so that additional tasks might be envisaged. Thus, hami could be involved in cell motility, such as mediated by some bacterial type IV pili (Mattick, 2002; Ayers et al., 2010). Those can be retractile, and thus allow the bacterial cells to move on surfaces ("twitching motility"; Semmler et al., 1999; Maier, 2005). Although motility on a surface has not been observed for the SM1 Euryarchaeon so far, the cells might be able to control and regulate the attachment and the cell-cell distance via directed assembly and disassembly of the filaments. Another function could be electron-transfer, as observed for bacterial *Geobacter* species, which could allow cell-surface and cell-cell interactions (Reguera et al., 2005). Noteworthy, the SM1 Euryarchaeon seems to seek contact to bacteria of a specific morphotype: filament-forming, rod-shaped bacterial cells are frequently grappled by hami, and sometimes even completely cocooned by the surface appendages (Probst et al., 2014a). This observation might pinpoint at a specific interspecies interaction (Näther et al., 2006; Fröls et al., 2008; Ajon et al., 2011; Bellack et al., 2011; Jarrell et al., 2011), but remains speculative at this point.

The SM1 Euryarchaeon possesses two membranes, which has rarely been described for Archaea. A typical archaeal cell wall is composed of a single membrane and an attached outer proteinaceous sheath (the S-layer), whose crystalline pattern can be used as a marker for certain genera and groups of Archaea (König et al., 2007; Rachel, 2010). It has been proposed that the S-layer represents the oldest cell wall structure (Albers and Meyer, 2011), since only few archaeal groups, such as several methanogens, members of the recently proposed *Methanomassiliicoccales* species (former classified as *Thermoplasmatales*, the seventh order of methanogens; Borrel et al., 2013; Iino et al., 2013) and *Ignicoccus* species

lack this protein layer. The latter possesses two membranes, where the outer cellular membrane (OCM) harbors the H_2:sulfur oxidoreductase as well as the ATP synthase, and therefore appears to be energized (Küper et al., 2010; see Supplementary Figure S1). *Ignicoccus hospitalis* is in direct physical contact with its ectosymbiont/ectoparasite *Nanoarchaeum equitans*, which obtains several cell components from its host in order to compensate for its own biosynthetic shortcomings. The nano-sized archaeon is interacting with the host's OCM, facilitating the transport of amino acids, lipids and—although not experimentally proven yet—ATP molecules and cofactors in an yet unknown process (Huber et al., 2012) . The unique cell architecture of all *Ignicoccus* species (Rachel et al., 2002; Junglas et al., 2008; Huber et al., 2012) in combination with the energized OCM demarcates *Ignicoccus* clearly from all known prokaryotic cell envelopes. To date, it is unknown whether the outer membranes of the Euryarchaeota *Methanomassiliicoccus* or SM1 are energized. This also remains unknown for the ultrasmall ARMAN cells, whose ultrastructure was interpreted as possessing an inner and OCM instead of an archaea-typical cell wal (Comolli et al., 2009; Baker et al., 2010). Except for the lipid composition, these membranes distantly resemble the dimensions and appearance of bacterial Gram-negative cell walls. It is not known whether such a cell wall architecture is rather a general feature of many archaea and was not recognized as such so far, or is an exception within this domain of life.

Strikingly, all archaea that possess a double membrane-based cell wall are involved in close interaction with other archaea, bacteria or their eukaryotic host. Bacteria which are participating in syntrophic partnerships are often found to be equipped with unique multiple membrane complexes (Orphan, 2009), and thus a positive effect on such interactions could be envisaged for several reasons: (1) An outer membrane is a suitable surface for anchoring proteins, lipids and carbohydrates, which could serve as contact sites for interactions (Mashburn-Warren et al., 2008). In contrast to S-layers, membrane architecture can be changed and regulated internally, allowing flexible responses to environmental changes. Within the SM1 Euryarchaeon, the double membrane also anchors the hami, which represent the major contact site of the cell toward biotic and abiotic surfaces. (2) The spanned periplasm provides additional space for metabolic products, chemosensors, signal cascades, storage compounds, and other molecules possibly involved in microbial interactions (Davidson et al., 1992; Wadhams and Armitage, 2004). Additionally two compartments provide the possibility of generating gradients and allow compartmentalization even within one single prokaryotic cell.

The finding of an increasing number of archaea with double-membrane cell walls could suggest this feature to be a general characteristic of a predecessor archaeon, and questions the S-layer as the (proposed) ancient cell wall type for Archaea. It shall be noted, however, that sample preparation and clear visualization of the undisrupted cell wall is challenging and, in most cases, has to include a careful interpretation of the obtained data. The question whether the double membrane is a general feature of Archaea emphasises the need for more detailed ultrastructural analyses of cultivated and uncultivated archaea, but also asks the community to reconsider the proposed models for archaeal cell division and formation of cell surface appendages. The latter includes the involvement of other (novel?) translocation machineries for cell surface molecules, including the transfer across two membranes and the periplasm. Overall, it becomes again clear that the archaeal domain is not humble in structure, organization, and function. The more we learn about this group of

microorganisms, the more we recognize the sophisticated, complex, and clever way of archaeal living.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00397/abstract and on the supporting CD (./Selected publications/03 Supplementary files).

III.2. S-layers at second glance? Altiarchaeal grappling hooks (hami) resemble archaeal S-layer proteins in structure and sequence

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Abstract

The uncultivated "*Candidatus* Altiarchaeum hamiconexum" (formerly known as SM1 Euryarchaeon) carries highly specialized nano-grappling hooks ("hami") on its cell surface. Until now little is known about the major protein forming these structured fibrous cell surface appendages, the genes involved or membrane anchoring of these filaments. These aspects were analysed in depth in this study using environmental transcriptomics combined with imaging methods. Since a laboratory culture of this archaeon is not yet available, natural biofilm samples with high *Ca*. A. hamiconexum abundance were used for the entire analyses. The filamentous surface appendages spanned both membranes of the cell, which are composed of glycosyl-archaeol. The hami consisted of multiple copies of the same protein, the corresponding gene of which was identified via metagenome-mapped transcriptome analysis. The hamus subunit proteins, which are likely to self-assemble due to their predicted beta sheet topology, revealed no similiarity to known microbial flagella-, archaella-, fimbriae- or pili-proteins, but a high similarity to known S-layer proteins of the archaeal domain at their N-terminal region (44–47% identity). Our results provide new insights into the structure of the unique hami and their major protein and indicate their divergent evolution with S-layer proteins.

Keywords: archaea, S-layers, Archaeal cell surface appendages, Hami, Nano-grappling Hooks, Double-membrane, Environmental transcriptomics, Electron cryo-tomography

Introduction

In the course of evolution, nature has developed simple and fascinating solutions for various challenges. Particularly microbial life seems thus to harbor an enormous potential of exploitable biomaterial, such as enzymes and other biomolecules. These compounds are thought to prove very useful for diverse applications, in e.g., medicine, pharmacy, or industry (Beg et al., 2001; Hasan et al., 2006; Dutta and Kundu, 2014). However, the majority of naturally occurring exploitable biomaterial remains to be explored, because a substantial amount of microorganisms resist cultivation in the laboratory and thus escape detailed characterisation of their metabolic potential and functional traits.

Cultivation-independent methods such as metagenomics enable scientists to directly access the genetic information of (entire) microbial communities. The sequence information retrieved can be used for assembly of near complete to complete genomes from key or underrepresented members of the communities (Tyson et al., 2004; Sharon and Banfield, 2013; Sharon et al., 2013). This information thus provides the basis for functional annotation of these novel microbial genomes. However, annotation of genes from lineages with only distant representatives is sometimes limited. Some cases have been reported in which approximately 50% of the predicted proteins could not be assigned a function (Baker et al., 2010; Kantor et al., 2013). Consequently, linking metagenomic data from uncultivated microorganisms with information retrieved by other molecular methods and/or imaging techniques in order to characterise such unknown predicted proteins is a promising approach. Imaging techniques, however, can currently not be conducted for highly complex microbial communities (e.g., those from soil) without substantial loss of information.

Nevertheless, populations with low and simple diversity and uneven abundance of its members, such as the uncultivated acid mine drainage microbial community, can be studied in detail using a variety of these techniques, enabling researchers to link metagenomics to cellular characteristics (Comolli et al., 2009; Baker et al., 2010; Yelton et al., 2013; Comolli and Banfield, 2014).

For instance, genomes of ARMAN cells have been linked to their ultrastructure (Baker et al., 2006; Comolli et al., 2009; Baker et al., 2010); the latter also revealed that their cell wall is composed of a double membrane—a highly unusual feature in the domain of Archaea (Klingl, 2014). This feature was described to be shared only with a few other members in this domain, which are represented by the genus *Ignicoccus (*Rachel et al., 2002; Junglas et al., 2008), the *Methanomassiliicoccus* species (Dridi et al., 2012; Iino et al., 2013; Borrel et al., 2014) and the recently described "*Candidatus* Altiarchaeum hamiconexum" (formerly known as SM1 Euryarchaeon; Rudolph et al., 2001; Probst et al., 2014b).

Ca. A. hamiconexum is a representative of the recently introduced euryarchaeal order "*Candidatus* Altiarchaeales", a widespread group of uncultivated archaea thriving in subsurface aquifers (Probst et al., 2014b). The tiny coccoid *Ca*. A. hamiconexum cells are washed up from the subsurface in extraordinarily pure biofilms (Henneberger et al., 2006; Probst et al., 2013b). In a very recent publication, metagenomic sequencing data of *Ca*. A. hamiconexum biofilms were combined with isotopic-based lipidomics to reveal its autotrophic metabolism, which may be the basis for substantial carbon dioxide fixation in the subsurface. Lipidomics has further shown that the archaeon's double membrane is composed of core diether lipids with either two phytanyl chains or a combination of one phytanyl and one sesterpanyl chain (Probst et al., 2014b). Anchored in this membrane, unique cell surface appendages called "hami" (singular: "hamus"; Moissl et al., 2005) were identified via ultrastructural analyses. Hundreds of these hami protrude from each cell and interlink with those of neighboring cells in order to form a biofilm (Probst et al., 2014b). Each filament is assembled from three protein sub-filaments that are wound up to a barbed-wire-like structure and a distal grappling hook. This unique structure is supposed to be composed of one major protein species (Moissl et al., 2005; Probst et al., 2014b) —similar to surface layer proteins (S-layer), which usually also consist of one or two protein species that assemble into a 2-dimensional array on cell surfaces (Sleytr and Messner, 1988; Eichler, 2003; Veith et al., 2009; for a detailed review on archaeal S-layers see Klingl, 2014, same Research Topic). It was proposed that individual hami subunits are expressed in the cytoplasm, transferred through the inner membrane by the Sec-pathway and then assembled in the periplasmic space between inner and outer membrane (Probst et al., 2014). Although six genes were annotated as putative hamus subunit-encoding genes, the actual gene that is expressed for hamus formation *in vivo* has not been identified so far (Probst et al., 2014b).

In this study a combination of -omic techniques with electron microscopy was applied in order to identify the *bona fide* gene sequence of the hamus subunit, shed light onto its phylogenetic evolution and further analyse its structure and the membrane in which it is anchored. Due to its barbed-wire-like structure and in particular its distal nano-hook, the hamus is considered an exploitable biomaterial and thus a tool for nanobiotechnology (Moissl-Eichinger et al., 2012a), for which we provide the basis in this communication.

Material and methods

Sampling and sampling processing. Archaeal biofilm samples were taken from the cold sulphidic spring, called Mühlbacher Schwefelquelle Isling (MSI), which is located in close vicinity to Regensburg, Germany (Rudolph et al., 2004b). The biofilms were harvested from double-opened flasks, which were incubated for 2–3 days in the water-flow of the spring, approx. 30 cm below the surface. The flasks were equipped with a polyethylene net, which proved useful to catch upwelling biofilm-pieces from the spring-water. After the incubation period, the entire flask was closed under water using rubber stoppers and transported on ice to the laboratory, where the samples were immediately processed (Probst et al., 2013b).

***TOF-SIMS (time of flight secondary ion mass spectrometry) chemical imaging*.** Archaeal biofilms were washed from the nets, and free-floating biofilm pieces were collected onto on gold-plated screens (hole 100 µm, G225G1, Plano GmbH, Wetzlar, Germany). Samples were immediately dried and the gold-coated aperture disks were placed onto silicon wafers and affixed along the edges with adhesive tape, with care to avoid contact with the biofilm.

Chemical imaging was performed with a modified commercial reflectron-type time-of-flight secondary ion mass spectrometer (TOF.SIMS V; IonTOF, Germany). Mass-selected Bi^+_3 ions with 25 keV kinetic energy impacted the sample surface at 45° with respect to the surface normal. Ejected cationic and anionic chemical species were collected in separate analyses. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) spectra were acquired with Bi^+_3 pulses in high current bunched mode, over an area of 500 µm × 500 µm, with a 256 pixel × 256 pixel raster scan at a repetition rate of 2.5 kHz, and secondary ions were extracted with a 10 µs long extraction −2000 V (positive ion mode) or +2000 V (negative ion mode) pulse. Electron charge compensation was not used.

***Electron cryo-tomography*.** Biofilm samples were centrifuged at 16,000 × g using an Eppendorf 5415 C table top centrifuge, and cell pellets were resuspended in an equal volume of KPH buffer (0.7 mM NaCl, 0.1 mM $MgCl_2$, 1.6 mM $CaSO_4$, 1 mM HEPES; the pH was adjusted with NaOH to 6.5; Moissl et al., 2005). Cellular suspensions were mixed with an equal amount of 10 nm colloidal protein-A gold (Aurion, Wageningen, The Netherlands), and 3 µl of this mixture were added to a glow-discharged Quantifoil (Quantifoil, Großlöbichau, Germany) grid, blotted for 3–5 s and plunged into liquid ethane.

Samples were transferred frozen into a Polara G2 Tecnai transmission electron microscope (FEI, Hillsboro, USA) operated at 300 kV. The TEM was equipped with a Gatan 4 × 4 k charge-coupled device (CCD) camera or a K2-Summit dierect electron detector as well as a Tridiem energy filter (Gatan Inc., Pleasanton, USA). Images were recorded using a magnification of 34,000 × on the CCD and 41,000 x on the K2 summit, corresponding to a pixel size of 0.6 or 0.54 in the final image, respectively. Zero loss filtered tomographic tilt series were collected in a range of −60° to +60°, at increments of 2°–2.5° and a defocus of 6–10 µm using the Gatan Digital Micrograph Latitude software (Gatan Inc., Pleasanton, USA) The maximum cumulative dose was 150 e^-/A^2.

For electron tomography at room temperature, samples were high-pressure frozen and embedded in epon resin as described in Perras et al. (2014). Tilt series were recorded at 120

kV on a JEOL JEM-2100, equipped with a LaB_6 cathode and a 2 × 2 k F214 fast scan CCD camera (TVIPS, Gauting, Germany) in a range of −60° to +60° in steps of 2°, a defocus of 3–6 µm and a magnification of 14,000 ×, which equals a pixel size of 1 nm in the final micrograph. Tilt series were reconstructed into tomograms with the IMOD software (Kremer et al., 1996), using weighted backprojection or SIRT and displayed in 3 dmod (IMOD).

Ultrastructural analysis using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For analysing the cell surface appendages, the unfixed, purified hami were deposited on a carbon-coated copper grid and negatively stained with uranylacetate [2% (w/v), pH 4.5]. The samples were examined with a CM12 transmission electron microscope (FEI Co., Eindhoven, The Netherlands) operated at 120 kV. All images were digitally recorded using a slow-scan CCD camera that was connected to a computer with TVIPS software (TVIPS GmbH, Gauting, Germany). Scanning electron microscopy was performed as described before (Probst et al., 2014a).

Particle characterisation within the cells. For element analysis of the particles within coccoid cells, *Ca*. A. hamiconexum biofilm flocks were embedded in TAAB embedding resin (TAAB, Aldermaston, UK) and thin sectioned as described before (Milić et al., 2015), followed by staining with platinum blue and lead citrate. Energy Filtered TEM (EFTEM) was performed with a Gatan GIF Quantum 963 energy filter using an FEI Tecnai 20 microscope at 120 kV acceleration voltage. To visualize the elemental distributions, elemental maps were made using the three window method at the standard losses provided by Gatan Digital Micrograph software (Teubl et al., 2015).

Energy Dispersive X-Ray spectroscopy was performed using an Edax silicum type ultrathin unit (SUTW) detector, as described bevore (Milić et al., 2015); the corresponding images were made with scanning transmission EM using a High Angle Annular Dark Field detector (HAADF).

Purification of hamus filaments and antibody generation. For the production of hamus-specific antibodies for protein analyses and structural investigations, hamus filaments were released from the archaeal cell surface. The purification procedure, as well as the production of hamus-specific antibodies, has been described elsewhere (Probst et al., 2014a). In brief, the archaeal biofilm cells were lysed using 0.1% (w/v) sodium dodecyl sulfate (SDS) and cell debris was removed via subsequent centrifugation and sucrose-gradient centrifugation steps.

Denaturating SDS-PAGE analysis and western blot. Hamus filaments were purified as described above and mixed with protein loading dye [Tris/HCl pH 7.5, 60 Mm; Glycerol 10% (v/v); SDS 2% (w/v); bromphenol blue 0.01% (w/v); 2-mercaptoethanol 5% (w/v)] and heated for 30 min in boiling water. Afterwards, proteins were separated via SDS-PAGE (Laemmli, 1970) using a Mini Protean 3 Cell [Bio-Rad Laboratories Inc., Munich; 12.5% (w/v) polyacrylamide linear gradient gel], at 15 mA followed by a higher current of 30 mA until the dye front reached the bottom of the gel.

The separated proteins were afterwards semi-dry-blotted onto a Roti®-PVDF (polyvinylidene fluoride) membrane (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) using a semidry transfer cell instrument (Bio-Rad, Munich, Germany) operated at 16 V for 1 h. Blocking was performed by incubation of the membrane in Tris buffered saline [including Tween 200.01%

(v/v), 3% milk powder (w/v); TBST-B] overnight. After a washing step with Tris buffered saline [including Tween 200.01% (v/v); TBST], the primary antibody (anti-hamus) was applied (1:5,000 dilution in TBST-B) and incubated for 3 h under agitation. The membrane was washed using TBST and the secondary antibody [anti-chicken coupled with horseradish peroxidase (1:1,000 in TBST-B; Sigma-Aldrich Chemie GmbH, Munich, Germany)], was applied for 2 h]. The reaction was visualized by applying a 3-amino-9-ethylcarbazole solution [20 mg of 3-amino-9-ethylcarbazole dissolved in 1 ml ethanol p.a., followed by mixing with 50 ml of potassium acetate, pH 5, 20 mM, 100 µl of triton X-100, 10% (v/v) and 10 µl of H_2O_2] after another washing step.

Liquid chromatography mass spectrometry of peptides. For identification of peptides in the band showing positive reaction in the western blot analysis, the corresponding band in the SDS-PAGE was cut out and trypsin digested. Obtained peptides were then subjected to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). HPLC was carried out using a Ultimate3000 RSLC nano-HPLC System (Thermo Fisher Scientific; at the facilities of Prof. Dr. R. Deutzmann, University of Regensburg) with a reversed phase chromatography analytical column (ReproSil Pur 120 C18-AQ, 75 µm × 25 cm). The mobile phase consisted of a linear gradient containing 0.1% (v/v) formic acid (eluent A) and 80% (v/v) acetonitrile, 0.1% (v/v) formic acid (eluent B). HPLC was coupled on-line to a maXis plus UHR-QTOF system (Bruker Daltonics) via nano-electrospray source and up to five most abundant precursors selected for fragmentation by collisional induced dissociation (CID). Identification of the obtained peptide mass fingerprints was performed by genome database searching using the PeptideMass software (Wilkins et al., 1997).

***Fluorescence immuno-labeling*.** For immuno-staining, the archaeal biofilms were fixed with paraformaldehyde (5%; v/v) at room temperature (1 h) and washed three times with PBS (phosphate buffered saline). The cell suspension was applied into a well of a gelatine-coated slide [P. Marienfeld KG, Lauda-Koenigshofen, Germany; slides were dipped into a 0.01% (w/v) gelatine solution and dust-free air-dried]. The dried cells were afterwards incubated in 16 µl of PBST [+0.1% (w/v) SDS] at 37°C and the PBST buffer was replaced with 16 µl PBST buffer containing the anti-hamus IgG (Davids biotechnology, Regensburg, Germany; dilution 1:2,000; 1 h, 37°C). Subsequently, the slide was washed 15 min in PBST [+0.1% (v/v) SDS], rinsed with H_2O and air dried. The secondary antibody (goat anti-chicken, Cy3-labeled; 0.64 mg/ml, Sigma Aldrich, Germany; dilution 1:500) was added and incubated for 1 h at 37°C. After washing two times with PBST [+0.1% (v/v) SDS], the slide was rinsed with H_2O again, DAPI counterstained and analysed by fluorescence microscopy (Olympus BX53F, Hamburg, Germany) with epifluorescence equipment and the respective imaging software (cellSens, Olympus).

Transcriptomic analysis of hamus gene(s). The presence or absence of specific hamus subunit transcripts was tested via specific mRNA detection in archaeal biofilm samples (see Supplementary Table S1, containing list of genes and primers). Three hamus subunits (i.e., MSIBFv1_A2980002, MSIBFv1_A2020020, MSIBFv1_A3210004; deposited in the European Nucleotide Archive; accession code: PRJEB6121 (Probst et al., 2014b) were examined in detail.

RNA was isolated using the PowerBiofilm™ RNA Isolation Kit (Mobio Laboratories Inc., Carlsbad, USA), following the manufacturers' instructions (DNA digestion was performed for

30 min). DNAse treatment was repeated after precipitation of nucleic acids. Contamination of the RNA extraction by residual DNA was excluded by PCR amplification, using a universal archaeal forward primer combined with a reverse primer binding in a non-transcribed region (e.g., primer: 344aF and 64R-23S, sequences in Supplementary Table S1). Samples showing no positive PCR signals were assumed to be free of contaminating DNA and were further processed. RNA was reverse-transcribed into cDNA (QuantiTect Rev. Transcription Kit, Qiagen, Hilden, Germany). Transcripts were amplified using specific primers, which were designed using the web tool Primer3v.0.4.0 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi; parameters: product size: optimum full length of gene, GC% 40–60%, annealing temperature: 60°C optimum). To confirm the specificity, primers were searched (Altschul et al., 1990) against NCBI NR and against the *Ca*. A. hamiconexum metagenome (Probst et al., 2014b). The designed primer pairs (see Supplementary Table S1) were used individually using cDNA as a template and tested for amplification success (denaturation time: 5 min 95°C; 30 cycles: 45 s 94°C, 45 s 60°C, 130 s 72°C; final elongation: 10 min 72°C). Positive PCR products were purified (HiYield® Gel/PCR DNA Fragments Extraction Kit; Süd-Laborbedarf GmbH, Gauting, Germany) and Sanger-sequenced (LGC Genomics GmbH, Berlin, Germany). Experiments were carried out in duplicates.

***Hamus protein analysis, structure determination, and phylogenetic tree reconstruction*.** The trans-membrane region of the hamus protein was predicted by TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The protein characteristics were analysed using GenScript's Peptide Property Calculator (https://www.genscript.com/ssl-bin/site2/peptide_calculation.cgi) and by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

Secondary structure was predicted using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/), alignment of the sequences was performed using the multi-sequence alignment program MAFFT (http://mafft.cbrc.jp/alignment/server/). PSIPRED prediction was combined with a fold recognition search using pGenThreader (Lobley et al., 2009).

Hamus protein region 5–81 was searched against the NCBI database (blastp; Altschul et al., 1990). The 50 mostly related protein sequences were retrieved and used for tree (Neighbor Joining and Maximum Likelihood) reconstruction via MEGA 6 (Tamura et al., 2013).

Results

The *Ca*. A. hamiconexum double membrane is composed of glycosyl-archaeol species. In positive ion TOF-SIMS spectra, several notable mass spectral peaks were observed in the high mass range [700–1,200 atomic mass units (amu) and around 2,000 amu]. Most prominent of these mass peaks is a cluster of peaks centered about ~1,000 amu, which was assigned to sodiated diglycosyl archaeol (Figure S1). 16 mass units lower and higher of the sodiated lipid peaks were additional clusters of peaks which could either reflect lithiated and potassiated variants, or, for the m/z ~1,016 peaks contribution from a core hydroxyarchaeol with sodiated diglycosyl polar head group. Minor contributions of sodiated triglycosyl archaeol and monoglycosyl archaeol were also detected at m/z 1,162 and 838, respectively.

In addition to the salt adducts of the mono-, di- and tri-glycosyl lipids, a significant contribution from m/z ~1,070 was observed. From LC/MS/MS data of additional samples of the SM1 biofilm, this lipid was assigned to a diglycosyl diether lipid with one C_{20} hydrocarbon chain and one C_{25} hydrocarbon chain.

In the mass range where dimers or tetraether species would occur, several groups of peaks could also be observed, albeit at relatively low intensity (Figure S2). Prominent among these species was a cluster of peaks with a maximum peak intensity observed at m/z 1,974.5 and 2,006.5 amu. M/z 1,974.5 was found too low in mass to correspond to a simple sodiated dimer of the diglycosyl lipid (strongest isotope peak would appear at m/z 1,977.6), and too high in mass to correspond to a sodiated diglycosyl dialkyl tetraether (which would have its strongest isotope peak at m/z 1,973.5). Based on the strong sodiated lipid contribution in the diglycosyl diether lipid-related peaks, it was assumed that the peaks represented a sodiated species. With this assumption, one possible structure could be a sodiated trialkyl tetraether lipid with four total glycosyl groups on the ends (Figure S2). It is not clear whether this species is native to the biofilm or a result of a dimerization process that occurs during the ion sputtering event.

Particles localized inside and outside of the* Ca*. A. hamiconexum cells reveal different elemental composition, indicating the presence of sulfur and phosphor deposits within the cells. TOF-SIMS analysis revealed inorganic species embedded in the biofilm, containing Na, K, Ca, Mg, and Fe (positive ion mode TOF-SIMS; Figure S3). Ca^{2+} and Mg^{2+} appeared to be concentrated within particles in the biofilm, which may indicate the formation of insoluble mineral carbonates. As revealed by scanning electron microscopy, such particles were easily visible in the preparations (Figure III.2-1A), and many particles were located in close vicinity or even touched by the hami of highly actively dividing cells (Figure III.2-1A).

Electron microscopic images of ultrathin-sectioned cells showed that most of the cells exhibited dark inclusions within their cells (Figure III.2-1B). Such dense areas were examined using energy dispersive X-ray spectroscopy and were identified containing most likely phosphor and sulfur (in some cells) (data not shown). This result was further confirmed using EFTEM (Figure S4).

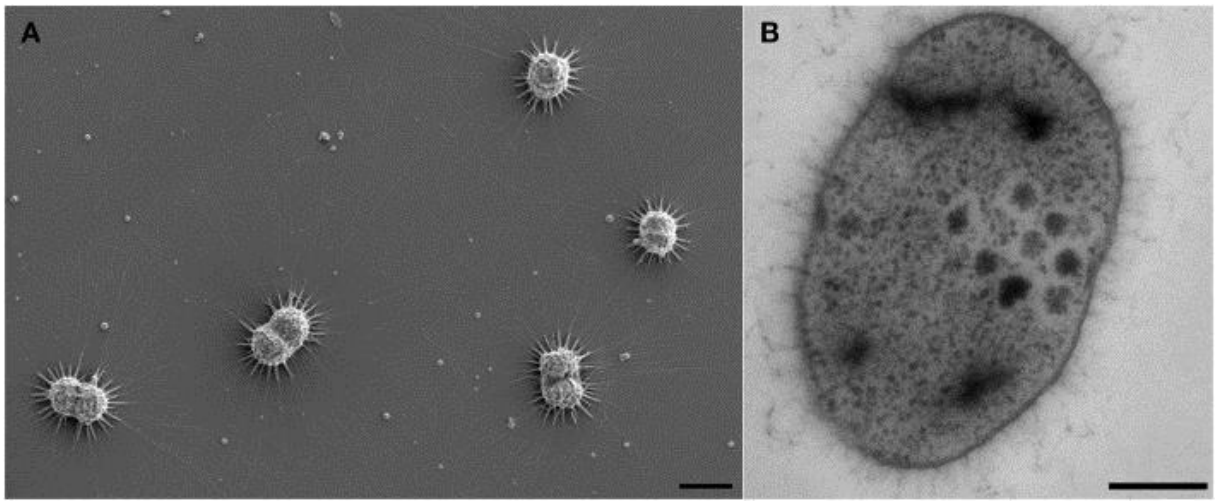

Figure III.2-1 Electron micrographs of *Ca.* A. hamiconexum cells within the biofilm**.** (A) Scanning electron micrograph of dividing cells in the biofilm, showing particles close to the hami and the cells. Bar: 1 µm. (B**)** Thin-section of one cell, showing dark-stained areas. Bar. 200 nm.

***Hamus-filaments are three-dimensional structures and span both membranes*.** Preliminary results of electron tomography from entire *Ca*. A. hamiconexum cells after high-pressure-freezing, freeze substitution and epon embedding resulted in a cell wall model (Figures III.2-2). Both the inner and the outer membrane were visualized in a 50 nm section through the cell (Figures III.2-2; Supplementary Movies 1, 2). Filamentous structures (8 nm in diameter), most likely representing the hami, passed through both membranes. At the cytoplasmic end of some of the filaments, an electron dense structure could be detected, representing a potential anchorage-associated structure of the filaments.

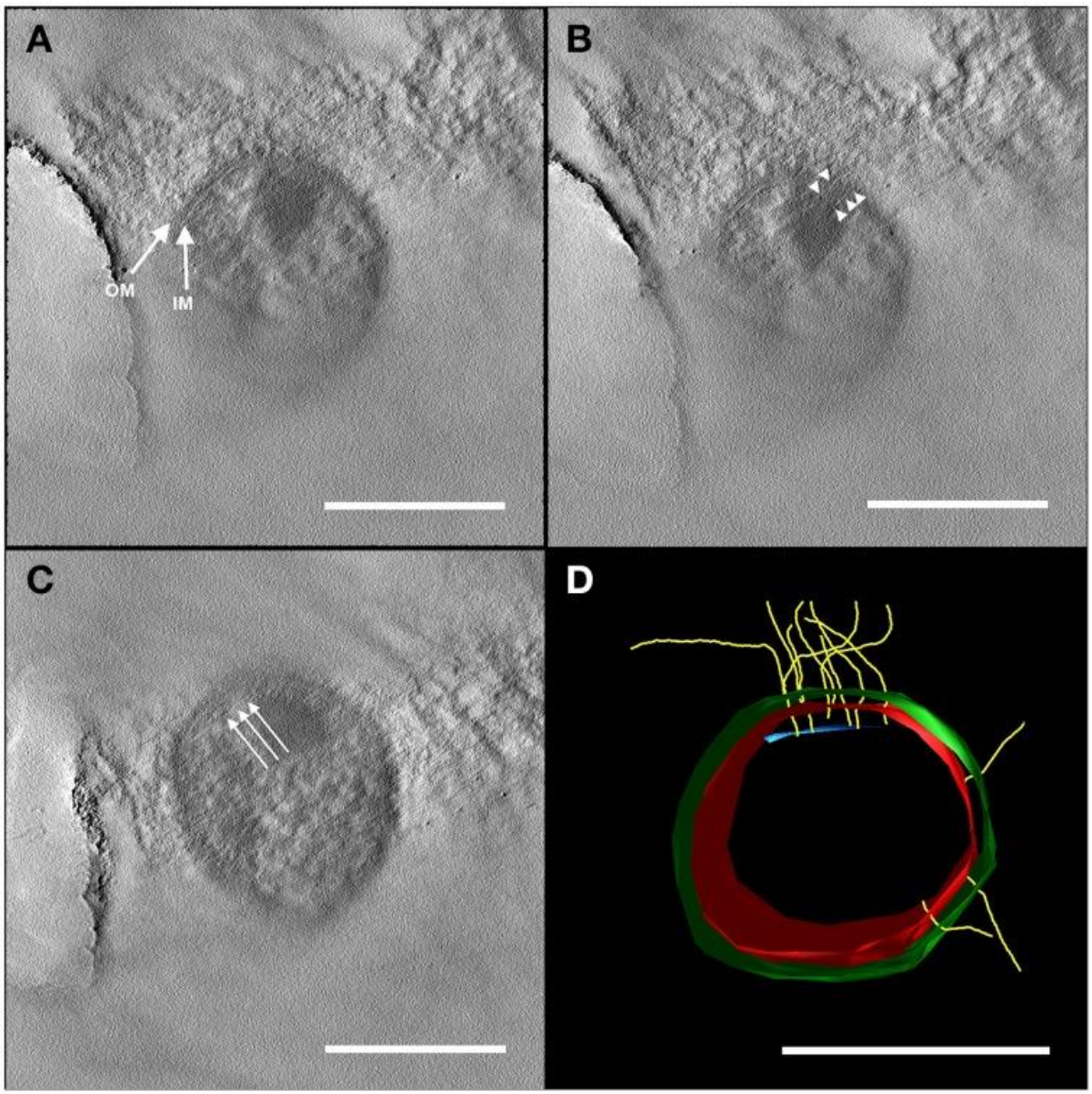

Figure III.2-2 Electron tomography of a 50 nm section of high-pressure frozen and freeze substituted cells**.** The tilt series was carried out at 120 kV from −60 to +60° with an increment of 2° and a final magnification of 14,000 x. The reconstruction of the tomogram was performed with IMOD. In selected virtual slices, several structures were indicated. **(**A**)** The final tomogram revealed the inner (IM) and outer membrane (OM) with a thickness of about 6 nm in each case. The overall thickness of inner and OM together with the periplasm was 44 nm. Therefore, the periplasm has a width of about 32 nm. **(**B**)** Further, two hami filaments with a diameter of approximately 8 nm were indicated spanning both the IM and OM (arrow heads). (C) Underneath the IM, another layer could be recognized (arrows). As the resolution of the tomogram is quite low, this could either be

kind of an anchoring structure/mechano sensor or a preparation/reconstruction artifact as it was just visible in a very low number of the virtual slices of the tomogram (continues on next page). With this tomogram, a model (segmentation) was constructed **(**D**)** illustrating the IM (red) and OM (green), the hami filaments (yellow) and the supposed anchoring-associated structure (blue). For simplicity, the membranes were illustrated as a monolayer. Scale bars: 500 nm.

Electron cryo-tomography of hami several µm away from the cell surface (Figures III.2-2, 3) revealed the typical barbed-wire structure as seen in micrographs of negatively stained samples (Figure III.2-4A; Moissl et al., 2005; Perras et al., 2014; Probst et al., 2014b).

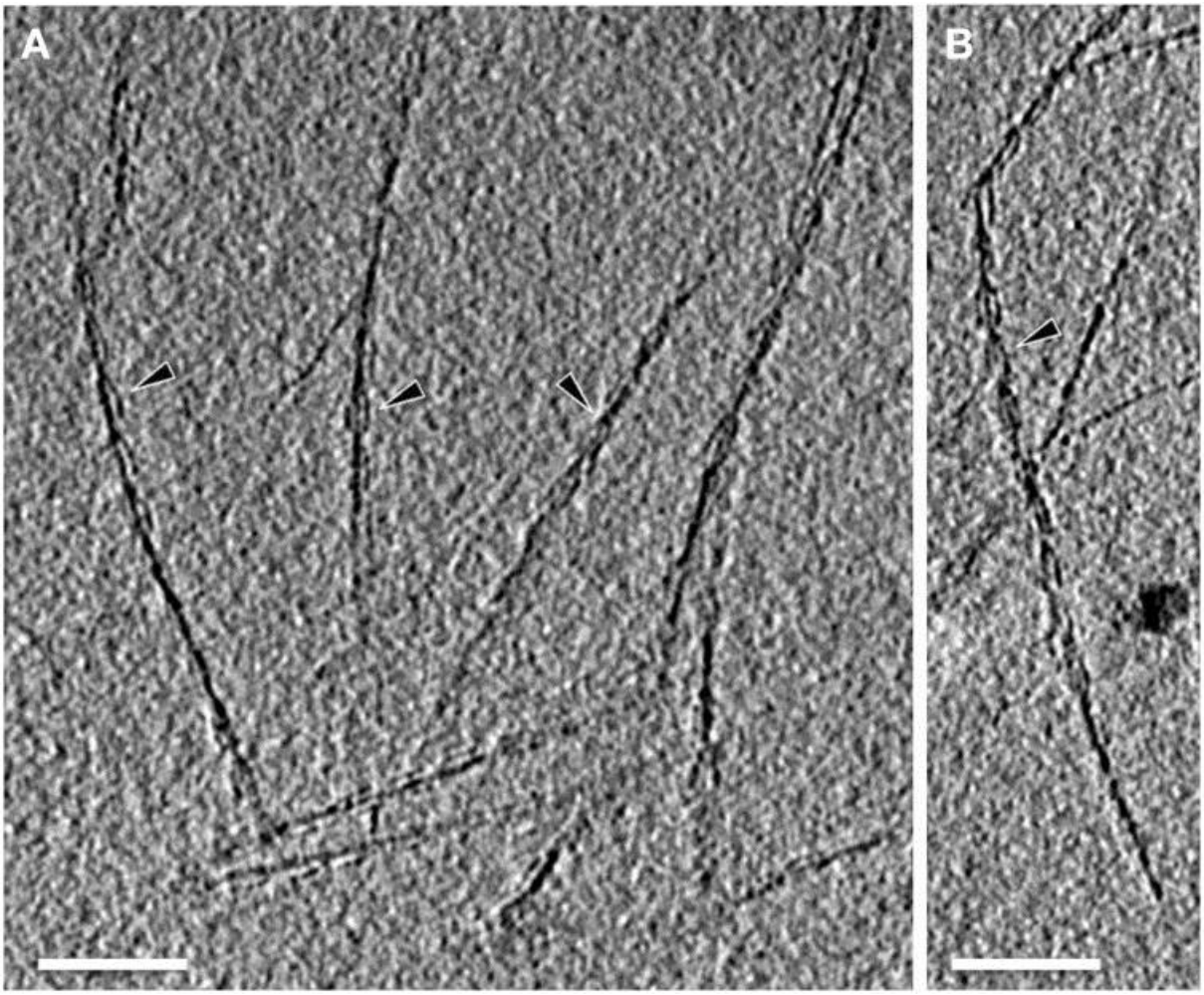

Figure III.2-3 Electron cryo-tomography of the hami**.** Tomographic slices through hami emanating from the cell surface. Hami occur as single barbed wire-like filaments (A,B; black arrowheads). Scale bar: 100 nm.

In the tomograms, the hami showed a repeating pattern of prickle triplets at an average center-to-center distance of 47 nm. Each prickle revealed an average length of 36 nm and emanated from the backbone of the filament. However, hami missing the typical barbed-wire pattern were also observed (Figures III.2-4A, B). They were partially plain filaments and could be identified as hami by their dimensions and typical hook (Figure III.2-4A).

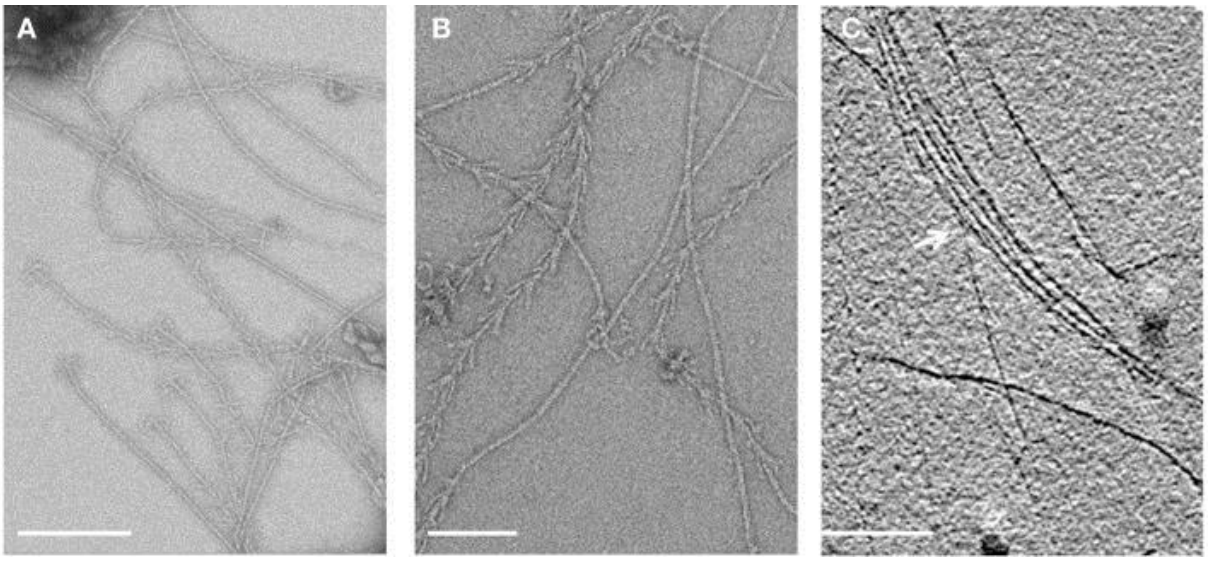

Figure III.2-4 Electron micrographs of isolated hami**.** (A,B) Overview of negatively stained hami with visible triple grappling hooks. The typical barbed-wire structure of the filament is shown, next to plain (probably) stretched filaments. Electron cryo-tomography shows the bundling of the hami (C, white arrow). Scale bar: 500 nm.

Apart from individual hamus filaments, bundles of such filaments were found (Figure III.2-4C), suggesting that they are capable of forming super-filaments that are interlinked by the prickles. Tomograms of the cell body showed a plethora of filaments emanating from the cells (Supplementary Movies 3, 4). Most of these filaments showed the barbed wire-like structure typical for hami. Due to the thickness of the cell body, filaments passing the membranes could not be clearly resolved

The major hamus subunit protein is encoded by one of six homolog genes**.** Purified hami were found to be composed of one major protein ("120 kDa protein"; Moissl et al., 2005). The antibodies generated against purified hamus filaments showed a strong and specific reaction against the surfaces of coccoid *Ca*. A. hamiconexum cells within the biofilms (Figure III.2-5).

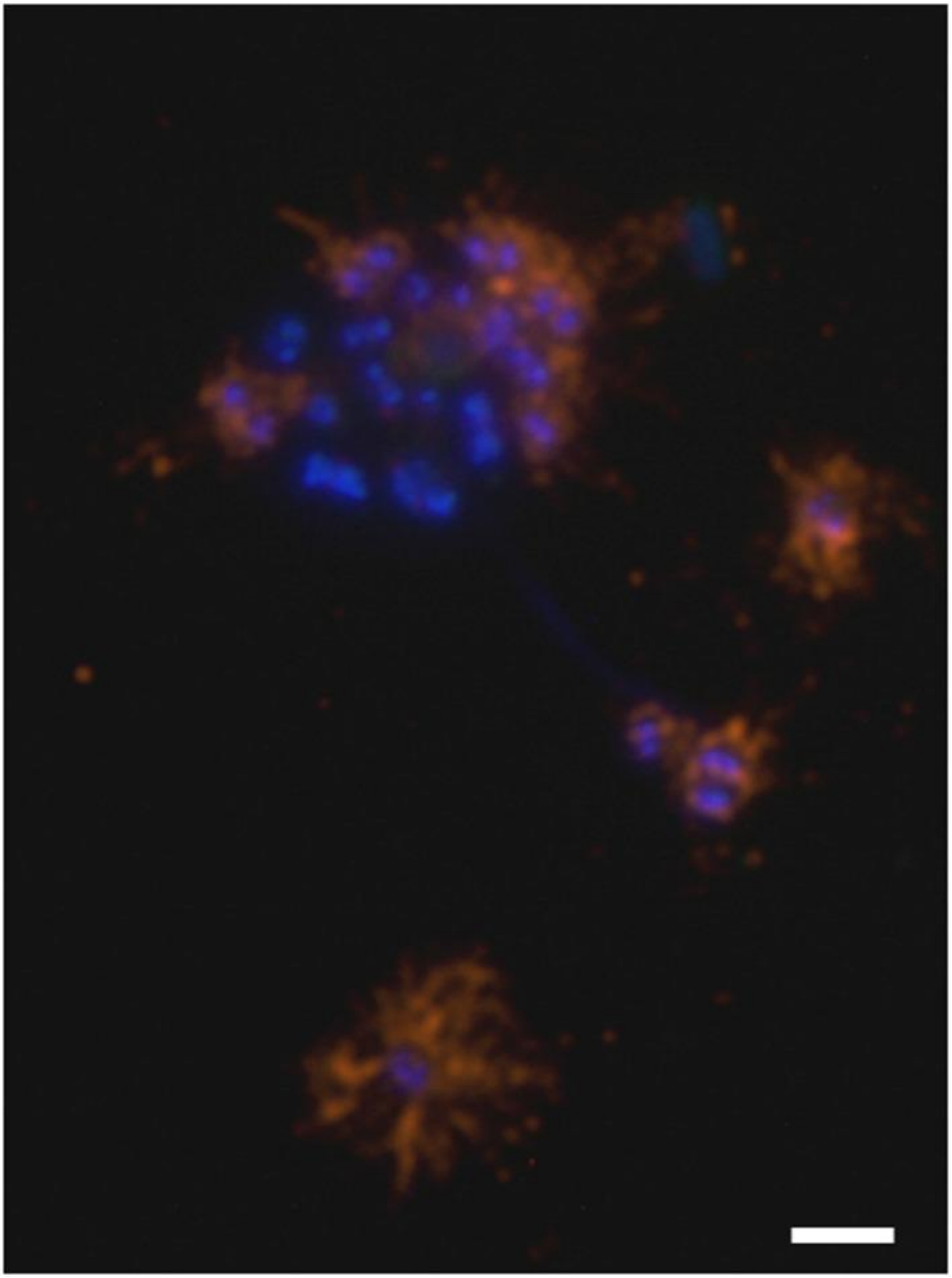

Figure III.2-5 Immuno-staining of cells embedded in the archaeal biofilm**.** DNA is stained blue (DAPI) and hami were visualized using a CY3-labeled anti-hamus antibody (orange). The hami formed a halo around the cell. Scale bar: 2 μm.

No signal was obtained from (filamentous) bacteria, occasionally enclosed in the biofilm. The same antibodies were used for a Western blot immuno-assay, showing a clear, strong signal

appearing on the gel—the previously identified "120 kDa protein" (Figure S5; Moissl et al., 2005). The LC-MS/MS fingerprint from this protein was compared with the metagenomic information of *Ca*. A. hamiconexum retrieved during a recent study (Probst et al., 2014b). Six homologous open reading frames (ORFs) potentially encoding for the major hamus protein were identified within the metagenomic data set. Supported by transcriptomic data (the retrieved RNA sequence revealed 100% identity in sequence and length), ORF MSIBFv1_A321004 was identified to code for the transcribed hamus subunit protein. This ORF was located on a relatively small contig (~5,600 nucleotides), containing only five ORFs in total. Transcription of the other homologous hamus genes could not be proven.

***The hamus protein carries glycosylation sites and an S-layer-like N-terminal region*.** The identified hamus ORF MSIBFv1_A321004 coded for an acidic, soluble 97 kDa protein (hydrophobicity: −0.28, PI 5.18) with major components Gly (9.71%) and Thr (9.49%). A sec-signal peptide was predicted (amino acid (aa) 1-27; Probst et al., 2014b), and in the same region of the gene, in congruence, TMHMM predicted a transmembrane helix (aa: 7-29). This sequence was predicted to be hydrophobic and charged positively (pI: 10.51). At least seven potential glycosylation sites were proposed for the hamus subunit protein. Thus, the discrepancy between the protein mass estimated from SDS-PAGE (120 kDa) and the gene-predicted mass (97 kDa) resulted most likely from post-translational glycosylation of the protein (Moissl et al., 2005).

The hamus subunit gene (ORF MSIBFv1_A321004) shared the contig with four additional ORFs (Figure S6), encoding for two proteins of unknown function, a glutamate-tRNA-ligase (closest related protein from *Methanobacterium formicicum*, DSM3637) and an acylphosphatase, with highest similarity to *Korarchaeum cryptofilum* (strain OPF8) acylphosphatase. One of the unknown proteins belongs to the TraB family, the other shows partial similarities (32%) to a *Sulfolobus solfataricus* (strain ATCC 33909) putative UDP-N-acetylglucosamine-dolichyl-phosphate N-acetylglucosamine-phosphotransferase.

Regarding the complete hamus subunit protein sequence, NCBI NR blastp search (Altschul et al., 1990) revealed no homologous sequences. However, three different architectures to be remotely related to parts of the protein sequence were revealed by the conserved Domain Architecture Retrieval Tool (http://www.ncbi.nlm.nih.gov/Structure/lexington/docs/cdart_about.html): Archaeal S-layer proteins, hypothetical proteins and acetyl-transferases. Patterns attributed to archaeal S-layer proteins were observed mainly at the N-terminal region of the protein ["S_layer_N": S-layer like family, N-terminal region (pfam 05123, aa 5-81) and "S_layer_MJ": S-layer protein, MJ0822 family (TIGR 01564, aa 5-98)]. Closest relatives were found to be N-terminal regions of S-layer proteins from *Methanotorris igneus* (WP_013799875.1; 47% identity, *e*-value: 6e-05; 56.6 bits), *Methanothermococcus thermolithotrophicus* (CAC83952.2; 44% identity, *e*-value 5e-04, 53.9 bits) and *Thermococcus eurythermalis* (AIU70131.1; 45%; *e*-value: 7e-04; 53.5 bits). In contrast to these and other known S-layer proteins, the hamus subunit-protein did not exhibit a typical S_layer_C- terminus pattern.

***The hamus protein exhibits a prominent β-sandwich fold and thus structurally resembles typical archaeal S-layers*.** The secondary structure prediction program PSIPRED identified about 56 beta strands connected by loops and flanked by helical segments in some cases (Figure S7). The first 110 amino acids showed sequence homologies to S-layer proteins from methanogenic Archaea (*Methanococcus voltae*, *Methanococcus maripaludis*,

Methanothermococcus okinawensis, and *Methanocaldococcus vulcanius*). None of these homologous S-layer proteins have yet been investigated structurally and they do not show any sequence identity to solved structures of S-layer proteins, from bacterial *Clostridium difficile* [Protein Data Bank (pdb) entry 3cvz; Fagan et al., 2009,], *Clostridium thermocellum* (pdb entry 4qvs) or archaeal S-layer proteins from *Methanosarcina acetivorans* (pdb entry 3u2h, 1l0q; Arbing et al., 2012). Therefore, it was not possible to find a suitable template for reasonable homology modeling just by using a BLAST algorithm.

Putative structural conservation patterns compared to the archaeal S-layer proteins were investigated by applying the multi-sequence alignment program MAFFT in combination with the topology prediction provided by PSIPRED (pdb entry 3u2h). A parallel overlap between sequence and topology conservation to the DUF1608 domain in the S-layer protein from *M. acetivorans* was identified, when only parts of the hamus protein sequence (Ala334-Asp665) were searched. The *M. acetivorans* S-layer protein MA0829 comprises 671 aa and, similar to the hamus protein, has an N-terminal signal peptide. In addition, it contains the tandem-duplicated DUF1608 domains exclusively found in methanogenic Euryarchaeota.

In a second step, a PSIPRED prediction combined with a fold recognition search (pGenThreader) was performed, which can be applied to individual protein sequences. Three hits were indicated with a p-score of 10^{-8} and an overall coverage of more than 50%. Interestingly, none of the proposed proteins were associated with S-layer proteins: (1) a cytoplasmic response regulator of two-component system, which controls heparin and heparan sulfate acquisition and degradation in the human gut symbiont *Bacteroides thetaiotaomicron* (pdb entry 4a2l; Lowe et al., 2012), (2) a xyloglucanase from *C. thermocellum* (pdb entry 2cn3 Martinez-Fleites et al., 2006), and (3) a human DNA-damage binding protein (pdb entry 3ei3 Scrima et al., 2008).

We have performed homology modeling based on the first three hits of pGenThreader (Figure III.2-6).

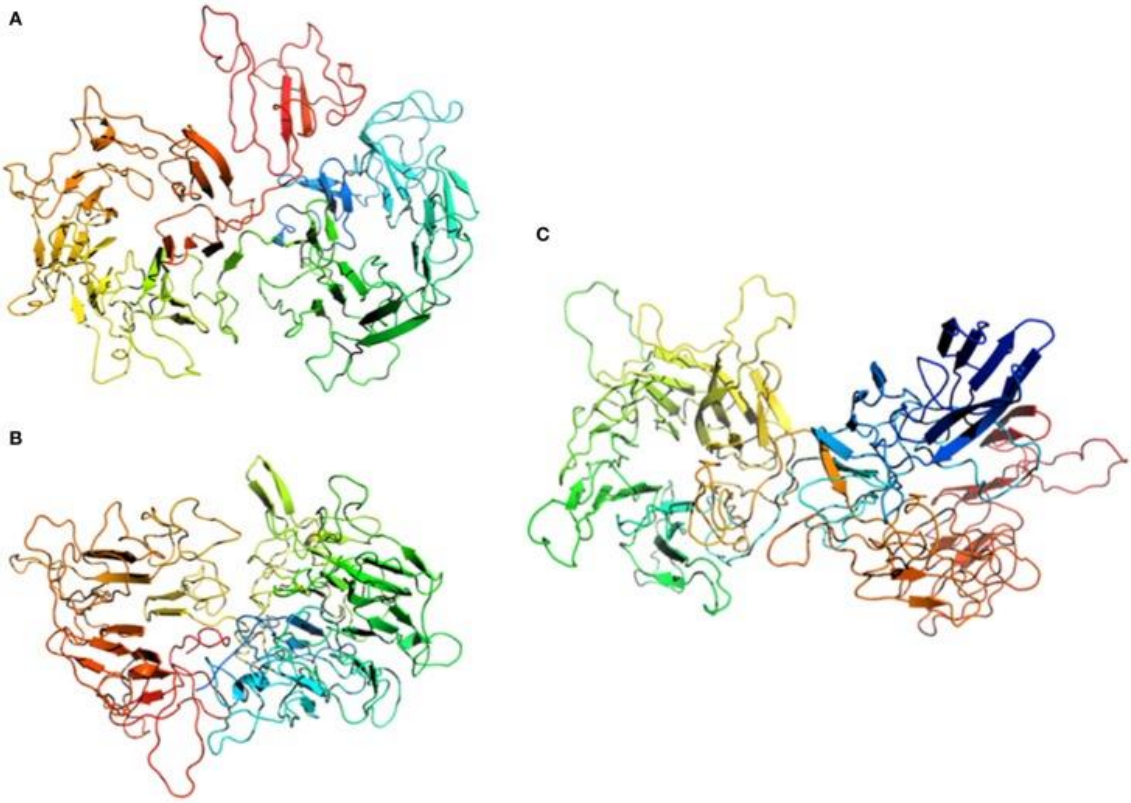

Figure III-2-6 Homology modeling of the Hami sequence on (A) a cytoplasmic response regulator of two-component system (pdb entry 4a2l), (B) a xyloglucanase from *Clostridium thermocellum* (pdb entry 2cn3), and (C) a human DNA-damage binding protein (pdb entry 3ei3)**.** The models are displayed as cartoons colored in spectrum mode with the N-terminal region in blue and the C-terminal region in red.

In all three models the first 110 aa were removed. Two of the models (Figures III.2-6A,C) show a propeller-like assembly reminiscent of a WD40 repeat. Although not being suitable for homology modeling, also the lower ranking hits proposed by pGenTHREADER revealed a consistent picture with an always returning motif of the WD40 propeller domain, which is often seen in S-layer proteins (Veith et al., 2009; Klingl et al., 2011) and represents one of the most conserved domains for protein-protein interaction. In the model of the hamus protein based on the template of the DNA-damage binding protein (pdb entry 3ei3) this motif would cover the region from aa 230-600, while in the model based on the template of the xyloglucanase from *C. thermocellum* (pdb entry 2cn3) the WD40 like array would correspond to aa 440-800.

Discussion

The filamentous cell surface appendages of the cold-loving, uncultivated SM1 Euryarchaeon, *Ca*. A. hamiconexum (Probst et al., 2014b) are composed of one major protein species. The sequence of this hamus subunit protein did not show any homologies to currently known

proteins involved in microbial fiber-, pili-, flagella-, or archaella formation, but showed similarities to known archaeal S-layer proteins: Besides a typical S-layer N-terminus pattern, the hamus subunit protein was found to be slightly acidic and most likely highly glycosylated, similar to S-layer proteins from e.g., *Acidianus ambivalens* and *Metallosphaera sedula* (*Veith et al., 2009)*. In addition, the hamus subunit protein revealed a prominent beta sheet topology and thus might be primed for self-assembly)Makabe et al., 2006). Previous modeling of beta-rich structures has shown that conformational diversity over a large number of repeats can lead to significantly different self-assemblies therein (such as the formation of fibrils, films, and ribbons) and that their final structure is determined by the way inherent flexibility is maintained via beta-sheet twists and bends (Makabe et al., 2006).

Although there is a remarkable lack of sequence similarity between archaeal S-layer proteins, which also limited the modeling possibilities, β-sandwich structures are obviously typical features of euryarchaeal S-layers (Arbing et al., 2012) and of S-layers from more distantly related archaea, such as Sulfulobales (Veith et al., 2009). Moreover, it is likely that the β-sandwich domains are structurally related to other proteins associated with enveloping functions not only in archaea but also in bacteria, fungi, and viruses, emphasising the general principle and self-assembly nature of beta-sheet rich proteins (Arbing et al., 2012). In general, S-layer proteins (with a size range between 40 and 210 kDa; Sleytr et al., 1997; Sára and Sleytr, 2000; König et al., 2007) are able to self-assemble into different lattices of oblique, tetragonal or hexagonal architecture—and can even exhibit complex, unusual structures, such as the tetrabrachion of *Staphylothermus marinus* (Engelhardt and Peters, 1998). This S-layer associated protein complex possesses umbrella-like thread morphology and distal branched quadrupled arms (Peters et al., 1995). The S-layer itself depicts p4-symmetry and the long stalks, which anchor the protein in the cytoplasmic membrane, form a 70 nm wide pseudoperiplasm. Furthermore, this membrane anchor is associated with a protease (STABLE; Peters et al., 1995), which might have a metabolic function for this species. Although the resemblance of tetrabrachion and hami appears striking, no similarity between both proteins could be shown on structural or sequence level.

The assembly and secretion process of bacterial and archaeal S-layers appears multifarious and obviously has evolved independently in some, even closely related microorganisms, such as *Aeromonas* species (Pugsley, 1993; Noonan and Trust, 1995; Thomas, 1995; Wattiau et al., 1996). By secreting the premature protein into the periplasm, multimerization of the mature proteins in the cytoplasm is prevented.

This mechanism of translocation strongly resembles the formation of type IV pili (Boot and Pouwels, 1996), where pilin precursors are inserted into the cytoplasmic membrane using the Sec translocation pathway (Arts et al., 2007; Francetic et al., 2007). After cleavage of the positively charged signal peptide the highly hydrophobic N-terminus of the mature pilin is exposed and provides a scaffolding interface for the assembly of the entire pilus structure (Bardy et al., 2003; Ng et al., 2007; Albers and Pohlschröder, 2009). A similar process was proposed for the formation of the hamus filaments (Probst et al., 2014b).

Although the hamus seems to be formed by one major protein, the presence of other proteins involved in its assembly cannot be excluded. Even supposedly simple systems, such as bacterial type IV pili, are usually composed by several pilins and require a certain set of membrane-associated proteins at the basis of the pilus structure (Mattick, 2002; Craig et al.,

2004; Nudleman and Kaiser, 2004). Additional hamus-associated proteins could possibly be identified via future co-immunoprecipitation assays, which could then help to understand assembly procedure of the hami and their potential function.

Possible functional traits of the hamus subunit protein were revealed by our combined PSIPRED prediction with a fold recognition search, which revealed three hits, a cytoplasmic response regulator, a xyloglucanase or a human DNA-damage binding protein. Although a functional relation to the DNA damage surveillance proteins serving in the initial detection of UV lesions *in vivo* is difficult to draw for the hamus protein, the first two fold-homologs can be prudently associated with a functional relationship. Xyloglucanases, for instance, hydrolyze polysaccharides from the cellulose microfibrils in plant cell walls (Hayashi and Kaida, 2011). The enzymatic reaction is central to the plant carbon cycle and might also indicate a role of the hami for cell wall degradation and/or carbon metabolism of *Ca*. A. hamiconexum.

Interestingly, the structure of the cytoplasmic response regulator revealed a substantial conformational change on ligand binding and signal transduction, which results in a scissor-like closing. This conformation resembles the barb-like assembly at the hook of the hamus fibril structure, indicative of a signaling role in cell-cell interactions—a possible function of the hami, which had been discussed earlier (Perras et al., 2014). To date it is unclear, whether the hami are involved in other processes apart from cell attachment and biofilm formation. In particular, the anchorage and organization of the hami within the cell wall could not further be resolved using electron cryo-tomography, although it remains without doubt that the hami span both membranes.

Due to the high similarity of the N-terminal amino acid sequence of the hamus subunit to known archaeal S-layer N-termini, one could even hypothesize about a divergent evolution of the hamus subunit protein from ancestral cell surface proteins and thus a conversion of a layered structure toward a filamentous arrangement—concomitant with a loss of the original surface layer and the development of a second membrane. However, it remains elusive if the two membranes of *Ca*. A. hamiconexum are different in their organization and whether a structural and compositional adaptation of the outer membrane has occurred due to the lack of an external S-layer. TOF-SIMS has confirmed that the membranes are mainly composed of diglycosidic diethers (C_{20}-C_{20} archaeol and C_{20}-C_{25} extended archaeol; Probst et al., 2014b). No clear indications of tetraether lipids are observed in the SIMS mass spectra, although trialkyl lipids may be present. Similar to the sodiated series of mono-, di- and tri-glycosylated diether lipid peaks revealed in this study are those of halophilic archaea, *Haloarcula marismortui*, by LC-atmospheric pressure ionization mass spectrometry (de Souza et al., 2009) .

In recent years, the field of nanobiotechnology advanced tremendously and is providing an increasing number of strategies to apply natural biomolecules in nanotechnology. For instance, spider silk proved to exhibit extraordinary properties such as strength, elasticity, biocompatibility, and biodegradability and is thus of major interest in nanobiotechnology (Gerritsen, 2002). The spider silk protein could be used in biomedical applications such as coating of implants and drug delivery or scaffolds for tissue engineering (Schacht and Scheibel, 2014). However, due to limited availability, experiments with natural spider silk proteins proved complicated (Fox, 1975) and thus the recombinant production of

engineered spider silk proteins was pushed forward (Heidebrecht and Scheibel, 2012), including testing of several protein expression systems, with different results in protein yield and property (Chung et al., 2012). Overall, the recombinant expression of the spider silk proteins took researchers decades until a satisfactory result was obtained. Since the hamus subunit protein and the spider silk protein share common features, such as elasticity, robustness and high molecular weight (hami: 97 kDa; Moissl et al., 2005; spider silk protein 250–320 kDa; Sponner et al., 2005; Ayoub et al., 2007), it is not surprising that preliminary overexpression attempts in *Escherichia coli* host strains failed so far. Recombinant expression of the spider silk protein resulted in insufficient yield, as conventional expression strains lacked the capacity of expressing proteins with high molecular weight (Chung et al., 2012) . This obstacle was finally overcome by a metabolically engineered *E. coli* expression host, which overexpressed and assembled the silk protein into a strong fiber (Xia et al., 2010). We suggest that a similar approach may be applicable to successful recombinant expression of the hami, which would pave the way for its exploitation in various fields of nanobiotechnology.

The results presented in this communication emphasise the uniqueness of the altiarchaeal hami: their major protein revealed no similarities in sequence and structure to known microbial filament-forming proteins, but showed relationship to archaeal S-layers (in sequence) and beta-sheet protein complexes (in structure), that are widely found in classical macromolecular self-assembled structures. Thus, the hami and the altiarchaeal cell wall (with two membranes and without S-layer) could represent a divergent form of cell organization or even a missing link between euryarchaeal ancestors and the current forms of euryarchaeal life.

Conflict of interest statement

Patent is pending on the “microbial nano-tool” (Pending European Patent Application No. EP15166985.0). This patent was filed jointly by the AKP, AJP and CME. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00543/abstract and on the supporting CD (./Selected publications/04 Supplementary files)

III.3. Resilient microorganisms in dust samples of the International Space Station – Survival of the adaptation specialists

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Abstract

The International Space Station (ISS) represents a unique biotope for the human crew, but also for introduced microorganisms. Microbes experience selective pressures such as microgravity, desiccation, poor nutrient-availability due to cleaning and an increased radiation level. We hypothesised that the microbial community inside the ISS is modified by adapting to these stresses. For this reason, we analysed 8-12 years old dust samples from Russian ISS modules with major focus on the long-time surviving portion of the microbial community. We consequently assessed the cultivable microbiota of these samples in order to analyze their extremotolerant potential against desiccation, heat-shock and clinically relevant antibiotics. In addition, we studied the bacterial and archaeal communities from the stored Russian dust samples via molecular methods (Next Generation Sequencing, NGS) and compared our new data with previously derived information from the US American ISS dust microbiome. We cultivated and identified in total 85 bacterial, non-pathogenic isolates (17 different species) and 1 fungal isolate from the 8-12 year old dust samples collected in the Russian segment of the ISS. Most of these isolates exhibited robust resistance against heat-shock and clinically relevant antibiotics. Microbial 16S rRNA gene and archaeal 16S rRNA gene targeting Next Generation Sequencing showed signatures of human-associated microorganisms (*Corynebacterium*, *Staphylococcus*, *Coprococcus* etc.), but also specifically adapted extremotolerant microorganisms. Besides bacteria, the detection of archaeal signatures in higher abundance was striking. Our findings reveal: (i) the occurrence of living, hardy microorganisms in archived Russian ISS dust samples, (ii) a profound resistance capacity of ISS microorganisms against environmental stresses and (iii) the presence of archaeal signatures on board. In addition, we found indications that the microbial community in the Russian segment dust samples was different to recently reported US American ISS microbiota.

Keywords: International Space Station, Microbiome, Confined habitat, Archaea, Extremotolerant

Introduction

The international space station (ISS) is a highly unusual working place. Completely sealed off from the outside, crews of three to ten astronauts and cosmonauts have routinely inhabited the modules since 2000 – as have billions of microorganisms. The ISS represents the most confined, man-made inhabited environment to date, characterized by radiation levels higher than on Earth, low nutrient levels due to reduced introduction of organic material, constant temperature (approx. 22°C), stable humidity (approx. 60%) and microgravity (Mora et al., 2016a).

The majority of the microorganisms detected on board the ISS are human-associated (as reviewed in Moissl-Eichinger et al., 2016a; Mora et al., 2016a) and the ISS microbiome thus resembles the microbiome of indoor environments on Earth (Coil et al., 2016). Next to human-derived (opportunistic) pathogens (Venkateswaran et al., 2014; Checinska et al., 2015), also technophilic microorganisms, which are able to corrode spacecraft materials (Novikova et al., 2001; Novikova, 2004; Alekhova et al., 2005; Alekhova et al., 2015), potentially inhabit the interior of the ISS. Therefore, the ISS microbial community is under constant surveillance to ensure the health of the human crew working on-board, as well as to evaluate potential risk factors for the integrity of the ISS materials and its function.

NASA (National Aeronautics and Space Administration) has identified that knowledge of the ISS microbiome is a major target for ongoing and future research studies. There is particular interest on the response of microbial communities to selective pressures such as microgravity, which could induce severe changes and adaptation processes (NASA, 2016).

Recent studies assessed the ISS dust microbiota using Next Generation Sequencing (NGS) techniques (Venkateswaran et al., 2014) and compared the microbial diversity with ground control samples (Checinska et al., 2015). The authors confirmed the anticipated composition of the ISS microbiota, as representing a typical human-associated community (Checinska et al., 2015). This observation appears logical due to the tremendous impact of the human microbiota on the environment (e.g. the human body spreads 10^6 bacteria per hour through breathing (Qian et al., 2012) and the severe restriction of other potential microbe sources. The major bacterial phyla detected by NGS-based methods were Firmicutes, Actinobacteria and Proteobacteria (Checinska et al., 2015), with the dominant genera *Corynebacterium* and *Propionibacterium*, representing typical human skin associated microorganisms (Grice et al., 2009). Besides NGS, cultivation-based approaches were also applied, which resulted in a high number of *Bacillus* and *Staphylococcus* isolates (Checinska et al., 2015).

Standardised monitoring of surface and air samples on board the ISS as well as more detailed post-flight investigations have been and are currently being conducted (e.g. NASAs Microbial Observatory Project). Another recent project, Merccuri (Microbial Ecology Research Combining Citizen and University Researchers on the ISS), studied 48 bacterial strains that were transferred from Earth environments to the ISS (Coil et al., 2016). For most of the cultures, the researchers found no significant change with respect to growth rate during the few days incubation time, except for *Bacillus safensis*, which grew 60% better in space than on Earth (Coil et al., 2016). However, selective pressures (desiccation, radiation, chemical and physical stresses) on board the ISS could cause an adaptation of the indigenous microbiota towards ISS conditions during a longer timeframe (Bruce et al., 2005; Ott et al., 2012).

The questions concerning a possible adaptation of microorganisms towards ISS stresses are addressed within an ESA flight project originally named “ARBEX” (Archaeal and Bacterial Extremophiles on board the ISS) now “Extremophiles” (Moissl-Eichinger et al., 2016a), which aims to analyze the adaptation processes of moderate and extremotolerant Bacteria and Archaea on the ISS. Thus far, Archaea have not been found in samples from the ISS (Checinska et al., 2015), but have frequently been detected in human-associated environments and clean rooms (Moissl-Eichinger, 2011), as they are integral part of the human skin and gut microbiota (Probst et al., 2013a; Gaci et al., 2014) and can therefore also be expected on board the ISS.

The ARBEX project focuses on the hardiest microorganisms inhabiting the ISS, and assessing their diversity and capabilities to resist certain stresses. For this study, we specifically selected dust samples from the Russian modules that were obtained 8-12 years ago, and stored since then under dried and sealed conditions on Earth. Targeting long-time survivors and spore-forming microorganisms, we consequently assessed the cultivable microbial community of these samples, in order to obtain model microbial strains that could be utilized in analyzing specific adaptation towards environmental stresses, such as desiccation and lack of nutrients. We analyzed these microorganisms with respect to their resistance to thermal stress and clinically relevant antibiotics. In addition, we assessed the bacterial and

archaeal communities from the stored dust samples via molecular methods (Next Generation Sequencing, NGS) and compared our new data with the previously derived information from the ISS microbiome (Checinska et al., 2015).

Materials and methods

Origin of samples. Extracts of different ISS samples obtained from the Russian Service Module of the ISS were provided by T. Alekhova and her team. Dust samples were retrieved during ISS-expedition 9 in October 2004 and during ISS-expedition 16 in April 2008: Sample 1: "Dust filter-1 (2004)", dust filter of ventilation system (internal abbreviation: RISS1); Sample 2: "Dust filter-2 (2004)", dust filter of ventilation system (internal abbreviation: RISS4); Sample 3: "Dust collector (2004)", from vacuum cleaner (internal abbreviation: RISS5); Sample 4: "Dust filter (2008)", dust filter of ventilation system (internal abbreviation: RISS3); Sample 5: "Dust collector (2008)", from vacuum cleaner (internal abbreviation: RISS2). During the entire time after retrieval, the vacuum cleaner bags and dust filters were stored sealed (never opened since sampling on the ISS), under dry conditions at ambient temperature. Culture controls done from an unused, sterile dust collector and dust filter were negative.

Dust Extraction protocol. For extraction, a 0.9% w/v NaCl solution was prepared using heat-treated NaCl (24 hours, 250°C, in order to degrade remnants of contaminating DNA) and autoclaved PCR-grade water (LiChrosolv, Merck Millipore). Three 5-10 cm^2 pieces of fabric were aseptically cut out of the vacuum cleaner bags and dust filters and submerged in 15 ml 0.9% DNA-free NaCl solution.

The fabric pieces in solution were then vortexed for 10 s, manually shaken for 15 s, ultra-sonicated at 40 kHz for 2 min and finally vortexed for 10 s to detach the dust from the fabric. The fabric was aseptically removed from the solution, and resulting suspension and solid fabric pieces were used for cultivation and molecular analyses.

Cultivation assays. The solid fabric was placed on aerobic R2A plates (pH7; BDH Prolabo®), whereas the homogeneous dust suspensions were used to inoculate different culture media in duplicates. The media used are given in Table III.3-1. Liquid media were inoculated once with 500 µl and once with 250 µl of the dust suspension and solid media were inoculated once with 200 µl and once with 100 µl of the dust suspension. Since the focus was to isolate bacterial and archaeal isolates, all aerobic media were supplemented with a final concentration of 50 µg/ml nystatin to suppress fungal growth.

Table III.3-1 List of used media and conditions

Medium	Phase	pH	Incubation temperature	Gasphase	Abbreviation	Target organisms	Medium reference
R2A agar pH5	Solid	5	30°C	aerobic (ambient)	pH5	Acidophiles	-
R2A agar pH9	Solid	9	30°C	aerobic (ambient)	pH9	Alkaliphiles	-
R2A agar pH7	Solid	7	30°C	aerobic (ambient)	pH7F	Heterotrophs	-
RAVAN pH7 for oligotrophs	Solid	7	30°C	aerobic (ambient)	RAV	Oligotrophs	Patolel and Phadnis, 2000 (modified*)
DSMZ_Medium97 for halophiles	Liquid	7.5	40°C	aerobic (ambient)	Halo	Halophiles	DSM 97 (www.dsmz.de)
R2A pH7 liquid	Liquid	7	30°C	N_2	pH7an	Anaerobes	-
Medium for methanogens	Liquid	7	40°C	H_2CO_2 (80:20)	MS	Methanogens	Balch et al., 1979
MS supplemented with 0.1% yeast extract and 0.1% acetate	Liquid	7	40°C	H_2CO_2 (80:20)	MS_sup	Methanogens	-
Archaea-supporting liquid medium	Liquid	7	30°C	N_2	ASM	Archaea	Stieglmeier et al., 2009**
ASM supplemented with 0.1% yeast extract and 0.1% acetate	Liquid	7	30°C	N_2	ASM_sup	Archaea	-
autotrophic all-rounder liquid medium	Liquid	7	30°C	N_2CO_2 (80:20)	AAM	Autotrophs	Stieglmeier et al., 2009**
autotrophic homoacetogen liquid medium	Liquid	7.5	30°C	H_2CO_2 (80:20)	AHM	Autotrophs	Stieglmeier et al., 2009**

*** 1:100 diluted, final concentration of 50 mg/l sodium pyruvate instead of 20 mg/l pyruvic acid**

**** Without addition of antibiotics**

Pure cultures were obtained via repeated dilution series in liquid medium and purification streaks on solid media. Positive enrichments of medium pH7 were transferred to anaerobic R2A plates and then purified by purification streaks.

Identification of isolates. Partial 16S rRNA genes of the isolates were amplified using the primers 9bF (5´- GRGTTTGATCCTGGCTCAG-3´) and 1406uR (5´- ACGGGCGGTGTGTRCAA-3´), applying the following cycling conditions: initial denaturation at 95°C for 2min, followed by 10 cycles of denaturing at 96°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 60s, followed by another 25 cycles of denaturing at 94°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 60s and a final elongation step at 72°C for 10 min (Stieglmeier et al., 2012). The template was either a small fraction of a picked colony in a colony-PCR assay or 5-20ng of DNA purified from culture via the peqGOLD Bacterial DNA Kit (peqlab, Germany). The 16S rRNA gene amplicons were Sanger-sequenced (Eurofins, Germany) and the obtained sequences were classified using the EzTaxon identification service at http://www.ezbiocloud.net/eztaxon (Kim et al., 2012).

The ITS sequence of one fungal isolate was sequenced using the primers ITS1F (5´-CTTGGTCATTTAGAGGAAGTAA-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) and following cycling conditions: initial denaturation at 95°C for 10min, followed by 35 cycles of denaturing at 94°C for 60s, annealing at 51°C for 60s, elongation at 72°C for 60s, and a final elongation step at 72°C for 8min. The amplicons were Sanger-sequenced (Eurofins, Germany) and the obtained sequence was classified using the EzFungi identification service at http://www.ezbiocloud.net/ezfungi/.

***DNA extraction of original samples and incubation experiment*.** After aliquots were removed for cultivation assays, the remaining dust suspension was centrifuged at 16000 g to concentrate the remaining dust particles and microorganisms, which were then re-suspended in three aliquots of 0.5 ml of the supernatant. One aliquot was directly frozen at -80°C, and one was treated with an end concentration of 50 µM propidium monoazide (PMA), to block free DNA of dead cells from downstream applications (Vaishampayan et al., 2013) before freezing.

One aliquot was mixed with 0.5 ml of pre-warmed 30°C LB medium and incubated at 30°C for 1.5 hr prior to direct DNA extraction with the aim of increasing the biomass and possibly triggering the germination of spores, which have been reported to resist state-of-the-art DNA extraction methods (Venkateswaran et al., 2014). DNA was extracted using the modified XS-Buffer method as described previously (Moissl-Eichinger, 2011). DNA concentrations were determined using Qubit, and DNA was afterwards subjected to PCR.

Molecular microbial diversity analysis using next generation sequencing methods. To investigate the detectable molecular diversity we used a "universal" and an Archaea-targeting approach. The 16S rRNA gene amplicons for the universal approach were amplified using Illumina-tagged primers F515 (5´-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGCCAGCMGCCGCGGTAA-3´) and R806 (5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGACTACHVGGGTWTCTAAT-3´; Caporaso et al., 2012). Archaeal amplicons were obtained via a nested approach (Probst et al., 2013a): First, a ~550 bp-long 16S rRNA gene amplicon was created via the primers Arch344F (5´-ACGGGGYGCAGCAGGCGCGA -3´) and Arch915R(5´-GTGCTCCCCCGCCAATTCCT -3´; Stahl and Amann, 1991; Raskin et al., 1994) and in a second PCR the amplicons for Illumina sequencing were generated by the tagged primers S-D-Arch-0349-a-S-17 (5´-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GYGCASCAGKCGMGAAW-3´) and S-D-Arch-0519-a-A-16 (5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TTACCGCGGCKGCTG-3; Stahl and Amann, 1991, using the purified product of the first PCR as template (Probst et al., 2013a).

The cycling conditions for the universal approach were: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturing at 94°C for 45s, annealing at 60°C for 60s and elongation at 72°C for 90s and a final elongation step at 72°C for 10 min. For the first PCR of the nested archaeal approach the cycling conditions were initial denaturation at 95°C for 2 min, followed by 10 cycles of denaturing at 96°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 60s, followed by another 15 cycles of denaturing at 94°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 60s, and a final elongation step at 72°C for 10 min. For the second amplification the cycling conditions were initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturing at 95°C for 40s, annealing at 63°C for 120s and elongation at 72°C for 60s, and a final elongation step at 72°C for 10 min.

Library preparation and sequencing were carried out at the Core Facility Molecular Biology at the Center for Medical Research at the Medical University Graz, Austria. In brief, DNA concentrations were normalized using a SequalPrep™ normalization plate (Invitrogen), and each sample was indexed with a unique barcode sequence (8 cycles index PCR). After pooling of the indexed samples, a gel cut was carried out to purify the products of the index PCR. Sequencing was done using the Illumina MiSeq device and MS-102-3003 MiSeq® Reagent Kit v3-600cycles (2x251 cycles).

Antimicrobial susceptibility tests. Nineteen of the isolates were selected for antimicrobial susceptibility testing and heat-shocks based on their phylogeny and on differences in phenotypical appearance. Antimicrobial susceptibility testing for selected, clinically relevant antibiotics (Table III.3-2) was performed using Etest® reagent strips (Biomérieux, Germany) according to instructions of the manufacturer. Since there were no species specific breakpoints available, MICs were interpreted according to EUCAST guideline table "PK/PD (Non-species related) breakpoints" (media/PDFs/EUCAST_files/Breakpoint_tables/v_6.0_Breakpoint_table.pdf, 2016).

Table III.3-2 Antibiotics used in this experiment (additional information: http://www.antibiotics-info.org/; Grayson, 2010).

Antibiotic substance	Type	Mechanism of action	Target group	Concentrations applied [µg/ml]
Amoxicillin/ clavulanic acid	β-lactam antibiotic (penicillin) and β-lactamase inhibitor	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.016-256
Ampicillin	β-lactam antibiotic penicillin	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.016-256
Cefotaxime	β-lactam antibiotic; Cephalosporin	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.016-256
Ceftriaxone	β-lactam antibiotic; Cephalosporin	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.016-256
Ciprofloxacin	Fluoroquinolone	Inhibits bacterial DNA gyrase; bactericidal	Gram+ and Gram- bacteria	0.002-32
Clarithromycin	Macrolide	Inhibits protein synthesis; bacteriostatic	Gram+ and Gram- bacteria	0.016-256
Clindamycin	Lincosamide	inhibits protein synthesis; bacteriostatic	Gram+ and anaerobic Gram- bacteria	0.016-256
Colistin	Polypeptide antibiotic; polymyxin	Attacks cell membrane; bactericidal	Gram- bacteria	0.016-256
Doxycycline	Polyketide antibiotic; tetracycline	inhibits protein synthesis; bacteriostatic;	Gram+ and Gram- bacteria	0.016-256
Gentamicin	Aminoglycoside	inhibits protein synthesis; bactericidal	Gram- and some Gram+ bacteria	0.016-256
Levofloxacin	Fluoroquinolone	Inhibits bacterial DNA gyrase; bactericidal	Gram+ and Gram- bacteria	0.002-32
Linezolid	Oxazolidinone	inhibits protein synthesis; bacteriostatic	Gram+ bacteria	0.016-256
Meropenem	β-lactam antibiotic carbapenem	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ and Gram- bacteria	0.002-32
Moxifloxacin	Fluoroquinolone	Inhibits bacterial DNA gyrase; bactericidal;	Gram+ and Gram- bacteria	0.002-32
Penicillin G	β-lactam antibiotic penicillin	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ bacteria	0.016-256
Trimethoprim/sulfamethoxazole	Dihydrofolate reductase inhibtor and sulfonamide	Inhibits tetrahydrofolate synthesis; bactericidal	Gram+ and Gram- bacteria	0.002-32
Vancomycin	Glycopeptide antibiotic	Inhibits cell wall synthesis; bactericidal against growing bacteria	Gram+ bacteria	0.016-256

In brief, overnight cultures (2-3 day cultures for slower-growing bacteria) were suspended in 0.9% saline to a turbidity of McFarland 0.5. 100 µl of this suspension was plated on standardized Müller-Hinton agar for antimicrobial susceptibility testing (Becton Dickinson). Etest® reagent strips were placed on the plates followed by aerobic incubation for 18 +/-2h at 34°C. Two strains were tested four times (in duplicate) on R2A pH7 and incubated for 48h at 34°C, because of their inability to grow on Müller Hinton medium and their intrinsic slow growth.

Heat-shock resistance test. The heat-shock test was carried out according to ESA standards (ECSS, 2008). This test is usually applied to quantify the bioburden of spacecraft, in order to identify hardy microorganisms that are potentially able to survive a spaceflight to other solar bodies. In brief, single colonies of 1-2 day old cultures were suspended in two test tubes containing 2.5 ml sterile phosphate buffered saline (PBS). As a control, one tube was kept at room temperature during the procedure. The other tube was placed in an 80°C water bath and exposed for 15 minutes. Samples were immediately cooled down on ice for 5 min after incubation time.

The temperature was monitored using a separate pilot tube containing 2.5 ml PBS also in the water bath. Afterwards 0.5ml of the heat-shocked suspension and 0.5ml of the room temperature suspension were plated and incubated at 30°C for three days (72 h).

Negative controls. Negative controls were performed thouroughly. Cultivation, extraction, PCR and sequencing controls were analysed in parallel with the processing of the samples. Cultivation controls were performed on two levels. First, the same, unused material (dust collector, dust filter material) as was used on the ISS was placed on cultivation medium. Secondly, extraction blanks were processed in parallel to the ISS material. All cultivation controls were negative (no growth of colonies). Extraction blanks used for DNA extraction, PCR and sequencing revealed a low number of ribosomal sequence variants (RSVs, see below). These RSVs were removed from all datasets, if present in the samples (the removed RSVs are highlighted in Supplementary Tables 1 and 2).

Bioinformatical analysis and data processing. Demultiplexed, paired reads were processed in R (version 3.2.2) using the R package DADA2 as described previously (Callahan et al., 2016a). In brief, sequences were quality checked, filtered and trimmed to a consistent length of ~270 bp (“universal” primer set) and ~140 bp (“archaea” primer set). The trimming and filtering was performed on paired reads with a maximum of two expected errors per read (maxEE=2). Passed sequences were dereplicated and subjected to the DADA2 algorithm to identify indel-mutations and substitutions. After merging paired reads and chimera filtering, the sequences were assigned to a taxonomy using the RDP classifier and the SILVA v.14 trainset. The visualization was carried out using the R package phyloseq (McMurdie and Holmes, 2013; Callahan et al., 2016b), metabolic pathways were predicted using the R package Tax4Fun (Aßhauer et al., 2015). Biostatistical analyses were performed using STAMP (Parks et al., 2014).

In contrast to previously described data processing pipelines such as QIIME (Caporaso et al., 2010) and mothur (Schloss et al., 2009) the DADA2 output Table was not produced based on a clustering step and thus no operational taxonomic units (OTUs) were generated. Each row in the DADA2 output Table corresponds to a non-chimeric inferred sample sequence, each with a separate taxonomic classification (ribosomal sequence variants; RSVs; Callahan et al., 2016a). In addition, the merging step occurs after denoising, which increases accuracy.

In order to compare our results to the recently published microbial community of US American ISS HEPA filter particulates and vacuum cleaner bag components (ISS Debris; Checinska et al., 2015) the protocol was changed as follows: Checinska et al. could not merge the forward and reverse reads using the software mothur (Schloss et al., 2009) and also the DADA2 approach, which we applied, did not result in a sufficient amount of merged sequences (data not shown). For the sake of comparability, we reanalysed the datasets of

Checinksa et al., 2015 containing dust samples (i.e. “ISS HEPA total”, “ISS HEPA viable”, “ISS Debris total” and “ISS Debris total”) and our dataset in parallel, by using only high quality forward reads (length ~130 bp, quality score: >30). This approach was in congruence with the data processing as described in Checinska et al., 2015.

For phylogenetic tree construction, the sequence data set was aligned and processed in MEGA 6 (Tamura et al., 2013). Alignment was minimized and cropped to the core area, on which tree calculation (maximum likelihood) was based on. The obtained tree and the data were visualized using iTOL (Letunic and Bork, 2007). The Venn diagram was created using the online tool InteractiVenn (Heberle et al., 2015) .

Data availability. Sequencing datasets as well as partial 16S rRNA gene sequences of bacterial isolates and ITS1 sequence of the fungal isolate were submitted to the European Nucleotide Archive and are publicly available. Sequencing datasets are assigned the study project number PRJEB14961 and samples are named according to internal abbreviations RISS1-5 as described in the chapter “Origin of samples”. The accession numbers assigned to the partial 16S rRNA gene sequences and ITS1 sequence are LT617056-LT617090 (http://www.ebi.ac.uk/ena/data/view/LT617056-LT617090).

Results

The International Space Station is an extreme working and living environment. It is completely sealed off from the outside and thus exhibits a unique combination of chemical and physical parameters that act on all abiotic and biotic matter. To date, the effect on the human body or on the microbial community therein is only sparsely studied. However, we hypothesise that the microbial community thriving and surviving inside of the International Space Station becomes adapted to desiccation and other stresses. For this reason, we analysed 8-12 year old dust samples from Russian ISS modules with respect to the cultivable portion of the microorganisms and the microbial community composition of these old samples. The retrieved microbial isolates were analysed with respect to their resistance towards heat-shocks and antibiotics. Overall, our data were compared to recently obtained results from present day ISS samples (Checinska et al., 2015).

Numerous bacteria survive long-term archiving of International Space Station dust samples. We applied a variety of different culture media to retrieve microbial isolates from ISS samples. The culture media supported slightly acidotolerant, alkalitolerant, and oligotrophic microorganisms, respectively, but also provided growth conditions for autotrophs and anaerobic microbes. Overall, 85 bacterial isolates were obtained (Table III.3-3) which could be assigned to eight genera. In spite of the nystatin applied to prevent fungal growth, we also obtained one single fungal isolate from “Dust filter-2 (2004)” on R2A pH9. The fungal isolate was classified according to its internal transcribed spacer (ITS) sequence as *Ulocladium botrytis.*

Table III.3-3 Number of bacterial isolates obtained on different cultivation media

Sample origin	Number of microbial isolates obtained on culture media							
	R2A pH5	R2A pH9	R2A pH7F	R2A pH7 anox	DSM97 „Halo“	MS_sup	ASM_sup	total
1: Dust filter-1 (2004)	5	0	3	0	0	0	0	**8**
2: Dust filter-2 (2004)	5	0	2	0	0	0	0	**7**
3: Dust collector (2004)	10	7	3	3	0	2	1	**26**
4: Dust filter (2008)	5	1	4	0	0	0	0	**10**
5: Dust collector (2008)	14	11	3	3	1	2	0	**34**
Total	**39**	**19**	**15**	**6**	**1**	**4**	**1**	**85**

The highest percentage of isolates (46%) was obtained from R2A agar with pH5, thus indicating a preference of slightly acidic growth conditions compared to pH9 (22%) and pH7 (18%). Additional growth was observed at pH7 under anoxic conditions, whereas only a few isolates were obtained under high-salt concentrations (18% NaCl, *Salinibacillus aidingensis*), or in liquid MS_sup or ASM_sup medium. Only 7 isolates were obtained from the "Dust filter-2 (2004)" sample, the highest amount of isolates was obtained from the "Dust collector (2008)" sample.

Overall, 34 bacterial isolates were found to be unique with respect to their 16S rRNA gene, which are displayed according to their phylogeny, preferred culture medium and sample origin in Figure III.3-1.

Notably, isolates from dust filter samples were non spore-forming Proteobacteria (*Cupriavidus, Methylobacterium, Bradyrhizobium*) and Actinobacteria (*Micrococcus*), whereas spore-forming species (Firmicutes, mainly *Bacillus* representatives) were isolated from dust collector samples only. These 34 different bacterial strains were assigned to 17 different species, which are listed in Supplementary Table 3, together with isolates obtained by Checinska et al. 2015.

ISS isolates were found to be resistant against desiccation, heat-shock and some common clinically applied antibiotics. All isolates obtained had been stored in dust/dust filters for at least 8 years before the cultivation experiments were performed. Since they were stored under dry conditions, all cultured strains can be assumed to be desiccation resistant.

For the heat-shock resistance and antibiotic susceptibility tests, 19 representative isolates were selected from our pool. Following the NASA and ESA guidelines for bioburden detection in clean rooms and on spacecraft (ECSS, 2008), cultures were exposed to a heat-shock (15 min, 80°C). This heat-shock is currently used by the named space agencies in order to determine the resistance to environmental stresses and to analyse whether microorganisms are possible candidates to survive space flight for planetary protection considerations. 16 out of 19 isolates survived the heat-shock treatment, as indicated in Figure III.3-1 (filled stars). All spore-forming microorganisms of the genera *Bacillus* and one *Paenibacillus* were able to survive the treatment, whereas *Methylobacterium*, *Cupriavidus* and *Micrococcus* could not be re-grown afterwards. Notably, a few cells (three colonies instead of bacterial lawn as observed for spore-formers and positive control) of

Bradyrhizobium, although representing a non-spore-forming Alphaproteobacterium, survived the heat-shock.

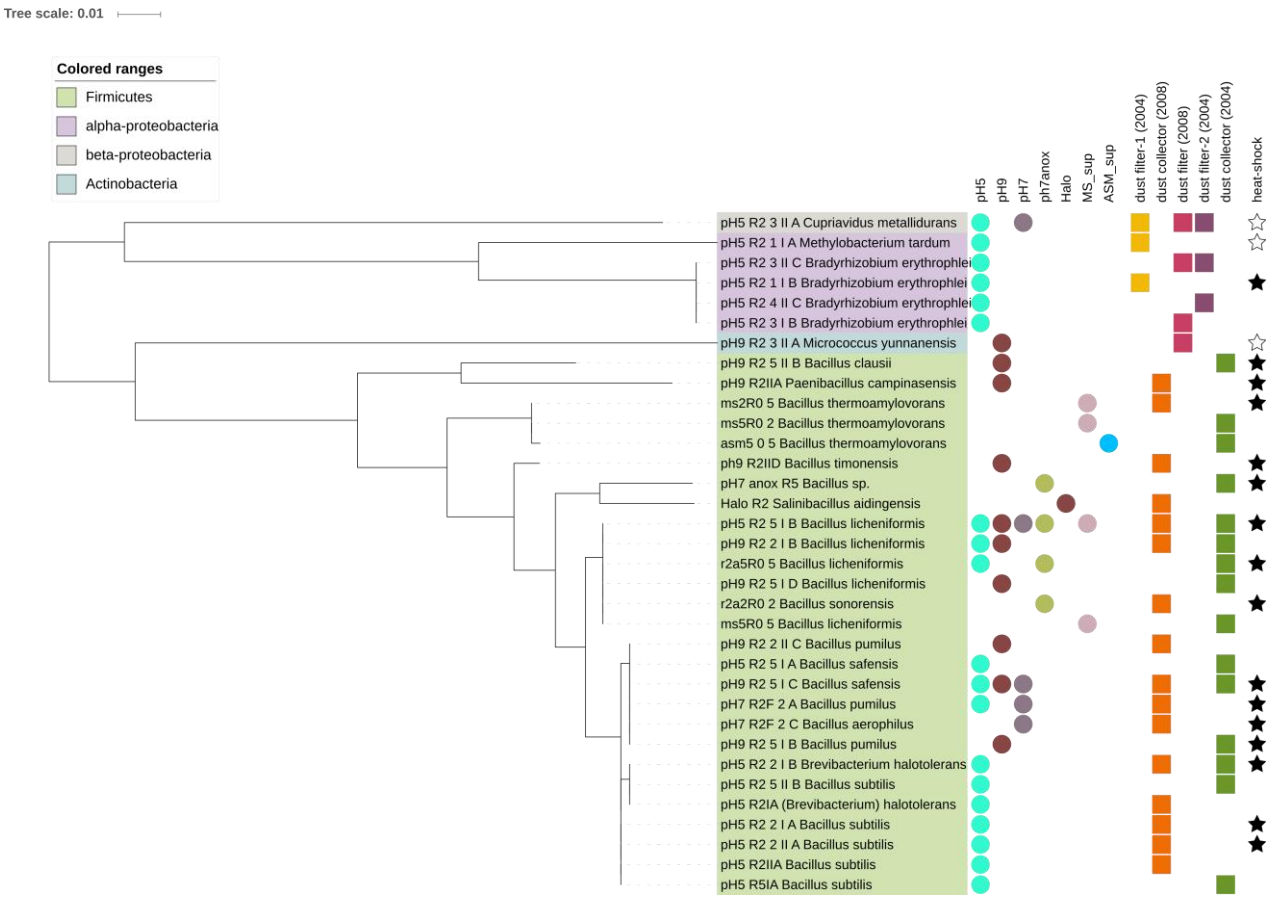

Figure III.3-1 Maximum-likelihood tree based on the unique 16S rRNA gene sequences of the ISS isolates. Circles indicate the medium they were cultivated in. Squares refer to the sample origin. Stars indicate the heat-shock resistance of the isolates (filled star: survived heat-shock at 80°C for 15 min; empty star: did not survive heat-shock; others were not tested). Tree was constructed using MEGA6 (Tamura et al., 2013) and displayed by iToL (Letunic and Bork, 2007).

Seventeen clinically relevant antibiotics (see Table III.3-4) were selected for susceptibility testing of the 19 selected microbial isolates. When applicable, the antimicrobial resistance was assessed using the most recent update of the EUCAST expert rules (www.eucast.org; Breakpoint Table for bacteria v6.0, January 2016). The results of the antibiotics-resistance tests are summarized in able III.3-2 (see also Supplementary Figure S1).

All strains were transferred to Müller-Hinton agar and antibiotic tests were performed using this medium, with the exception of two strains: *Methylobacterium tardum* and *Bradyrhizobium erythrophlei*, as they resisted growing under these conditions. For these two strains, we used R2A and an incubation time of 48 hours. Both strains revealed, under adapted conditions, robust resistances against numerous antibiotics. All strains revealed resistance against at least one antibiotic compound above the non-species specific EUCAST threshold, except *Micrococcus yunnanensis*.

Antibiotics / Isolate	pH5_R2_1_I_A *Methylobacterium tardum*	pH5_R2_1_I_B *Bradyrhizobium erythrophlei*	pH5_R2_1_II_A *Cupriavidus metallidurans*	pH5_R2_2_I_B *Brevibacterium halotolerans*	pH5_R2_2_I_A *Bacillus subtilis*	pH5_R2_2_II_A *Bacillus subtilis*	pH5_R2_5_I_B *Bacillus licheniformis*	pH7_R2F_2_A *Bacillus pumilus*	pH7_R2F_2_C *Bacillus aerophilus*	pH9_R2_3_II_A *Micrococcus yunnanensis*	pH9_R2_5_I_B *Bacillus pumilus*	pH9_R2_5_II_B *Bacillus clausii*	pH9_R2_5_I_C *Bacillus safensis*	r2a5R0,2 *Bacillus licheniformis*	r2a5R0,5 *Bacillus licheniformis*	ms2R0,5 Bacillus *thermoamylovorans*	pH7_anox_R2 *Bacillus licheniformis*	pH9_R2IIA *Paenibacillus campinasensis*	pH9_R2IID *Bacillus timonensis*
Max. Concentration: 256 µg/ml																			
Amoxicillin/ clavulanic acid	2	**256***	*1.5*	2	3	*0.5*	7	3	*0.25*	*0.75*	2	*0.19*	3	**16**	**12**	*0.19*	3	**256***	2
Cefotaxime	**12**	**256***	1	2	**8**	**16**	**64**	**256***	**128**	*0.75*	**16**	**32**	**256***	**24**	**32**	**32**	**256***	**12**	**32**
Ceftriaxone	1.5	**256***	*0.5*	1.5	*0.38*	**12**	**256***	**32**	**16**	*0.19*	**4**	**12**	**24**	**24**	**12**	**8**	**64**	**12**	**48**
Linezolid	**256***	**256***	**256***	*1*	*1*	*0.75*	*1*	2	*1.5*	*1*	*0.75*	*1*	2	*1.5*	*1*	*1*	2	**6**	*1*
Penicillin G	**3**	**256***	**3**	**8**	0.75	0.5	**256***	0.38	*0.064*	*0.094*	**256***	**24**	0.25	**24**	**6**	**4**	0.75	**24**	1
Ampicillin	**256***	**256***	*1.5*	2	3	**16**	**256***	2	*0.25*	*0.5*	*1.5*	**24**	2	**24**	8	2	3	**256***	2
Colistin	256*	256*	24	32	32	64	64	48	12	2	32	3	24	32	32	64	24	48	16
Clarithromycin	2	256*	2	0.094	0.064	0.064	0.38	0.125	0.064	4	0.064	256*	0.5	0.064	256*	256*	0.032	256*	0.032
Clindamycin	256*	256*	256*	0.5	0.75	0.5	12	3	2	0.25	24	256*	4	4	8	1	4	256*	2
Doxycycline	1.5	256*	0.064	0.047	0.094	0.19	0.25	0.19	0.094	0.25	0.064	0.25	0.125	0.125	0.25	0.75	0.38	0.125	0.19
Gentamicin	8	96	32	0.094	0.094	0.75	0.38	0.125	0.064	0.38	0.5	0.064	0.094	0.19	0.75	0.125	0.064	0.75	0.064
Vancomycin	256*	256*	0.75	256*	1	2	3	1.5	1	0.19	2	0.75	0.75	1	8	2	2	4	1.5
Max. Concentration: 32 µg/ml																			
Ciprofloxacin	**6**	**32***	*0.016*	*0.047*	*0.064*	*0.064*	*0.125*	*0.19*	*0.094*	0.75	*0.032*	0.75	*0.094*	*0.094*	*0.047*	*0.19*	*0.125*	*0.125*	*0.125*
Levofloxacin	**3**	**32***	*0.047*	*0.094*	*0.125*	*0.094*	*0.19*	*0.25*	*0.125*	1.5	*0.094*	2	*0.125*	*0.094*	*0.064*	*0.38*	*0.125*	*0.094*	*0.094*
Meropenem	**32***	**32***	*0.125*	*0.064*	*0.19*	*0.094*	*0.19*	*0.25*	*0.094*	*0.47*	*0.094*	*1*	*0.25*	**19**	**19**	*0.38*	*0.38*	3	*0.38*
Moxifloxacin	0.25	**32***	*0.023*	*0.016*	*0.047*	*0.023*	*0.032*	0.640	*0.016*	*0.38*	*0.008*	0.5	*0.023*	**16**	*0.012*	*0.032*	*0.025*	*0.047*	*0.023*
Trimethoprim/ sulfamethoxazole	32*	32*	0.064	0.125	0.19	0.19	0.19	0.004	0.004	0.5	0.032	0.047	0.094	0.032	0.032	0.094	0.006	0.75	0.004

Table III.3-4 Minimal inhibitory concentrations for the tested isolates.

Italic underlined: sensitive according to non-species related breakpoints of EUCAST; Bold: resistant according to non-species related breakpoints of EUCAST; * grew at the tested maximum concentration of the respective antibiotic; concentrations given in: µg/ml. Slow growing M. tardum and B. erythrophlei were tested on R2A medium with 2 days incubation time since they did not grow at all on MH medium.

We tested six different β-lactam antibiotics of which the cephalosporins cefotaxime and ceftriaxone as well as penicillin G were found to be most ineffective against the ISS isolates, since almost all microbial strains exceeded their resistance breakpoints or at least the intermediate breakpoints (see Table III.3-4 and Supplementary Figure S1A).

Molecular, NGS-based analysis revealed the presence of a broad bacterial and archaeal diversity. Aliquots of the same samples that were used for the cultivation approach were subjected to molecular analyses. We followed three different approaches. A) Samples were processed untreated, B) Samples were exposed to liquid growth medium (LB) for 1.5 h (30°C) in order to increase biomaterial and trigger spore germination (incubated samples), C) Samples were treated with propidium monoazide (PMA) to mask background DNA from disrupted cells (Nocker et al., 2007). PMA treated samples (C) did not reveal any signals after DNA extraction and PCR, using "universal" and archaea-targeting primer sets, although cultivation from these samples was successful. However, all samples that underwent the incubation treatment (B) resulted in reasonable PCR product yields. Untreated samples (A) resulted in positive archaeal amplicon generation for 4 out of 5 samples, namely "dust filter-1 (2004)", "dust collector (2004)", "dust filter (2008)" and "dust collector (2008); 3 out of 5 samples resulted in positive "universal" amplicon generation ("dust filter-1 (2004)", "dust filter (2008)", "dust collector (2008)").

"Universal" and "archaeal" amplicons were subjected to next generation sequencing (Illumina MiSeq). Raw reads were processed using DADA2. It should be noted that DADA2 does not perform a clustering step, thus does not produce operational taxonomic units (OTUs). Each sequence obtained corresponds to a unique taxonomic classification (ribosomal sequence variant; RSV).

In total, 203,667 high quality sequence counts were obtained of the four positive archaeal approaches (length >140 bp), representing 9 different RSVs. Amongst the four samples, the "dust collector (2004)" yielded the highest number of sequence counts (102,782). The "dust filter-1 (2004)" sample and the "dust collector (2008)" sample resulted in 71,203 and 29,600 archaeal sequence counts, respectively, whereas the lowest number was observed in the "dust filter (2008)" sample (82 sequence counts). As a consequence, the "dust filter (2008)" sample revealed the lowest richness, the lowest Shannon Index, and the lowest InvSimpson Index (Supplementary Figure 2). The highest archaeal richness was observed in the "dust filter-1 (2004)" sample (8 RSVs).

Overall, sequences assigned to Thaumarchaeota (*Nitrososphaera* sp.), Euryarchaeota (*Methanobrevibacter* sp.) and Woesearchaeota were found in the ISS samples (Figure III.3-2).

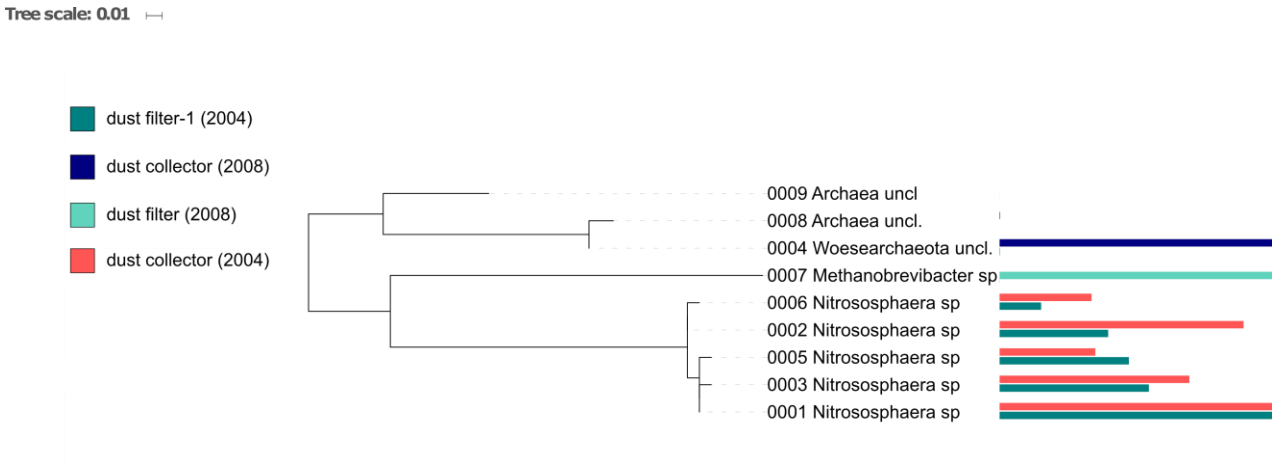

Figure III.3-2 Archaeal maximum likelihood tree: detected taxa in different samples of ISS.

Methanobrevibacter sequences could be detected in a very low abundance in "dust filter-1 (2004)" and also in "dust filter (2008)", where all obtained 82 sequence reads belonged to the genus *Methanobrevibacter* (see also Supplementary Table 4). Unclassified Woesearchaeota signatures were found in "dust filter-1 (2004)" at very low abundance (<0.1% of sample) and in "dust collector (2008)" with a very high abundance (>99.9% of sample; 14.5% of all archaeal sequence counts). *Nitrososphaera* signatures (Thaumarchaeota) were detected in two samples in a high abundance ("dust filter-1 (2004)" and "dust collector (2004)"), but were not observed in other samples. Furthermore, thaumarchaeal signatures were also detected in sequence data derived from amplicons produced with the universal primer pair. In particular, they were detected in untreated samples of "dust filter-1 (2004)", in agreement with the archaea-targeting approach mentioned above. 48.5% of the universal 16S rRNA gene sequences derived from this sample were assigned to Thaumarchaeota, soil crenarchaeotic group (SCG), with *Nitrososphaera* as the main genus. However, all other samples containing archaeal reads revealed only very low abundances (<1%). Those were mainly assigned to Euryarchaeota (Methanobacteria), represented by *Methanobrevibacter* (0.6% of incubated "dust filter-2 (2004)"), *Methanosphaera*, *Methanobacterium* or not further classified members of the Woesearchaea (0.5 % of "dust collector (2008)"; in congruence with the high amount of woesearchaeal reads obtained by the archaeal primer set for this sample). "Dust collector (2004)", the other sample with a high abundance of *Nitrososphaera* when sequenced with archaeal primers, did not deliver any sequences with the universal primer pair. In the incubated "dust filter-1 (2004)" and "dust collector (2004)" we could also not detect Thaumarchaeota with the universal primer set. In total, NGS based on the "universal" primer set generated 227,439 high quality sequences (Supplementary Table 5). Sequences obtained by the universal primer approach were classified using the SILVA database (Quast et al., 2013) and community composition was summarized (see Figure III.3-3).

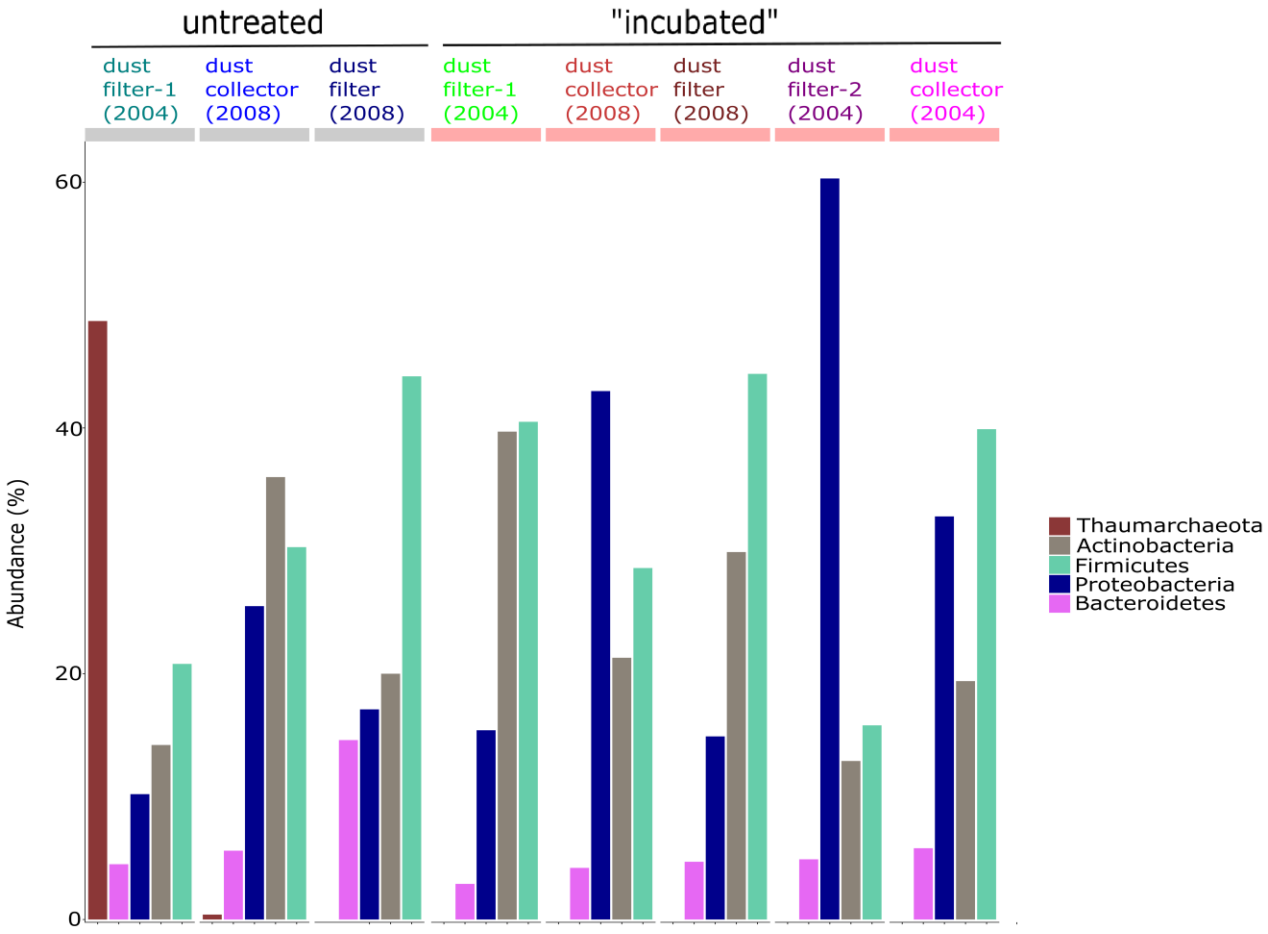

Figure III.3-3 Taxonomic profiles of the microbial communities from Russian ISS samples at phylum level. The five most abundant phyla are depicted. We discriminate between untreated and incubated samples. Total counts are given in % ("Abundance").

In the following, we distinguish between untreated and incubated samples, referring to treatment A) and B), respectively, as indicated above. In untreated samples, most signatures were assigned to phyla Thaumarchaeota (48.7%; "dust filter-1 (2004)"), Actinobacteria (36% in "dust collector (2008)") and Firmicutes (44.2% in "dust filter (2008)" sample)). In incubated samples, the dominant phyla were Actinobacteria, Firmicutes and Proteobacteria. Signatures of Bacteroidetes (lowest abundance in incubated "dust filter-1 2004" sample; highest abundance in untreated "dust filter 2008" sample) were also found in all samples. Further details are shown in Figure III.3-3.

At class level, the most abundant taxa were assigned to thaumarchaeal Soil Crenarchaeotic Group (SCG), Actinobacteria, Bacilli, Gammaproteobacteria and Betaproteobacteria. In incubated and untreated "dust filter (2008)" samples, there was no remarkable difference with respect to the microbial community composition. Five genera were identified whose abundance appeared significantly different in incubated and untreated samples (paired White Test, $p>0.001$,

confidence >0.95, see Supplementary Figure 3): *Facklamia* (Lactobacillales; higher abundance in untreated sample), *Coprococcus_1* (Clostridiales; higher abundance in untreated sample), *Leuconostoc* (Bacilli; higher abundance in untreated sample); *Coproccocus*_3 (higher abundance in incubated sample) and an unclassified member of the family *Ruminococcaceae* (higher abundance in incubated sample).

In total, we could identify signatures of 23 microbial genera shared by untreated and corresponding incubated samples (Figure III.3-4).

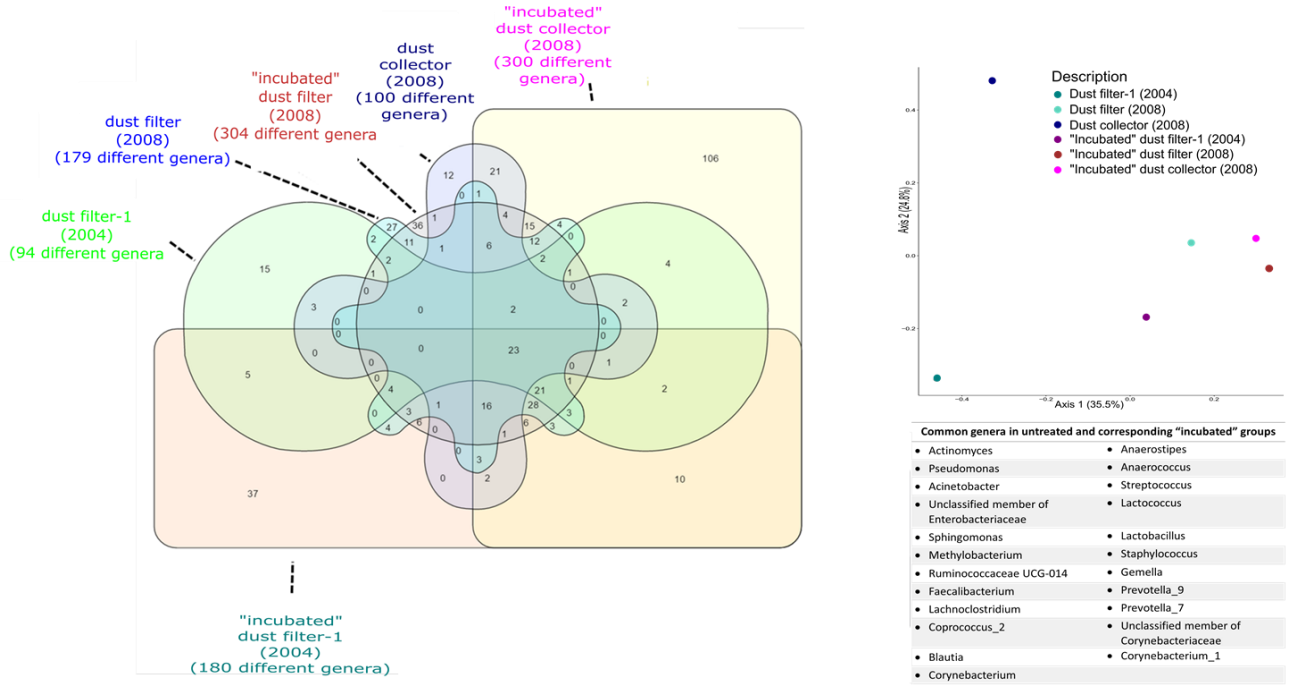

Figure III.3-4 Venn diagram depicting common genera in untreated groups and their corresponding incubated counterparts. In total, signatures of 23 genera were common in all 6 samples. The PCoA plot on the right side is depicting the dissimilarity between incubated and untreated samples using the unweighted Bray-Curtis distance. No clear cluster pattern is visible between the two groups.

These taxa were mostly assigned to Actinobacteria (4), Clostridia (6), Bacilli (5) and Alpha/-Gammaproteobacteria (2 and 3, respectively). To compare community composition among samples, a beta diversity matrix (i.e. Bray-Curtis distance (unweighted)) was computed and evaluated using principal coordinate analysis (PCoA, see Figure III.3-4). Untreated “dust filter (2008)” was found to reveal a similar microbial community composition as the incubated “dust filter (2008)” sample and the incubated “dust collector (2008)” sample. In contrast, the microbial community of the untreated “dust filter-1 (2004)” sample and the “dust collector (2008)” sample appeared to be distinct.

Comparison between culture-based microbial diversity and molecular analysis emphasizes the need of cultivation. For the comparison of culture-based microbial diversity with the overall microbial diversity, we focused on the 34 unique isolates. The 16S rRNA sequences of individual, unique isolates were compared pairwise with all Illumina sequences belonging to the same genus. The sequences were considered to belong to the same species if they exceded the similarity threshold of 99%. Almost all isolates could be retrieved in the sequencing results (see Figure III.3-5), but the isolates belonging to the genus *Bradyrhizobium* and *Salinibacillus* could not be detected in the sequencing pool.

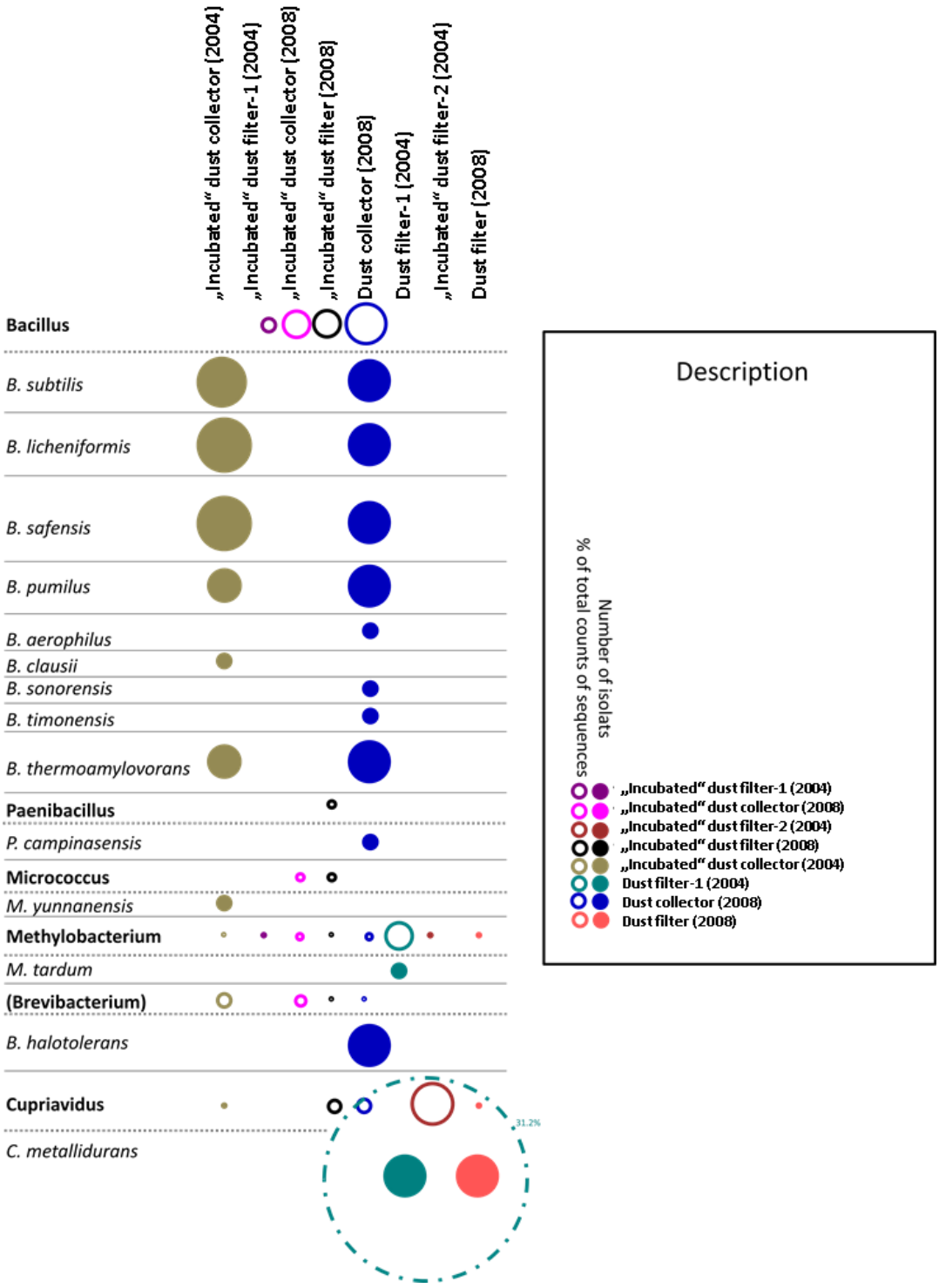

Figure III.3-5 Comparison of the number of retrieved isolates with molecular data. Donuts indicate retrieved sequences on molecular level, filled circles are indicative for cultivated isolates. The larger the donuts/circles are, the more counts of sequences/isolates were obtained. Every sampling site has a different color (see legend). If no circle/donut appears, no sequences/isolates were obtained. It has to be noted, that no NGS data for the untreated "dust collector (2004)" could be retrieved, however, isolates from this sample could be cultivated.

In general, most isolates could be obtained from the “dust collector (2004)” and the “dust collector (2008)”, whereas the highest counts of sequences could be obtained in the “dust filter-1 (2004)” (*Cupriviadus metallidurans*, 31.2% of all sequence reads). Remarkably, a high proportion of different *Bacillus* species could be isolated, in accordance with the total sequence count retrieved for all samples. However, the half of the isolates were cultivated out of the “dust collector (2004)”, where no sequences for *Bacillus* were detected at all. The non-conformity between sequencing data and isolated cultures is also true for species of *Paenibacillus* and *Micrococcus*. *Brevibaterium* signatures could be obtained in 4 of 6 sampling sites and *Brevibacterium* isolates could be cultivated out of one sampling site. *Methylobacterium*, in contrast, was detected throughout all sampling sites but could be isolated out of only one sampling site.

Differences between the microbial communities of archived ISS dust samples (this study) and freshly retrieved samples (Checinska et al., 2015). Very recently, a dataset of microbial community composition of comparably “fresh” dust samples from the US American modules of the ISS has been published (ISS Hepa filter particulares, vacuum cleaner bag components of ISS (ISS Debris); retrieved 2011 and 2012; Checinska et al., 2015). These data were retrieved and used for comparison by beta-diversity matrices (Bray-Curtis distance (unweighted); see also Supplementary Table 6).

We want to emphasize here, that for the sake of comparibility, we exclusively analysed the forward reads of each study, since in contrast to our study, the reads in the study of Checinska et al., 2015 were not mergeable. However, the PCoA plot in Supplementary Figure 4 shows a clear clustering of the US American dust samples (US-ISS; Checinska et al., 2015) and the Russian dust samples (RISS, this study), which indicates, despite insufficient possibility of data analysis, a certain dissimilarity of the microbial community in the two different ISS settings. In Table III.3-5, we compared the data derived from Checinska et al., 2015 with our dataset, containing merged and further processed forward and reverse reads. A deeper look into the overall community shows a clear difference in the abundance of the dominant phyla. First, in contrast to the previous study, we were able to detect archaeal sequences, mainly classified as Thaumarchaea. Second, although both studies detected Actinobacteria, Firmicutes and Proteobacteria as the dominant phyla in all samples, the mean abundance varied. The phylum Actinobacteria was observed to constitute ~64% of all samples in the US american ISS samples, whereas the older, archived russian samples only harboured ~24 %. In addition, the number of dominant genera was two-fold reduced (mean US-ISS: 58 genera, mean R-ISS (Russian ISS samples): 28 genera). In contrast, the average abundance of Proteobacteria was increased ~8.7 times (mean values US-ISS: 3.255%, R-ISS: 27.4%), however, the number of genera was higher in US American ISS samples (mean number of genera R-ISS: 44.125 and mean number of genera US American ISS: 69.78). The amount of classified Firmicutes sequences and genera was comparable in both US American ISS and R-ISS samples (sequences mean: 25.78% and 28%, respectively and mean number of genera 79 and 71.5, respectively).

Table III.3-5 Comparison of microbial community composition of US-American ISS dust samples Checinska et al., 2015 and Russian ISS dust samples (this study).

		US- American ISS dust samples				Russian ISS dust samples							
		ISS HEPA total	ISS HEPA viable	ISS DEBRIS	ISS DEBRIS viable	Incubated dust filter-1 (2004)	incubated dust collector (2008)	incubated dust filter (2008)	incubated dust filter-2 (2004)	incubated dust collector (2004)	Dust filter-1 (2004)	Dust collector (2008)	Dust filter (2008)
	Total number of reads	553,176	587,569	1,148,047	1,116,419	24382	61571	49956	7278	39289	15021	7599	22343
	Percentages of sequences of all dominant phyla (Archaea and Bacteria)	90.92	99.65	92.35	98.26	95.6	92.9	89.2	89	92.1	93.9	92.2	81.3
	Percentages of sequences of dominant bacterial phyla (without Archaea)	90.92	99.65	92.35	98.26	95.6	92.9	89.2	89	92.1	45.2	91.8	81.3
Actin o-	Percentage of sequences	63.28	95.28	40.52	66.54	39.7	21.3	29.9	12.9	19.4	14.2	36.0	20.0
	Number of Genera	78	55	62	38	21	50	28	21	54	13	21	22
	Number of dominant genera**	16	7	28	16	8	16	11	2	15	5	4	5
Firmicutes	Percentage of sequences	24.83	3,97	45.67	28.48	40.5	28.6	44.4	15.8	39.9	20.8	30.3	44.2
	Number of Genera	118	67	100	31	89	105	92	23	99	41	39	84
	Number of dominant genera**	50	17	65	18	39	29	33	2	33	7	6	24
Proteo- bacteria	Percentage of sequences	2,81	0,41	6,16	3,24	15.4	43.0	14.9	60.3	32.8	10.2	25.5	17.1
	Number of Genera	95	65	89	30	35	82	43	20	88	19	26	40
	Number of dominant genera**	22	7	49	10	9	25	13	8	22	4	5	10
Thaum- archaea	Percentage of sequences	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	48.7	0.4	0.0
	Number of Genera	0	0	0	0	0	0	0	0	0	2	1	0
	Number of dominant genera**	0	0	0	0	0	0	0	0	0	1	0	0

** >100 sequence counts

***Prediction of resistance capacities*.** Next to the phylogenetic diversity of microorganisms, we also wanted to retrieve information on which genes might be essential to their adaption to this extreme environment. It has to be emphasized that that we did not apply a metagenomics approach to assess the entire set of functional genes but used the *in-silico* tool Tax4Fun (Aßhauer et al., 2015) to predict functional genes derived from our 16S rRNA amplicon dataset (universal primer set). In total, we obtained 6,558 predicted single genes and 281 pathways (KEGG3 level). We focused on predicted genes/pathways responsible for antibiotic synthesis/resistance, transporters in general, resistance in general (e.g. resistance against metals or sporulation ability) and compared the individual relative abundances throughout all samples (Figure III.3-6).

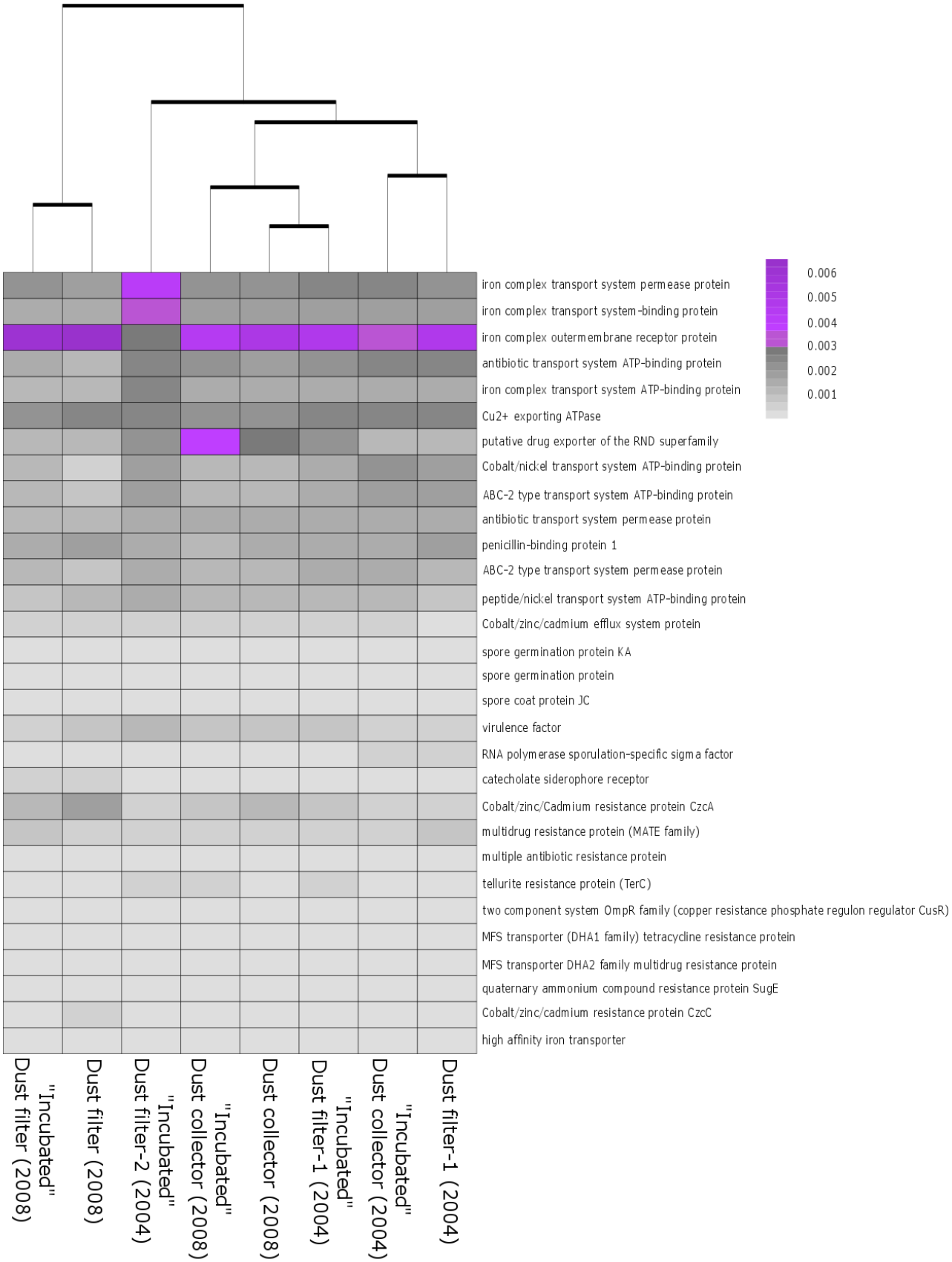

Figure III.3-6 The profiles clustered based on sampling site. The colour scale reflects relative abundance of genes in % (black: low abundance, violet: high abundance).

First, hierarchical clustering of selected functional genes resulted in two main clusters, consisting of the sample “Dust filter (2008)” with the incubated respective sample and the other samples forming the other cluster. There was no cluster pairing specifically incubated or untreated samples. The gene encoding for the iron complex “outer membrane receptor protein” was predicted to be highly abundant throughout all samples with the exception of the incubated sample “dust filter-2 (2004)”. In general, genes encoding for resistances/adaptions were predicted to be equally distributed throughout all samples. These in-silico-based predictions are not obligatorily reflecting the actual gene pool and need to be verified experimentally in future work.

Discussion

In this communication, we found novel information on the resistance capacities of resilient microorganisms derived from archived ISS dust samples. Our work allows deeper insight into the extremotolerant and adapted microbial community therein, revealing the presence of archaeal signatures as well as a robust microbial resistance machinery.

All 85 bacterial isolates survived for a prolonged time period of 8-12 years in desiccated dust. This implies a desiccation resistance achieved by different strategies, such as spore-forming capability or optimized DNA repair mechanisms (Table III.3-6). The origin of the bacterial isolates remains unknown, but many of them have already been detected in ISS or spacecraft- associated clean rooms or are typical human-associated microorganisms (Table III.3-6).

Table III.3-6 Summary of proposed survival strategies of the isolates and their possible origin.

Isolated microbial genus	Possible origin	Proposed survival strategy	References
Bacillus	HA, CR, ISS	Endospores	(Nicholson et al., 2000; Castro et al., 2004; Novikova et al., 2006; La Duc et al., 2007; Hong et al., 2009a; Ghosh et al., 2010; Moissl-Eichinger et al., 2013)
Paenibacillus	CR, ISS	Endospores	(Castro et al., 2004; La Duc et al., 2007; Ghosh et al., 2010; Moissl-Eichinger et al., 2013)
Salinibacillus	HA, ENV	Endospores	(Ren and Zhou, 2005; Endesfelder et al., 2016)
Micrococcus	HA, CR, ISS, IA	Intrinsic desiccation resistance	(Castro et al., 2004; Novikova et al., 2006; Grice et al., 2009; Bonetta et al., 2010; Kooken et al., 2012; Moissl-Eichinger et al., 2012b)
Cupriavidus	CR, ISS	Various DNA-repair mechanisms; adapted to extreme, metal-rich, anthropogenic environments	(Moissl-Eichinger et al., 2012b; Monsieurs et al., 2014)
Methylobacterium	CR, ISS, IA	Intrinsic desiccation resistance	(Hugenholtz et al., 1995; Bruce et al., 2005; Novikova et al., 2006; Vaishampayan et al., 2013; Venkateswaran et al., 2014)
Bradyrhizobium	CR, ISS	Intrinsic desiccation resistance	(Bruce et al., 2005; Novikova et al., 2006; Cytryn et al., 2007; Vaishampayan et al., 2013; Venkateswaran et al., 2014)

Legend: HA= human-associated; CR= reported in spacecraft assembly clean room(s) before; ISS= reported in ISS before; ENV= environmental; IA= indoor air.

It has to be pointed out that the origin of the halophile isolate *Salinibacillus* is very unclear. It has not been detected on board the ISS or in spacecraft assembly clean rooms before and is also not a typical human-associated bacterium. However, it has recently been detected in human stool, although the authors did suspect an erroneous classification (Endesfelder et al., 2016).

Nineteen representative ISS isolates were tested for their ability to survive a heat-shock and their susceptibility to 17 clinically relevant antibiotics *in vitro*. As expected, all spore-forming isolates survived the heat-shock and non-spore forming isolates did not, except for *Bradyrhizobium erythrophlei*. A few colonies appeared after the heat-shock at 80°C for 15 min. It has been reported that *B. japonicum*, a close relative of *B. erythrophlei*, possesses

multiple small heat-shock proteins that support survival of naturally occurring heat peaks of more than 40°C (Münchbach et al., 1999). However, since only a few survivors were found after the 80°C treatment, one might assume that such heat-shock proteins, if present in *B. erythrophlei*, are not a reliable protection at these elevated temperatures.

The same strains were tested for their susceptibility against 17 clinically relevant antibiotics. It should be stressed that none of the isolates obtained was judged to be an (opportunistic) pathogen, and the antibiotic resistances remain without clinical relevance in this regard. However, the isolates revealed a remarkable pool of antibiotic resistance. Only the isolate *Micrococcus yunnanensis* showed no resistance towards all tested antibiotics. The most resistant isolate which could be evaluated according to the EUCAST standard (see materials and methods section for more details) was *Paenibacillus campinasensis*, showing resistance against 8 of the 17 tested antibiotics.

During testing of the cephalosporines cefotaxime and ceftriaxone, we observed a high number of resistant isolates (16/19: cefotaxime and 14/19: ceftriaxone). However, most of the resistant isolates were *Bacillus* representatives, whereas our few Gram-negative isolates were mostly rated not resistant. Overall, *Bacillus* species appear to be rather resistant against cefotaxime and ceftriaxone, as reported before for e.g. *B. anthracis* (Doğanay and Aydin, 1991; Mohammed et al., 2002; Turnbull et al., 2004).

However, of the two organisms that were tested under adapted conditions (due to no growth on Müller Hinton agar), *Methylobacterium tardum* exceeded the non-species related resistance breakpoints of 11 of the 17 antibiotics, whereas the *Bradyrhizobium erythrophlei* isolate appeared to be even unaffected by almost all of the tested antibiotics except gentamicin. Of course these results have to be evaluated with extreme caution since not all of the EUCAST evaluation criteria could be met. *Bradyrhizobium erythrophlei* was recently isolated from root nodules of Ironwood in south China and subsequently described as a new species (Yao et al., 2015). Notably, *Bradyrhizobium* sp. (Accession number AY599676), now classifiable as *B. erythrophlei*, was also recently observed in propidium monoazide treated samples of spacecraft assembly facilities (Vaishampayan et al., 2013). *Bradyrhizobium* signatures were reported in high abundance even in intensive care units and hospital biofilms. (Oberauner et al., 2013; Soto-Giron et al., 2016). To date, the impact of *Bradyrhizobium* species (except for *B. enterica*) on human health remains elusive, but this genus has obviously a robust strategy for survival under stressful conditions. However, although *B. erythroplei* is not reported to be pathogenic, it could act as a reservoir for resistance genes on the ISS that might, under selection pressure, be passed on via horizontal gene transfer to infectious microorganisms.

A number of studies have been conducted on the reaction of bacteria to human spaceflight conditions, focusing on the changed pathogenic potential or resistance development (Rosenzweig et al., 2014). For some microorganisms, an elevated virulence has been found, whereas others remained unaffected (Rosenzweig et al., 2014). Recently it has been shown for *Staphylococcus* species, that even a short-term stay in space can trigger the development of antimicrobial resistance (Fajardo-Cavazos and Nicholson, 2016). In addition, decreased susceptibility of microbes to antibiotics under space-flight conditions have been reported (Fajardo-Cavazos and Nicholson, 2016). Notably, bacterial infections ocurring during human space-flight on Mir or spaceshuttle have been observed earlier, such as infections of the urinary tract, upper respiratory tract and subcutaneous tissue, as well as an increased

reactivation of latent viral infections due to the deterioration of the astronaut´s immune system (Mehta et al., 2014; Fajardo-Cavazos and Nicholson, 2016).

Of the antibiotics we tested in this study, amoxicillin (without clavulanic acid), ceftriaxone, ciprofloxacin, clindamycin, doxycycline, levofloxacin, sulfamethoxazole/trimethoprim, and moxifloxacin are also ingredients of the ISS medical inventory (Mehta et al., 2014) and can thus be used for treatment of bacterial infections aboard. In our tests (see Table III.3-4), the environmental isolates from ISS were susceptible to amoxicillin (with clavulanic acid), ciprofloxacin, doxycycline, levofloxacin, sulfamethoxazole/trimethoprim, and moxifloxacin. We confirmed that ceftriaxone is not very effective against *Bacillus* sp. but it was effective against most non spore-forming isolates. When comparing the measured clindamycin MICs to resistance breakpoints defined for other species, 16 of 19 isolates could be rated potentially resistant against clindamycin: For all aerobic microorganisms, for which the clindamycin resistance breakpoint is defined in the EUCAST breakpoint table v6.0, it is *">0.5 µg/ml"* (e.g. *Staphylococcus* sp. or *Corynebacterium* sp.). However, due to missing specific resistance breakpoints for non-pathogenic microbial isolates, this finding cannot be used for risk estimations.

The cultivable diversity of our older dust samples (17 bacterial species) was found to be lower than the cultivable diversity of the US American study (26 bacterial species), although a broader variety of cultivation media was used. In both studies, the genus *Bacillus* was the most prominent genus. However, on species level, no overlap between the isolates of these studies was found, which strongly indicates a difference in the microbial communities of the analysed samples – either caused by longer storage, or an overall difference in the microbiota composition of the Russian modules in 2004/2008 and of the US American modules in 2008-2012 (US American HEPA filter was installed from 2008 to 2011 and US American vacuum cleaner samples were taken 2012 [4]). Because of the use of nystatin in our study, we isolated only one eukaryotic isolate, *Ulocladium botrytis*, retrieved from medium with a pH of 9. *U. botrytis* was also not among the 10 different fungi isolated by Checinska et al. 2015 ([4], Supplementary Table 3).

Besides the analysis of the isolates, we carried out a comprehensive sequencing study to shed light onto the microbial diversity that was present 8-12 years ago in the Russian ISS segment.

Notably, PMA treatment did not result in positive amplification of 16S rRNA genes, although cultivation efforts confirmed the presence of viable cells. Either those were present only in very low numbers, so that the PMA treatment resulted in DNA below detection limit, and/or the microbes were present as hardy spores. The latter observation is in accordance with the sequencing results showing a high proportion of Bacilli (17.4 %) and Clostridia (13.7 %), whose spores require a harsh DNA extraction method (Kuske et al., 1998).

In order to increase the amount of available DNA in the samples, and possibly also to trigger spore germination, we incubated the samples in warm LB medium before DNA extraction. Although the shift of the microbial community caused by the incubation was found to be substantial, we were able to retrieve enough DNA for a positive amplification of 16S rRNA genes in all samples. Without incubation, only three out of five samples gave a positive signal.

Besides spore-formers, our dataset revealed a high presence of signatures belonging to human-associated bacteria, including *Pseudomonas* (Trautmann et al., 2005), *Acinetobacter* (Hartzell et al., 2007), *Sphingomonas* (White et al., 1996) and *Corynebacterium* (Soriano et al., 1995) throughout all samples. Most of these microbes have been detected on human skin (such as *Corynebacterium* and *Staphylococcus*) and in the human gut (such as the strictly anaerobic *Faecalibacterium*). These findings confirm that the indoor airborne microbial community is derived directly from the astronauts' presence as discussed in other studies (Hospodsky et al., 2012; Adams et al., 2015; Checinska et al., 2015).

We also found signatures of other strictly anaerobic genera were found, such as *Anaerococcus* and *Anaerostipes,* which can, however, tolerate oxygen when dormant. An intensive study of all taxa revealed a high proportion of extremotolerant microorganisms such as (**i**) spore-forming bacilli, with known resistance against radiation, pressure, desiccation, and space and Mars-simulation conditions (Dose and Klein, 1996; Tauscher et al., 2006; Horneck et al., 2012; Vaishampayan et al., 2012 and references therein), (**ii**) signatures of *Rhodococcus*, known for high resistance against desiccation and ultraviolet radiation (LeBlanc et al., 2013), and (**iii**) members of *Cyanobacteria*, which are considered to be highly resistant against extreme conditions (Rampelotto, 2010 and references therein). The presence of extremotolerant microorganisms is also reflected in the predicted metabolic capacities. A various range of predicted genes encoding features which allow organisms to withstand extreme conditions such as the "two-component-system", several transporters, iron acquisation, antibiotic resistance and biosynthesis could be detected and were distributed equally throughout all samples.

In order to gain insight into the overall microbial community of the ISS, we compared the microbial community of the US segment (Checinska et al., 2015) and the Russian segment (our study) by performing a joint data analysis, using NGS raw reads from both studies, processed by DADA2. We observed a high dissimilarity in the microbial composition between both segments, potentially caused by the different location, sampling time-frame or methods used to gather the data. However, in the US segment, the microbial community was also dominated by human-associated microorganisms and the same core taxa on phylum level (Actinobacteria, Firmicutes and Proteobacteria), although with a different relative abundance. It should be mentioned that a deeper comparison of both settings was not possible, due to the different primers used in the US segment study, as well as the short reads obtained (~130 bp are considered borderline for proper classification; paired reads could not be stitched; Checinska et al., 2015).

The most striking difference found during our comparison of already available data and that from our new study was the presence of archaeal signatures in the Russian samples. Overall, the presence of archaea on the ISS has not been reported before, or previous attempts to detect them were negative (Venkateswaran et al., 2014; Checinska et al., 2015). Archaea are generally known to be widely distributed in extreme environments, and are specifically well-adapted to biotopes with energy-constraints. In our samples, we mainly found *Nitrososphaera* signatures, belonging to a group of chemolithoauthotrophic, ammonia-oxidizing archaea, distributed in soil and hot springs, but also abundant on human skin (Hatzenpichler et al., 2008; Probst et al., 2013a). Interestingly, in spite of known mismatches in the used primers (Probst et al., 2013a), *Nitrososphaera* was also detected with universal primers, indicating a high abundance in the dust samples.

Signatures of human-associated *Methanobrevibacter* could also be observed. *Methanobrevibacter* species are described as anaerobic, extreme human gut commensals (Miller et al., 1982). Notably, the (rare) presence of Woesearchaeota signatures can also be reported. Their detection has been occasionally reported in samples from soils and aquatic environments (Dojka et al., 1998; Großkopf et al., 1998). Castelle et al. analysed their genomic potential, which revealed a small genome size and limited metabolic capacities, which suggests that these Archaea have a symbiotic or parastic lifestyle (Castelle et al., 2015). Although the detection of archaeal signatures cannot inform on the role and activity of these microbes yet, we can state that the International Space Station is/was indeed populated by all three domains of life.

Conclusion

The ISS dust microbiome analysed in this study contained living, hardy microorganisms and showed the presence of archaeal signatures. Numerous resistance capabilities towards environmental stresses were either predicted on a molecular level or shown by retrieved isolates. It should be stressed, that, although these findings raise many questions and require discussion, the International Space Station is and has always been a safe workplace (Van Houdt et al., 2012) and no severe infections or disease outbreaks have been reported thus far. The specific resistance capacities of our non-pathogenic ISS isolates against desiccation, heat-shock and some antibiotic compounds refer to samples that have been collected around a decade ago and the findings need to be reconfirmed with novel microbial isolates. Nevertheless, it remains without doubt that microorganisms on the ISS experience selective pressures, and that a number of microbes adapt to these stresses. Our findings and those of previous publications in this regard can now be considered for the planning of future, crewed long-term spaceflights, but also for potential habitats on the moon or other planetary bodies.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets generated and analysed during this study are available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena), the Sanger sequences are available via accession numbers LT617056-LT617090. Ilumina MiSeq reads are accessible via study accession number: PRJEB14961.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

MM and AP contributed equally to this study. MM planned and executed the laboratory work, evaluated the data and wrote the paper. AP performed bioinformatics, biostatistics and wrote the paper. TAA, AA and TN provided and pre-processed the samples, provided metadata. LW performed experiments and provided input for writing the paper. RK provided critical input for execution and evaluation of the antimicrobial susceptibility tests. CME, principal investigator of the ARBEX project, initiated, planned the study, evaluated experiments, performed bioinformatics, wrote the paper.

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Supplementary information

Supplementary information and a description can be found online (http://microbiomejournal.biomedcentral.com/articles/10.1186/s40168-016-0217-7) on the supporting CD (./ Selected publications/06 Supplementary files)

III.4. Mars exploration begins on Earth: Systematic comparison of the anaerobic, intact and cultivable microbiome of extreme, anoxic, Mars-analogue environments

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Abstract

Fueled by the general interest of detecting life beyond Earth's biosphere, the chemical and physical limits of life and thus borders of habitability remain a burning question. Extreme terrestrial environments and their endogenous life are currently studied in order to decipher basic information on evolution and adaptation capabilities of microorganisms - a prerequisite for life detection on other planetary bodies.

Nowadays, Earth's ecosystem contains oxygen, a feature that probably distinguishes our planet from potential habitable extraterrestrial systems. Motivated by the identified significant lack of information on microbial communities from Mars-analogue, anoxic settings, and the desire to understand the characteristics and limits of anaerobic life, we carried out the first systematic microbiological study from a number of terrestrial anoxic environments with space-relevant extremes. We investigated the inhabiting, living bacterial and archaeal diversity by amplicon sequencing and compared the outcome with the results from a wide-scale cultivation approach, in which 1034 enrichments were processed. We uncovered a highly adapted bacteriome and archaeome, thriving under harsh conditions, and a cosmopolitan group of microorganisms thriving in all Mars-analogue environments. The study provides highly valuable information on microbial communities in anoxic environments, with a strong impact for the search for life in the universe.

Keywords: Mars analogues, Extreme environments, Microbiome, Anaerobic microorganisms, MASE

Introduction

Environmental microbiology has made enormous strides in understanding what microbial communities inhabit some of Earth's most extreme biotopes. Fuelled by recent reports on numerous habitable planets and the general interest of detecting life beyond Earth's biosphere, the main research questions focused on the chemical and physical limits of life as well as the general habitability of extraterrestrial environments. These environments, as identified so far, have one characteristic in common, namely the lack of available oxygen. The Great Oxygenation Event of Earth 2.3 billion years ago has caused tremendous changes in the overall evolution of life, with oxygen being one of the strongest triggers for changes in microbial community composition and function. Studying microbial communities from the present-day oxygenated Earth's biosphere as potential models for Early Earth and extraterrestrial life thus seems inappropriate, emphasizing the need for a more detailed survey of the anaerobic microbial communities and their physical and chemical limits of the biosphere. As a consequence, we carried out the first systematic study of extreme anoxic environments, motivated by a desire to understand the characteristics of anaerobic life at the limits of Earth's biosphere and the potential habitability of Mars.

Nowadays, Mars is considered a hostile, dry planet, with a thin, oxygen-limited atmosphere, low temperatures and sterilizing radiation on the surface (Moissl-Eichinger et al., 2016a). Nevertheless, 4.1-3.7 billions of years ago, the planet was most likely characterized by a carbon dioxide-rich atmosphere, partially stable and relatively warm surface temperatures (probably due to volcanism or cosmic impacts) and thus conditions conducive to the presence of liquid water on the surface (Bibring et al., 2006; Carr and Head, 2010). Available

chemical (in)organic compounds could have served as energy and carbon sources for life (Hofmann and Bolhar, 2007; Martin and Russell, 2007; Nisbet et al., 2007; Duprat et al., 2010; Westall et al., 2011; Westall et al., 2015).

Environmental conditions changed dramatically between ~4.0 and 3.6 Ga, when Mars began to lose its atmosphere, until it reached 1% of the density of the Earth's atmosphere today. As a consequence, the surface of Mars became desiccated and turned into a very extreme environment, highly challenging for any potential microbial life that could, theoretically, have remained in (radiation-) protected, habitable areas (so called "special regions"; Rummel et al., 2014; Rettberg et al., 2016).

As indicated by geochemical data obtained from numerous laboratory-based analyses of Martian meteorites and several instruments on rovers and orbiters, habitable conditions are most likely restricted to the subsurface, where a permafrost-like setting seems to occur (Boynton et al., 2002). Another possibility are brine flows, which are closer to the surface. This phenomenon is known as recurring slope lineae (RSLs; McEwen et al., 2011; Grimm et al., 2014; McEwen et al., 2014; Stillman et al., 2014; Ojha et al., 2015), which could provide aqueous, high salty biotopes, suitable only, however, for adapted halophilic microorganisms (Litchfield, 1998; Davila et al., 2010; Leuko et al., 2010; Bryanskaya et al., 2013; Oren et al., 2014). Although methane outbreaks in the Martian atmosphere support speculations on potential subsurface Martian life (Kotsyurbenko et al., 1996; Paull and Dillon, 2001; Formisano et al., 2004; Atreya et al., 2007; Sloan Jr and Koh, 2007; Geminale et al., 2008; Mumma et al., 2009; Fonti and Marzo, 2010; Geminale et al., 2011; Kral et al., 2011; Webster et al., 2015), so far no proof has been found of past or present life on Mars, and considerations on the potential morphology, biochemical properties or metabolisms of Martian life remain speculative. Consequently, Mars-analogue research has become an important pre-requisite for the planning and preparation of life detection missions, such as ExoMars (Montroni et al., 2016). Such terrestrial, analogue biotopes, which are studied with respect to their chemical, physical characteristics and their indigenous microbial life, resemble either Early or Present Mars conditions and exhibit usually a number of typical parameters, such as: (**i**) low water activity, (**ii**) low temperature, (**iii**) restricted availability of (complex, organic) nutrients, (**iv**) oxygen limitation and other stressors for microbial survival (i.e. high salinity, high acidity, radiation).

However, none of the terrestrial Mars-like locations is capable of combining all Martian stresses at once (Moissl-Eichinger et al., 2016a). This shortcoming has to be circumvented by systematic analyses of various analogue sites that resemble Early and Present Mars conditions, considering general principles as driving factors to shape their microbial communities. The outcome can help to interpret potential information on biosignatures that could be expected on Mars.

A number of studies have already addressed the diversity of microorganisms thriving in Mars-analogue sites using cultivation-based approaches and cultivation-independent analysis based on the 16S rRNA gene sequences (Amils et al., 2007; Blank et al., 2009; Mormile et al., 2009; López-Lozano et al., 2012; Boetius et al., 2015; Parnell and McMahon, 2016). However, the available cultivation-based studies did not address the fact that the Martian atmosphere lacks available oxygen. With respect to more or less anoxic conditions on Mars, an intensive study based on the diversity and functional capacities of strict

anaerobic microorganisms is missing in the current body of literature. Extreme environments harbor harsh conditions and most likely contain many dead cells derived from not-adapted, introduced microorganisms. Standard molecular methods fail to distinguish viable and dead fractions of microbial communities and reports on microbial diversities from Mars-analogue sites may be falsified by the detection of DNA released from biological debris.

Based on such considerations, the MASE project (Mars Analogues for Space Exploration; http://mase.esf.org/) was initiated. Bringing together more than 20 experts from seven European countries in the frame of the 7th European framework funded project, major goals were to design a standard operation procedure (SOP) to systematize the **a)** anaerobic sampling of selected Mars analogue sites and **b)** the anaerobic cultivation of Mars-relevant organisms (Cockell et al., 2016b). We collected anoxic soil, water and sediment samples from different Mars-analogue sites, namely **i**) cold sulphidic springs, **ii**) two different acidic environments, **iii**) a hypersaline, subsurface environment, and **iv**) frozen sites represented by glacier and permafrost sites.

In addition, we aimed to elucidate the full spectrum of bacterial and archaeal signatures of the living portion of the Mars-analogue microbial communities by applying the chemical compound propidium monoazide (PMA) to mask free DNA before processing the samples via molecular methods (Next Generation Sequencing, NGS).

This study concentrated on the systematic comparison of the anaerobic, intact and cultivable microbiome of extreme, anoxic environments that served as Mars-analogue systems. This would enable to retrieve and provide novel model microorganisms and to determine general principles of anaerobic microbial communities with impact on the habitability assessment and the search for life on Mars and beyond.

Materials and methods

Sample collection. Mars-analogue sediment, water and soil samples were obtained during a number of sampling campaigns performed in the years 2014 and 2015. The sampling sites are described in more details elsewhere (Cockell et al., 2016b), in Supplementary Table 1 and are summarised in Table III.4-1.

Table III.4-1 Summary of all sampling sites (more details are available in Supplementary Table 1). A star (*) in the sample abbreviations indicates pre-treatment of the samples with propidium monoazide (PMA).

Sampling sites		**Abbreviation in main article**	**Mars-analogue condition**
Sulphidic springs (Bavaria, Germany)	Sippenauer Moor	"sulphidic spring SM"; "sulphidic spring SM*"	Early Mars: anoxic, low in organics, sulfur-rich
	Islinger Mühlbach	"sulphidic spring IM"; "sulphidic spring IM*"	
Hypersaline environment (Boulby mine, United Kingdom)	Sampling site 1	"hypersaline environment"	Present Mars: anoxic brine slopes
Acidic lake (Lake Grænavatn, Iceland)	Sampling site 1	"acidic lake SS1"; "acidic lake SS1*"	Early Mars: anoxic, low in organics, acidic
	Sampling site 3	"acidic lake SS3"; "acidic lake SS3*"	
Acidic river (Rio Tinto, Spain)	Lago Peligroso	"acidic river LP"; "acidic river LP*"	
	Galdieras	"acidic river Gal"; "acidic river Gal*"	
Kaunertaler Glacier (Austria)	Sampling site 1	"glacier SS1"; "glacier SS1*"	Early and Present Mars: low temperature environment
	Sampling site 2	"glacier SS2"; "glacier SS2*"	
Permafrost setting (Canada and Russia)	SlpD14-PS1-11	"permafrost SlpD14-1"; "permafrost SlpD14-1*"	Early and Present Mars: low temperature environment, anoxic
	SlpD14-PS3-11	"permafrost SlpD14-3"; " permafrost SlpD14-3*"	
	SOB-14-06-A-37	"permafrost SOB"; "permafrost SOB*"	
	TSD-14-IW1-01	"permafrost TSD"; "permafrost TSD*"	

Sampling sites included (i) two sulphidic springs (Moissl et al., 2002; Rudolph et al., 2004a; Henneberger et al., 2006; Probst et al., 2013b; Perras et al., 2014; Probst et al., 2014b; Perras et al., 2015; Probst and Moissl-Eichinger, 2015), (ii) one subsurface, hypersaline environment (Woods, 1979; Norton et al., 1993), (iii) one acidic lake (Thorarinsson, 1953), (iv) one acidic river (Sánchez-Andrea et al., 2011; Sanz et al., 2011) (v) two permafrost environments and (vi) one glacial environment in high altitude. The glacier samples were retrieved during a Mars simulation event of the Austrian Space Forum (ÖWF) in 2015 (AMADEE15; Groemer et al., 2016a; Groemer et al., 2016b), where four analogue astronauts were trained to take samples in a full space suit (Groemer et al., 2016a; see also www.http://oewf.org/portfolio/oesterreich-amadee-15/; Supplementary Information Figure 1). A detailed analysis of the geochemical setting of each sampling site is given in Cockell et al., 2016b.

The sampling, additional samples, the cultivation and physiological tests of the isolates and the procedures for fluorescence in situ hybridization (FISH) are described in more detail in the Supplementary Information.

Processing of the samples for molecular analyses: PMA treatment, DNA extraction and amplicon sequencing. In order to discriminate between viable (i.e. cells with an intact cell membrane) and dead cells (cells with a disrupted cell membrane) in subsequent molecular analyses, 0.25 g of each sample was treated with propidium monoazid (PMA, VWR) before DNA extraction. In the following, all PMA treated samples are marked with a star (*), e.g. the sample "sulphidic spring IM*" refers to the PMA treated sample, whereas sample "sulphidic spring IM" refers to the PMA untreated, simultaneously processed sample. More details are given in Supplementary Information.

Library preparation, Illumina paired-end sequencing, community profiling and inferred metagenomics. These procedures are described in full detail in Supplementary Information. Reads were processed using R (version 3.2.2) and the packages DADA2 (Callahan et al., 2016a) as already described elsewhere (Mora et al., 2016b), following the SOPs as recommended by the developers (Callahan et al., 2016a). DADA2 does not implement a clustering step and does not generate operational taxonomic units (OTUs) as known by other data processing tools. Here, each row in the DADA2 output table corresponds to a non-chimeric inferred sample sequence with a separate taxonomic classification (ribosomal sequence variants, RSVs).

Data availability. Sequence datasets were submitted to EBI and are publicly available (Study project number: PRJEB18706).

Results

In this study, we analysed the microbial community of samples from various, nutrient-poor, anoxic Early and Present Mars analogue sites, ranging from hypersaline environments, cold sulphidic springs, acidic aquatic settings, to permafrost and glacier environments. In addition to an extensive cultivation effort, we applied a live/dead marker to our molecular microbial community analyses to distinguish between signatures of organisms possessing an intact cell wall and residing in the Mars analogue settings, and signatures of dead cells. It should be emphasised that the sample from the "hypersaline environment" could not be treated with our life/dead marker, due to cross-reactions between salts and PMA (Barth et al., 2012).

General observations. All samples retrieved were subjected to microscopic examination. However, due to the low amount of microbial cells present, and numerous particles in the sediment samples, the identification of microbial cell structures proved difficult. Similar results were obtained when fluorescence in situ hybridisation was performed. Clear microbial signals were obtained from sulphidic spring samples (Supplementary Information Figure 2). Here, the bacterial cells of diverse morphologies (cocci, rods, filaments) outnumbered the archaeal cells by far. We assume that chemical properties of the other samples (high salt, low pH), hindered cell fixation and visualization via FISH (Antón et al., 1999; Dedysh et al., 2006), so that this method was not considered to be reliable with respect to the determination of microbial community composition.

***Sixty-nine microbial enrichments and 31 isolates were obtained from the MASE sites*.** For enrichments of microbial anaerobes, we used a standardized medium ("MASE I", "MASE II"), supplemented with compounds supporting the growth of (i) fermenting microbes, (ii) sulfate reducers, (iii) C1 utilizers, (iv) nitrate reducers, (v) methanogens, (vi) polycyclocaromatic hydrocarbon degraders, (vii) amino acid utilizers and (viii) iron reducers (a comprehensive description can be found in Cockell et al., 2016b. In total, we performed 1034 enrichment attempts with varying supplements (95% targeting heterotrophs and 5% targeting autotrophs) and were able to obtain 69 enrichments. Growth was detected in 6.9% of all enrichment attempts targeting heterotrophs and in 1.9% of all enrichments targeting autotrophs. In further purification steps, 31 pure isolates (see Table III.4-2) were obtained. 31 isolates belonged to the domain of the Bacteria (mostly Proteobacteria), and 1 isolate was affiliated to the domain of Archaea (*Methanomethylovorans* sp. MASE-SM-1). All of them grew under anoxic conditions and required organic medium supplements

Table III.4-2 Isolates obtained within the frame of the MASE project and their respective environmental source. The taxonomic affiliation on phylum and genus level is given. Furthermore, characteristics of close relatives (same genus) are added, either inferred from own observations or literature review. So far unclear situations are marked with ✦. Additional details can be found in Cockell et al., 2016 (continues on page 109).

Source [total number of obtained isolates]	**Phylum (no. of isolates)**	**Genus [no. of individual strains if >1] (strain abbreviation)**	**Characteristics**	**DSM number (if available)**
Hypersaline environment [1]	Firmicutes (1)	*Halanaerobium* sp. (MASE-BB-1)	Halophilic, (strictly✦) heterotrophic, strictly anaerobic (Abdeljabbar et al., 2013; W Roush et al., 2014)	
Sulphidic springs [18]	Firmicutes (5)	*Clostridium* sp. [4] (MASE-IM-4, -6, MASE-SM-2, -4)	Mixotrophic, strictly anaerobic, spore-former	
		Trichococcus sp. (MASE-IM-5)	(Strictly✦) heterotrophic, (facultatively) anaerobic (Pikuta et al., 2006)	
	Proteobacteria (12)	*Desulfovibrio* sp. (MASE-IM-7)	Mixotrophic (Mechalas and Rittenberg, 1960), obligate anaerobic (Postgate and Campbell, 1966)	
		Citrobacter sp. [2] (MASE-IM-8; MASE-SM-7)	Mixotrophic (Johnson, 2006), (facultatively) anaerobic (Huang and Tseng, 2001)	
		Buttiauxella sp. (MASE-IM-9)	(Strictly✦) heterotrophic (facultatively) anaerobic (Cui et al., 2012),	
		Obesumbacterium sp. (MASE-IM-3)	(Strictly✦) heterotrophic, (facultatively) anaerobic	
		Aeromonas sp. [2] (MASE-IM-1 and MASE-SM-5)	(Strictly✦) heterotrophic, (facultatively✦) anaerobic	
		Yersinia sp. [4] (MASE-SM-8 and (MASE- SM-6,-9-,10))	Mixotrophic, (facultatively) anaerobic (Buzolyova and Somov, 1999)	102846, 102991, 102848, 102849
		Hafnia sp. (MASE- SM-3)	(Strictly✦) heterotrophic, (facultatively✦) anaerobic	
	Euryarchaeota (1)	*Methanomethylovorans* sp. (MASE-SM-1)	(Strictly✦) heterotroph, (obligate) anaerobic (Wang et al., 2014)	
Acidic lake [9]	Actinobacteria (3)	*Cellulomonas* sp. [3] (MASE-LG-6,7,8)	Mixotrophic, (facultatively) anaerobic (Dermoun and Belaich, 1988)	
	Firmicutes (3)	*Pelosinus* sp. (MASE-LG-2)	(Strictly✦) heterotrophic, (facultatively) anaerobic (Moe et al., 2012), spore forming (Yutin and Galperin, 2013)	
		Clostridium sp. (MASE-LG-3)	Mixotrophic, obligate anaerobic, spore-former	
		Desulfosporosinus sp. (MASE-LG-5)	Mixotrophic, obligate anaerobic, spore-former (Robertson et al., 2001)	
	Proteobacteria (3)	*Yersinia* sp. (MASE-LG-9)	Mixotrophic, (facultatively) anaerobic (Buzolyova and Somov, 1999)	
		Serratia sp. (MASE-LG-10)	Mixotrophic, (facultatively) anaerobic(Bidlan and Manonmani, 2002)	
		Yersinia intermedia (MASE-LG-1)	(Strictly) heterotrophic, (facultatively) anaerobic	102845

Source [total number of obtained isolates]	**Phylum (no. of isolates)**	**Genus [no. of individual strains if >1] (strain abbreviation)**	**Characteristics**	**DSM number (if available)**
Permafrost [1]	Proteobacteria (1)	*Sodalis* sp. (MASE-SOB14-1)	Mixotrophic✦, (facultatively) anaerobic (Dale and Maudlin, 1999)	
Glacier [2]	Firmicutes (1)	*Enterococcus* sp. (MASE-Glacier [SS1]	Mixotrophic✦, (facultatively) anaerobic	
	Proteobacteria (1)	*Rahnella* sp. (MASE-Glacier [SS3])	Mixotrophic✦, (facultatively✦) anaerobic	

Four isolates (*Halanaerobium* sp. (MASE-BB-1), *Trichococcus* sp. (MASE-IM-5), *Yersinia intermedia* (MASE-LG-1), *Clostridium* sp. (MASE-IM-4)) were selected for more detailed physiological analyses (temperature, salinity, pH tolerance and growth range; this study), fossilisation experiments, genomic and proteomic analyses, as well as comprehensive stress tests (experiments ongoing). These representatives were selected based on their reliable and fast growth to high cell numbers. The isolates obtained from the sulphidic spring preferred moderate temperatures (temperature range: 25°C-30°C for *Trichococcus* sp. (MASE-IM-5) and 25°C-35°C for *Clostridium* sp. (MASE-IM-4)), but grew within a wide range of pH (pH5-8 for *Trichococcus* sp. (MASE-IM-5) and 3-9 for *Clostridium* sp. (MASE-IM-4); Table III.4-3). No growth was observed beyond a NaCl concentration of 0.1 M (*Trichococcus* sp. (MASE-IM-5) and 0.3 M (*Clostridium* sp. (MASE-IM-4)). The isolate *Yersinia intermedia* (MASE-LG-1), obtained from the acidic lake, grew at moderate temperatures (25°C-35°C) and neutral to slightly alkaline conditions (pH7-8). The limit of NaCl concentration for growth was 0.5 M. All three described isolates showed their optimal growth (shortest generation time) in the presence of 0 M NaCl, at 30°C, and pH7. The growth of the fourth isolate *Halanaerobium* sp. (MASE-BB-1) occurred at neutral pH values (7-8), higher temperatures (30-45°C) and higher NaCl concentrations (up to 3 M). Optimal growth was observed at pH7, 45°C and 3 M NaCl.

Table III.4-3 Characteristics of selected, representative isolates.

Isolate strain	Source	PH range	Temperature range	NaCl range	Optimum pH	Optimum temperature	Optimum NaCl
Halanaerobium sp.(MASE-BB-1)	Hypersaline environment	pH7-8	30°C-45°C	1M-3 M	pH7	45°C	3 M
Trichococcus sp. (MASE-IM-5)	Sulphidic springs	pH5-8	25°C-30°C	0-0.1 M	pH7	30°C	0 M
Yersinia intermedia (MASE-LG-1)	Acidic lake	pH7-8	25°C-35°C	0-0.5 M	pH7	30°C	0 M
Clostridium sp. (MASE-IM-4)	Sulphidic springs	pH3-9	25°C-35°C	0-0.3 M	pH7	30°C	0 M

In general, physiological optima of tested microbial isolates did not fully reflect the environmental conditions of their origin. In particular the *Yersinia* species, although retrieved from various sites (sulphidic springs and acidic lakes) seemed to tolerate extreme conditions, but preferred moderate conditions for optimal growth.

***The MASE sites harbor a diverse archaeome comprising 14 archaeal phyla*.** In total 787,842 archaeal raw sequences were obtained using the Archaea-targeting primer set, resulting, after processing, in 1,502 archaeal RSVs. On average, 37,516 raw sequences were obtained from each sample. However, the number of retrieved raw sequences varied strongly across the samples, although DNA as well as PCR products were normalized before NGS. The lowest count was achieved for the sample “acidic lake SS3” (72 raw sequence counts), whereas the highest read count was obtained for “sulphidic spring IM*” (PMA treated sample of sulphidic spring Islinger Muehlbach; 106,622 raw sequence counts). Noteworthy, no archaeal signal could be observed for the samples obtained from the glacier (PMA treated and untreated), which indicates either a low amount of archaeal 16S rRNA genes (below detection limit) or an insufficient primer match (Hong et al., 2009b; Tremblay et al., 2015). A barchart displaying the archaeal community composition of all samples (PMA treated and untreated) is given in Supplementary Information Figure 3.

Since this study focused on the viable proportion of microbial communities, we will, in the following go into detail on PMA treated samples, but also include the PMA untreated “hypersaline environment” sample (PMA treatment is hindered in high-salt samples, see explanation in materials and methods; Barth et al., 2012).

The majority of RSV counts (PMA treated samples and “hypersaline environment”) were assigned to Euryarchaeota (39.3%), Thaumarchaeota (22.7%), unassigned Archaea (20.8%) and Woesearchaeota (DHVEG-6; 10.9%).

Signatures of Euryarchaeota were detected throughout all archaea-positive samples (except samples from the acidic lake SS1*), with highest proportion of Euryarchaeota in the

hypersaline environment (14.5% all euryarchaeal sequence counts from all samples) which affiliated to Halobacteriales (99% of all Euryarchaeota signatures within this sample). Other sequences from the hypersaline environment sample were classified as Nanohaloarchaeota (12.6% of all archaeal sequences), Thaumarchaeota and Woesearchaeota (both rarely present: below 1%). Notably, 99.9% of the sequences detected in the acidic lake SS1* remained unclassified within the archaeal phylum and thus might represent a new taxon.

In contrast, all sequences of the second sampling site of the acidic lake (SS3*) could be assigned on phylum level and were classified as Bathyarchaeota (Meng J, 2014), which were recently revealed as methane-producers (Evans et al., 2015; He et al., 2016; Lever, 2016; 19.4% of all sequence counts within this sample), ammonia-oxidizing Thaumarchaeota (Brochier-Armanet et al., 2008; 72.9% of all sequence counts within this sample), Euryarchaeota (7.6% of all sequence counts within this sample) and a very minor proportion of Woesearchaeota DHVEG-6 (<1% of all sequence counts within this sample).

Sequences of the acidic river* were mainly assigned to unclassified Archaea, Thaumarchaeota and Euryarchaeota (71.4%, 10.1%, and 16.5% in the acidic river Gal* sample, respectively) and a high proportion of Euryarchaeota (98.8% in the acidic river LP* sample). Further details can be found in (Figure III.4-1a). Signatures of the Altiarchaeales cluster were found in the acidic river LP* sample, which were also detected in samples of the sulphidic spring*, a result that was in agreement with previous findings (Probst et al., 2014b; Probst and Moissl-Eichinger, 2015).

In the spring biotopes, a remarkably high proportion of signatures assigned to Woesearchaeota (DHVEG-6) was detected (36.5% and 21.2% of all sequence counts in SM* and IM* samples, respectively). Other sequences were assigned to Euryarchaeota and unclassified Archaea. Notably, only the sulphidic spring biotope revealed signatures of Archaea belonging to the group of Aenigmarchaeota (<1%).

Permafrost samples contained mainly signatures of Euryarchaeota with high proportions of methanogenic Archaea (Methanobacteria and Methanomicrobia) and Thaumarchaeota (Soil Crenarchaeotic Group; SCG and Marine Benthic Group; MBG; Brochier-Armanet et al., 2008; Spang et al., 2010). Further details are given in Figure III.4-1a, and b.

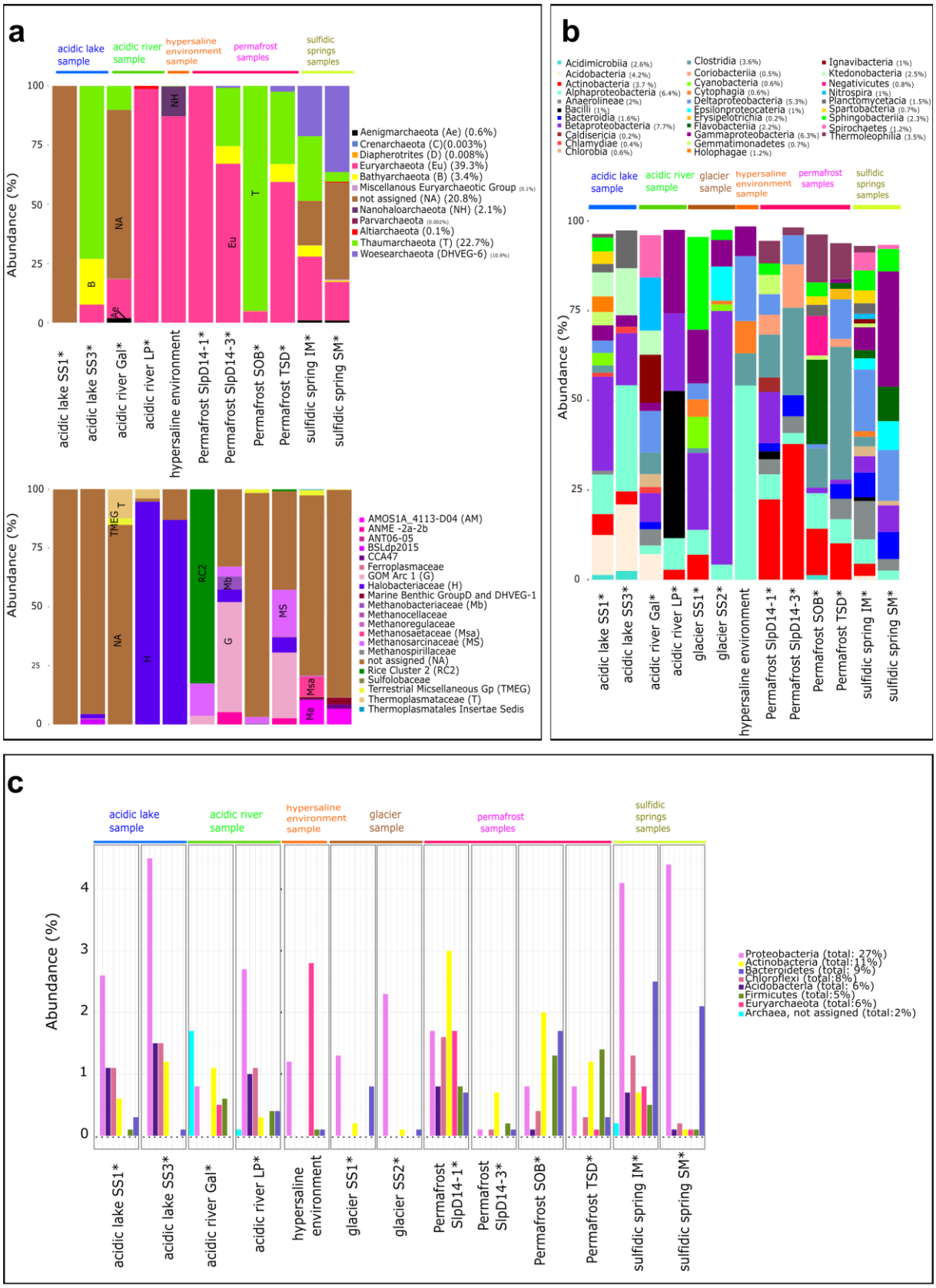


Figure III.4-1 The microbiome of MASE environments. Panel (a) shows the taxonomic profile obtained using Archaea-targeting primer on phylum and family level. (continues on next page)

Figure III.4-1 (continuation) Panel (b) shows the according bacterial composition (using an universal primer approach; abundance of bacterial classes in each sampling site >1%). Panel (c) displays the top phyla of the microbial community ("universal" primer set, microbiome). The relative abundance of each taxon within the sample sites was calculated and is shown on the y-axis. The total relative abundance was summed up for all samples and is shown for each phylum in the legend.

Notably, the "universal primer" approach revealed slightly more archaeal taxa than the Archaea-specific approach. In comparison, using the "universal primer" approach, archaeal signatures of 14 different phyla and 92 different genera were detected, while the Archaea-specific primer set detected 12 different phyla and 87 different genera. Most of the genera were covered using both primers, however, 20 genera were only covered with the universal approach and 16 genera were only covered with the archaeal-specific approach. Strikingly, sequences assigned to the candidate phyla Aigarcheaota and the Ancient Archaea group (both phyla with signatures detected in hydrothermal systems; Takai and Horikoshi, 1999; Hirayama et al., 2005; Goertz and Hedlund, 2013; Rinke et al., 2013) were obtained only by using the "universal approach". The main archaeal taxa retrieved using the universal approach were methanogenic Euryarchaeota (mainly in permafrost samples, sulphidic spring samples) and Thermoplasmata (mainly from acidic environments). The "hypersaline environment" harboured mostly signatures belonging to archaeal Halobacteria. A summary of all retrieved archaeal taxa is shown in Supplementary Information Figure 4.

The microbiome of MASE sites harbors a vast diversity of hardy and anaerobic organisms. Apart from Archaea, we were also interested in the microbial community and thus used a "universal" primer set (as proposed by the Earth microbiome consortium; Gilbert et al., 2014) that targeted the entire microbiome.

Also here, we amplified both 16S rRNA genes pools of PMA treated and PMA untreated samples. We obtained a total number of 1,523,276 sequences (minimum count: 10,819, "permafrost SlpD14-3*"; maximum number of counts: 119,379, "acidic river Gal*"; mean counts: 60,851). After processing the raw sequences using the DADA2 algorithm, in total, 15,945 different RSVs were obtained. In the following, we will concentrate on the intact microbial proportion (PMA treated samples, and the PMA untreated "hypersaline environment"). The taxonomic profile of all PMA untreated samples is given in Supplementary Information Figure 5.

"Viable" microbiomes in MASE environments were generally characterized by Proteobacteria (27%), followed by Actinobacteria (11%), Bacteroidetes (9%), Chloroflexi (8%), Acidobacteria (6%), Firmicutes (5%), Euryarchaeota (6%) and a minor proportion (2%) of unassigned Archaea (mean values of total amount of RSV counts; Figure III.4-1c).

Signatures of Proteobacteria were present throughout all sampling sites, but in different proportions. Signatures of Actinobacteria were also detected in each environment with the exception of the "hypersaline environment". This biotope also revealed only a minor proportion of Firmicutes signatures, which were found completely absent in samples from the acidic lake* environment, but were present in all other samples.

In the datasets, we identified the sequences affiliated to a diversity of anaerobic microorganisms, such as methanogens (Prieur, 2004; e.g. *Methanobacterium*; Kral et al., 2004 and *Methanomassilicoccus*; Teske, 2014), halophiles (Leuko et al., 2010), autotrophs (McKay, 1997), phototrophs (e.g. Cyanobacteria; Cabrol and Grin, 1995), acidophiles (e.g.

Acidithiobacillaceae; Amils et al., 2011), psychrophiles (e.g. *Pseudomonas*; Hoover et al., 2002*, Oscillatoria*, *Phormidium* and *Arthrobacter;* Gilichinsky et al., 2007), iron reducers (Boston et al., 1992; e.g. *Geobacter*; Lovley et al., 1993, *Shewanella*; Nealson and Cox, 2002), sulfate reducers (Banfield et al., 2001; e.g. *Desulfobacterium*, *Desulfovibrio*, and members of Desulfuromonadaceae and Desulfovibrionales) all of them equipped with the capability for an anaerobic metabolism.

The abundance of different taxa of anaerobic microbes in the MASE samples varied according to the respective environmental conditions, i.e. sulfate-reducers were most abundant in sulfur-rich sulphidic springs, cyanobacterial signatures were most abundant in high-altitude glacier samples and signatures of psychrophiles and methanogens were mainly detected in permafrost samples (more details are shown in the RSV Tables, given in Supplementary Table 2 and 3). Due to our focus on anaerobic microorganisms, we specifically looked for the presence of sequences belonging to taxa known for their strictly anaerobic metabolism. For instance, signatures of *Aerophobetes*, *Latescibacteria*, *Anaerolina*, *Halanaerobium* members of Thermoanaerobacterales *Desulfosporosinus*, Desulfarculales, Desulfobacterales, *Clostridium* and Desulfuromonadales were detected, however, not equally abundant in each environment. A minor number of RSVs classified as *Desulfovermiculus* (1371 sequence counts) and *Limimonas* (4123 sequence counts) for instance were exclusively detected in the "hypersaline environment". Phototrophic cyanobacteria, with members representing extremely desiccation-resistant microbes on the other hand were absent in this specific environment but present in all other environments as the hypersaline saline is light-secured and thus might not support their growth. Radiation-resistant microbes such as members of Deinococcus-Thermus (*Deinococcus*, *Truepera* and additional unclassified members), were detected in the glacier samples*, sulphidic spring* samples, permafrost* samples and acidic river* samples. Also spore-formers, such as *Bacilli* and *Clostridia*, in general resisting harsh environments, were found in all environments.

***Cultivation and microbiome analyses reveal different microbial communities that overlap only partially*.** 16S rRNA gene sequences retrieved by NGS and from cultivated isolates were compared and searched for an overlap on genus level. 27% of all cultivated genera could be recovered by NGS analysis (in both PMA treated and untreated samples) in their respective environments. We compared the viable proportion (thus potentially cultivable) with the cultivated part and not all environments harbored signatures of their cultivated representatives (Table III.4-3). Except *Clostridium*, none of the cultivated microbial genera were found to be represented in a substantial amount within the microbiomes of the different biotopes.

Table III.4-3 Comparison of NGS data and cultivation efforts. * marks PMA treated samples.

Cultivation			**Next generation sequencing**		
Isolate (genus level)	**Number of isolates**	**Isolated from biotope(s)**	**Number of RSVs detected**	**Detected in PMA untreated samples from:**	**Detected in PMA treated sample from:**
Aeromonas	2	Sulphidic spring	-	-	-
Obesumbacterium	1	Sulphidic spring	-	-	-
Buttiauxella	1	Sulphidic spring	-	-	-
Clostridium	5	Sulphidic spring, acidic lake	49	Acidic lake, sulphidic spring, glacier, permafrost	Acidic lake, acidic river, sulphidic spring, glacier, permafrost
Trichococcus	1	Sulphidic spring	3	Permafrost, sulphidic springs	Permafrost, sulphidic springs
Desulfovibrio	1	Sulphidic spring	4	Permafrost, sulphidic springs, acidic lake, acidic river	Permafrost, sulphidic springs, acidic lake
Citrobacter	2	Sulphidic spring	-	-	-
Hafnia	1	Sulphidic spring	-	-	-
Yersinia	6	Sulphidic spring, acidic lake	1	Sulphidic springs	Sulphidic springs
Pelosinus	1	Acidic lake	-	-	-
Desulfosporosinus	1	acidic lake	6	Permafrost, glacier, acidic lake, acidic river	Permafrost, glacier, acidic lake
Cellulomonas	3	Acidic lake	4	Permafrost, sulphidic springs, acidic lake, acidic river	Permafrost, sulphidic springs
Serratia	1	Acidic lake	-	-	-
Enterococcus	1	Glacier	-	-	-
Rahnella	1	Glacier	-	-	-
Methanomethylovorans	1	Sulphidic spring	1	Sulphidic springs	Sulphidic springs
Halanaerobium	1	Hypersaline environment	1	Hypersaline environment, sulphidic springs	-
Sodalis	1	Permafrost	-	-	-

The sulphidic springs harbor the most diverse microbial community. Within the archaeome (following the approach with specific Archaea-targeting primer sets), the highest richness and diversity was observed in the sulphidic spring biotopes (observed species: 529.5, Shannon index: 4.95; mean of all samples of the sulphidic spring biotope; PMA treated and untreated); on the contrary, samples from the acidic lake (pH 3) obtained an overall very low microbial richness and diversity (observed species: 14.5, Shannon index: 1.30; mean of all samples of the acidic lake; PMA treated and untreated). More details can be found in Figure III.4-2a and Supplementary Table 4. With respect to PMA treated and the PMA untreated counterparts, the archaeal richness and diversity values were very similar. Also on general microbiome level, the samples from sulphidic springs showed the highest diversity (observed species: 2589, Shannon index: 6.28 (mean all samples (PMA treated and untreated) of the sulphidic spring biotope)), whereas the lowest diversity was observed in samples from the hypersaline environment (observed species: 155, Shannon index: 3.8; Figure III.4-2d, see also Supplementary Table 5).

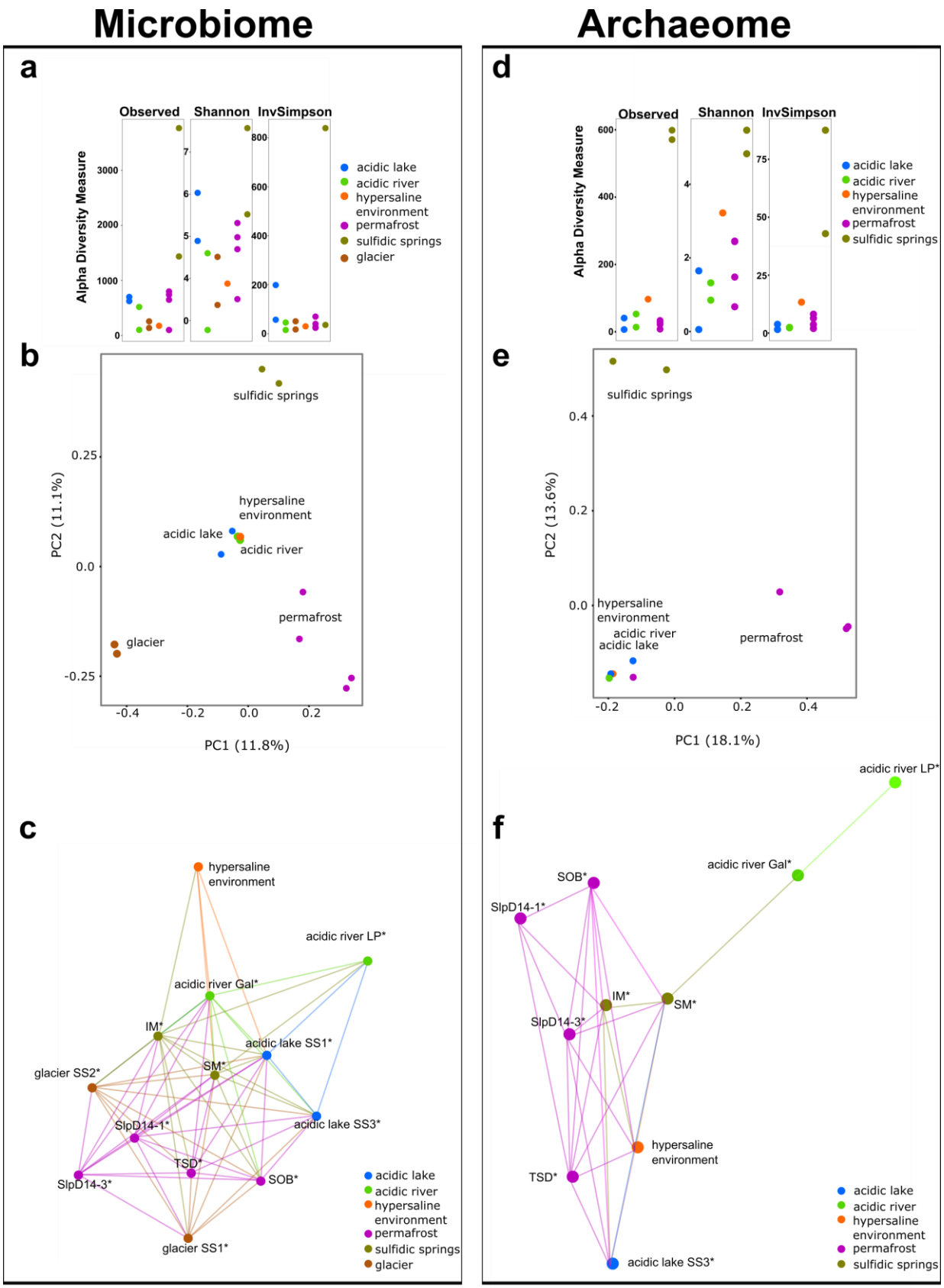


Figure III.4-2 Microbial diversity and network analysis of microbial communities in samples from MASE sites. The left panel gives information on the general microbiome, whereas the right panel displays results from the archaeome analyses. The alpha diversity (a, d), beta diversity (PCoA plots; unweighted Bray-Curtis distance; b,e) and the connections between in the sampling sites is shown as a network (c,f).

The beta diversities of the archaeome and the general microbiome were visualized via PCoA plots (based on unweighted Bray-Curtis distances; Figure III.4-2b, e). The microbial communities derived from the acidic environments such as the acidic river and acidic lake (although obtained from geographically remote sampling sites in Iceland and Spain) showed high similarity on both PCoA plots (Figure III.4-2b, e), along with the sample from the "hypersaline environment". The permafrost samples (with one exception), the sulphidic spring samples and the glacier samples (only "universal" primer set) grouped into own clusters.

To analyse the order of connectivity amongst sampling sites, networks based on archaeome and microbiome were constructed (max. dist=1.0, line weight=0.4; Figure III.4-2c, f). Regarding the archaeome, the samples derived from acidic environments (acidic lake and acidic river) were not highly connected to other sampling sites (only 1 edge connected; Figure III.4-2f). The permafrost samples showed a high level of connection to the sulphidic springs and one acidic lake sample.

The microbiome network (max. dist=1.0, line weight=0.4), was overall similar to the archaeome network, namely a low connection of acidic environmental samples, but a high connection of non-acidic environments (Figure III.4-2f). Notably, the "hypersaline environment", representing an environment with very pronounced physical and chemical parameters (dissimilar to the other sampled environments), connected primarily with acidic environmental samples. Overall, the hypersaline environment clustered strongly with the acidic environments on the microbiome level. This observation could point to a potential microbial relationship of both environmental types.

In order to display the connectivity between the hypersaline environment and the acidic environments in more detail, the core genera were determined. Eight overlapping taxa were resolved to genus level (*Paludibacter*, *Aquabacterium*, *Geobacter*, *Sulfurovum*, *Beggiota*, *Thiothrix*, *Spirochaeta_2*, and *Opitutus*). Most of the core taxa were observed to be affiliated to Proteobacteria (Supplementary Information Figure 6).

A comparison between MASE sites and sites with no astrobiological relevance (i.e. Austrian streamlet; "Ragnitzbach" and human stool samples) revealed a remote clustering of the MASE sites (Supplementary Information Figure 7). This indicates strongly that there is a specific community in our MASE sampling sites, which is distinct from sites with no astrobiological relevance.

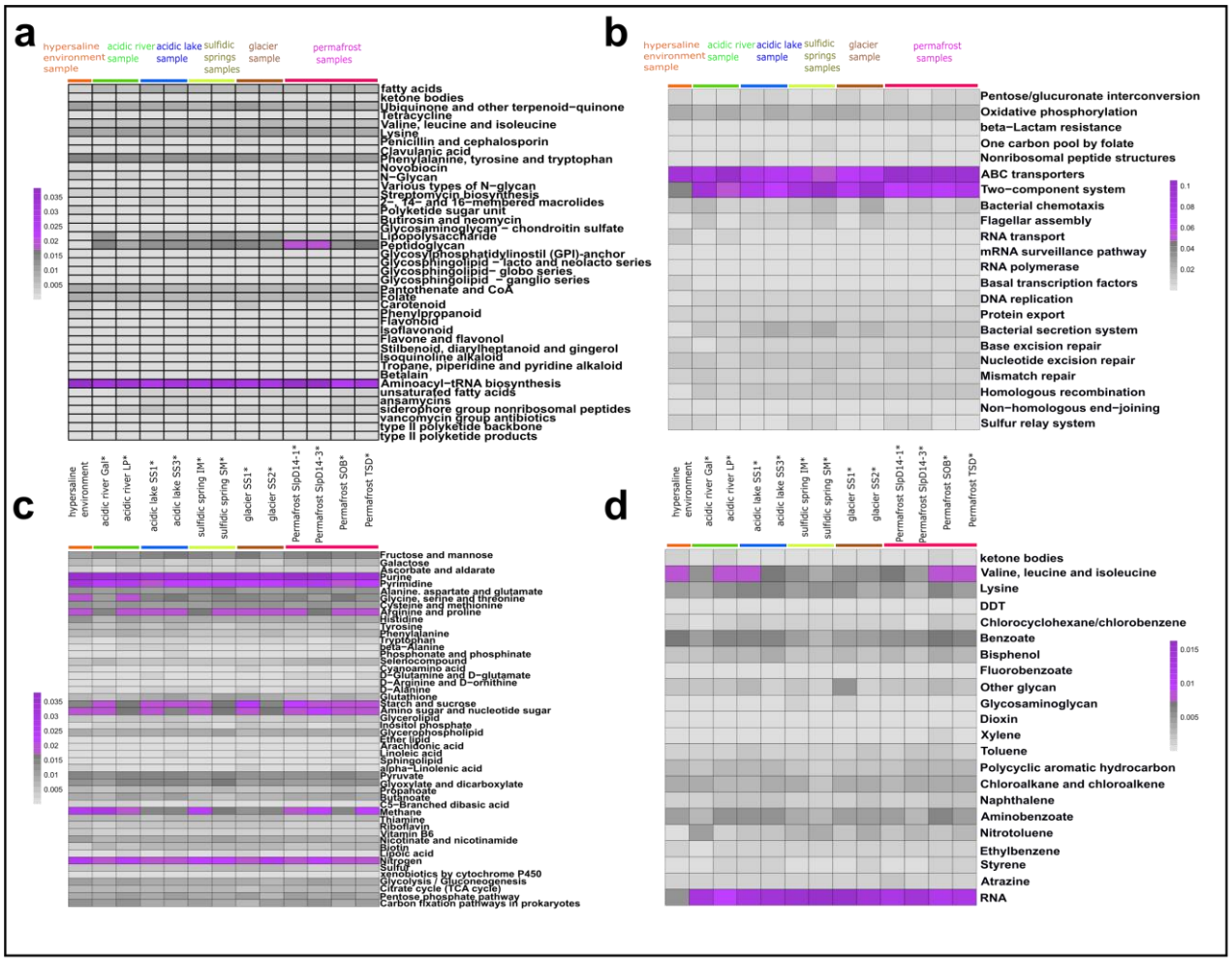
The prediction of Mars/space-relevant metabolic capacities. Furthermore, we were specifically interested in the functional capabilities of the microbial communities of the different Mars analogue sites. The major focus was set on resistance, adaptation capability and metabolic pathways, with particular interest in the conversion of organic and inorganic compounds.

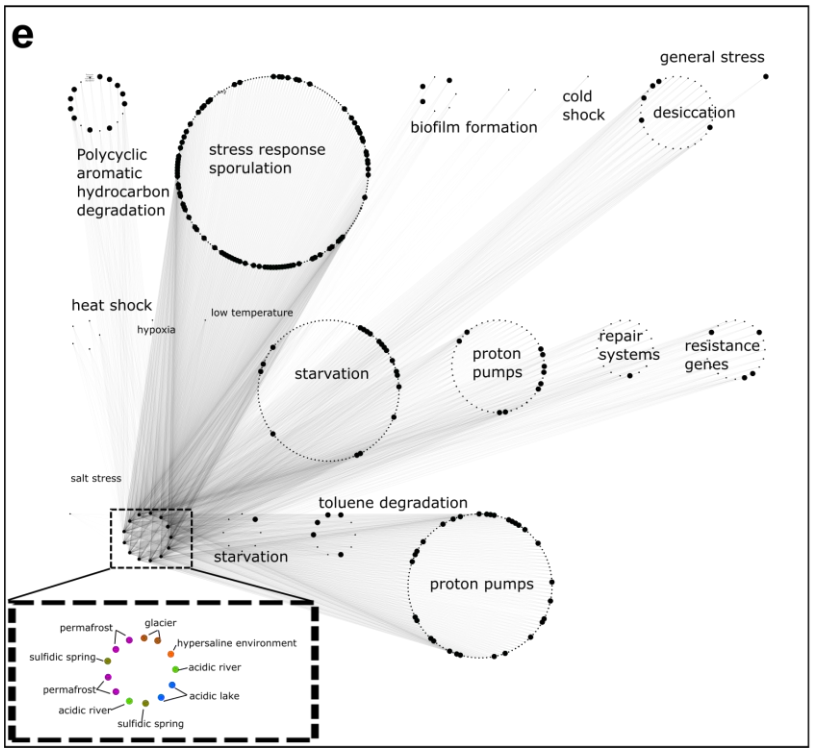
We predicted the entire set of present pathways (284) and genes (6600) for PMA treated samples (including the "hypersaline environment") using the R tool Tax4Fun (Aßhauer et al., 2015), and relevant data were extracted from the dataset for further detailed analyses. In particular, we were interested in pathways involved in stress resistance, stress responses and adaption genes in general. Interestingly, genes encoding functions involved in the basic stress and adaption system (i.e. the two-component system) were predicted for all biotopes having a general high abundance. This was also true for genes belonging to the family of

ABC-transporters. Other stress response systems, such DNA repair (e.g. homologous recombination, mismatch repair, base excision repair) could be detected as well and were present in each environment (Figure III.4-3a-d).

Microorganisms thriving in Mars analogues sites were found to potentially exhibit a wide variety of genes enabling the degradation of diverse chemical compounds ranging from toluene, naphthalene, styrene, glycan and benzoate. Also genes responsible for the degradation of amino acids (in particular, valine, leucine, isoleucine, and lysine) were present. Their gene abundance was predicted to be the highest in permafrost samples, the hypersaline environment and the acidic environment.

In order to withstand extreme conditions such as desiccation and radiation microorganisms must possess several genes responsible for protection/ and or repair mechanisms. In an extensive survey of the predicted functional genes set, several genes with that capacity were found (i.e. genes responsible for stress response, sporulation, response to starvation, heat shocks, hypoxia etc.), but they differ in their abundance (Figure III.4-3e). The most abundant predicted genes (>0.005% of selected, stress-relevant predicted genes) comprised genes encoding for sporulation, motility, proton pumps, DNA repair (e.g. *RadA*, *RecN*) and others and are depicted in Figure III.4-3f and Supplementary Information Figure 8.





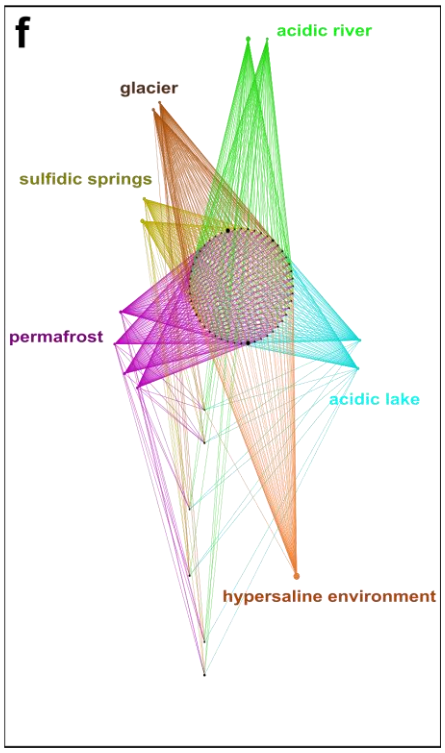
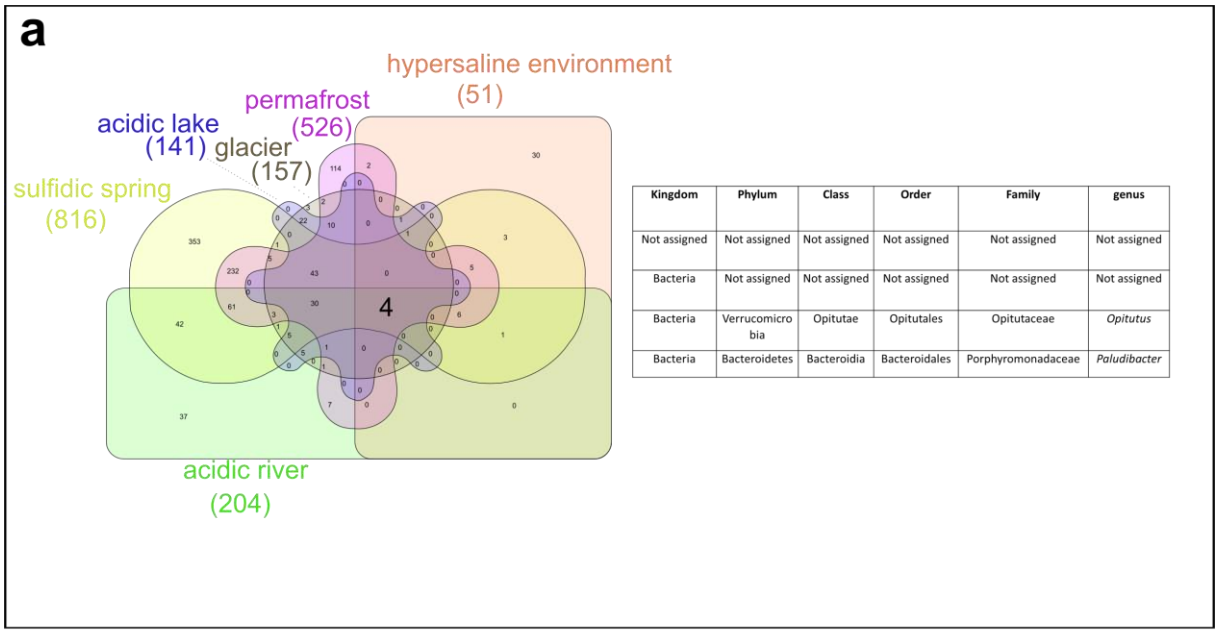


Figure III.4-3 Predicted KEGG3 pathways and network of functional genes of the MASE "universal" microbiome (continues on next page).

Figure III.4-3 (continuation) The heatmaps in (a-d) display the relative abundance of predicted pathways in each sample. (a) shows the genes involved in various synthesis pathways, (b) genes encoding for transporters and repair systems (amongst others), (c) genes encoding for the metabolic pathways, and (d) genes encoding for degradation pathways . In (e) and (f), genes were selected individually and their abundance is shown as networks. Each node represents the summed relative abundance throughout all samples. The size of the nodes is representative for the relative summed relative abundance. The edges display the connection to corresponding sampling sites (close-up). For (f) genes with a rel. abundance >5% were selected to generate a network. The genes are described in detail in Supplementary Information Figure 8.

***Physical and chemical parameters that shape the microbiome*.** Fitting environmental variables, namely water activity, temperature and pH values, onto the NMDS ordinations revealed that the microbial community variation might be best explained by temperature (Supplementary Information Figure 9) followed by pH values. Water activity measurements were similar in each site, except for the "hypersaline environment", which had the lowest value (0.7).

The MASE core microbiome. The core microbiome of all sampling sites comprised (PMA treated samples + "hypersaline environment") four taxa: one taxon could not be assigned on the kingdom level, one taxon was classified as bacterium but not further, and two taxa could be resolved on genus level: *Paludibacter* (12 different RSVs present in all sampling sites, 0.3% of all RSV counts) and *Opitutus* (34 different RSVs present in all sampling sites; 0.1% of all RSV counts). One RSV deriving from the "sulphidic spring SM*" was found to be very closely related to the type strain *Paludibacter propionicigenes*, an obligate anaerobic bacterium producing propionate (Ueki et al., 2006) and two RSVs classified as *Opitutus* were branching very close to the type strain *Opitutus terrae*, an obligately anaerobic microorganism (Chin et al., 2001; "acidic river Gal*" and permafrost*; Figure III.4-4a, b).



Kingdom	Phylum	Class	Order	Family	genus
Not assigned	Not assigned	Not assigned	Not assigned	Not assigned	Not assigned
Bacteria	Not assigned	Not assigned	Not assigned	Not assigned	Not assigned
Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	*Opitutus*
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Paludibacter*

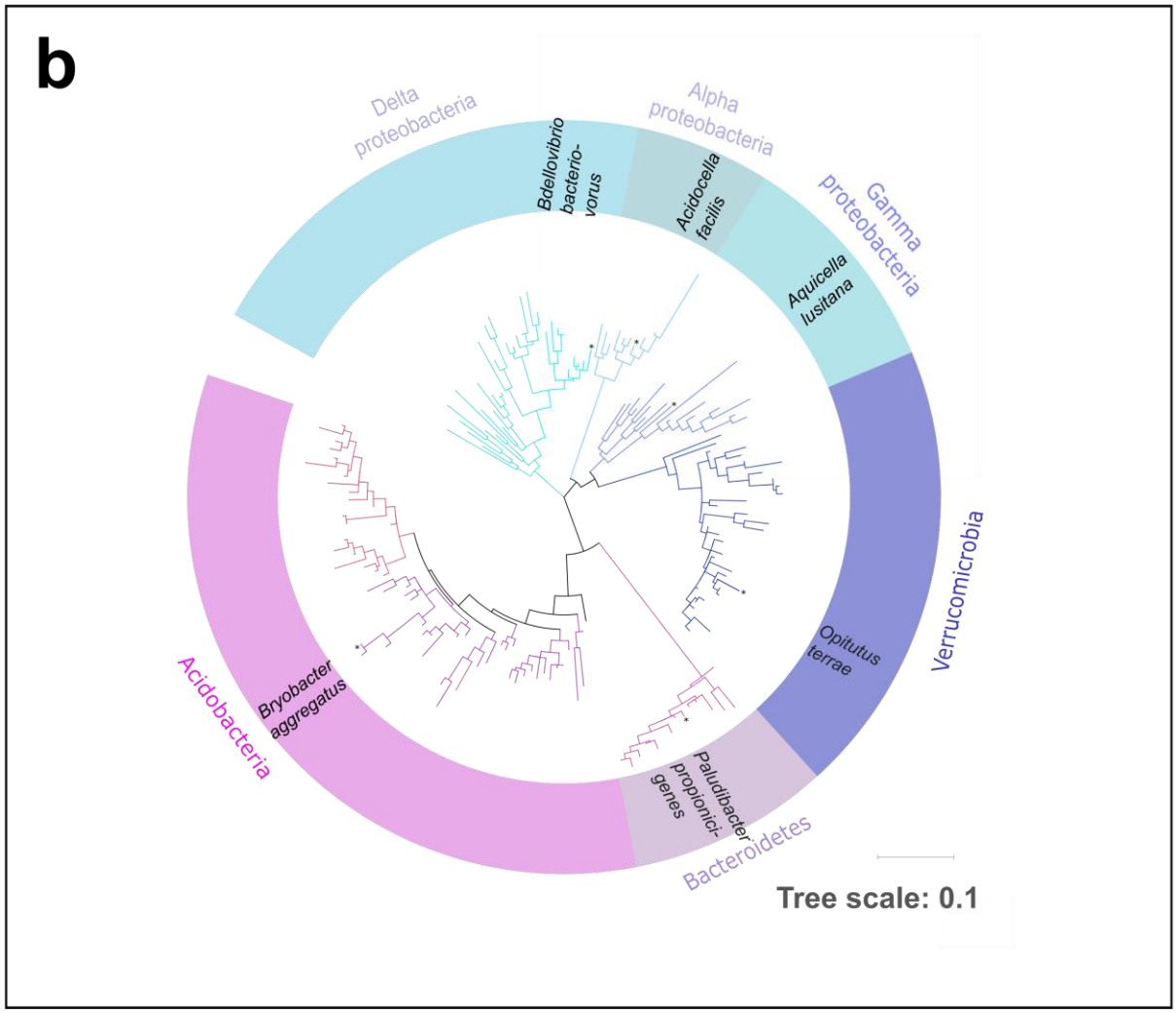


Figure III.4-4 The MASE core microbiome. (a) displays a Venn diagram including all genera of samples treated with PMA (represented in corresponding sampling sites) and the PMA untreated hypersaline environment. Four genera were abundant in all samples. In (b), the phylogenetic tree displays all RSVs, which were common in all sampling sites. Available type strain 16S rRNA gene sequences of corresponding genera were included in the calculation.

The viable core microbiome of all environments (i.e. excluding the PMA untreated "hypersaline environment") exhibited 34 common taxa. Seven of them could be resolved on the genus level (namely *Bryobacter* (35 different RSVs), Candidatus *Solibacter* (22 different RSVs), *Acidocella* (9 different RSVs), *Bdellovibrio* (34 different RSVs), *Aquicella* (16 different RSVs), *Opitutus* (34 different RSVs) and *Paludibacter* (14 different RSVs) and 20 could be resolved on the order level. Most RSVs belong to the family Anaerolinacaea (239 different RSVs), followed by members of the order Gaiellales (92 different RSVs) and the family Planctomycetaceae (88 different RSVs; Supplementary Information Figure 10).

The core microbiome of PMA untreated samples representing the Early Mars environments and the core microbiome of all PMA untreated samples is shown in Supplementary Information Figure 11 and 12. Here 20 common genera were observed and three could be resolved to the genus level (*Bdellovibrio*, *Bryobacte*r and *Anaeromyxobacter*) in PMA treated samples. When including the "hypersaline environment", two taxa are shared (Gammaproteobacteria and unassigned Bacteria).

Notably, we could not detect any core archaeal taxa.

None of the mentioned genera in the core microbiome could be retrieved by cultivation. The MASE core microorganisms are of uttermost interest, since they could represent excellent model organisms for studying adaptation and resistance properties. In order to identify potential drawbacks in our cultivation procedures and selected methods, the functional capacity of the core microbiome was predicted *in silico* and compared with the predicted functions of the cultivated microorganisms.

The comparison of predicted metabolic capabilities (Supplementary Information Figure 13) revealed a major difference in the need of specific carbon sources (Glycolyse/Gluconeogense) and Cofactors. Obviously, due to the faster growth of heterotrophs, they were supported by our enrichment procedures, as they were able to overgrow other, slow growing (autotrophic?) microorganisms. This explains the low number of autotrophic isolates but is encouraging for future cultivation attempts. In the anticipated experiments, a higher amount of vitamins and Cofactors, as well as less organic compounds should be used.

Discussion

Here we report, for the first time, on the comprehensive investigation of the viable microbiomes hosted by anoxic, Mars-analogue environments exhibiting a wide range of physical and chemical extremes. Our study combined a comprehensive site description, broad sampling and an extensive molecular NGS-based analysis, supported by a wide-ranging cultivation approach. The latter comprised 1034 enrichment attempts, that resulted in 69 stable enrichments and 30 anaerobic, pure bacterial isolates and one archaeon (*Methanomethylovorans* sp.; Cockell et al., 2016b). Our restricted time-frame, however, did not allow, the cultivation of slow-growing autotrophs, which are of higher interest with respect to the scarce amount of organics on Mars (Freissinet et al., 2016; Sutter et al., 2016).

To our knowledge, our study is the first, using propidium monoazide (PMA) to mask background DNA from dead microorganisms in Mars-analogue extreme settings. This method allowed us to retrieve information on a vast diversity of the microbiomes and

archaeomes of the intact and thus probably living microbial communities. However, the diversity found was only partially covered by cultivated isolates, a phenomenon already described earlier (Ellis et al., 2003; Donachie et al., 2007; Lagier et al., 2012; Dubourg et al., 2013). Information on a diverse set of (anaerobic) taxa, which were present in all sampling sites could be retrieved, and also several signatures of taxa with astrobiological relevance were detected (Rivkina et al., 2004; Ganzert et al., 2007; Blank et al., 2009; Amaral-Zettler et al., 2011; Sánchez-Andrea et al., 2011). Noteworthy, a large fraction of signatures could not be phylogenetically assigned and the role of respective microorganisms in these extreme environments remains unclear.

In-silico analysis predicted each microbiome to be equipped with resistance and adaption capacities, which might enable the microorganisms encoding the corresponding genes to survive in their respective extreme biotopes. A cosmopolitan group of (mostly) mixotrophic and anaerobic microorganisms was able to reside in Early Mars analogue settings and a small fraction thereof (namely *Opitutus* and *Paludibacter;* Chin et al., 2001; Ueki et al., 2006) was also observed to withstand potential Present Mars analogue conditions. However, the predicted metabolism of the core genera was not targeted by our standard cultivation medium and, thus, as a logic consequence, most core microorganisms escaped our cultivation efforts.

Hardy and versatile microbes are highly relevant in astrobiology, and their cultivation deciphers new insights into their potential for resistance to extremes. A number of potential model organisms was retrieved, which directly feed into the other goals of the MASE project, namely studying the limits of growth of selected isolates, deciphering the molecular principles of resistances, analysing the genomic inventory of representative microbes, studying the fossilisation processes and detectability of biomarkers during artificial fossilisation, and the optimisation of automated life detection (Cockell et al., 2016b).

Overall, our study showed that anaerobic microbial communities from Mars-analogue settings on Earth possess an impressive machinery to withstand physical and chemical pressures. Notably, a small subset of signatures from viable microorganisms was found in all different environments analysed, revealing the presence of poly-extremotolerant allrounders, that could be perfectly well-suited for inhabiting also extraterrestrial environments with variable conditions. Overall, the outcome of the MASE project will certainly be a highly valuable catalogue for microbial community analysis in anoxic environments, with a strong impact for the search for life in the universe.

Conflict of Interest

The authors do not declare a conflict of interest.

Author`s contribution

AKP planned the study, participated during sampling trips, performed experiments, bioinformatics and wrote the manuscript. LW and SD assisted in performing physiological tests and cultivation. EM participated during sampling, provided geochemical context data and post-sampling analyses; proof read manuscript. PS participated during sampling, performed cultivation and experiments, contributed to writing and editing of the manuscript. CC helped writing the manuscript, participated during sampling trips and conceptualized the MASE project as a whole. KB-V provided assistance during elaboration and interpretation of the physiology data, contributed to the writing and editing of the manuscript. FG and FW contributed to writing and editing of the manuscript. PV participated during sampling trips, performed cultivation and experiments, contributed to writing and editing of the manuscript. PR participated during sampling (Iceland, glacier), assisted during

elaboration and interpretation of the physiology data, contributed to the writing and editing of the manuscript. MBo provided assistance during elaboration and interpretation of the physiology data. NW, PC, FG, LG-D, PE, MBa, AM proof read the manuscript. MM provided samples from Río Tinto, performed enrichment cultures for iron oxidisers and reducers for different sampling sites. RA provided samples from Río Tinto, supervised culture enrichment procedures, proof read the manuscript. VM participated during sampling trips, performed cultivation and experiments, contributed to the writing and editing of the manuscript. AE provided assistance in FISH experiments. CME participated in sampling, planned and supervised the study, supported interpretation of the data and wrote the manuscript.

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Supplementary information

Supplementary information can be found on the supporting CD (./Selected publications/08 Perras et al., submitted)

IV. General Discussion

This thesis reports on the results of a comprehensive investigation upon microbial communities hosted by extreme environments. Cold sulphidic springs harboured a fascinating archaeal biofilm, formed by the SM1 Euryarchaeon. The cells exhibited unique cell surface appendages ("hami") and a double-membrane, positioning it into an extraordinary place amongst the Archaea (Perras et al., 2014; Probst et al., 2014b; Perras et al., 2015). Their biofilms present in two distinct spring systems were investigated and confirmed strain specific variation on ultrastructural level (Perras et al., 2014; Probst et al., 2014a). Furthermore, the hamin protein was speculated to diverge from S-layer proteins.

A second extreme environment was represented by the International Space Station (ISS). The ISS orbits Earth as a man-made confined extreme biotope and primarily harbours well-adapted human-associated microorganisms (Checinska et al., 2015). Eight to twelve year old dust samples derived from the Russian ISS modules contained archaeal signatures and cultivated ISS microorganisms showed an extraordinary range of stress resistances (Mora et al., 2016b). A large set of anoxic terrestrial Mars-analogue settings delivered novel model organisms to support the search for extraterrestrial life. A comprehensive NGS and cultivation-based survey within the frame of the MASE (Mars Analogues for Space Exploration) project described a high diversity of microorganisms. A cosmopolitan group of viable, anaerobic novel model organisms was observed to reside in all settings (Perras et al., 2016). The findings provide new aspects for the search of extraterrestrial life.

1. Small size matters! Recent findings on the grappling SM1 Euryarchaeon

The uncultivated SM1 Euryarchaeon is dominating subsurface biofilms within two sulphidic springs in Bavaria, Germany (Islinger Mühlbach and Sippenauer Moor). The biofilms underwent an extensive genetic, chemical and ultrastructural analysis with results emphasising the outstanding position of the SM1 Euryarchaeon within the archaeal world.

Although the SM1 Euryarchaeon positions within the group of methanogens in phylogenetic trees (Rudolph et al., 2001), it is not capable of methanogenesis which is a characteristic trait of methanogens (Balch et al., 1979; Probst et al., 2014b). Based on metagenomic studies, the SM1 Euryarchaeon genome is missing genes for the complete methanogenic pathway.

Intriguingly, unique metabolic features including a novel autotrophic pathway, namely a reductive acetyl-CoA pathway, was revealed (including two independent C1 carrier pathways; Probst et al., 2014b). This feature is common in methylotrophic bacteria (Chistoserdova et al., 1998), but is rarely described in this form for Archaea. The pathway was confirmed by transcriptomic studies and is speculated to serve for carbon dioxide fixation and assimilation into activated acetic acids. The energy metabolism was hypothesised to rely on CO and H_2 (Probst et al., 2014b), but the pathway was not fully uncovered and potential electron donors/acceptors have yet to be elucidated (Probst et al., 2014b). This is of uttermost interest, since the knowledge of a fully resolved metabolism delivers valuable hints for its cultivation.

Next to metabolic pathways, the state of division was investigated. Amongst others, the cell division triggering gene *FtsZ* was identified to be encoded and its transcription was confirmed. A prerequisite for an active cell division is the aggregation of FtsZ proteins forming a ring-like structure. Immuno-staining experiments targeting FtsZ homologues proved the forming of these ring-like structures in ~73% of all SM1 Euryarchaeon cells. This result was confirmed by TEM and FIB-SEM imaging. Thus, the majority of SM1 euryarchaeal cells are in a continuous growth and viable (95% of all cells within the biofilm; Henneberger et al., 2006). It remains to be proven if their growth is restricted to the (deep) subsurface or also occurs at the surface, as it was assumed earlier (Moissl et al., 2003).

These findings motivated further cultivation attempts. In the frame of the MASE project, the sulphidic spring were assigned as Mars-analogue environments due to their cold, organic-poor and sulphate-rich conditions (Cockell et al., 2016b). In particular the ability to form biofilms suggests the SM1 Euryarchaeon as a promising model organism for astrobiology. Biofilms can cope better with chemical and oxidative stress compared to their free-living counterparts and show an enhanced rate of survival when exposed to Martian simulations (Bridier et al., 2011; Burmølle et al., 2006; Geier et al., 2008; Baqué et al., 2013). Cultivation attempts of the SM1 Euryarchaeon were extensively performed, however, none of the attempts were successful (Cockell et al., 2016b; Perras et al., 2016).

In very recent studies, multiple protein markers replaced 16S rRNA environmental markers and merged the SM1 Euryarchaeon and close relatives into a new monophyletic euryarchaeal order: *Candidatus* Altiarchaeales (“deriving from the deep”; Probst et al.,

2014b; Probst and Moissl-Eichinger, 2015), which branches within the novel phylum DPANN (Rinke et al., 2013). The SM1 Euryarchaeon is proposed to be named "*Candidatus* Altiarchaeum hamiconexum sp. nov." and is described in detail in Probst et al., 2014b. The proposed name will replace the term "SM1 Euryarchaeon" throughout the following text.

Members of the order *Candidatus* Altiarchaeales are widespread and were also very recently detected in the Crystal Geyser spring (USA; Probst et al., 2014b) and deep anoxic methane and sulphide rich sediments (USA; Bird et al., 2016). Genome and metagenomic analysis confirmed an order-wide and novel autotrophic metabolism. Based on further phylogenetic investigation, the order was subdivided into the Alti-1 and Alti-2 subclades (Bird et al., 2016). Alti-1 contains organisms from actively flowing springs (Islinger Mühlbach, Sippenauer Moor and the Crystal Geysers), equipped with genes encoding for hami structures and biofilm formation. In contrast, the more diverse and widespread clade Alti-2 contains planktonic, free-living members lacking hami-encoding genes (Bird et al., 2016, Figure IV.1-1).

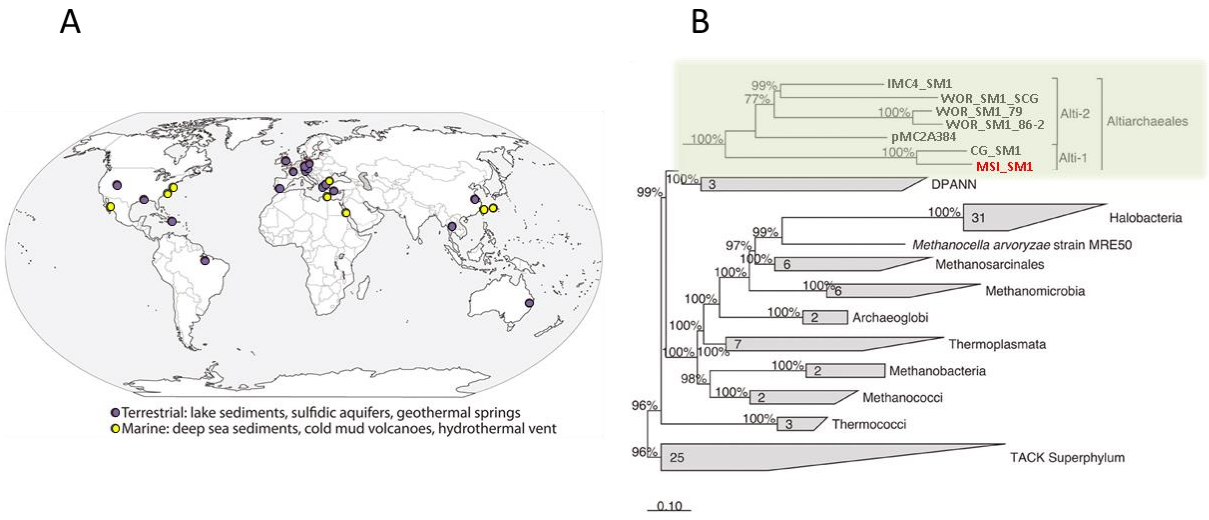

Figure IV.1-1 Distribution of Altiarchaeales signatures present in the NCBI database is shown on a global map (A). The phylogenetic tree (B) is based on conserved universal proteins and shows the new subdivision of Altiarchaeales in Alti-1 and Alti-2. The images were taken from Bird et al., 2016

The biofilms of the Islinger Mühlbach are highly dominated by multiple *Candidatus* Altiarchaeum hamiconexum strains (relative abundance: 87.7%; Probst et al., 2014b) and a minor proportion of a close relative (IM-C4; relative abundance: 1.3%, branching within the Alti-2 clade; Probst et al., 2013b; Probst et al., 2014b).

Within the biofilm, different species of sulphate-reducing bacteria represented 3.7% of the microbial community (mainly *Desulfocapsa*; Deltaproteobacteria). The bacterial community of the two biofilms varied at species and strain level, but the overall Archaea-predominated microbial composition remained similar (Probst et al., 2014a; Probst et al., 2014b). This microbial composition was constantly observed over a period of ten years and strongly suggests an interaction of the two domains (Probst et al., 2014a). Such an inter-domain interaction was exclusively observed in the anaerobic, methane-oxidizing archaea consortia thus far (ANME; Lloyd et al., 2006) .

As mentioned before, molecular analyses based on unique gene markers proved two site-specific strains (Probst et al., 2014a). In other words, *Candidatus* Altiarchaeum hamiconexum deriving from the Sippenauer Moor (*Ca.* A. hamiconexum-SM) is phylogenetically distinct from the strain hosted by the Islinger Mühlbach (*Ca.* A. hamiconexum-MSI). Furthermore, cells from both sites exhibit significantly different sizes as confirmed by comprehensive ultrastructural analysis (Perras et al., 2014).

A typical archaeal cell envelope is composed of one membrane often covered by a highly ordered, crystalline sheath, the S-layer. Both *Ca.* A. hamiconexum strains possess a double membrane which is composed of glycosyl-archaeol lipid species (Probst et al., 2014b; Perras et al., 2015). In general, the dimensions of the membranes reflect bacterial Gram-negative cell membranes (Perras et al., 2014). Only few archaeal species namely *Methanomassilicocus luminyensis* (Dridi et al., 2012; Lang et al., 2015), *Ignicoccus hospitalis* (Rachel et al., 2002), *Ca*. Methanoplasma termitum (Lang et al., 2015) and ultrasmall ARMAN cells (Comolli et al., 2009) exhibit a double membrane system. This trait might be more widespread amongst Archaea, since a recent study demonstrated the challenging preparation of the sensitive archaeal outer membrane (Klingl, 2014; Lang et al., 2015). Presumably, archaeal outer membranes might have been disrupted along the preparation procedure and the composition might be incorrectly considered as one single membrane. This emphasises the need of a detailed and careful re-examination of accessible archaeal membrane layers (Perras et al., 2014; Lang et al., 2015).

Double membranes may have many benefits, such as the intra- and inter-species interaction. These are particularly found in members of syntrophic partnerships (Orphan, 2009). The importance of the *Ca.* A. hamiconexum double membrane is not clear, however, it is

speculated that it provides an advantage in the potential interaction with bacterial partners. Interaction with Bacteria was observed to be primed by the hami. Occasionally, filamentous bacteria were observed to be cocooned almost completely by hami (Perras et al., 2014; Probst et al., 2014b). Remarkably, contact with only one filamentous morphotype was observed, indicating a certain type of interaction whose role remains unclear. Noteworthy, the cells of some of these bacteria often showed numerous perforations. These pictures created the impression of a predatory archaeon, which perforate the cell wall by using the anchor-like hami as instruments and nourish bacterial substances. This is highly speculative and would assign hami as weapons. A number of predatory bacteria were frequently found in the past (Makkar and Casida Jr, 1987). *Bdellovibrio* for instance, uses its polar flagellum to attack, kill and feed on their prey (Starr and Baigent, 1966). The most striking advantage of this behaviour is an enhanced accessibility of nutrients. *Ca.* A. hamiconexum might eliminate bacterial competitors entrapped within the biofilm and simultaneously obtain valuable energy sources in its nutrient-poor environment. The ability of biofilm formation is restricted to Alti-1 members and has only been observed in the Bavarian spring waters. It appears to be advantageous within this specific biotope, however, is it unknown how planktonic cells cope with their environment without hami.

The hami are intriguing from any point of view. Filaments were confirmed to be composed of one major protein subunit (120 kDa; Moissl et al., 2005; Perras et al., 2015), and the protein sequence was not homologues to any known prokaryotic cell-surface appendage (e.g. pili, flagella, fiber, curli, archaella). *In silico* analysis predicted the secondary structure of a prominent beta sheet topology, which is known to prime for self-assembly (Makabe et al., 2006). The hamus structure is unique in its full extent, however, the prickle region strongly resembles the trimer symmetry of S-layer proteins SlaB expressed by the archaeon *Sulfolobus solfataricus* (Baumeister and Lembcke, 1992; Figure IV.1-2).

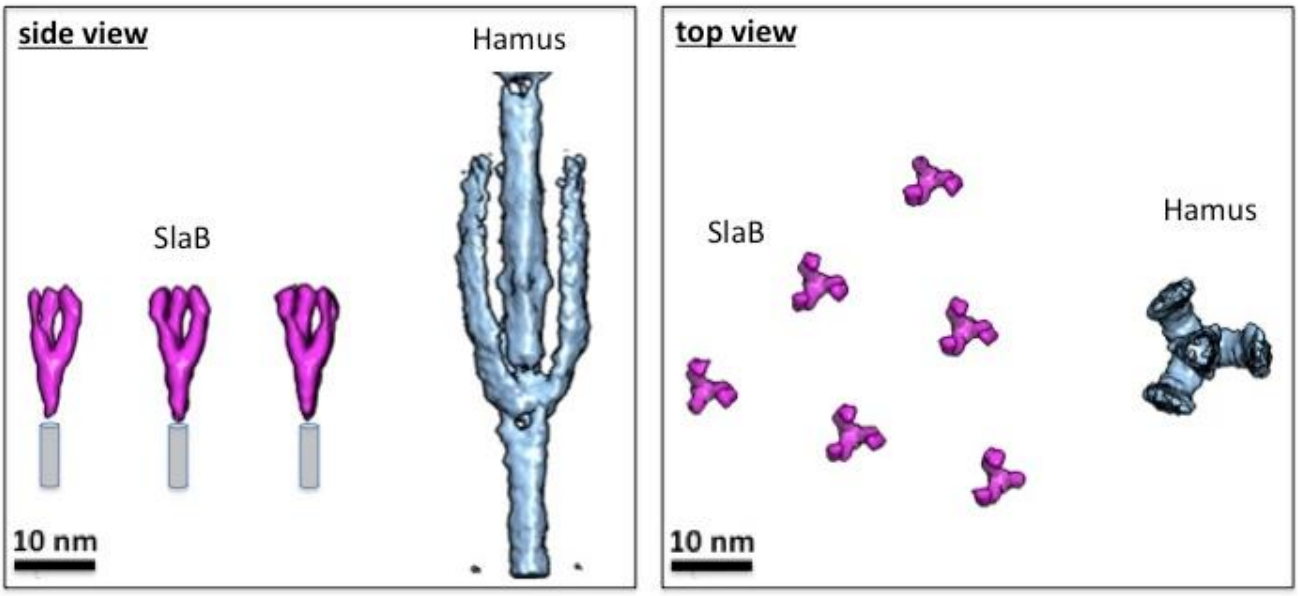

Figure IV.1-2 The tomographs show the side and top view of SlaB proteins and of the hamus prickle region. The grey cylinders indicate SlaB anchor stalks (not fully resolved) and are known to be 20nm in length (Baumeister and Lembcke, 1992). The images were kindly provided by Dr. Bertram Daum, MPI (Frankfurt).

The smaller dimensions of the SlaB proteins may be explained by the lower molecular weight of the protein subunits (45 kDa). The SlaB proteins are anchored in the cytoplasmic membrane and form the stalk region. Coiled-coil regions form a trimer and extend into the surface (Veith et al., 2009). In addition, the secondary structure was described to contain the self-assembly priming beta sheet topology typical for euryarchaeal S-layers (Arbing et al., 2012). Three-dimensional structures, which resemble hami on first sight, were also observed on the surface of *Staphylococcus marinus*. The tetrabrachion protein organizes into an umbrella-like morphology and distal-branched quadruple arms (Peters et al., 1995; Engelhardt and Peters, 1998).

The full-length sequence of the hamus-assembling major protein species lacks a remarkable homology to known S-layer proteins. A highly similar S-layer pattern was exclusively given in the N-terminal region of the hamus protein. Considering the structural resemblance to certain S-layer structures, it seems likely that the hamus structure diverged from ancient S-layer proteins. S-layers are the proposed ancient cell wall type for Archaea. Such protein layers provide an extraordinary protection in osmotically challenging and high-temperature environments (Völkl et al., 1993). S-layers are also assumed to be precursors of more complex cell walls and might therefore have helped to stabilise early cells (Sleytr and

Plohberger, 1980). As soon as stable cell envelope polymers evolved, the significance of such functions might have been lost. By acquiring a protective double membrane, an ordered mono-protein layer might have evolved to well-defined hami filaments with similar temperature and pH resistance properties in *Ca.* A. hamiconexum. S-layers provide porosity, depending on the pore size. It can be speculated that a loss of the S-layer was complemented by a periplasmic space within the double membrane, which provides regulated permeability besides mechanical protection (Engelhardt, 2007).

In general, control of translocation and anchoring of cell surface appendages depends on cell envelope composition (e.g. single membrane or double membrane) and the nature of the protein. In Archaea, natural association of S-layers with the cytoplasmic membrane occurs only by hydrophobic stretches of proteins (e.g. SlaB stalks; Baumeister and Lembcke, 1992), which act as anchors to immobilise the proteins and lipids. Hami span both membranes and their anchor system is most likely located within the inner cell membrane (Probst et al., 2014b). Released hami, which were examined by transmission electron microscopy showed a supporting stalk-like anchoring structure (“thorn”), which are assumed to protrude into the cytoplasm of the host cell (Probst et al., 2014b). However, this results needs to be confirmed using, for instance, ultrathin sectioning combined with immunogold labelling (Meyer et al., 2014). Based on these observations, a convergent bacterial type 4 pili formation was envisaged (Probst et al., 2014b; Perras et al., 2015). The bacterial type 4 secretion system involves the outer membrane and translocates pilin precursors into the cytoplasmic membrane by the Sec pathway (Arts et al., 2007; Francetic et al., 2007). Subsequently, the positively charged signal peptide triggers cleavage, which results in mature pilins with a highly hydrophobic N-terminus providing the scaffolding interface (Bardy et al., 2003; Pohlschröder et al., 2005; Ng et al., 2007; Albers and Pohlschröder, 2009). A similar leader peptide was identified in the hami aminoacid sequence (position 1-27), which suggests a similar assembly of hami filaments.

The major hamus-forming protein was identified; however, it is very likely that more associated proteins are involved. Preliminary experiments revealed a pore-like structure, which might facilitate the secretion of hami proteins (Figure IV.1-3). The identification of assembly- and anchor-associated proteins might be possible by co-immunoprecipitation assays and is anticipated in future experiments (Perras et al., 2015).

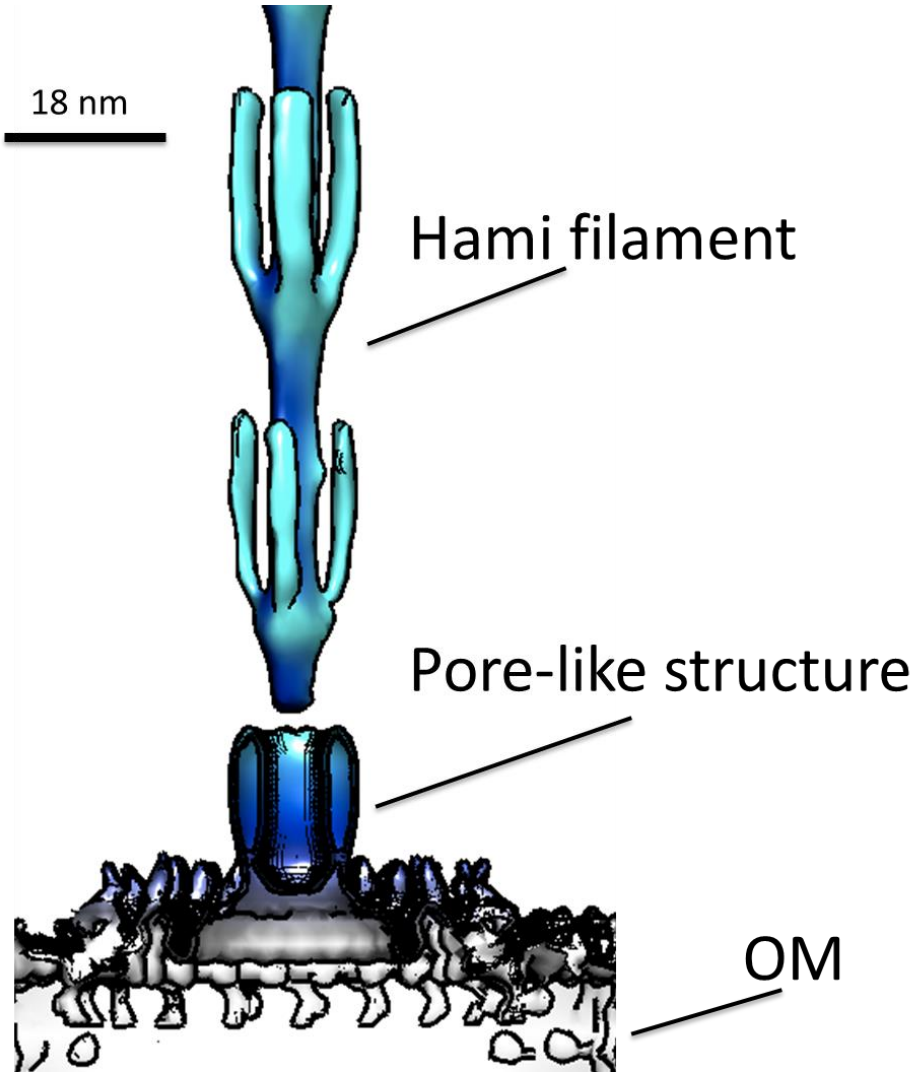

Figure IV.1-3. The tomograph depicts a hamus filament and the associated pore-like structure. The pore-like structure warrants the translocation of hamus proteins (OM= outer membrane). The electron image was kindly provided by Dr. Bertram Daum, MPI (Frankfurt).

The exceptional traits of the hami filaments (nano-grappling tripartite hook, high stability and flexibility) provide them as ideal tools in nanobiotechnology. As already mentioned, the hami host refused any laboratory cultivation so far. Consequently, for sufficient hami production, heterologous expression systems were necessary. The fundament for these experiments relies in the resolution of the full-length gene sequence encoding the hamus major subunit. This was facilitated in recent studies based on metagenome data (Perras et al., 2015). The *Ca*. A. hamiconexum-MSI possesses six homologues open reading frames (ORFs) potentially encoding the hamus gene (Probst et al., 2014b). Transcriptomic studies combined with MALDI-TOF analysis revealed the encoding gene with a length of 2691 bp

(Perras et al., 2015). This gene sequence and the resulting amino acid sequence were patented ("Microbial nano-tool"; publication number: WO2016180762 A1; Moissl-Eichinger et al., 2016b). The hamus major subunit protein is accordingly named "hamin protein". The heterologous expression of the hamin protein failed in commercially available *E. coli* strains (Perras et al., 2015) and was also not feasible using human embryonic kidney cells (Freestyle^{TM} 293-F cells, the experiments were carried out by LifeSciences, Regensburg). However, hamin-transformed eukaryotic Baculovirus System (IF9 cells) expressed recombinant proteins having an expected weight as shown by immunoblot analysis (data not shown, the experiments were carried out by LifeSciences, Regensburg). Another suitable host system is represented by *Pichia pastoris*. *P. pastoris* is preferable, because the expression imparts various functional advantages including ease of purification. In preliminary experiments the expression and even secretion in the medium was observed, however, only in low rates (data not shown). In future experiments, a high scale production of hamin proteins is anticipated. Further steps include heterologous expression as well as purification and correct folding of the polypeptide, which will be monitored by electron microscope imaging. The expression and folding of similar high-molecular proteins proved to be challenging in the past (e.g. spider silk, proteins; Heidebrecht and Scheibel, 2012; Schacht and Scheibel, 2014) and might be also true for hamin proteins.

However, the range of potential industrial applications for hamus filaments is convincing. Their properties (high stability, high flexibility, highly adhesive, biological degradable) offer the implementation for medical (e.g. coating of medical devices as implants and transplants, use as biologically degradable patches), and non-medical purposes (e.g. adhesive glue system in particular in moist systems, nano-robots etc.). The results presented in this thesis pave the way for the exploitation of hami filaments in various fields of nanobiotechnology.

2. Survival specialists deriving from the ISS

The ISS represents a multifunctional research platform, which is used to perform a wide range of scientific experiments. This sealed environment is providing a habitable environment for human beings and their accompanying microorganisms for more than 16 years. To ensure a safe and secure working place onboard the ISS as well as for future long-termed space missions, a broad knowledge on microorganisms and their interaction with the environment and humans onboard the ISS is essential.

Cultivation combined with cultivation-independent analysis revealed a comprehensive microbial picture of remarkably long-term stored (8-12 years) dust samples deriving from the Russian modules of the ISS (Mora et al., 2016b). Investigation was carried out in the frame of the European Space Agency (ESA) flight project "Extremophiles" (former: "ARBEX" – Archaeal and Bacterial Extremophiles onboard the ISS; Moissl-Eichinger et al., 2016a;) and revealed (**i**) the presence of hardy microorganisms equipped with a wide range of resistances and (**ii**) signatures of Archaea onboard the ISS. The latter were detected for the first time onboard the ISS and hence, in an extraterrestrial setting (Venkateswaran et al., 2014; Checinska et al., 2015; Mora et al., 2016b). In particular, reported archaeal *Nitrososphaera* and *Methanobrevibacter* ISS-signatures were also found in and on the human body as well as in cleanroom environments, which underlines crewmembers as their main track-back source (Probst et al., 2013a; Gaci et al., 2014; Moissl-Eichinger, 2011). The archaeal role and impact on the human body is still unclear and so far, no study reports a clear and fundamental evidence for a positive or negative influence of Archaea. Archaea are essentially not proven to be pathogenic, and therefore human-associated studies received less attention (Cavicchioli et al., 2003; Gill and Brinkman, 2011). Only assumptions on potential harmful or beneficial impacts on health were drawn and still more evidence is needed (Triantafyllou et al., 2013; Brugère et al., 2014). It also remains to be elucidated if Archaea onboard the ISS threaten/ favour the health of crewmembers or do not have any influence at all. Noteworthy, no archaeal cultivated isolate deriving from the ISS was obtained yet (Mora et al., 2016b). Furthermore, respective samples for molecular analysis treated with propidium monoazide (PMA), a marker for cell wall integrity, resulted in negative signals in 16S rRNA gene amplification. These observations may include that the archaeal proportion was not viable anymore due to a lack of desiccation resistance. However, the analysis is based on old dust samples, which give no indications on the situation at the time point of sampling. The analysis of freshly taken samples is therefore of uttermost interest and is included in the scope of future projects. This will include thorough cultivation efforts focusing on archaeal isolates.

Cultivated isolates enable the analysis of metabolic activity and responses to stressors. Cultivation attempts retrieved bacterial and fungal representatives (in total 85 isolates belonging to 34 unique strains) equipped with a high desiccation resistance. The majority of genera were also detected in other parts of the ISS and spacecraft assembly cleanrooms

(Castro et al., 2004; Novikova et al., 2006; Ghosh et al., 2010; Moissl-Eichinger et al., 2013; Koskinen et al., 2016; Checinska et al., 2015). Onboard the ISS, the community of the US and the Russian module was heterogeneous on species level, which was in line with results from molecular analysis, and underlines a distinct microbial community in the modules. Nineteen strains were tested upon their susceptibility against a wide range of clinically-relevant antibiotics, which partly belong to the medical inventory onboard the ISS (NASA, 2016). Similar studies reported an altered resistance to antibiotics, when the microorganisms were grown in space (Tixador et al., 1985b). In our study, eighteen isolates exhibited resistance against at least one of the tested antibiotics.

It is long known that some bacteria exhibit an altered behaviour in microgravity when compared to their growth characteristics on Earth. This includes biofilm formation (Kim et al., 2013), enhanced growth rates (Coil et al., 2016), development of a thicker cell envelope (Tixador et al., 1985a), and enhanced pathogenicity (Wilson et al., 2007). Noteworthy, an increased rate of bacterial conjugation was observed as well (Ciferri et al., 1986). All obtained isolates from our study were non-pathogenic, but one might speculate that an enhanced antibiotic resistance may be transferred from non-pathogenic to potentially present pathogenic microorganisms, which would challenge the treatment of infections. Thus, alternatives to antibiotic treatment of diseases such as antibacterial peptides are subject of intensive research (François et al., 2016).

The presented findings include viable microorganisms deriving from long-term stored and space-related dust samples with outstanding resistances. In future experiments, these results will be compared to fresh samples from the ISS. Upcoming findings will help to decipher potential discrepancies within the microbial community, which might result of more than a decade of storage. The ISS exemplifies similar vehicles, which will travel to Mars and beyond. Microbiological findings in this regard help to plan future manned long-termed missions and to provide a healthy and microbiologically secure workplace for the crew members.

3. Terrestrial extreme, Mars-like habitats: Implications for extraterrestrial life?

In 2004, as methane outbreaks were detected on Mars (Formisano et al., 2004), researchers enthusiastically speculated on methanogens as a potential source (Moran et al., 2005). Present Mars might even provide habitable subsurface environments ("hot spots"; Rettberg et al., 2016), which are protected from radiation and other harmful life-limiting factors. Nevertheless, Mars is characterised by a variety of other extreme conditions and it remains to be elucidated if life is or was able to withstand them.

Mars changed its appearance with respect to climate change and associated water availability. Three different chronological climatic changes are speculated: (i) a water rich era in the early years, (**ii**) a subsequent cold and semi-arid era, and (**iii**) a final, still lasting very cold desert-like era (Fairén et al., 2010) The first two eras are referred to as Early Mars and the third condition as Present Mars. Terrestrial Mars-analogue studies assist to get an understanding of possible Mars lifeform evolution throughout the eras.

Early Mars resembling terrestrial settings are, for instance, represented by acid sulphate lakes in Western Australia (Baldridge et al., 2009), and the Rio Tinto basin in Spain (Amils et al., 2007; Fernández-Remolar et al., 2008). Permafrost settings in the Antarctica resemble conditions later into the history of Early Mars (Dickinson and Rosen, 2003). Conditions of the hyper arid Present Mars are found for instance in the Atacama Desert (Navarro-González et al., 2003) and in Australian deserts (Mann et al., 2004).

These environments on Earth partly exhibit the conditions prevalent on Mars, however, none of them reflect all of them in a combined manner (Moissl-Eichinger et al., 2016a). Geochemistry and biodiversity of Mars-analogue environments were extensively studied to elucidate implications for extinct or extant life on Mars. One crucial but widely ignored condition on Mars is the lack of oxygen. This is of uttermost interest, since the growth of microorganisms on Present and Early Mars is limited on (facultatively) anaerobic microorganisms. Another limiting factor concerns life on Present Mars: In the course of losing the atmosphere, Mars became unprotected against radiation. This was not severe in the Early history of Mars and is also not severe for protected subsurface areas, but remains a crucial life-limiting fact for any life on the surface of Present Mars. Knowledge on terrestrial,

anaerobic microorganisms capable of thriving in Mars-like, anoxic environments is sparse and thus needed to be extended.

Within the frame of the MASE project (Mars Analogues for Space Exploration) we focused on the anaerobic microbial community of diverse extreme environments to determine the phylogenetic and (possible) functional diversity of the anaerobic microorganisms thereof (Perras et al., 2016). In order to get a comprehensive picture of the microbial diversity we combined NGS with cultivation. The selected extreme environments were similar to **Early Martian conditions**, namely acidic environments, sulphidic environments, frozen environments and **Present Martian conditions**, namely brines, which reflect recurring slope lineae on the surface (RSL; Chevrier and Rivera-Valentin, 2012; Ojha et al., 2014; Ojha et al., 2015). It has to be emphasised that all above mentioned Martian analogue sites do not cover all environmental conditions prevalent on Mars. However, these sites reflect some of the closest possible terrestrial Martian conditions. In addition, the reaction of cultivated microorganisms to Martian stressors were simulated in laboratory facilities (Beblo-Vranesevic et al., 2016).

Our cultivation attempts recovered 31 primary mixotrophic and facultatively anaerobic isolates (Cockell et al., 2016b; Perras et al., 2016). High cultivation success was achieved in the sulphidic spring environment. Almost 58% of all isolates were retrieved from this environment. The least successful cultivation was obtained from the permafrost samples (3.2%). Microorganisms obtained from very cold environments exhibit generally a slow growth rate in their natural environment and also under laboratory conditions due to slow metabolism at low temperatures (Panikov and Sizova, 2007). We expect growth in further enrichment approaches, however, the restricted time-frame has not yet allowed obtaining further cultivated isolates.

Most of the cultivated microorganisms belong to the phyla Proteobacteria (51%) and Firmicutes (32%) with a high proportion of *Yersinia* and *Clostridium* species. Clostridia are spore-formers, known to resist a wide variety of harsh conditions, and consequently have a high astrobiological relevance (Mendez et al., 2005). In contrast, members of non-spore forming *Yersinia* genera are rather known from a medical perspective. Relatives of the MASE isolate *Yersinia intermedia*, such as *Yersinia pestis* and *Yersinia pseudotuberculosis* are the causative agents of diverse and severe diseases (Perry and Fetherston, 1997; Koornhof et al.,

1999; Smego et al., 1999). Genomic (data not shown) and phylogenetic analysis (Boghenbor et al., 2006) suggests that our MASE *Yersinia intermedia*, which has been isolated from of an acidic environment, is non-pathogenic for humans. The isolate was subjected to various Mars-simulated stressors such as desiccation, radiation, nutrient starvation, high amounts of perchlorates and combinations thereof. Noteworthy, this isolate did not show high tolerances against radiation, but surprisingly, the bacterium was particularly high tolerant against desiccation and perchlorate ions compared to other *Yersinia* species and other model organisms (Beblo-Vranesevic et al., 2016; Gut et al., 2016). Perchlorate ions are prevalent in Martian soils (Hecht et al., 2009) and were considered to hamper the persistence of life in very high concentrations (0.4 M is the limit for some halophilic archaea and bacteria; Oren et al., 2014; Matsubara et al., 2016). However, even a high concentration of 2.4 M did not limit the survivability of the MASE *Yersinia* strain.

Radiation and desiccation stressed MASE *Yersinia intermedia* cells were artificially fossilised and tested upon its potential for preservation and biomarker detection (Gaboyer et al., 2016). Artificially fossilised microorganisms give valuable information on biosignature detection on Mars as microbial fossils are speculated to be prevalent on the radiation-exposed surface (Westall, 1999). As shown recently, preservation of MASE Yersinia was possible, however, no biomarkers were detected using state-of-the-art techniques (i.e. SOLID-Chip; Parro et al., 2005).

In summary, our MASE *Yersinia intermedia* isolate is supposed to be a new model for astrobiology. The MASE *Yersinia* genome underwent shotgun-sequencing and future experiments include investigations on its genetic repertoire. The outcome may elucidate potential resistance machineries. Several other MASE *Yersinia* isolates were obtained from the pH neutral sulphidic springs as well. The question remains, if the resistance is promoted by the acidic environment or if it is also applicable on other sulphidic spring *Yersinia* isolates. Further resistance tests on these isolates will elucidate the answer.

NGS analysis allowed a more comprehensive insight into the overall microbial community. The overlap of genera obtained by cultivation and by NGS was 27%. A large portion of the cultivated isolates remained hidden in NGS approach and *vice versa*. This phenomenon was already observed earlier (Koskinen et al., 2016; Mora et al., 2016b) and emphasises once

more the importance of combining high-throughput sequencing methods with cultivation approaches.

The cultivation-independent approach using NGS recovered a broad archaeal and bacterial diversity. The microorganisms are potentially pre-armed with a wide variety of resistance mechanisms, which enable them to reside in extreme environments. By using NGS in combination with the chemical compound propidium monoazide (PMA), we revealed the viable part of microorganisms (meaning microorganisms having an intact cell wall). We identified 34 genera, which were present in all MASE Early Mars settings. Assigned genera were classified as *Opitutus*, *Paludibacter*, *Candidatus* Solibacter, *Acidocella, Bdellovibrio, Aquicella*, and *Bryobacter.* These core genera represent a cosmopolitan group of microorganisms, which are able to tolerate a range of Early Mars analogue conditions. We included the hypersaline environment into our calculations and obtained two (assigned) common genera throughout Early Mars and Present Mars settings: *Opitutus* and *Paludibacter*. Both genera comprise anaerobic species and thus fulfil one essential prerequisite to thrive on Mars. We elucidated the microbial core community which might be able to thrive in Early Mars settings and might withstand conditions on Present Mars. Early Mars, which offered life-friendly conditions, allowed the emergence of microbial life. In the course of the Martian climatic change, microorganisms either adapted to the drastically changing environment or became extinct. Our model organisms for outlasting microorganisms are speculated to resemble *Paludibacter* and *Opitutus*, since they withstand both, Early and Present Mars-like conditions.

Noteworthy, we need far more evidence to prove this warranted speculation. First, *Paludibacter* and *Opitutus* are in the scope of cultivation approaches. Their tolerance capacity against desiccation, radiation and other Mars-conditions need to be tested on first hand, which is only possible with cultivated isolates. Second, it remains to be proven whether *Opitutus* and *Paludibacter* can be also obtained from other, non-MASE Mars analogue sampling sites. Our Present Mars analogue environment comprises only one setting so far and more are needed for strong evidence. They might include Present Mars analogues like the Atacama Desert (Navarro-González et al., 2003) and brine lenses in permafrost settings (Dickinson and Rosen, 2003).

In the course of this thesis, the taxonomic assessment of three not-MASE-related extreme environments was accomplished. These environments resemble various Mars conditions with respect to radiation dose and water accessibility and harbour dissimilar abiotic conditions.

a) The first environment, namely the **Yellow-Oily Lake, Dallol**, is a hydrothermal (~40°C), hypersaline spring, which is characterised by low pH values. In cooperation with Karen Francis-Olsson (UK) and Barbara Capaccioni (Italy), DNA was extracted using an appropriate protocol (Moissl-Eichinger, 2011) and sequenced. Data processing was carried out as described earlier (Mora et al., 2016b, Perras et al., 2016).
b) The second environment is located in the East Antarctica (**Central Queen Maud Land, Lake Untersee**). The lake is described as ultra-oligotrophic and sediments resemble permafrost-like settings. The project was carried out in cooperation with Birgit Sattler and Klemens Weisleitner (Austria). Sampling was performed by Klemens Weisleitner. Sample processing, sequencing and data processing was carried out as described earlier in the course of this thesis.
c) The third location was settled in **Brasilia**. Sampling was performed by Petra Schwendner (UK). The sampling site was exposed to high doses of radiation (pers. communication Petra Schwendner).

Ribosomal Sequence Variants (RSVs, Callahan et al., 2016a) obtained from Dallol samples, Lake Untersee samples, Brasilia samples, and MASE samples by an “universal approach” (primer set targeting Bacteria and Archaea; Caporaso et al., 2012) are depicted in a PCoA plot (unweighted Bray-Curtis distance) in Figure IV.3-1. Further details on data processing can be found in Mora et al., 2016b and Perras et al., 2016.

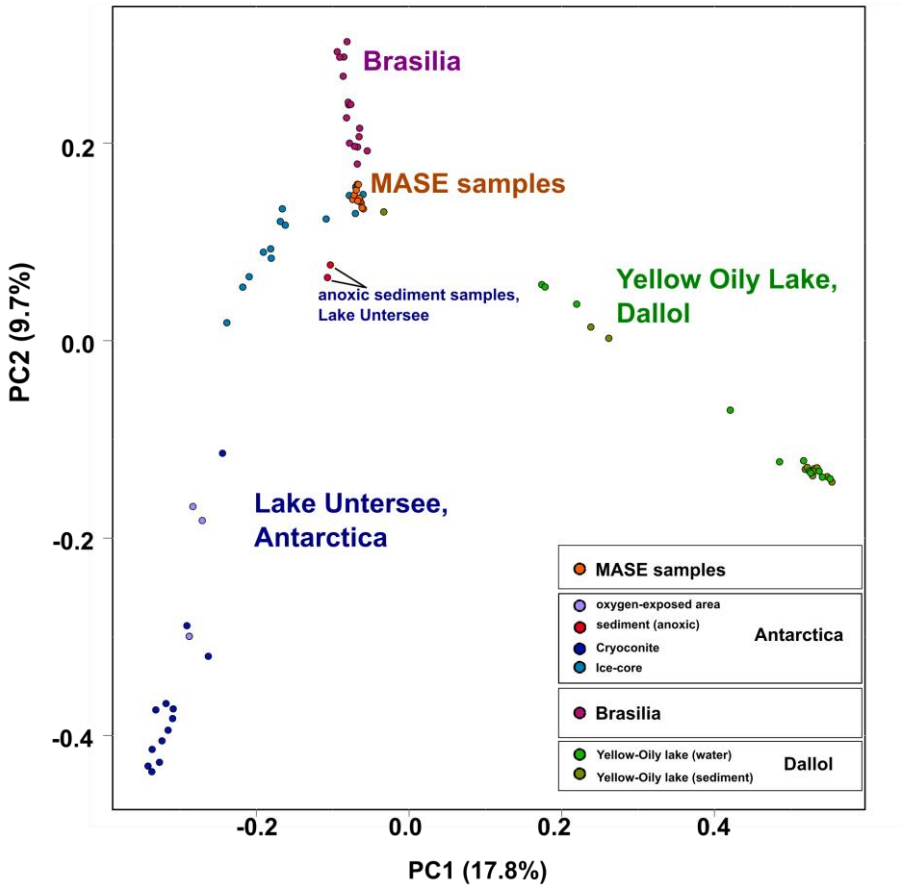

Figure IV.3-1 The PCoA plot (unweighted Bray-Curtis distance) summarises the similarity of all Mars-analogue samples processed in the frame of this work. The samples were not treated with PMA, and extracted DNA was amplified using the "universal" primer set as suggested by Caporaso et al., 2012. Raw data was processed using the protocol as described in Perras et al., 2016.

A strong clustering of all MASE samples (orange) highlights a close microbial similarity. Antarctica samples (blue), Dallol samples (green) and Brasilia samples (pink) form their own clusters, however, scattered along the PC2 axis rather than forming strong groups. Some samples show very high similarity to the MASE samples: (**i**) four Antarctica samples deriving from an ice-core cluster directly along with the MASE samples, (**ii**) two anoxic sediment samples cluster very close to the MASE cluster and (**iii**) one sediment sample of the Dallol site exhibits a similar microbial community. These samples consequently show a similar microbial composition. A deeper analysis on non-MASE sites will shed insights into the taxonomy and possibly reveal further model organisms.

As described in Perras et al., 2016, a close up view exclusively on MASE samples displays site-specific dissimilarities within the microbial composition, which disappears upon using the large-scale view shown above. Noteworthy, the Antarctica setting, the Dallol setting and the

Brasilia setting were not sampled along the strict, oxygen-avoiding MASE guidelines (Cockell et al., 2016b). This might explain the microbial composition dissimilarities.

A closer analysis on these samples will reveal further insights into Martian life-like candidates and is in the scope of future experiments.

In summary microbes living in such extreme Mars-analogue environments are attractive candidates for the existence of life on other planets. The prospect of finding extraterrestrial life is thrilling and supports our understanding on the origin and evolution on Earth as well. Certainly, the situation on Mars is more complicated than in the most extreme settings on Earth. Nevertheless, the described study represents an important puzzle piece, and contributes to complete the full picture of the origin and evolution of the biological existence.

Conclusion

In the course of this thesis, the microbial diversity of various extreme environments was investigated. Each environment was observed to harbour a distinct community of “survival specialists” adapted to the prevalent conditions. Extensive cultivation effort delivered novel, extremo-tolerant model organisms for astrobiology, which allows further investigation on their genetic and metabolic capacities.

The International Space Station as well as Mars-like settings proved the omnipresence of a hardy bacterial and archaeal community. Depending on the setting, we face distinct communities and specified adaptations. The work of this thesis strongly emphasises the combination of state-of-the-art cultivation independent methods with cultivation techniques in order to get a comprehensive microbial picture. Both methods have drawbacks; however, in combination they deliver the best possible result.

Molecular analysis enabled us to speculate a possible transition of microorganisms from Early Mars-like settings to Present Mars-like settings. We might even face similar extant/extinct microorganisms on Present Mars. The development of detection systems of these microorganisms is in the future scope of the MASE project. Although we were able to gain extensive knowledge into anoxic Mars-like settings, it has to be emphasised that the investigation on further anoxic environments is indispensable. The presence of our MASE

core genera has to be confirmed and further validated. In addition, only their cultivation will give insights into their behaviour when exposed to Mars-like stress.

The findings on *Ca.* A. hamiconexum, thriving in a Mars-analogue site confirms that this archaeon is exceptional and outstanding with respect to its ultrastructure. The hami of this microbe provide an excellent and promising source for nanobiotechnology with various applications. The outcome of this thesis delivers valuable knowledge on extreme terrestrial settings with implication on astrobiology and nanobiotechnology.

V. Zusammenfassung

Extreme Biotope sind durch eine Vielzahl von physischen und chemischen Stressvariablen gekennzeichnet und bieten eine hervorragende Möglichkeit die Grenzen des Lebens zu verstehen – auch außerhalb der Erde z.B. auf der **International Raumstation (ISS)** oder auf dem Mars. Einige terrestrische, extreme Lebensräume ähneln stark ausgewählten Bedingungen auf dem Mars und lassen daher Rückschlüsse auf dessen potenzieller Bewohnbarkeit zu. Ein wesentlicher Faktor ist der Mangel an Sauerstoff auf dem Mars. Allerdings erweisen sich die Probennahme und mikrobielle Kultivierung aus sauerstoffarmen Umgebungen als sehr herausfordernd und werden nicht sehr oft praktiziert. Es benötigt besonders die Betrachtung solcher sauerstoffarmen Umgebungen und deren assoziierten Mikroorganismen, um einschätzen zu können, ob extraterrestrisches ausgestorbenes und/oder noch bestehendes Leben auf dem Mars existieren könnte. Bemannte Langzeitzeitmissionen zum Mars bedürfen zudem sowohl Wissen über die mikrobielle Diversität in Raumfahrtzeugen als auch über deren Anpassungsfähigkeit und Stress-Toleranzen. Nur dadurch kann ein sicheres Arbeitsklima sichergestellt werden. Die vorliegende Arbeit umfasst eine tiefreichende Untersuchung extremer Biotope mit Hilfe von Kultivierungsmethoden und molekularen Methoden und eine detaillierte Analyse einiger ausgewählter mikrobiellen Bewohner.

Eines dieser extremen Biotope umfasst das kontrollierte Innenleben ISS. Frühere Studien detektierten bereits eine hohe Abundanz an Mensch-assoziierte Bakterien, allerdings blieb der Nachweis von Archaeen erfolglos. Archaeen sind allgegenwärtig auf der Erde und werden zahlreich in Reinräumen detektiert, in welchen die Raumschiff Hardware assembliert wird. Im Rahmen dieser Arbeit wurden 8-12 Jahre alte Staubproben auf deren mikrobielle Diversität untersucht. Molekulare Analysen bestätigten ein mit dem Mensch assoziiertes Bakteriom. Zudem konnten zum ersten Mal auch archaelle Signaturen nachgewiesen werden. Kultivierte, bakterielle Einzelisolate ermöglichten es, Stress-resistente Experimente durchzuführen. Neben der Austrocknungsresistenz, zeigte eine Vielzahl von Isolaten eine hohe Resistenz gegen zahlreiche Antibiotika. Diese Ergebnisse erweitern unser Wissen über die archaelle und bakterielle Diversität und deren Adaptionen im Weltraum.

Terrestrische, Mars-ähnliche Systeme ermöglichen es, Modellorganismen zu finden, welche potenziell auf dem Mars gelebt haben und/oder immer noch dort leben könnten. Zahlreiche Studien beschäftigten sich mit deren Untersuchung, allerdings fehlte bisher ein profundes Wissen über sauerstoffarme Systeme. Im Rahmen des **MASE Projektes** (*Mars Analogues for Space Exploration*) wurden gezielt sauerstoffarme Biotope auf deren mikrobielle Diversität untersucht. Einige dieser Biotope zeigen eine hohe Ähnlichkeit zu Marsbedingungen in der Vergangenheit (vor ca. ~4 Milliarden Jahren), während ein Biotop den Bedingungen auf dem heutigem Mars reflektiert. Auch hier konnten mit Hilfe eines umfassenden Kultivierungsansatzes zahlreiche Anaerobier erhalten werden, die als astrobiologische Modelle dienen. Molekulare Methoden prognostizierten eine außerordentliche Adaptionsfähigkeit und hohe Diversität in den untersuchten Lebensräumen. Alle untersuchten, dem frühem Mars entsprechenden Lebensräume, so unterschiedlich sie auch sind, zeigten einen gemeinsamen taxonomischen Kern, welcher sich teilweise in dem heutigem Mars-ähnlichem Setting wieder spiegelte. Ähnliche Lebensformen könnten die extremen klimatischen Änderungen im Laufe der Jahrmillionen Jahre überdauert haben und sowohl als Fossilien wie auch als lebende Organismen zu finden sein.

Ein unterirdisches Biotop, welches sich unter den MASE-Biotopen befand, beherbergt ein schon länger bekanntes, außergewöhnliches Archaeum, das **SM1 Euryarchaeon** (jetzt: „*Candidatus* Altiarchaeum hamiconexum“). Biofilme dieses Archaeums werden an die Oberfläche der Nährstoffarmen Quellen gespült und ihr Lebensstil und ihre Ultrastruktur wurden ausgiebig untersucht. *Candidatus* Altiarchaeum hamiconexum weist eine Doppelmembran auf - ein sehr untypischer Zellmembranaufbau in der Domäne der Archaeen. Zudem besitzt es hoch-organisierte, einzigartige Zellanhängsel („hami“), welche höchstwahrscheinlich ähnlich zu bakteriellen Typ 4 Pili assembliert und verankert werden. Der N-terminale Teil der Proteinsequenz zeigte überraschenderweise hohe Ähnlichkeit zu S-layer Proteinen (geordnete Proteinstrukturen, die sich zu einer Außenhülle an die Membran anlagern), wodurch eine divergente Evolution der beiden Strukturproteine spekuliert wird. Die gesamte Hami-kodierende Gensequenz wurde entschlüsselt und diente als Vorlage in heterologen Expressionsversuchen. Die außergewöhnliche Struktur erweist sich als vielversprechend in der Bionanotechnologie und die Resultate dieser Arbeit setzen die wesentlichen Fundamente für weitere Experimente.

Diese Arbeit bietet verblüffende Einblicke in die Welt außergewöhnlicher Mikroorganismen. Die vorgestellten Ergebnisse führen zu einem besseren Verständnis der Diversität und Biologie von Mikroorganismen aus extremen Lebensräumen.

VI. Bibliography

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VII. Content of supporting CD

The following table explains the data structure on the supporting CD. The CD includes all publications (published and submitted), selected publications (see Chapter III.), respective Supplementary Information, and a pdf version of this thesis.

Table VII-1 Data structure on the supporting CD

Folder	All publications		Selected publications
	Published manuscripts	Submitted manuscripts	0* Supplementary files
Content	All published manuscripts as pdf	All submitted manuscripts as pdf	All Supplementary files, descriptions and the manuscripts as pdf. The selected publications can be found in Chapter III.

VIII. Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Regensburg, 13.01.2017 _____

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