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Ph.D. THESIS

SUMMARY

”Cold adaptation mechanisms in psychrophilic microorganisms”

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Introduction

A great extent of the Earth's biosphere is exposed to lasting or seasonal temperatures below 5 °C (Margesin et al., 2007). These cold environments characterized by the presence of ice in extensive masses comprise deep seas (90% of the oceans exhibit temperatures < 5 °C), cold deserts, and glacial habitats (Margesin and Miteva, 2011). Although the ice-covered environments were previously considered abiotic or as a potential archive created by the stochastic transportation by wind (Cowan and Tow 2004), they have been found to harbor a surprising variety of microorganisms detected and recovered by cultivation techniques (Van de Vossenberg et al., 1998). Despite the challenge represented by the water scarcity in cold environments, researchers were able to demonstrate that bacterial cells are metabolically active and able to actively proliferate, as resulting from isotopes incorporation studies (Christner, 2002; Junge et al., 2004) and microscopic visualization (Bakermans et al., 2003). Nowadays cold environments are the poorest investigated on Earth (Buzzini et al., 2012).

A multiplicity of cold environments on our planet, together defined as cryosphere, have been found to host microorganisms belonging to all three domains of life (Feller, 2013) Bacteria (Morita, 1975), Archaea (Cavicchioli, 2006) and Eukarya including algae (Morgan-Kiss et al., 2006) and fungi (Buzzini et al., 2012). Icy habitats include snow (Maccario et al, 2019), glacier ice (Miteva, 2008), supraglacial cryoconites (Edwards et al., 2014) and subglacial sediments (Hamilton et al., 2013), sea ice (Bowman et al., 2011), sea water (Han et al., 2014) and permafrost (Mackelprang et al., 2011). Low temperature linked with the water shortage activated a peculiar process of natural selection favouring the most resilient species with a consequent atypical shaping of the cold adapted microbial community (Anesio and Bellas, 2011).

Aims and contribution

This thesis aims to contribute to the understanding of cold adaptation mechanisms in microorganisms from frozen habitats at different organization levels, revealing a series of structural adaptations of aspartate transcarbamoylase (ATCase), the key enzyme of the pyrimidine nucleotide biosynthesis, from the psychrophilic bacteria *Glaciibacter superstes* and *Rugamonas sp.*, alongside thermal responses of bacterial cells and ice microbiome when changing habitat due to glaciers retreat. The corroborated data on the temperature dependence of total and active ice microbiomes, the differential expression of heat-shock and DNA synthesis-related genes, and structural properties of extremozymes contributed to deciphering the microbial resilience and response to steep temperature

variations mechanisms in order to cope with extreme conditions and climate variations in the context of global warming.

Glacier ice covers a noteworthy area of the planet and is presently facing an accelerated melting rate, combined with a potential remodeling of the microbial composition as a result of exposure to a temperature variation when moving to a contiguous soil. In this context, this thesis focused on investigating thermal adaptation mechanisms of psychrophilic microorganisms and the heat shock impact on microbiomes from frozen environments at community, cellular and molecular levels in the context of the climate changes impact on ice microbiomes associated to glacier melting (Figure 1).

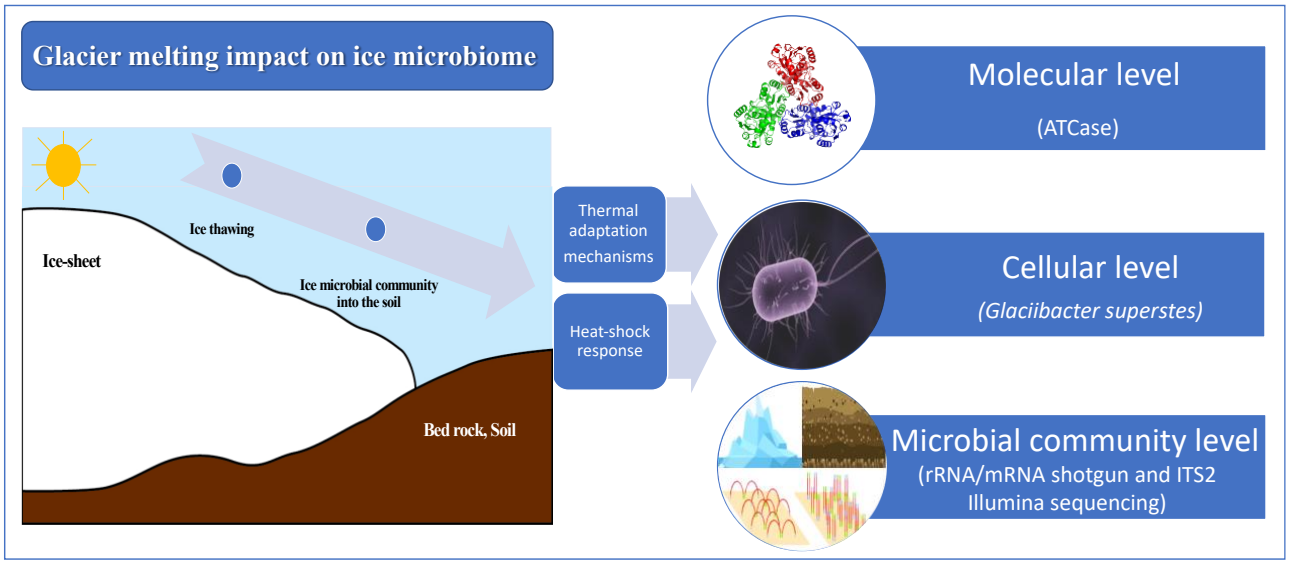

Figure 1. Conceptual model of cellular response to warming

The investigation of the temperature dependence of ice microbiome, the differential gene expression and structural/functional properties of the key enzyme catalyzing the pyrimidine nucleotides *de novo* biosynthesis in psychrophilic bacteria contributed to deciphering the microbial adaptation mechanisms to frozen environments and to steep temperature variations in order to cope with extreme conditions and climate variations.

Chapter I: Molecular cold-adaptation mechanisms

Psychrophiles have evolved by producing cold-active enzymes able to cope with the reduction of chemical reaction rates induced by low temperatures and must count on an effective and intricate mechanism network to reduce the damages using a key adaptive strategy modifying the structure and the functionality of their enzymes. To unravel the thermal adaptation mechanisms of DNA synthesis in psychrophilic bacteria, we investigated the structural properties of the key enzyme catalysing the first steps of pyrimidine biosynthesis, aspartate transcarbamoylase (ATC) from the *Rugamonas sp.* strain isolated from an Antarctic fresh water lake, and *Glaciibacter superstes* isolated from Alaska ice wedge. Primary, secondary and tertiary structure, as well as hydrophobic clusters distribution in both these cold-active enzymes were analyzed in comparison with other psychrophilic, mesophilic and hyperthermophilic homologous enzymes, in order to unravel cold-adaptation structural features and particular structural adaptations to cold environments of this key enzyme of pyrimidine nucleotides biosynthesis related to their function at low temperatures.

Aspartate transcarbamoylase (ATCase EC:2.1.3.2**)** is a cytosolic enzyme involved in the first committed step in the pyrimidine de novo biosynthetic pathway (Berg et al., 2002). This enzyme is able to perform the condensation of *L*-aspartate and carbamoyl phosphate (CP) to produce *N*-carbamoyl-*L*-aspartate (CAA) with the final yield of CTP (Lipscomb and Kantrowitz, 2011). The holoenzyme is formed by two trimeric catalytic subunits (c3)2 (34 kDa each) and three regulatory dimeric subunits (r2)3 (17 kDa each) which combine to form a dodecameric complex (310 kDa) with the following subunit composition 2(c3):3(r2) (De Vos et al., 2004). The dodecameric active structure follows an allosteric behavior (Macol et al., 2001) and has been extensively studied using *E. coli* enzyme as a model (Helmstaedt et al., 2001).

This study comprises the structural analysis of the ATCase from the psychrophilic bacteria *Glaciibacter superstes* belonging to microbacteriacee family and isolated from Alaska's ice wedge, and *Rugamonas sp.* belonging to the family of pseudomandacee and retrieved from a fresh water lake located on King George Island (NW Antarctica) in comparison with other psychrophilic, mesophilic and hyperthermophilic homologous enzymes. The *pyrB* gene coding for the ATCase catalytic chain of G. superstes was cloned and expressed in E. coli. In the case of Rugamonas sp., the recombinant enzyme was purified by affinity chromatography from the heterologously expressed fused protein in *E. coli*.

The aim of this study is to determine the structural changes at molecular level of the key enzyme of the *de novo* pyrimidine nucleotides biosynthetic pathway in cold-adapted bacteria in order to understand the adaptation mechanisms to extreme environmental conditions of this class of enzymes essential for DNA synthesis in the cell.

Conclusions

This study offered a comparative structural analysis of ATCases from the psychrophilic *Rugamonas sp.* and *G. superstes* species evaluating the structural cold adaptations relative to homologous enzymes from mesophilic, psychrophilic and hyperthermophilic species. Primary structure homology assessment revealed a full conservation of the active site residues in all the investigated prokaryotic ATCases, in accordance with the ubiquity and the common reaction catalyzed by this enzyme in all organisms, in support of the conservation of pyrimidine biosynthesis pathway during the evolutionary processes. A noteworthy dissimilarity in their amino acid composition consists of the absence of cysteine, the reduced content of glutamic acid residues for both *Rugamonas sp.* and *G. superstes* and the enhanced content of histidines and prolines in *Rugamonas sp.* ATCase as compared with the mesophilic and hyperthermophilic counterparts.

The absence of cysteine residues and the estimated lower number of ionic interactions in both psychrophilic *Rugamonas and Glaciibacter* ATCases indicated the lack of disulfide bridges along with a lower content of salt bridges in the ATCases catalytic chain suggesting an increased flexibility of the cold-active enzymes.

Secondary structure pattern in psychrophilic enzymes revealed a higher presence of coils, especially in the case of ATCase from *G. superstes*, suggesting improved interactions between the catalytic subunits bearing more flexibility in the catalytic area.

The analyzed psychrophilic ATCases revealed a reduced number and size of hydrophobic clusters and a specific distribution along the PyrB chain favoring an increased flexibility of this class of cold adapted proteins.

The 3D model of both *Rugamonas sp. and G. superstes* ATCases suggested changes at subunits interfaces as compared to the *E. coli* and *P. abyssi* enzymes which might affect the trimer stability and a different spatial orientation of residues in the catalytic site.

The recombinant ATCase from *Rugamonas sp.* was successfully expressed in *E. coli* and purified by affinity chromatography.

The observed changes in the primary, secondary and tertiary structures of the psychrophilic ATCases as compared to the mesophilic and hyperthermophilic homologs appear to favor the catalytic process through an enhanced flexibility of enzyme domains harboring the active site and subunits within the trimer's interaction. The greater plasticity established for the enzymes in cold environment could allow the psychrophilic enzyme to be active at very low temperatures and might lower the **Δ**G of activation by reducing the distance between the substrate binding site residues.

The current structural investigation of the ATCase from both psychrophilic bacteria contributed to understanding the cold adaptation mechanisms emphasizing a series of molecular features favoring catalysis at low temperatures in one of the key enzymes involved in DNA synthesis and responsible for the resilience of bacteria thriving in frozen environments. Further investigations on the psychrophilic ATCase functional characteristics and crystal structure will corroborate our hypothesis and extend the assessment of specific cold-active enzyme thermal adaptations. Solving the x-ray crystallographic structure of this ATCase will help unravelling further molecular adaptations of this enzyme to low temperatures in comparison to mesophilic and (hyper)thermophilic counterparts, in order to extend the general understanding of the structural strategies of key catalysts from extremophiles adapted to cold environments.

Chapter II: Heat shock response of microbial communities from cold environments and psychrophilic model bacterium

Glacier melting implies the transfer of ice-contained microbial communities to new type of habitats such as the adjacent soil and possible exposure to high temperature variations during circadian phases. We investigated the microbial thermal response during a 7-day heat/freeze cycles experiment examining the changes in the gene expression pattern in the microbiome of ice core from Scarisoara ice cave, subglacial Icelandic soil and the psychrophilic bacterial strain *Glaciibacter superstes*, in order to determine an enzymatic biomarker for the microbiome resilience to heat shock treatment. The gene expression pattern coding for the key enzyme of pyrimidine nucleotides biosynthesis aspartate transcarbamoylase (ATC) and of the *dnaK* family (HSP70) was determined by qPCR and their response to daily thermal cycle and cell viability were compared in the cases of the model psychrophilic bacterium and the two types of cold habitats in order to assess an enzymatic biomarker for the microbial community response to temperature variations when changing habitats due to glacier retreat.

The anthropogenic impact on the climate has a remarkably negative effect on the ecosystem, triggering a loss of biodiversity (Cavicchioli et al., 2019). While the macroscopic endangered species gained interest during last years, no evaluation of the climate change on the "microscopic world" was carried out so far regarding the influence of microbiomes structure alteration on the nutrients and carbon cycles. During the last decades, the global warming produced an increase of glacier downturn (Weller et al., 2005) provoking a higher loss of ice with the subsequent movement of the ice embedded community to the neighboring soil. Once the microbial community reaches the soil, it becomes exposed to higher temperatures and direct sunlight. The speeded melting rate could then alter the microbial microcosm with also a revision of the genes expression pattern. Studies have proved the warming effect on soil microbial community change (Frey et al., 2008). Temperature is unquestionably one of the biggest stress factors challenging the microbial growth. Therefore, understanding the microbial thermotolerance strategies leading to resilience and adaptation to extreme environments (Deegenaars and Watson, 1998) could be crucial to evaluate the microbiomes survival and structural shaping.

In order to understand the response of microbial community from ice habitats to environmental temperature variations, we investigated the effect of heat shock treatment on the gene expression pattern of different cold habitats microbiomes. The experiment consisted of applying a daily heat

shock cycle followed by incubation at 4ºC constant temperature, during 1 week on ice from Scarisoara ice cave and subglacial Icelandic soil while a culture of psychrophilic bacterium *Glaciibacter superstes*, following the daily heat-shock, was alternatively incubated at both and 4ºC and -18℃ constant temperature. Evaluation of changes in gene expression of the treated microbiomes and bacterial model was quantified through RT-PCR using the cDNA constructed library. The selected genes were *pyrB* encoding for the aspartate transcarbamoylase (ATC), the key enzyme for pyrimidine nucleotides biosynthesis, and *dnaK* coding for the heat shock protein Hsp70 related to thermal stress defense mechanisms (Richter et al., 2010). Changes in the ATC and Hsp70 coding genes were correlated with the viability of thermal treated samples after evaluating the live/dead cell content after each heat shock step.

Comparative quantification of these genes' expression to repeated heat shock cycles highlighted long and short-term microbial responses to temperature variations related to DNA synthesis and specific thermal defense strategies depending on the microbiome habitat (ice and soil) and in a psychrophilic model bacterium.

Conclusions

The thermal response after 7-day heat shock cycles applied to Icelandic glacier forefield soil, Scarisoara cave ice core and the psychrophilic bacterial model *Glaciibacter superstes* revealed complementary expression profiles of the genes coding for aspartate transcarbamoylase (ATC), a key enzyme in the pyrimidine nucleotide de novo biosynthesis, and the HSP70 heat shock family depending on the type of habitat (soil vs. ice) and microbial complexity (culture vs. microbiome).

The microbial communities submitted to thermal shock showed an upsurge of the genes transcription as compared with the non-treated samples. RT-PCR thermal response investigation of the gene coding for HSP70 family disclosed an up-regulation for both *G. superstes* culture and ice microbial community indicating a short-term adaptation process. The ice microbiome thermal stress response revealed an up-regulation of the genes involved in the processes of cellular protection such as HSP70 and a down-regulation of the *pyrB* gene involved in the cellular duplication processes. Unlike the ice microbiome, soil microbial communities were less sensitive to the thermal shock application possible due to their thermal preadaptation in accordance with their glacier ice origins prior to ice melting. Assessment of cell viability provided an indication of how the increased *pyrB* expression might be correlated with the cellular duplication. The heat shock response of *pyrB* gene expression delineated the ATCase as putative enzymatic biomarker for the environmental temperature impact on ice and

soil microbial communities providing a putative tool to evaluate the microbiome resilience when exposed to temperature variations.

Comparative analyses of variations in the expression profile of ATC and HSP70 genes after seven days of heat shock treatment contributed to understanding the balance between the short-term and long-term strategies for microbial cells survival after environmental disturbances depending on the type of habitat.

Corroboration of the altered genes expression pattern and structural/functional properties of the key enzyme of the *de novo* synthesis of pyrimidine nucleotides in cold-adapted bacteria will supply our knowledge on the adaptation mechanisms to extreme environmental conditions when changing habitats due to glaciers melting.

Chapter III: Impact of heat-shock stress on the structural and functional diversity of total and active ice-embedded microbiomes

In order to unravel the impact of environmental thermal variation on the ice microbiome at both taxonomic and metabolic levels, we carried out a metatranscriptomic analysis of the total rRNA extracted from 900 years old ice deposits of Scarisoara ice cave. The RNA extraction was carried out based on a specific protocol developed in this thesis, and cDNA libraries for each step were sequenced using an Illumina NextSeq platform. Shotgun metatranscriptomics of millennium-old ice microbiome from Scarisoara ice cave submitted to a 3-day heat-shock cycling treatment constituted the first characterization of the active microbial community from this type of habitat providing taxonomical and functional information on the variation of potentially active Bacteria, Archaea and Eukaryotic ice contained species and metabolic pathways and genes based on rRNA and mRNA analyses.

This survey tries to fill the gap in the unknown microbial processes after the ice thawing in this peculiar secluded habitat providing a first glimpse on the ice embedded microbiome post-disturbance distribution, genes expression and predation processes in order to obtain a stable ultimate community.

Ice can be considered as a storage matrix for microorganisms, representing a source of genomic diversity and a reservoir of new microbial species (Ma et al., 2000). Recently, investigations of the microbial communities from a series of icy environments were performed including permafrost (Schostag et al., 2019), Antarctic ice sheets (Abyzov et al., 2005), polar ice (Ma et al., 1999) sea ice (Nichols et al., 2005) and also subglacial lakes (Rogers et al., 2013). Among these habitats, ice caves provide limited information regarding the microbiome from the perennial subterranean ice deposits accumulated in these secluded habitats (Purcarea, 2018). The presence of ice-contained microorganisms in Scarisoara cave was first mentioned in the ice-stalagmites formed in the Little Reserve area (Hillebrand-Voiculescu et al., 2013), followed by studies of the cultured/uncultured microbial communities from the perennial ice block (Hillebrand-Voiculescu et al., 2014), and chronological distribution of cultured bacteria in ice layers old up to 900 years (Itcus et al., 2016). Although the culturing method provided a step forward in the microbial screening, the scientists became aware of the limitations of the culture dependent techniques due to the uncultivability problems in describing the diversity of microbiomes (Hug et al., 2016). To overcome this problem, studies were conducted using denaturing gradient gel electrophoresis (DGGE) to unravel the fungal diversity (Brad et al., 2018) while a more advanced sequences identification was achieved with the application of molecular techniques including 454 pyrosequencing of prokaryotic community (Itcus

et al., 2018), and Next Generation Sequencing of fungal communities along the 1500 years old ice based on ITS2 Illumina sequencing (Mondini et al., 2019).

These reports based on DNA sequencing provide information of the total communities, while no data on the metabolically active microbiome from this habitat was provided so far. Recently, the total and potentially active bacterial communities from a 13,000-years old ice core from Scarisoara were determined by 16S rRNA Illumina sequencing (Paun et al., 2019), suggesting the existence of active microbial community in this habitat. In this context, one of the studies developed in this thesis focused on investigating the active microbiome from Scarisoara cave ice using RNA shotgun Illumina sequencing and metatranscriptomic reconstitution of total and active prokaryotic and eukaryotic microbial communities. The 900-O cave ice sample was selected according to the high prokaryotic (Itcus et al., 2018) and fungal (Mondini et al., 2019) diversity previously determines. The major complexity of the microbiome in 900-O sample can be attributed to the specific warm and wet climate during the ice layer deposition characteristic for the Medieval Warm Period which could have led to a higher accumulation of sediments and organic matter entrained by the heavy rains (Persoiu et al., 2017).

Although reports on metatranscriptomes from frozen habitats indicated the presence of active microorganisms in ice (Rogers et al., 2013), this is the first study unraveling the active prokaryotic and eukaryotic microbial communities from an underground perennial ice from a cave based on the metagenome rRNA and mRNA Illumina sequencing. Moreover, our data report the changes in the total and active microbial communities at taxonomic and metabolic levels as response to a 3-day heat-shock treatment followed by incubation at 4°C over 14 days, in order to understand the impact of glacier melting on the ice-embedded microbiome.

Conclusions

To date, this is the first evidence of an active microbiome in perennial ice from caves, and of the thermal treatment effect on total and active ice-entrapped microbiomes. Temperature changes are known to disturb the microbial homeostasis alongside an altered taxa distribution while little is known about the genes response. This characterization of the microbial community structural and functional changes generated by a temperature increase and prolonged incubation is expected to increase our understanding of changing environments and their ecological impact due to ice melting.

The current investigation of the microbiome entrapped in Scarisoara cave ice deposits revealed a complex potentially active (rRNA) community in this habitat and confirmed the presence of active microorganisms (mRNA) in 900 years old ice strata. The potentially active microbiome from this icy

environment revealed a complex community with a distinctive composition sustaining thermal shock resistance and specific functional response to 4°C-25°C thermal cycling variations. Putative active microbial community was dominated by copiotroph taxa able to quickly use the carbon source released by the ice thawing. Proteobacteria and Bacteroidetes dominated the community indicating higher resistance to thermal stress whereas Firmicutes and Actinobacteria phyla showed a decline in the relative abundance and therefore a reduced ability to cope with the temperature raises.

Archaea, mainly characterised by Methanomicrobia class belonging to the Euryarchaeota phylum, were scarcely represented in T0 resulting to be highly affected by heat-shock without displaying increase in the relative abundance even after prolonged incubation. Fungal community appeared to be the most affected by the thermal shock with the highest number of reads assigned to adapted aquatic Blastocladiomycota and Chytridiomycota phyla whereas Basidiomycota and Ascomycota phyla showed a decline in their relative abundance thus indicating a reduced resistance to thermal shock. Microeukaryotes, although disturbed by the temperature increase, exhibited (after prolonged incubation) a positive upsurge on their relative abundance. Heterotrophic flagellates, such as Stramenopiles, showed the highest presence after a week and the peek after 14 days. Proliferation of this protozoa cluster designated a high predation process upon bacteria favored by their smaller dimensions (compared with other protozoa) speeding up the duplication time.

Analysis of the differential genes' expression pattern helped unravelling the resilience factors involved in the microbe's adaptation. The heat-shock treatment activated the transcription of genes coding for chaperons (HSP60 family), superoxide dismutase and the alternative SigmaE factor for the polymerase activity conferring to the microbial cells more resistance to thermal stress. According to the presence of dominant bacterial classes endowed with a copiotroph lifestyle, an increase in the genes associated with the Tricarboxylic Acid cycle (TCA cycle) followed by a slight reduction after 14 days was observed. Polyhydroxyalkanoates (PHAs) genes expression was high in all samples indicating an elevated energy storage activity. Further up-regulation was visible in the genes involved in the Carbon and nitrogen regulation and cellular motility considering the water environment. Prolonged incubation (T14) showed an upsurge in the transcripts for activating the defense mechanism, suggesting the start of the competition for similar food sources.

Chapter IV: Climate and geochemical impact on ice-embedded fungal communities

Our investigation provides the first high-throughput Illumina MiSeq ITS2 sequencing of Scarisoara Ice Cave microbiome across 1500 years old ice, in order to shed light on the temporal distribution of fungal communities in cave ice deposits in relation with the climate variation during perennial ice deposition and geochemical configuration. The taxa distribution in ice layers formed during warmer periods (Medieval Warm Period) and colder periods (Little Ice Age) was analyzed in order to identify fungal biomarkers for climate variations in this perennial ice cave, consolidating the relationships between microbial population and environmental characteristics. Correlation of fungal taxa distribution across the cave ice block with the geochemical parameters of the ice was also carried out to assess the impact of the substrate chemical composition on the fungal community structure. This work addresses the lack of knowledge of ice entrapped microcosm distribution in perennial cave ice deposits allowing us to understand the fungal role in the icy habitat and providing a potential fungal biomarker to comprehend the local climate and substrate geochemistry effect on the microbial composition in icy habitats.

The increasing interest in icy environments in the last two decades led to investigation of the ice-inhabiting microbial communities (Brinkmeyer et al., 2003) and their temporal and spatial distribution (Itcus et al., 2016) in different frozen habitats.

Contrarily to exposed terrestrial and marine icy environments, ice caves are among the poorest investigated cold habitats (Kern and Perșoiu, 2013), hence little is known so far on their microbial communities (Purcarea, 2018). Recent studies, based on 18S rRNA conducted on Scarisoara ice cave, Romania, reported the presence of microbial eukaryotes belonging to phototrophic and heterotrophic species (Hillebrand-Voiculescu et al., 2014).

The interest for these secluded and highly preserved ice-contained niches resides in the potential of accumulated ice strata for paleoclimate reconstitution (Perșoiu et al., 2017). Various geological (Racoviţă and Onac, 2000) and paleoclimatic (Perșoiu et al., 2017) studies of ice deposits from Scarisoara ice cave conducted in the last decades, provide climatic and geochemical data associated with the cave ice strata formation that were further used for studying the impact of these parameters on the diversity and microbial community structure from corresponding ice layers (Purcarea, 2018). These studies revealed the climate variations undergone during the last millennium, alternating cold and dry periods during the Little Ice Age (LIA; 1250-1860 AD) and Dark Ages Cold Period (DACP, 400–800 AD) with warm and wet intervals during the Medieval Warm Period (MWP; 800-1250 AD)

(Perșoiu et al., 2017). More recently, the microbial diversity across the perennial ice block of Scarisoara cave was investigated, revealing the presence of uncultured bacteria (Hillebrand-Voiculescu et al., 2014; Itcus et al., 2018; Paun et al, 2019), cultured bacteria (Itcus et al., 2016) and cultured fungi (Brad et al., 2018). Although fungi retain viability in ice strata for at least a thousand years old (Abyzov, 1993; Ma et al., 1999), culture-dependent techniques failed to detect a high diversity of fungal taxa (Kochkina et al., 2012). In this respect, utilization of molecular techniques allowed the identification of these microbes at genus and species levels (Ghosh, 2017).

This work addresses the lack of data on the uncultured fungal community composition in Scarisoara cave ice block up to 1500 years old ice layers, based on Illumina MiSeq sequencing of the internal transcribed spacer 2 (ITS2) region of ribosomal RNA genes, in relation with the different geochemical configuration shaped during the previous climate-associated records. To our knowledge, this is the first report on the distribution of uncultured fungal diversity in perennial cave ice using high-throughput sequencing.

Conclusions

This study provided the first-time deeper sequencing across 1500 years old ice chrono sequence revealing a broader fungal diversity in Scarisoara ice. Illumina MiSeq ITS2 sequencing targeting the ice-entrapped fungal community from this secluded ice cave revealed a total of 1751957 sequences which corresponded to 182 fungal OTUs suggesting a climate- and geochemical-driven distribution during deposition across the perennial ice accumulation and a decreasing diversity directly correlated to the ice age due to a DNA degradation process. At phylum level Ascomycota was present in all the ice strata appearing to dominate the ice layers deposited during the Little Ice Age (LIA) and Dark Ages Cold Period. On the contrary, Basidiomycota was the predominant phylum in 900 years old ice layer accumulated during the Medieval Warm Period (MWP). To our knowledge this is the first report of Chytridiomycota phylum in ice environment. The assignment at genera level revealed *Cryptococcus victoriae* as the only fungal strain present in all the analyzed ice strata suggesting a strong adaptation and resilience related to this order. Furthermore, the concentration of dissolved organic carbon (DOC) appeared to play an important role in the distribution of Basidiomycota taxa highly present in ice strata characterized by low DOC content, whereas outcompeted by saprotrophic species in organic rich ice.

This report helped unravelling the global climatic effect on fungal distribution in icy habitats providing a first evidence of the potential identification of a fungal biomarker for climate variations correlating the change of biodiversity with historic environmental events at each of the time points.

General conclusions and perspectives

Enzyme structural adaptation to low temperatures

Structural characterization and comparison of the recombinant ATCases from the psychrophilic *Glaciibacter superstes* and *Rugamonas sp.* strains with those of the mesophilic *Escherichia coli* and *Pseudomonas aeruginosa* and hyperthermophilc *Pyrococcus abyssi* and *Aquifex aeolicus* revealed specific modifications of the primary, secondary and tertiary structures, of residues forming subunit and domain interfaces, and of hydrophobic clusters distribution contributing to a higher stability and a more flexible structure in order to perform the enzymatic reaction at low temperatures.

The main results regarding the structural analysis of *Glaciibacter superstes* and *Rugamonas sp.* ATCases are further summarized:

- All ATCase active site residues from the analyzed psychrophilic, mesophilic and hyperthermophilic strains were conserved, in support of the common reaction catalyzed in all organisms independent of the environmental temperature.
- In comparison to mesophilic and thermophilic homologues, the primary structure of both *Glaciibacter superstes* and *Rugamonas sp.* ATCase showed the complete absence of cysteine (Cys) residues resulting in a more flexible structure by lack of disulfide bridges
- Both psychrophilic ATCases showed a reduced glutamic acid (Glu) content suggesting a reduced number of salt bridges that confers an increased enzyme flexibility
- *Rugamonas sp.* ATCase displayed an increased histidine (His) content that could be responsible for structure stabilization through ionic interactions
- The higher number of Proline (Pro) residues in *Rugamonas sp.* ATCase could facilitate the protein folding
- The higher number of coils in the secondary structure of *G. superstes* ATCases and the position revealed by the 3D model could favor the interaction between catalytic subunits to favor catalysis at low temperature and leading to increased interactions with the solvent
- Presence of reduced hydrophobic clusters in the CP-ASP domains interface has a possible contribution to an enhanced chain plasticity of the domains closure during catalysis
- Cloning and heterologous expression in *E. coli* of the recombinant *pyrB* gene from *G. superstes* and purification of the recombinant ATCase from *Rugamonas sp.* were successfully performed for further functional characterization

Bacterial cell response to heat shock

The cellular resilience related to the pyrimidine nucleotide synthesis and heat shock proteins response to heat shock treatment of a psychrophilic bacterium *G. superstes* was evaluated by RT-PCR quantitation of the genes coding for the ATCase and HSP70. Their comparative expression pattern during 7 daily heat shock cycles, led to assigning the ATCase as a promising marker for cellular adaptation to environmental thermal stress.

- Changes in the microbial gene expression pattern depended on the cell freeze/non-freeze status during thermal cycles for ATCase expression, while HSP70 gene expression was independent of the temperature interval (30°C / 4°C or 30°C / -18°C) of the thermal shock
- Change of the cooling temperature from 4°C to -18°C after heat shock induced a 1,5-fold increase in the *pyrB* gene expression, suggesting an enhanced response of DNA synthesis in the cell for a faster adaptation to environmental changes
- *G. superstes* revealed a short-term adaptation strategy expressing higher level of genes coding for the HSP70 family after the thermal shock followed by a stabilization after 5 days.
- Opposite response of ATCase and HSP70 gene expression was observed independent of the temperature variation interval. The highest HSP70 gene induction occurred after 48 h and 96h heat shock cycles, while the ATCase gene expression decreased during the first 48 h and peaked after 72 h and 120 h, suggesting a complementary response of the cell leading to an efficient cell protection in the first stage followed by increased DNA synthesis for cellular duplication.
- ATCase quantitation revealed a direct connection with the cellular viability during thermal treatment, outlining this enzyme as a potential marker for cellular response to environmental stress.

Thermal adaptation and heat shock response of microbiomes from cold habitats

The current study on the microbial community structure from frozen habitats in relation with the geochemistry and climate variations during ice deposition, and its response to heat shock treatment provided new data on the impact of environmental temperature on the diversity and resilience of the fungal community from Scarisoara perennial ice accumulated during the last 1500 years, and on the taxonomic, metabolic and molecular responses of total and active microbiomes from ice and soil to temperature increase and prolonged incubation, respectively.

Screening of the fungal distribution in the ice chronosequence from Scarisoara ice cave based on ITS2 Illumina sequencing revealed a geochemical and climate related distribution of fungal taxa providing evidence for potential identification of a fungal biomarker for climate variations. The main results are:

- Basidiomycota taxa prevailed in ice strata characterized by low dissolved organic carbon (DOC) concentrations based on their ability to degrade complex carbon sources from habitats with resources scarcity.
- High DOC content favored the presence of Ascomycota suggesting the presence of copiotrophs taxa outcompeting the oligotrophic species
- The *Cryptococcus victoriae* phylotype was found in all the analyzed ice strata suggesting a high metabolic versatility of this fungal species towards various geochemical and climate conditions.
- Basidiomycota phylum was more abundant in ice layers formed during warmer and wetter periods (Medieval Warm Period) associated with the high occurrence of *Picea abies* tree needles that could have created a higher acidic environment suitable for the high versatility of this phylum
- Ascomycota phylum dominated the ice deposited during the colder and dryer “Little Ice Age” period possibly associated with a higher dispersion of spores due to reduced precipitations and increased ventilation in the cave from the surrounding habitats
- This is the first report of Chytridiomycota phylum in icy habitats
- This study provided evidence of the potential identification of a fungal biomarker for climate variations in this habitat

The response to heat shock treatment of microbiomes from glacier cave ice and Icelandic glacial forefield soil assessed by the differential genes' expression between the ATCase and the HSP70 family revealed a complex and complementary transcription pattern of these genes that varied with the type of habitat.

- Soil microbial community exhibited an enhanced resilience to thermal shock due to the preadaptation to increased temperatures after ice thaw, as resulted from the reduced transcription of the HSP70 gene during heat shock treatment due to the presence of molecular chaperons in the cells.
- Ice microbial community revealed a higher HSP70 gene expression within the first 4 days of thermal treatment followed by a reduced expression after a week, suggesting a slower adaptation to heat shock
- Evaluation of the general microbial disturbance based on ratio between ATCase and HSP70 gene expression showed similar pattern with that observed in the case of *G. superstes* cells, indicating complementary gene expression of these two genes at microbiome level.
- ATCase constitute a putative enzyme biomarker of the microbial community resilience in response to extreme temperature variations directly coupled with the higher cell viability

Shotgun metatranscriptomic of 900 years-old ice microbiome from Scarisoara ice cave submitted to a three-day heat-shock (4 - 25°C) cycling treatment and further incubation at 4C for 11 days constituted the first characterization of the active microbial community from this type of habitat. Both rRNA and mRNA data revealed a major microbial structure variation immediately after the heat shock application, where the fungal and microeukaryotes communities were the most affected. Shotgun metatranscriptomics analysis on both rRNA and mRNA data revealed a major microbial structure variation immediately after the heat shock treatment indicating the presence of microbial taxa with a different resilience to temperature increase and a different adaptation process as proved by the different genes' transcription.

- The potentially active microbial community was dominated by bacterial taxa with a copiotroph metabolism such as Proteobacteria and Bacteroidetes able to grow fast and use the present carbon source quickly and therefore outcompeting the oligotrophic taxa.
- Among bacterial taxa, Firmicutes, Actinobacteria and Chlorobi showed a decrease in relative abundance ranging from 5-5,5 and 19-fold respectively, with the highest response after the 3-day heat shock treatment from Proteobacteria and Bacteroidetes with 1,5- and 2,5-fold respectively.

- Eukaryotes community was the most affected by temperature raise with an 8-fold reduction during the heat shock cycles and 10,5-fold recovery after 14 days.
- Archaea were scarcely present in all the samples (<0,2%), represented by Euryarchaeota phylum showing a 9-fold reduction of their relative abundance after the heat shock cycles
- Microeukaryotes revealed a high abundance in heterotrophic flagellates, such as Stramenopiles after prolonged incubation, suggesting their ability to recover after a thermal shock. A hypothesis for this relative abundance recovery is based on the predatory nature of protozoa upon bacteria, highlighting their role in the natural environment control.
- Increased abundance up to 2-fold of transcripts for cellular protection against temperature stress with the presence of genes coding for molecules with chaperone activity in the protein correct folding and enzymes with the ability to reduce the negative effect of reactive oxygen species (ROS)
- Higher abundance up to 4-fold of the alternative sigma factor for the polymerase indicating a direct thermal stress response
- Increased expressions of genes involved in the Tricarboxylic Acid cycle (TCA) up to 2,8-fold substantiating the higher presence of microorganisms with copiotroph metabolism
- Up regulation of the genes involved in the cellular motility up to 2-fold considering the water environment
- Higher expressions up to 1,3-fold of genes involved in the carbon and nitrogen regulation favoring the processes of substrates liberation
- Incubation for 11 days post heat-shock treatment showed a 2,5-fold increase in the expression of genes coding for defence mechanisms against secondary metabolites, suggesting the start of the battle to outcompete the taxa using similar sources as substrates

Future efforts should be pointed towards the functional characterization of the investigated cold-active ATCase and determining the ATCase x-ray crystallographic structure from these psychrophilic bacteria, in order to deepen the understanding of structure-function relationship in this class of enzymes adapted to catalyze the synthesis of key metabolites at low temperatures. Analysis of specific genes and metabolic pathways expression variation as a response to heat shock based on the cave ice metatranscriptomic data obtained from different cold environments will contribute to understand the impact of glacier melt on the active microbiomes and expected biosphere modifications.

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