Universität für Bodenkultur Wien

Department für Biotechnologie

SURVIVAL STRATEGIES OF ROCK INHABITING FUNGI IN EXTREME ENVIRONMENTS

Dissertation

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Посвящаю эту работу своей семье и друзьям
To my family and friends

“Человек — это то, во что он верит“. Антон Павлович Чехов

“Man is what he believes”. Anton Chekhov

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Abstract

Black microcolonial fungi (MCF) and black yeasts are among the most stress resistant eukaryotic organisms on Earths. They are known to inhabit bare rock surfaces in hot and cold deserts of all regions of the Earth, moreover showed ability to survive harsh conditions of Outer space. Some of them have a close phylogenetic relation to human pathogenic black fungi which makes them important model organisms also with respect to clinical mycology.

In this work we tried to understand cellular mechanisms underlying the resistance of MCF against harsh environments on Earth and Mars-like conditions. We selected three organisms representing different environments: two species of microcolonial rock inhabiting fungi – *Cryomyces antarcticus* from cold deserts, *Knufia perforans* from hot environments and a species of black yeasts – *Exophiala jeanselmei* which is closely related to human pathogen species of the genus.

First of all we investigated the survival strategies under poikilohydric conditions. To this aim selected strains of MCF were subjected to desiccation and re-hydration in climate chambers and subsequently RNA, sugar content and the protein pattern (using 2D-gel electrophoresis) were analyzed. The ability to survive after complete desiccation and the speed of re-hydration as well as changes of the whole cell protein pattern were demonstrated. Data on intracellular sugar suggest that both trehalose and mannitol might play a cell protective role in those fungi.

The next study was carried out in order to investigate the survival of the fungi in Mars-like environments. The organisms were exposed to thermo-physical Mars-like conditions in the simulation chamber of the German Aerospace Center (Berlin). In this study the alterations at the protein expression level from various fungi species under Mars-like conditions were analyzed for the first time using 2D-gel electrophoresis (2DE). The comparative analysis of the protein patterns shows that the fungi not only survive exposure to Mars-like conditions: after one week of exposure they acclimatize and seem to be metabolically active.

In order to get a better understanding about the cellular processes behind the stress resistance, *Cryomyces antarcticus* was selected as model organism for protein identification. To explore the protein repertoire of this fungus 2DE and MALDI-

TOF/TOF mass spectrometry were performed. Only a limited number of proteins could be identified by using sequence homologies in public databases. Due to the rather low identification rate by sequence homology, this study reveals that a major part of the proteome of *C. antarcticus* varies significantly from other fungal species.

The results obtained from a variety of analysis were mostly focused on proteomics. The results are discussed in details and speculate about applications of the study and future prospects.

Keywords: black microcolonial fungi, extremophiles, Mars-like conditions, desiccation, proteomics

Kurzfassung

Schwarze mikrokoloniale Pilze (MCF) und schwarze Hefen gehören zu den Stress resistentesten Organismen der Erde. Es ist bekannt, dass diese Pilze nackte Gesteinsoberflächen in heißen und kalten Wüstengebieten besiedeln; darüber hinaus konnte gezeigt werden, dass sie auch im Weltall überleben können. Einige der schwarzen Pilze sind nahe verwandt mit humanpathogenen Spezies, daher gelten sie auch in Bezug auf klinische Mykologie als wichtige Studienobjekte.

In der vorliegenden Arbeit wurden die zellulären Mechanismen, die der Stressresistenz dieser Pilze in Extremhabitaten der Erde aber auch unter Mars-ähnlichen Bedingungen, zugrunde liegen näher untersucht. Es wurden dazu zwei mikrokoloniale Pilze – *Cryomyces antarcticus* aus kalten Wüsten, *Knufia perforans* aus warmen Habitaten - sowie die schwarze Hefe *Exophiala jeanselmei* als Modellorganismen ausgewählt. *Exophiala jeanselmei* ist nahe verwandt mit human pathogenen Arten dieser Gattung.

Zunächst wurde das Überleben der Pilze unter wechselfeuchten – poikilohydrischen – Bedingungen untersucht. Dazu wurden die ausgewählten Pilzstämme völliger Trockenheit ausgesetzt und anschließend in einer Klimakammer rehydriert. Vor und nach der Austrocknung beziehungsweise der Rehydrierung wurde der RNA-Gehalt und der Zuckergehalt der Zellen gemessen und das Proteinmuster mit 2D-Gel Elektrophorese (2DE) analysiert. Es konnte gezeigt werde, dass die Zellen völlige Austrocknung überleben; das Proteinmuster zeigt, dass sich die Zellen bei Rehydrierung rasch erholen und metabolisch wieder aktiv werden, Messungen des Zuckergehaltes bestätigen, dass sowohl Trehalose alsauch Mannitol eine wichtige Rolle für den Zellschutz spielen.

In einer weiteren Untersuchung wurde die Überlebensfähigkeit der Pilze unter Mars-ähnlichen Bedingungen getestet. Die Pilze wurden in der Mars-Simulationskammer des Deutschen Zentrums für Luft- und Raumfahrt (Berlin) Mars-ähnlichen thermophysikalischen Bedingungen ausgesetzt. Erstmals wurden die Veränderungen im Proteinmuster der Pilze unter Marsbedingungen analysiert. Die vergleichende Analyse der Proteinmuster konnte zeigen, dass die Pilze die Marsbedingungen nicht nur überleben: vielmehr kam es nach 7 Tagen zu einer

Akklimatisierung und das Proteinmuster gab einen deutlichen Hinweis darauf, dass die Pilze sogar metabolisch aktiv waren.

Um besser zu verstehen, welche Proteine an der Stressresistenz maßgeblich beteiligt sein können, wurden Identifizierungen der Proteine des Modellorganismus *C. antarctikus* basierend auf 2D-Gelen und anschließender MALDI-TOF/TOF Analyse durchgeführt. Durch Homologiesuche in öffentlichen Datenbanken konnte nur eine begrenzte Anzahl der Proteine identifiziert werden. Die sehr niedrige Identifizierungsrate deutet an, dass das Proteom von *C. antarcticus* sich erheblich von dem anderer Pilze unterscheidet.

Die in der vorliegenden Arbeit gezeigten Ergebnisse basieren in erster Linie auf Proteomanalysen. Die Ergebnisse werden im Detail dargestellt und ihre Bedeutung für die zukünftige Forschung und mögliche Anwendungen diskutiert.

Schlüsselwörter: schwarze mikrokoloniale Pilze, extremophile Organsimen, Mars-ähnliche Bedingungen, Austrocknung, Proteomics.

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1. General introduction

1.1 Fungi in deserts

For a long time desert regions including the hot deserts of North America (Great Basin Desert), Africa (Sahara desert, Kalahari desert), Asia (Gobi desert), the Middle East (Syrian desert), Australia (Great Victoria desert) as well as the permafrost environments up to the cold, dry valleys of Antarctica, Arctic (Fig. 1) and Patagonia were believed to be too hostile for any forms of life (Cary *et al.*, 2010). However, results from recent studies showed that there are several groups of organisms which are able to live 'at the edge' of life.

Figure 1. Arctic desert (photo by K. Sterflinger)

For instance, Antarctic ice-free deserts are among the closest analogues of Mars due to a very cold and very dry conditions, extremely poor nutrient availability, and large fluxes of UV-light (Onofri *et al.*, 2007). Despite of the hostile conditions life is present in cryptic niches: microorganisms are organized in cryptoendolithic

communities mostly dominated by lichens and by non-lichenized fungi and cyanobacteria (Friedmann, 1982). Some of the components of those communities are black meristematic fungi, also known as microcolonial fungi (Selbmann *et al*., 2008).

Black microcolonial fungi (MCF) and black yeasts together with lichens and cyanobacteria are among the most stress-tolerant organisms living on the Earth (De Hoog, Grube, 2008). Detailed study presented in part **5.4 "Fungi in hot and cold deserts with particular reference to microcolonial fungi"** shows that MCF are found not only in the cold Antarctic deserts but also in extreme environments of the hot deserts of Arizona (USA), as well as in Mediterranean countries as e.g. Italy and Greece (Sterflinger *et al*., 2012).

The extreme environments of deserts are characterized by a combination of dry, cold, oligothophic extremes along with huge fluxes of UV radiation (Robinson, 2001). Moreover, those conditions are characterized by rapid and extreme instability during the day and the season. Therefore rock inhabiting fungi are facing periods of stress-induced dormancy, which are suddenly interrupted by the occasional return of growth-favourable conditions. In order to survive such harsh conditions organisms have to have a very broad range of tolerance to multiple stresses. The term poikilo-tolerant (resistant to variable stress; from poikilos: various) describes the behaviour of living organisms in environments where in order to survive they have to tolerate multiple and fluctuating stress (Gorbushina, 2007).

Among other stressors, desiccation is the major challenge to survive in the desert conditions. As it was reported, there are no cultivable bacteria found in one of the driest and Mars-like environments on Earth, for example the area around the Yungay station of the hyperarid Atacama Desert (Chile). However, hyphomycetes survive in this region in a re-cultivable state by the formation of thick-walled spores (Conley *et al.,* 2006). Fungi are known to be survival specialists of complete desiccation due to ability to produce spores; they also prosper – even though slowly – at extremely low levels of water availability (Sterflinger *et al.,* 2012).

Organisms which are able to tolerate complete dehydration are known to be in anhydrobiosis, therefore black rock inhabiting fungi supposed to be anhydrobiotes. It is reported that anhydrobiotic organisms – during the process of desiccation – enter a state of metabolic arrest which is reversible on re-hydration (Goyal *et al*., 2005).

As it is supposed by Sterflinger and colleagues (Sterflinger *et al*., 2012), in the polar environment rock inhabiting fungi are in dormant state most of the year and that they are only active during the short summer period when temperature rises and melting water is available. On the contrary, hot desert fungi might profit from dewfall that develops in deserts during a short time before sunrise (Ninari *et al*., 2002). This process would necessitate a very fast re-hydration and up-regulation of the metabolic activity. Our first study was focused on verifying these hypotheses and described in details in part **5.1 "Microcolonial fungi on rocks: a life in a constant drought?".**

1.2 Stress adaptation

The enormous stress resistance of MCF makes them important models for understanding stress resistance and adaptations in Eukaryotes in general.

MCF form black, clump-like colonies consisting of isodiametrically dividing cells on rock surfaces, in cracks, pores and fissures of the rock and in micropits, created by their own deteriorative activity. Colony of rock-inhabiting microcolonial fungus is shown in figure 2. Morphology of MCF is interpreted as response to multiple stress factors, therefore they are keeping the surface-volume ratio optimal which allows them to decrease the water loss and minimize the colony surface with direct exposure to sun light and other physical and chemical stressors (Sterflinger, 2010). Sampling in natural environment is shown in figure 3.

Figure 2. Colonies of MCF on a rock sample (photo provided by K. Sterflinger)

The special characteristics of the black fungi can be summarized as follows: (1) the cell composition of the fungi which consists mostly of very complex lipids (e.g. mono-, di-and triacylglycerols, phosphotidilcholine, phosphatidylethanolamines,

sterols, sterol ethers, phosphatidilrthanolaminesand free fatty acids); (2) pigments with their various protective effects as e.g. melanins, carotenoids in colourless and brown-red formations and (3) the dense cell wall built of chitin, melanin and polysaccharides (Selbmann *et al.,* 2008). Melanin plays a special role in MCF: exposure of melanin to ionizing radiation and, possible also other forms of electromagnetic radiation, changes its electromagnetic properties. As it was reported, melanized fungal cells grow faster than non-melanized after exposure to ionizing radiation (Dadachova *et al*., 2007). It was also shown that melanin plays a role in radioprotection (Revskaya *et al*., 2012).

Recent experiments showed that the stress-resistance of MCF against solar radiation, radioactivity, desiccation and oligitrophic conditions even allows them to survive space and Martian conditions. Our second study shows results after exposure of selected MCF to simulated Mars-like environments. Eventually all fungal samples were able to survive 7 days of exposure to those conditions and were able to grow and develop after returning to normal conditions. More on this topic is in part **5.2. “Protein patterns of black fungi under simulated Mars-like conditions“.**

Figure 3. Sampling of rock-inhabiting black fungi in Arctic desert (photo by K. Sterflinger)

At the same time those special characteristics of MCF make it difficult to perform analysis on the cellular level. One of the few studies on protein patterns of MCF is described in part **5.5 "Alteration of protein patterns in black rock inhabiting fungi as a response to different temperatures".** For instance, the cell wall is the main obstacle in protein extraction from fungal biomass. Moreover, due to rigid cell wall and melanin content, also DNA extraction and purification have not been convenient to obtain genomic data of MCF (Marzban, *et al*., 2013). Up to now whole genome sequences of only two strains of black microclonial fungi – *Coniosporium apollinis* and *Cryomyces antarcticus* – exist in the public databases, however without annotation. In addition, the studied MCF strains have an extremely slow growth: they need at least 30 days to reach the minimal quantity of biomass level for any type of experiment.

Along with extreme environments occurring in nature there are some which are results of a human activity. For instance, zone of Chernobyl nuclear power plant accident is inhabited by melanized fungi which survive chronic irradiation from multiple radionuclides and spore germination and hyphal growth of some of those fungi are stimulated by ionizing radiation. Melanin-dependent and melanin-independent mechanisms are involved in elaboration of radiation-induced fungal growth 'and implicate direct and/or indirect interaction of melanin with high energy ionizing protons as an important pro-proliferable factor' (Shuryak *et al*., 2014).

1.3 Black fungi in Astrobiology

The discovery of extreme Earth's environments and the organisms that inhabit them has awakened curiosity of humans on the limits of life on and outside our planet and even through the Universe.

Earth's biosphere has evolved in more than three billion years while protected by the planet's magnetosphere and the atmosphere from the hostile environment of outer space. Conditions of Outer space are characterized by combination of space vacuum, thermal extremes, cold, solar UV radiation, ionizing radiation and galactic radiation Such environments are real challenges for any form of life (Horneck *et al*., 2010). Large quantities of highly melanized spores have been found in early Cretaceous period deposits when many species of animals and plants become extinct. This period matches with Earth's crossing the 'magnetic zero event' when the planet lost its protection against cosmic radiation (Hulot and Gallet, 2003).

The question about possible existence of life on other planets, survival in outer space and possible interplanetary transfers are in the focus of Astrobiology. It was reported that organisms which inhabit extreme environments – the so-called extremophiles – are generally considered to be the best model for exobiological studies (Cary *et al*., 2010; Rivkina *et al*., 2000). Extremophiles can not only survive extreme thermo-physical conditions, but can also tolerate extreme saline, acidic, alkaline or other conditions which can induce DNA damage in less resistant organisms (Onofri *et al*., 2007).

In our second study we investigated how the model organisms could react to Mars-like environments on molecular level which is shown in part **5.2**. It is believed that Martian climate conditions are too cold and the atmosphere too thin for allowing bulk water to be stable. The water availability and interaction between regolith and near-surface atmosphere would be a precondition for habitability on Mars (Stoker *et al*., 2010; McKay *et al*., 2013).

Although organisms need water activities of a_w=0.8-0.9 for active metabolism, there are some special life forms which are also able to live in much drier conditions with a temporary saturation of atmospheric water vapor, for instance black microcolonial fungi (MCF) and some lichens. Such temporary saturation can occur on Mars (de Vera *et al.,* 2013), (Fig. 4). Our first study which is described in part **5.1**

showed that fungi could survive successfully seven days of desiccation by adjusting their metabolism.

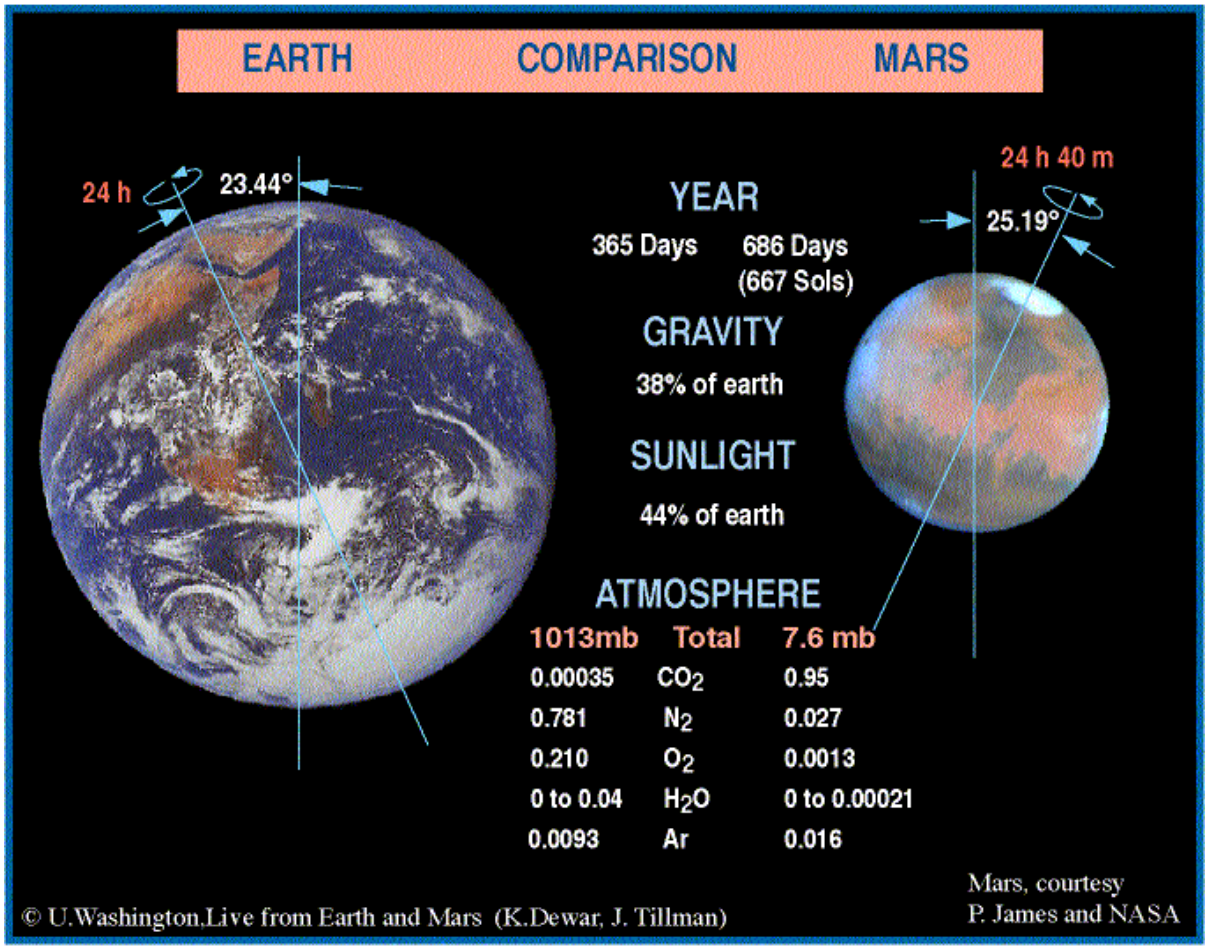

Figure 4. Comparison Earth and Mars characteristics (source: http://www-k12.atmos.washington.edu/k12/resources/mars_data-information/mars_overview.html)

Therefore microcolonial fungi – and particularly *C. antarcticus* – have been suggested as eukaryotic models for studies on the habitability of Mars (de Vera *et al*., 2010) and the biological exploration of Mars (Onofri *et al*., 2008). Results of our study which are shown in part **5.2** support fully this statement.

1.4 Model organisms

For our study we chose three model organisms, each of them represents different environment: (1) *Cryomyces antarcticus* MA 5682 represents a group of extremophilic fungi living in Antarctic dry deserts; (2) *Knufia perforans* MA 1299 is a mesophilic but highly stress tolerant fungus found in hot and arid environments and (3) *Exophiala jeanselmei* MA 2853 represents a rock inhabiting black yeast closely related to opportunistic pathogens in humans. The strains were obtained from the ACBR culture collection (Austrian Center of Biological Resources and Applied Mycology, www.acbr-database.at).

Cryomyces antarcticus (Dothideomycetes, Ascomycete) was found in Linnaeus Terrace, McMurdo Dry Valleys, Southern Victoria Land, Antarctica the desert of Antarctica and described for the first time by Selbmann, de Hoog, Mazzaglia, Friedmann and Onofri in 2005.It was indicated that colonies on CZA (Czapek agar) attaining up to 7 mm in 3 months, flat with very irregular margin, diffusing black-brown pigment in the agar. Microscopy: Description based on strain CCFEE 534 on MEA at 10 °C: mycelium often not observed, where present rarely meristematic or more frequently composed of short hyphae, closely septate, rarely branched, brown, thick walled, torulose, 4–9 µm wide. Torulose hypae and meristematic mycelium often resolved into 1-celled conidia globosa, dry, brown to dark brown, very thick-walled, coated by fragmented incrustations, roughed, acrogenous when produced in torulose chains, 4–12 µm diam, produced by schyzolitic secession (Selbmann *et al*., 2005).

Knufia perforans is specie of the genus *Knufia* which is black yeasts, belonging to the family *Chaetothyriaceae (Chaetothyriales, Ascomycota). Knufia perforans* is orginating from marble in the Mediterranean basin, are described as new species of rock inhabiting microcolonial fungi which was introduced by Sterflinger and colleagues (Sterflinger *et al.,* 1997*).* Previously *K. perforans* was named *Coniosporium perforans* (Tsuneda *et al.,* 2011). Morphological characters of *Knufia* species in culture indicate (1) slow-growing, black colonies; (2) blastic, and endogenous conidia in undifferentiated hyphae or multicellular bodies; and (3) darkly pigmented, enlarged multicellular bodies are reliable characteristics to differentiate species (Tsuneda *et al., 2011*).

Exophiala jeanselmei has been described in the beginning of 20th century and its final classification was made by De Hoog and Hermanides-Nijhof in 1977: *E. jeanselmei* belongs to the class *Ascomycetes*, order *Chaetothyriales*, family *Herpotrichiellaceae*. It was recorded that colonies growing slowly or moderately slowly, greenish-grey, black, olivaceous-green or dark brown; appearing smooth or velvety, often initially mucous at the centre; becoming raised and developing tufts of aerial mycelium with age, often becoming dome-shaped and suede-like in texture. Numerous ellipsoidal, yeast-like, budding cells are usually present, especially in young cultures. Scattered amongst these yeast-like cells are larger, inflated, subglobose to broadly ellipsoidal cells (germinating cells) which give rise to short torulose hyphae that gradually change into unswollen hyphae. Cultures grow at 37°C but not at 40°C (De Hoog and Hermanides-Nijhof, 1977).

Clinical significance: *E. jeanselmei* has a world-wide distribution and is a recognised causative agent of mycetoma and phaeohyphomycosis in humans. *Exophiala* species are common environmental fungi often associated with decaying wood and soil enriched with organic wastes. However, several species notably *E. jeanselmei*, *E. moniliae* and *E. spinifera*, are well documented human pathogens (Fig. 5).

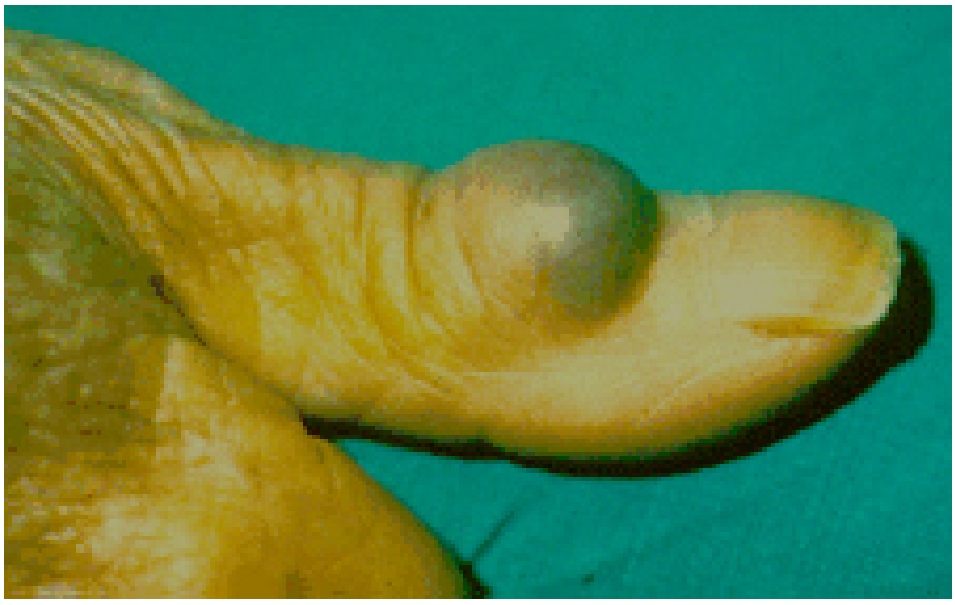

Figure 5. Subcutaneous phaeohyphomycosis caused by *Exophiala jeanselmei (source:* http://www.mycology.adelaide.edu.au/Mycoses/Subcutaneous/Phaeohyphomycosis/ *)*

Clinical manifestations include mycetoma (especially for *E. jeanselmei*), localized cutaneous infections, subcutaneous cysts, endocarditis and cerebral and disseminated infections. Phaeohyphomycosis caused by *Exophiala* species has been reported in both normal and immunosuppressed patients (De Hoog and Hermanides-Nijhof, 1977).

2 Aims of the study

Analyzing metabolic activity of the MCF is a key to understand their abilities to adapt and survive in extreme environments. We aimed to investigate the mechanisms which make the fungi such successful survivors. In our first work which is described in part **5.1** we focused on achieving three aims: (1) to test how fast MCF can react to changes of humidity; (2) to examine under which conditions these fungi are active in their natural environments and (3) to analyze how they respond to dehydration and rehydration on the proteome and RNA level. By doing this we tried to understand how fungi maintain their living in conditions which might naturally occure in deserts.

Next step was the exploring those fungi in simulated Mars-like environments which is described in details in part **5.2**. So far cellular mechanisms underlying the resistance of MCF against harsh space and Mars conditions are not yet fully understood. As it was mentioned before there is only limited information on genomic sequences due to some obstacles occurring during DNA extraction and purification. In this study we carried protein profiling of the fungi using 2D-gel electrophoresis; we were aiming to get more precise impression of cellular and metabolic activity of these fungi under simulated Mars-like conditions.

Until now there is no identification of any single protein of those fungi, therefore we decided to 'take it as a challenge' and performed our third study shown in part **5.3 "Global proteomics of the extremophile black fungus *Cryomyces antarcticus* using 2D-Electrophoresis''**. The aim of the study was to perform the first protein identifications of the very particular fungal species - *C. antarcticus* - cultivated under laboratory conditions as a basis for a deeper understanding of the systems biology of this fungus and related extremophile genera.

3 Methods

3.1 Sample preparation

Inoculi were prepared as cell suspensions and drop-inoculated onto a sterilized cellophane membrane, which allowed much more sufficient biomass harvesting. The membranes were placed on the surface of 2% malt-extract agar (MEA). Fungi were grown at 20°C for *E. jeanselmei* and *K. perforans* and 15°C and for *C. antarcticus* for 30 days. Fresh biomass was harvested by scratching the material from the plates using a scalpel, then transferred to a sterile tube and immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

3.2 Desiccation experiments

In order to analyze the response to desiccation, the cellophane membranes with well grown fungal colonies were detached from the MEA and transferred into empty Petri dishes, which were subjected to dehydration in a climate chamber containing silica gel and dried to their constant weight, which was reached after 6 days (this was tested in pre-experiments). The water loss, estimated by weight, was about 90% of fresh biomass, as a consequence of the severe water loss involved, this treatment was defined as desiccation.

Part of the dried biomass was immediately frozen in liquid nitrogen for the further analysis. Other cellophane membranes were replaced onto fresh MEA and immediately placed into a climate chamber at 98% rH. During the process of re-hydration samples were taken at 3 time points: after 3 min, 10 min and 1 hour. After each sampling the biomass was immediately frozen in liquid nitrogen for further experiments.

Samples subjected to desiccation and re-hydration in climate chambers were subsequently proceeded with 2DE in order to analyze the protein pattern.

Total RNA was extracted from frozen samples as is reported in part **5.1** in details. RNA quality and quantity was analyzed using a Nano Drop 1000 Spectrophotometer according to the manufacture's instruction.

Detection and quantitative analysis of saccharides and polyols was carried out by HPLC (High Performance Liquid Chromatography). The autosampler (Waters, 717 plus autosampler) injected 10 µl per sample and peak integration and calculation was performed with Empower software delivered. As standards trehalose, mannitol, glucose, glycerol, erythritol and arabitol were used.

3.3 Mars-simulation

The experiment was carried out in the Mars Simulation Facility (MSF) at the Institute of Planetary Research of the German Aerospace Center (DLR, Berlin). The MSF simulates Mars-like atmospheric conditions and is used to perform laboratory experiments with controlled time-profiles using experimental Mars Simulation Chamber (Fig. 6).

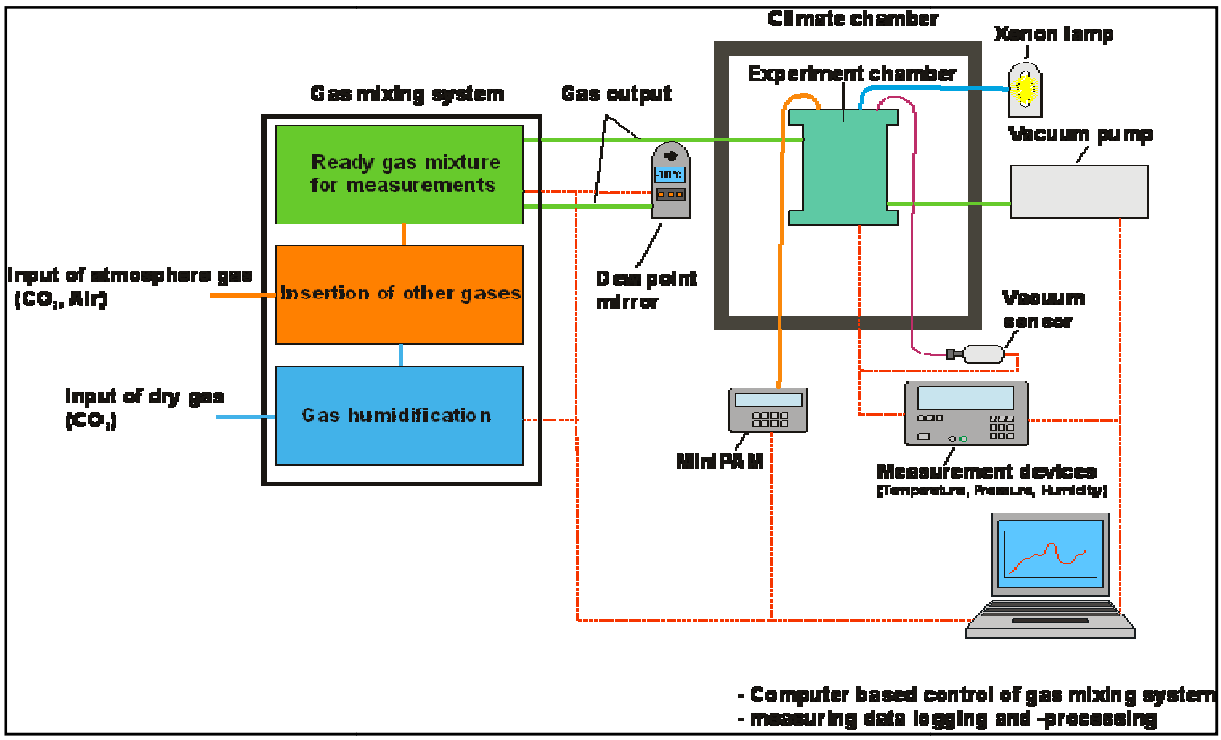

Figure 6. Scheme of Mars Simulation Chamber (provided by Lorek A. and de Vera J-P)

Due to the specific technical characteristics of the experimental Mars-simulation chamber, samples for the experiment were prepares as followes: from the Perti dishes with well grown colonies onto cellophane membranes 3 cm diameter fragments were cut out (Fig. 7).

Figure 7. Sample of MCF: A: preparation for the exposure to Mars-like environments; B: sample of *E. jeanselmei* after exposure to Mars-like environments.

The experimental chamber could be cooled, evacuated and filled with defined gas mixture and humidity in a controlled manner (Fig. 8).

Figure 8. Experimental chamber: *right*: for simulating day and night conditions part of the samples were covered with foil; *left*: all the samples were placed into the chamber.

All relevant experimental parameters and data were PC-controlled by software programs, which were LabView based (Lorek and Koncz, 2013).

After each experiment a small inoculum was transferred onto a Petri dish containing 2% MEA media to check the viability of the fungi. Remaining biomass was immediately frozen in liquid nitrogen for 2DE.

3.4 Proteomics

Since 1975, when O'Farrell published his study, polyacrylamide gel electrophoresis has been extremely useful as an analytical tool for the separation and quantification of protein species from complex mixtures (O'Farrell, 1975). 2DE analysis are able to identify the protein composition of the cell, tissue or whole organ at a given time showing all physiological implications of particular condition (Otto *et al.,* 2014), which is in our study desiccation, exposure to Mars-like conditions. Moreover 2DE method has a great value due to the unique ability of 2D gels to be a high resolution method separating complete proteins with all their modifications (Rabilloud *et al*., 2010).

Eventually, the 2DE is a successful technique has been used in our work, especially considering the fact that we attempt to identify the proteins by *de novo* sequencing and our model organism has not yet genome sequence available (Marzban *et al*., 2013; Rogowska-Wrzesinska *et al.,* 2013).

Protein extraction from biomass and the procedure of 2DE was optimized for MCF. We have made some modifications in the protein extraction protocol in order to improve it. The final version, containing all the changes, explained in details in part **5.3.**

Protein determination was performed using the Bradford protein Assay (Bradford, 1976). The concentration of proteins in fungal extracts was determined according to manufacturer's recommendation. Reactions were carried out in 96-welled microtiter plates. Iso-electric focusing was performed using Protean IEF Cell according to the manufacturer's instructions overnight.

All 2DE experiments were carried out in triplicates. For the analysis 13 cm strips non-linear pH 3-10 were used; protein concentration value was calculated and for each gel 20µg of proteins was used in our first study **5.1.** However, during the work on our third study **5.3** (on *Cryomyces antarcticus*) we discovered that application of 20µg of total protein to each gel was too low for the protein identification of this fungus using mass spectrometry. Therefore 200µg of total protein were used to generate preparative 2D gels.

Additional advantage of using 2DE is that 2D gel-based proteomics is the only proteomic setup in which there is a readout before mass spectrometry, as it was said by Rabilloud and colleagues: 2D gels are not gridded blindly with each other and every piece of gel sent out for digestion and MS analysis' (Rabilloud *et al*., 2010). We have chosen a high sensitive detection technique - a silver stain - which is able to operate at nanograms ranges as a protein visualization method (Shevchenko *et al*., 1996).

After staining, the gels were scanned as 16 bit TIFF images and stored at 4°C in MilliQ water for further analysis. Gel images were matched by warping using Image Master 2D Platinum software and the sum of all spots which were present at least in two gels was taken into account for the protein pattern analyses.

3.5 Protein identification

The typical workflow for protein identification and characterization starts with a protein sample, which is digested by the enzyme, often trypsin, to peptides. Each isolated peptide is then induced ti fragment and then MS/MS spectrum is captured. For each MS/MS spectrum software determines which peptide sequence in a database of protein or nucleic acid sequences gives the best match (Cottrell, 2011). In our study shown in part **5.3** the protein spots were excised by scalpel, washed, destained, reduced with DTT (D, L-Dithiothreitol) and alkylated with IAA (Iodoacetamide). In-gel digestion was performed with trypsin. Dried peptides were concentrated and desalted using Zip-Tips C18 (microbed) according to the manufacturer's instructions.

In order to enhance the quality of tandem mass spectrometry (MS/MS) spectra for de novo sequencing, N-terminal chemical modification using 4-sulfophenyl isothiocyanate (SPITC) was carried out. Then desalted peptides (0.5 µl) were spotted onto a disposable AnchorChip MALDI target plate pre-spotted with a-cyano-4-hydroxycinnamic acid. Data were acquired on a Matrix Assisted Laser Desorption Ionisation Tandem Time-of-Flight (MALDI-TOF/TOF) mass spectrometer in MS and

MS/MS modes. Spectra processing and peak annotation were carried out using FlexAnalysis and Biotools.

For data analysis and *de novo* peptide sequencing we used standard databases in order to search for processed spectra. Each spectrum was compared via an in-house Mascot server (Matrix Science) to the Swiss-Prot database, NCBInr and to a generic database, based on genomic data of the organism. All the search parameters described in details in part **5.3**. Peptide sequencing was carried out manually using FlexAnalysis and homology search by MS-homology (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology) was carried out.

4 Results and conclusions

Hereafter we describe in details all the results and conclusions which have been derived from our three studies. The experiments on desiccation reported in part **5.1** have showed that the fungi tested did not exhibit a remarkable change of the morphology even after 6 days of desiccation. After desiccation to the level of water loss 90% fungi did not gain their original fresh weight. However, after transferring to fresh medium, all three strains were able to grow again which manifests that they are alive and active after desiccation. There were no differences in morphology being observed in any of the sample (Fig. 9).

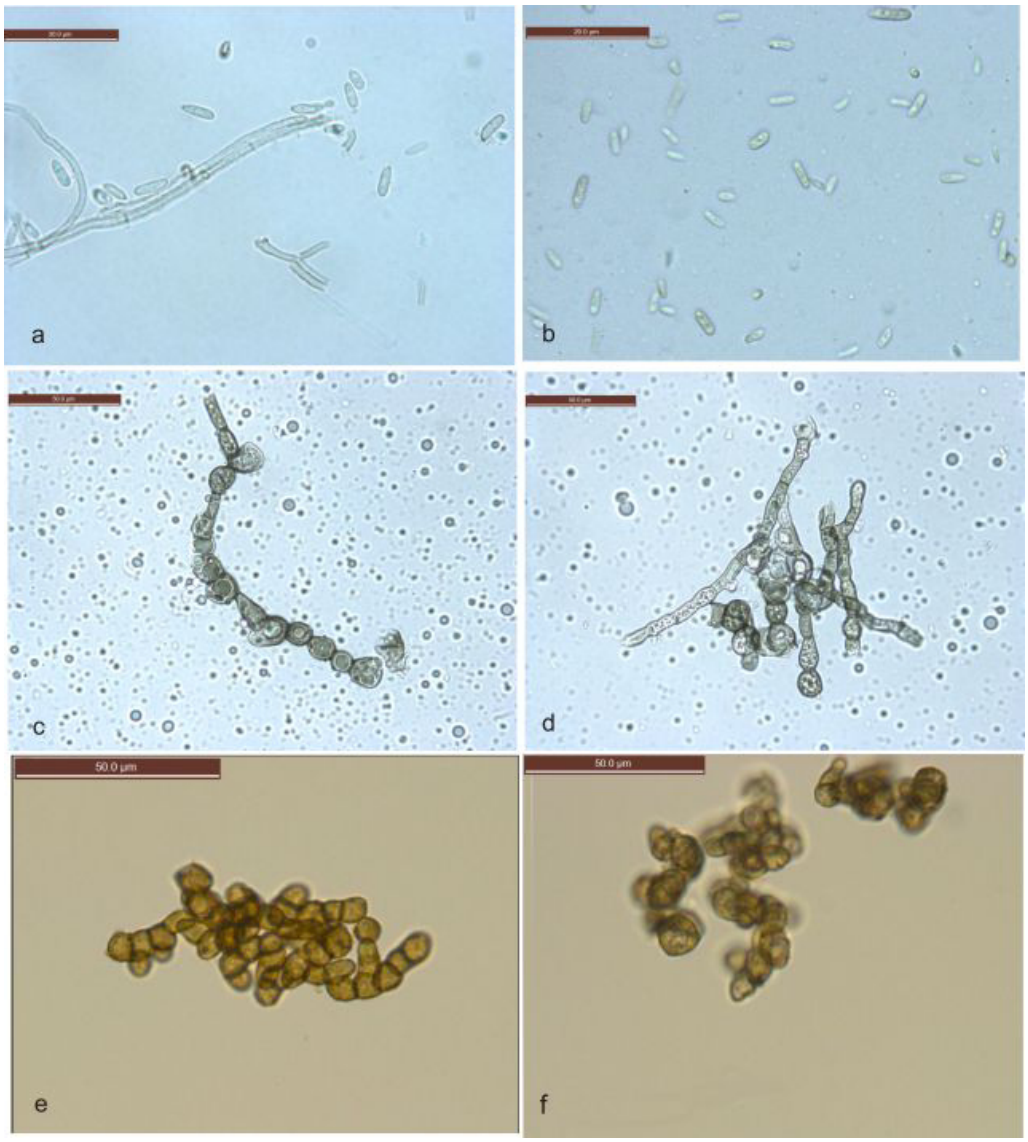

Figure 9. Cells before and after desiccation for 1 week: a: *E. jeanselmei* (before); b: *E. jeanselmei* (after); c: *K. perforans* (before), d: *K. perforans* (after); e: *C. antarcticus* (before), f: *C. antarcticus* (after).

This could mean that the fully hydrated state is never reached in nature – growth on laboratory medium and with levels higher than 90% rH is artificial; those levels of nutrient availability and humidity are never reached in the natural environment of MCF.

Also the protein profiles and the RNA quantification shown is this study, which is illustrated in part **5.1**, support the hypothesis that MCF and black yeasts are able to be active with low levels of intracellular water and are able to react to re-hydration after periods of desiccation in a short time.

The protein content that could be released from the fungal samples significantly increased upon drying during the desiccation period of 6 days. The protein content of the wet cell is very low. This indicates massive amounts of other materials in the cell, as cell walls or solutes, which was shown by Sterflinger (Sterflinger *et al.*, 1998). Samples that are replaced on MEA show invariably lower levels of protein release even compared with the fresh samples before drying.

In the fungal samples protein profile clearly changed during the process of desiccation and again during the process of re-hydration both in the number of protein spots and in the protein pattern.

The results of this study clearly show that the mesophilic fungi *E. jeanselmei* and *K. perforans* show a different response to desiccation and re-hydration than the extremophilic fungus *C. antarcticus*. During desiccation proteins of *E. jeanselmei* and *K. perforans* were changed and some additional proteins were detected. *C. antarcticus* did not show a significant change in the protein pattern but a dramatic decrease in the number of spots. Additionally, *C. antarcticus* showed slower reaction to re-hydration than *K. perforans*. It is interesting, because being from Antarctica, *C. antarcticus* most probably reacts to yearly cycles of anhydrobiosis and its activity is depending on the Antarctic seasons. Meanwhile *K. perforans* lives in the moderate and semi-dry environment with short cycles of dryness and humidity due to rain events and dew fall thus necessitating a faster response to re-hydration.

It is important to mention, that the cells, after drying for 2 weeks – an additional experiment was carried out – and being inoculated onto MEA, showed the ability to grow.

Trehalose and mannitol are both known to accumulate upon exposure to various types of stress, and are the most abundant compatible solutes in conidia of *Aspergillus* (Sterflinger *et al*., 1998; Ruijter *et al*., 2003). Sugar analysis shows that trehalose and mannitol are also the main solutes in the three fungal species of this study. Obtained results suggest that accumulation of these compounds is a normal aspect of vegetative growth of these fungi.

In our second work, which is described in part **5.2**, we focused on studying the mechanisms of three MCF to survive Mars-like environments. The results of the experiments showed that the number of expressed proteins has changed during the experiment in all fungi tested. *Exophiala jeanselmei* did not express any changes of protein patterns, almost all the spots were matched among the gels of different conditions, thus indicating there was no expression of new proteins after 24 hours (day simulation) or 4 or 7 days of exposure. After 7 days the pattern and number of proteins resembles the cellular state at the starting point (the biomass from normal conditions) of the experiment, which indicated that no novel or stress-related proteins were expressed.

Also in *K. perforans* the number of proteins decreased after 24 hours before increasing during the following days of the experiment. The induction of new proteins was not detected in this fungus; however, many proteins were down-regulated and could not be visualized by silver-staining.

In *C. antarcticus* after incubation for 24 hours at day and night simulation the number of protein spots dropped dramatically. There were no new protein spots detected but over-expression of several proteins was observed.

After re-inoculation and incubation for 2 weeks at room temperature the increase of biomass of all the tested organisms was observed. The biomass growth of all the tested strains manifested reproduction ability and survival for all fungal species after Mars-simulated treatments.

The results of this study are the first approach to obtain a better understanding of survival mechanisms of MCF under Mars-like conditions. Although the experiments did not allow us to extract enough protein amounts for the identification of a single protein spot (due to technical reasons allowing only very low amounts of the biomass

to be placed inside the Mars simulation chamber), the results give some astonishing insights into ecology of MCF.

The results achieved from our study led to the conclusion that black microcolonial fungi can survive in the Mars environment.

In order to visualize and investigate the proteome of *C. antarcticus*, 2DE was performed using an optimized protein extraction protocol for MCFs as described in details in part **5.3**. We achieved our aim to separate the total protein repertoire of *C. antarcticus*, which was grown under laboratory conditions as a major basis to study the adaptations of the fungus to the extreme environment of Antarctica. Taking into consideration all possibilities and limitations, our results allowed a first insight into the protein expression pattern in *C. antarcticus.*

The 2DE delivered 200 well defined protein spots by the orthogonal properties of isoelectric point and molecular weight. All protein spots were analyzed by MS-MALDI-TOF/TOF, however, only 11 proteins could be identified based on sequence homology with other species.

Although missing an annotated genome of *C. antarcticus* as a reference, some protein spots could be identified by de novo sequencing with high reliability (Mascot score 84- 531%). The identified proteins are divided into three main categories: (1) heat shock proteins and (2) functional enzymes and (3) other proteins. Detaled description on each group is present in part **5.3**.

The identified proteins belong mostly to highly conserved protein families, which are encoding key proteins guaranteeing the maintenance of the central metabolism for growth and regeneration after state of dormancy. However, the rest of the protein spots remained unidentified, since they show no similarities to the available data. This suggests that the proteome of *C. antarcticus* must comprise a high number of novel proteins with unknown functions.

5 Publications

5.1 Microcolonial fungi on rocks: a life in constant drought?

Kristina Zakharova, Donatella Tesei, Gorji Marzban, Jan Dijksterhuis, Timon Wyatt, Katja Sterflinger.

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Microcolonial Fungi on Rocks: A Life in Constant Drought?

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Abstract Black microcolonial fungi (MCF) and black yeasts are among the most stress-resistant eukaryotic organisms known on Earth. They mainly inhabit bare rock surfaces in hot and cold deserts of all regions of the Earth, but some of them have a close phylogenetic relation to human pathogenic black fungi which makes them important model organisms also with respect to clinical mycology. The environment of those fungi is especially characterized by extreme changes from humidity to long periods of desiccation and extreme temperature differences. A key to the understanding of MCF ecology is the question about metabolic activity versus dormancy in the natural environments. In this study, the time lag from the desiccated state to rehydration and full metabolic activity and growth was measured and defined in accordance with simulated environmental conditions. The ability to survive after desiccation and the speed of rehydration as well as changes of the whole cell protein pattern are demonstrated. Whereas both mesophilic strains—*Exophiala jeanselmei* and *Knufia perforans* (=*Coniosporium perforans*)—show a clear reaction toward desiccation by production of small proteins, *Cryomyces antarcticus*—the extremotolerant MCF—does not show any response to desiccation but seems just to down-regulate its metabolism. Data on intracellular sugar suggest that both trehalose and mannitol might play a cell protective role in those fungi.

Keywords Black yeast · Anhydrobiosis · Two-dimensional gel electrophoresis · Protein profiling · Trehalose

Introduction

Black microcolonial fungi (MCF) and black yeasts together with lichens and cyanobacteria are among the most stress-tolerant organisms on the Earth [6]. They are found in the hot deserts of Arizona (USA) [24, 27], the cold Antarctic deserts [20, 22], as well as in Mediterranean countries, for example, Italy and Greece [10, 30]. Many of those rock inhabiting fungi—especially species of the genera *Exophiala* and *Knufia* (=*Coniosporium*)—cluster within the order *Chaetothyriales* and *Hysteriales* and thus have a close phylogenetic relation to human and animal pathogens like *Cladophialophora* spp., *Exophiala dermatitidis* or

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Knufia epidermidis (=*Coniosporium epidermidis*) [2, 34].

MCF form black, clump-like colonies consisting of isodiametrically dividing cells on rock surfaces, in cracks, pores and fissures of the rock and in micropits, created by their own deteriorative activity. Their morphology is interpreted as response to multiple stress factors: keeping the surface-volume ratio optimal decreases the loss of water and minimizes the colony surface with direct exposure to sun light and other physical and chemical stressors [28, 29, 31]. Recent experiments showed that the stress resistance of MCF against solar radiation, radioactivity, desiccation and oligotrophic conditions even allows them to survive space and Martian conditions. Therefore, black fungi are promising organisms for investigating the life in outer space and for gamma radiation experiments [23]. Humidity, temperature and solar radiation, deposition of organic/inorganic and nutrients on rock surfaces influence the settlement, growth and development of microorganisms. The stress resistance in those fungi might have played a significant role for the evolution of virulence factors in human pathogen species. Although other habitats may offer more extreme conditions of temperature, pH and salinity, they are rarely subjected to such rapid and extreme instability in physical and chemical conditions. It is known that on the rock surfaces, where microbial interactions occur, there are constant changes in atmospheric conditions. In this sense, as assumed by Gorbushina [11], ubiquitous subaerial biofilms are bioindicators that are continually subjected to climate change. Any changes in the composition of the atmosphere and climate are sensed by life on the rocks, which in turn affects atmospheric composition through its metabolic activity and biologically induced weathering.

The changing environmental conditions force rock inhabitants into periods of stress-induced dormancy, which are suddenly interrupted by the occasional return of growth-favorable conditions. Only organisms which have a very broad range of tolerance to multiply and fluctuating stress can survive under these harsh conditions. The term poikilo-tolerant (resistant to variable stress; from poikilos: variegated) has been used to describe the behavior of living organisms in environments where tolerance to multiply and variable parameters is essential for survival [11].

The real challenge to survive in the desert environments is desiccation, and neither bacteria nor archaea are specialists of survival under conditions of matrix stress. One of the driest and Mars-like environments on Earth—the area around the Yungay station of the hyperarid Atacama Desert (Chile)—is nearly free of any cultivable bacteria. Nevertheless, several hyphomycetes survive in this region in a re-cultivable state by the formation thick-walled spores [5]. That is why fungi are really survival specialists of complete desiccation by producing spores and also prosper—even though slowly—at extremely low levels of water availability [32].

Organisms, which are able to tolerate essentially complete dehydration, are known to be in anhydrobiosis; black rock inhabiting fungi can therefore also supposed to be anhydrobiotes. It is reported that anhydrobiotic organisms—during the process of desiccation—enter a state of metabolic arrest which is reversible on rehydration [13, 19].

As it is supposed by Sterflinger et al. [32], in the polar environment, it is well possible that the fungi are in dormant state most of the year and that they are only active during the short summer period when temperature rises and melting water is available. On the contrary, hot desert fungi might profit from dewfall that develops in deserts during a short time before sunrise [21]. This process would necessitate a very fast rehydration and up-regulation of the metabolic activity. For this reason, the aims of the study was: (1) to investigate how fast MCF can react to changes of humidity, (2) to examine under which conditions these fungi are active in their natural environments and (3) to analyze how they respond to dehydration and rehydration on the proteome and RNA level.

To answer these questions, selected strains of MCF were subjected to desiccation and rehydration in climate chambers and subsequently the protein pattern was analyzed by 2D gel electrophoresis. Protein expression profiles characterize states of dormancy, activity and growth related to different ecological conditions [14].

Materials and Methods

Model Organisms

The model organisms used for this study are as follows: (1) *Exophiala jeanselmei* MA 2853, a rock inhabiting black yeast closely related to opportunistic pathogens in

humans; (2) *Knufia perforans* MA 1299, a mesophilic but highly stress-tolerant fungus found in hot and dry environments, like the Mediterranean, formerly named *Coniosporium perforans* [34]. Both, *E. jeanselmei* and *K. perforans,* can therefore also be addressed as extremotolerants, (3) *Cryomyces antarcticus* MA 5682, an extremophilic fungus from Antarctica. The strains were obtained from the ACBR culture collection (Austrian Center of Biological Resources and Applied Mycology, www.acbr-database.at).

Rehydration Experiments

In nature, the fungal biomass is presented in such limited amount that it is impossible to perform any analysis. This study is a first attempt to find out the survival mechanisms of these fungi; therefore, the model system which reflects normal conditions was created.

All experiments were done in triplicate (2 biological and 3 technical replicates), and the average was taken. Inoculi were prepared as cell suspensions and drop-inoculated onto a sterilized cellophane membrane (Model 583 gel dryer Backing. Catalog# 1650963, Bio-Rad), which were placed on the surface of 2 % malt-extract agar (MEA, Applichem GmbH, Darmstadt, Germany). *E. jeanselmei* and *K. perforans* were grown at 20 °C and *C. antarcticus* at 15 °C for 30 days. Fresh biomass was harvested by scratching the material from the plates using a scalpel, then transferred to a sterile tube, and immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

In order to analyze the response to desiccation, the cellophane membranes with well grown fungal colonies were detached from the MEA and transferred into empty Petri dishes, which were subjected to dehydration in a climate chamber containing silica gel and dried to their constant weight which was reached after 6 days (this was tested in pre-experiments, see supplementary). The water loss, estimated by weight—measured using scales (Non-automatic weighing instrument ME235S-OCE, Sartorius mechatronics)—was about 90 % of fresh biomass, as a consequence of the severe water loss involved; this treatment was defined as desiccation.

Part of the dried biomass was immediately frozen in liquid nitrogen for the further analysis. Other cellophane membranes were replaced onto fresh MEA and immediately incubated in a climate chamber at 98 % rH. During the process of rehydration, samples were taken at 3 time points: after 3 min, 10 min and 1 h. After each sampling, the biomass was immediately frozen in liquid nitrogen for further experiments: (1) RNA extraction and quantification and (2) 2-D gel electrophoresis.

RNA Extraction

Total RNA was extracted from frozen samples (150-200 mg) according to manufacturer's guidelines (TRIzol reagent, Invitrogen). The RNA pellet was air-dried and then dissolved in distilled water (DNase/RNase Free; Invitrogen) during incubation for 10 min at 69 °C.

RNA quality and quantity was analyzed using a Nano Drop 1000 Spectrophotometer according to the manufacturer's instruction.

Protein Extraction and 2D Gel Protein Profiling

Extraction of whole cell protein and 2-D gel electrophoresis was carried out according to a protocol which was specially adapted for black fungi by Isola et al. [14]. The Bradford protein Assay [4] was performed to determine the concentration of proteins in fungal extracts. Reactions were carried out in microtiter plates according to the manufacturer's instructions. A standard curve was established using serial dilutions from 0.8 to 100 μg ml^{-1} of bovine serum albumin (BSA). The resulting optical density (OD) at 595 nm was analyzed with a plate reader (Magellan; Tecan Austria, Grödig, Austria). All experiments were carried out in triplicate. For absolute amounts of RNA and proteins in fresh—non-desiccated—samples, the relative amounts of protein and RNA were calculated in relation to the dried biomass of the samples. The weight loss was quantified according to the weight change during desiccation. In the same way, the relative amounts in fresh biomass were calculated from absolute values in desiccated biomass. For each gel, 20 μg of protein was applied. IEF separation was performed using 13-cm strips pH 3-10NL. 2D gels were made in triplicate for each condition (fresh, dried, after 3 min and 1 h of rehydration), the 3 gels were matched by warping (Image Master 2D Platinum version 5.0, Amersham Biosciences, Swiss Institute of Bioinformatics,

Geneva, Switzerland) and the sum of all spots which were present at least in two gels was taken into account for the protein pattern analysis.

Isolation of Sugars and HPLC Analysis

The dry and fresh mycelium samples (50–450 mg) were frozen in liquid nitrogen and placed in a stainless steel grinding jar (Qiagen, Venlo, The Netherlands. Catalog# 69985) and pulverized with the Qiagen Tissuelyser® (2 min at 30 strokes/s). Before a second pulverization step (2 min at 30 strokes/s), 1–2 ml MQ was added. After the grinding, the sample was transferred into eppendorf tube and centrifuged (10,000× *g* at 4 °C, for 30 min). The supernatant was collected, heated for 30 min at 95 °C and centrifuged another 30 min at 10,000×*g*. Again, the supernatant was collected, filtered (Pall Life Science, Acrodisc Cr13 mm Syringe filter) and stored at −20 °C until used for sugar analysis by HPLC.

Detection and quantitative analysis of saccharides and polyols was carried out by HPLC [36] equipped with an IR detector (Waters, 2414 refractive index detector), a Ca+ cation-exchange column (Waters, SugarPak I column) and a mobile phase of MQ with 0,1 mM Ca EDTA (Sigma. Catalog# 340073). The column temperature was kept at 50 °C with a column heater (Waters), and a flow of 0.5 ml min^{-1} was maintained during separation (Waters, 515 HPLC pump). The autosampler (Waters, 717 plus autosampler) injected 10 µl per sample, and peak integration and calculation was performed with Empower software delivered. As standards trehalose, mannitol, glucose, glycerol, erythritol and arabitol were used.

Results

After a desiccation period of 6 days, the maximum water loss was reached in the three fungal strains tested: water loss was 90 % for *E. jeanselmei*, 70 % for *K. perforans* and 70 % for *C. antarcticus*. After rehydration, *E. jeanselmei* re-gained 14.8 %; *K. perforans,* 36.4 %; and *C. antarcticus,* 33.9 % of the weight within 1 h. However, after 1 day of rehydration, *E. jeanselmei* gained 16.6 %; *K. perforans,* 37.3 %; and *C. antarcticus,* 35.8 % of the original biomass weight and stopped at this level (Fig. 1). An additional experiment for 14 days, when dried biomass was replaced into empty Petri dishes and exposed to rehydration at 98 % rH, was carried out. The results showed that the fungi did not gain their original fresh weight again; however, all three strains were able to grow again, after transferring to fresh medium, and thus proved to be alive and active after desiccation and rehydration.

In *E. jeanselmei* and *K. perforans,* the amount of proteins in relation to the whole fungal biomass significantly increased upon drying due to the water loss within a period of 6 days (Fig. 2). The protein content increased from 4,000 to 16,800 µg g^{-1} for *E. jeanselmei* and from 4,100 to 13,200 µg g^{-1} for *K. perforans*. However, in *C. antarcticus,* the protein

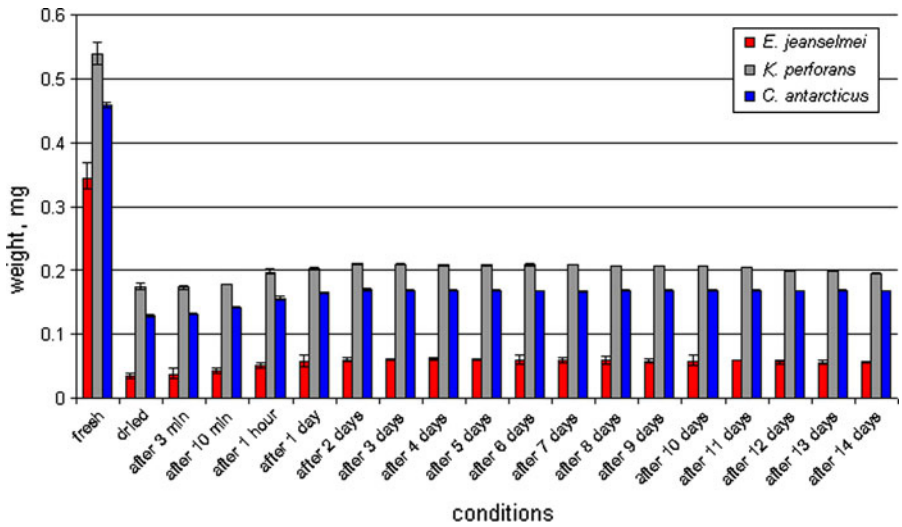

Fig. 1 Rehydration of MCF and *black* yeast. Fresh biomass was subjected to the desiccation until the constant weight was reached (on 6th day), and then dried biomass was rehydrated for 14 days. The weight was estimated for all the samples at different conditions: starting from 3 min of rehydration till the 14th day of rehydration period

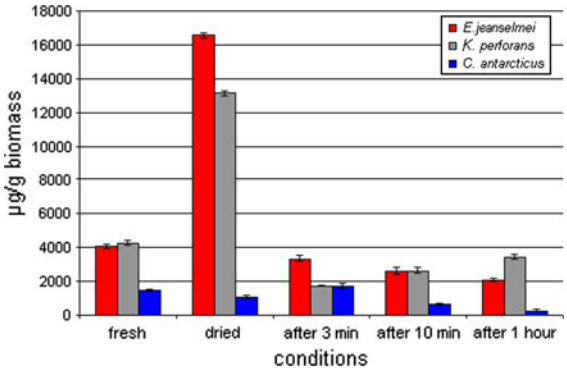

Fig. 2 Absolute protein concentration of MCF and *black* yeast. Protein amount of the biomass was measured before and after desiccation, and also at different conditions: after 3 min, 10 min and 1 h of rehydration

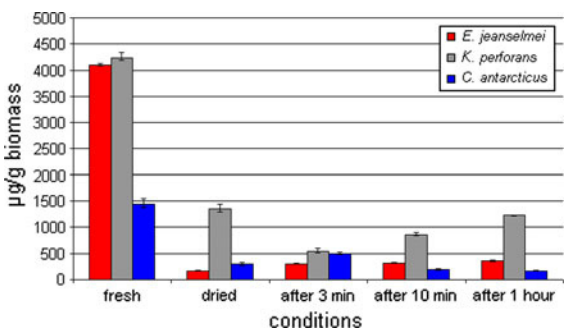

Fig. 3 Relative protein concentration of MCF and *black* yeast. Protein content was re-calculated to the hydrated biomass according to the water loss of the samples

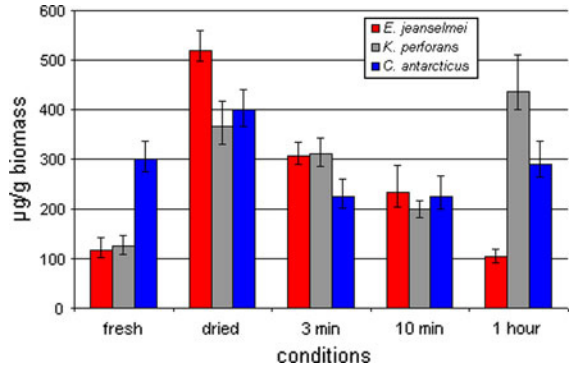

Fig. 4 Absolute RNA concentration of MCF and *black* yeast. RNA concentration of the biomass was measured before and after desiccation and also at different conditions: after 3 min, 10 min and 1 h of rehydration

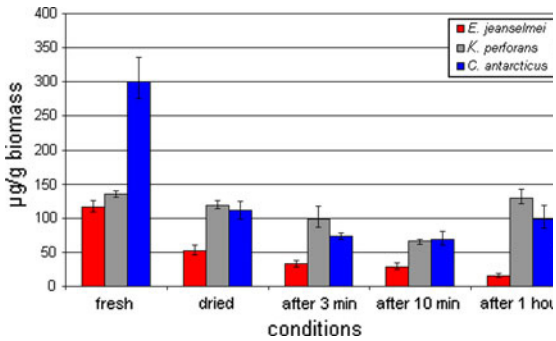

Fig. 5 Relative RNA concentration of MCF and black yeast. RNA concentration was re-calculated to the hydrated biomass according to the water loss of the samples

content decreases even in relation to the dry biomass from 1,700 to 1,500 µg g^{-1}. The protein content of the fully hydrated cells was low: 4,000 µg g^{-1} for *E. jeanselmei*, 4,100 µg g^{-1} for *K. perforans* and 1,700 µg g^{-1} for *C. antarcticus* (Figs. 2, 3) and when protein values were calculated back to the fully hydrated state, it can be seen that the values did not show a significant increase during the rehydration period.

The absolute amount of RNA (Fig. 4) was lower in fully hydrated colonies—when grown on MEA—than in the fully desiccated biomass and also in the rehydrated biomass. In contrast, the relative amount of RNA (Fig. 5)—recalculated according to the loss of water—shows that the cell activity decreased during desiccation. In *E. jeanselmei,* the RNA concentration was decreasing within all measurements. In colonies grown on MEA, it was 117 µg g^{-1}, after drying the concentration decreased to 52 µg g^{-1} and after 1 h of rehydration 16 µg g^{-1}. However, the cells, after drying for 2 weeks—this additional experiment was mentioned before—and being inoculated onto MEA, showed the ability to grow. In contrast, in *K. perforans,* RNA concentration decreased from 125 µg g^{-1} in fresh biomass to 119 µg g^{-1} after drying (absolute and related to fresh biomass). The minimal concentration 66 µg g^{-1} was reached after 10 min of rehydration. Then, concentration increased and reached 159 µg g^{-1}, thus indicating up-regulation of the metabolism after 10 min of rehydration. In *C. antarcticus,* the results demonstrated that RNA concentration decreased that 300 to 111 µg g^{-1} after desiccation, and the minimal concentration was 69 µg g^{-1} after 10 min of rehydration. After 1 h of rehydration, the concentration was increasing thus indicating up-regulation of the metabolism after 1 h of rehydration. The re-activation of the metabolism after 10 min in *K. perforans* and 1 h,

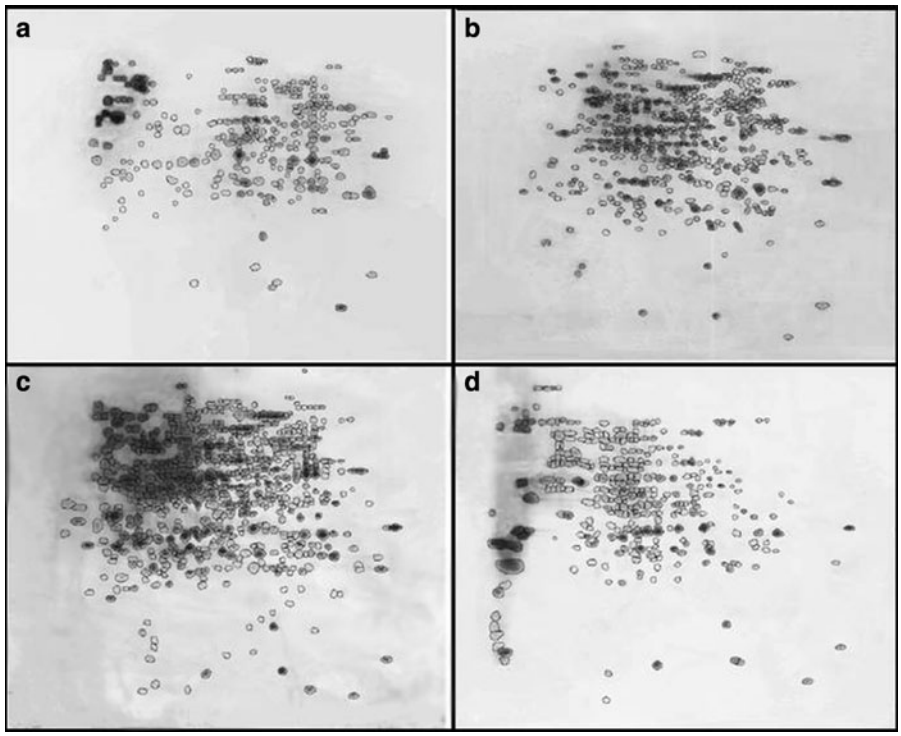

Fig. 6 Comparison of 2D gel protein expression profiles of *K. perforans*: **a** fresh biomass, **b** after desiccation for 6 days, **c** after rehydration for 3 min and **d** after rehydration for 1 h. For each gel, 20 µg of protein was applied. IEF separation was performed using 13-cm strips pH 3-10NL

respectively, in *C. antarcticus* is also indicated by the whole cell protein content and the changes of the protein patterns as follows:

In all fungi tested, the protein profile clearly changed during the process of desiccation and again during the process of rehydration both in the number of protein spots and in the protein pattern. During desiccation, *K. perforans* shows a significant increase in protein spots from 275 to 474. Especially, the number of proteins in the higher pH range and with lower molecular weight is increased (Fig. 6). While 262 spots were matching in the fresh biomass compared with the dried biomass, 212 new spots were induced. In addition to small proteins, also some spots with higher molecular weight were detected as reaction to dehydration. After 3 min of rehydration, an increase from 474 to 589 spots was detected, indicating that the cell already after this short time period reacts to the availability of water. After 1 h of rehydration, the amount of spots decreased to 323 spots. Since especially those spots that were expressed after desiccation disappeared again after 1 h of rehydration, it can be concluded that the status of the cells normalizes during this period.

In *E. jeanselmei,* there is a smaller increase in protein spots during desiccation as compared to *K. perforans*. Among the 280 spots shown after desiccation, 156 are pair with the 233 spots expressed in the fresh biomass (Fig. 7). However, also in this fungus, the protein pattern suggests that there is a strong response toward water loss since the pattern in the desiccated fungus differs in 122 spots from the fully hydrated colonies when grown on MEA. After 3 min in 98 % rH, a high number of proteins was detected, but after 1 h, the protein number is decreasing (showing 213 spots) and the protein pattern goes back to the level of the fresh biomass from MEA medium.

In contrast to *K. perforans* and *E. jeanselmei, C. antarcticus* reacts to desiccation by reducing the number of protein spots from 323 in fresh biomass to 52 in the dried cells (Fig. 8). In the dried state, 34 big spots were exhibited.

Sugar analysis shows that trehalose and mannitol are the main solutes in the three fungal species of this study. Accumulation of the solutes is already prominent in wet samples and is increased in dried samples (Table 1; Fig. 9). The increase in the amount of sugars was most

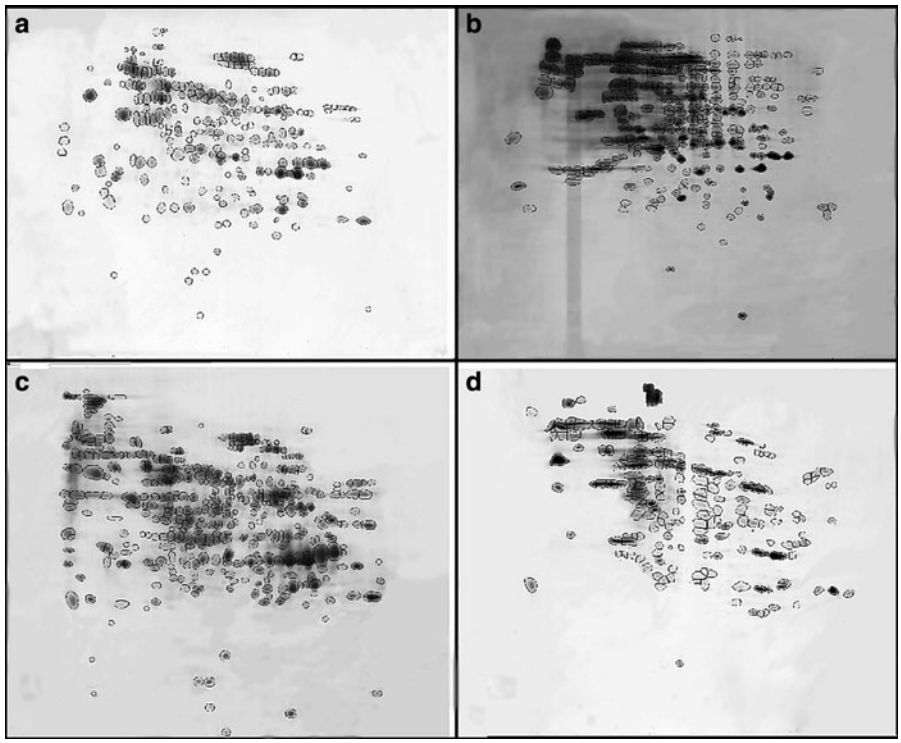

Fig. 7 Comparison of 2D gel protein expression profiles of *E. jeanselmei*: **a** fresh biomass, **b** after desiccation for 6 days, **c** after rehydration for 3 min and **d** after rehydration for 1 h. For each gel, 20 µg of protein was applied. IEF separation was performed using 13-cm strips pH 3-10NL

extensive with *E. jeanselmei*, which is corresponding with the amount of weight loss after drying of this fungus.

Discussion

Until now, the only factors that are known to be involved in stress adaptation of MCF are the strong melanisation of the multilayered cell walls, the microcolonial morphology, as well as the production of trehalose which helps to stabilize enzymes during periods of desiccation and the production of glycerol that is induced by osmotic stress [12, 29, 38]. The ability to survive in a desiccated state was shown to be an important feature to withstand periodical high temperatures that might occur on the rock in deserts and in the Mediterranean climate [29]. However, protein patterns and RNA levels as influenced by desiccation and rehydration were analyzed in this study for the first time.

The results of this study clearly show that the extremotolerant fungi *E. jeanselmei* and *K. perforans* show a different response to desiccation and rehydration than the extremophilic fungus *C. antarcticus*. The fact that both MCF fungi (*K. perforans* and *C. antarcticus*) lost less weight than *E. jeanselmei* can be explained by the bigger portion of dry biomass in MCF which is mainly due to their multi-layered cell walls but also by the EPS layers of *E. jeanselmei,* which normally retains high amounts of water [29]. Thus, the absolute amount of water in MCF is lower than in the thin-walled cells and in the EPS of *E. jeanselmei*. It can also be concluded that the fully hydrated state may never be reached in nature—growth on laboratory medium and with levels higher than 90 % rH is artificial; those levels of nutrient availability and humidity are never reached in the natural environment of MCF.

The protein profiles and the RNA quantification shown in this study support the hypothesis that MCF and black yeasts are able to be active with low levels of intracellular water and are able to react to rehydration

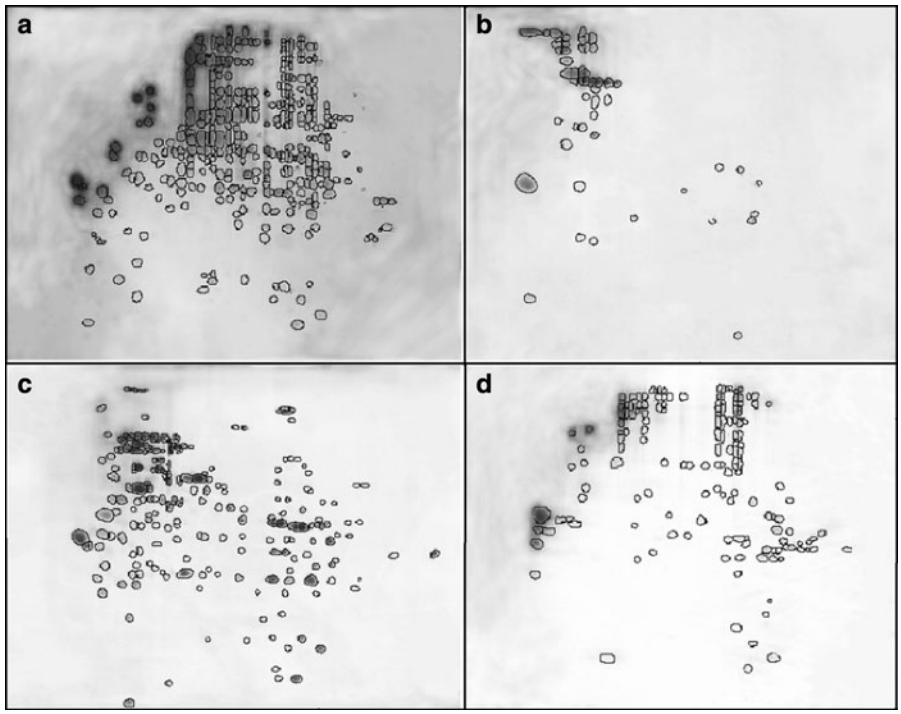

Fig. 8 Comparison of 2D gel protein expression profiles of *C. antarcticus*: **a** fresh biomass, **b** after desiccation for 6 days, **c** after rehydration for 3 min and **d** after rehydration for 1 h rehydration for 1 h. For each gel, 20 μg of protein was applied. IEF separation was performed using 13-cm strips pH 3-10NL

Table 1 Sugars (% w/w) present in fresh (fully hydrated) biomass and dried biomass after desiccation for 6 days

Species	Trehalos	Glucose	Mannitol	Total sugars
E. jeanselmei fresh	0.76	0.07	0.26	1.10
E. jeanselmei dried	3.85	0.00	2.59	6.44
K. perforans fresh	2.71	1.14	1.38	5.23
K. perforans dried	4.77	2.57	3.69	11.03
C. antarcticus fresh	1.28	0.62	1.24	3.13
C. antarcticus dried	3.73	0.41	1.68	5.83

after periods of desiccation in a short time. The protein content of the wet cell is very low, which indicates massive amounts of other materials in the cell, as cell walls or solutes, which was shown by Sterflinger [28]. Trehalose and mannitol are both known to accumulate upon exposure to various types of stress and are the most abundant compatible solutes in conidia of Aspergillus [7, 8, 25, 29, 37]. Conidia are survival structures [17] and have medium resistance to heat, drought and other stressors [37]. The accumulation of these compounds is of the same order of magnitude as those of the conidia [18]. These data suggest that accumulation of these compounds is a normal aspect of vegetative growth of these fungi. The data of this study also show that the basic level of sugar in the fully hydrated cells is already very high, thus necessitating no induction of sugar production when desiccation is starting. The even higher amount of sugars inside of the dried biomass is rather due to the weight loss during desiccation than to a real increase in the sugars in sense of additional sugar production.

The protein patterns of *E. jeanselmei* and *K. perforans* indicate a modification of existing proteins and an expression of additional protective proteins during the process of desiccation. There is an obvious increase in large proteins suggesting the formation of clusters from protein plus chaperon. Again, the increase in the spot patterns after 3 min of rehydration in *K. perforans* can probably be

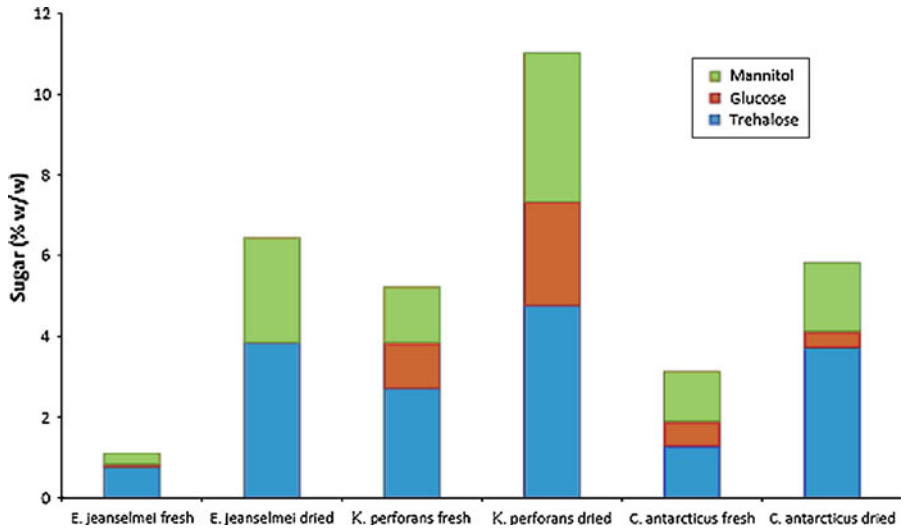

Fig. 9 Sugars (% w/w) present in fresh (fully hydrated) biomass and dried biomass (after desiccation for 6 days). The amount of mannitol, glucose and trehalose estimated for each sample

interpreted as a release of the protective chaperons from the clustered proteins.

In contrast to *E. jeanselmei and K. perforans*, *C. antarcticus* did not show a significant change in the protein pattern but a dramatic decrease in the number of spots. This indicates that *C. antarcticus* rather down-regulates the metabolism than inducing a number of protective proteins. This would also mean that the proteins necessary for the survival of the colony are either desiccation resistant without any additional protective proteins or that other cellular components—for example, sugars or fatty acids—are involved in the protein protection. However, this fungus seems to have a slower reaction to rehydration than *K. perforans*. This can possibly be explained by the fact that *C. antarcticus* reacts to yearly cycles of anhydrobiosis and its activity is depending on the Antarctic seasons while *K. perforans* lives in the moderate and semi-dry environment with short cycles of dryness and humidity due to rain events and dew fall thus necessitating a faster response to rehydration. Also from the phylogenetic point of view, *Cryomyces* is highly distinct from the genera *Knufia* and *Exophiala*. While *Knufia* and *Exophiala* cluster within the order *Chaetothyriales* [34], *Cryomyces* forms a distinct clade without any obvious direct ancestor [26].

In general, desiccation together with anhydrobiosis—defined as a complete loss of "free" water from an organism—is an extreme stress [35]. Desiccation tolerance is characterized by the physical and chemical adjustments in order to withstand the dehydration and resume the biological activity after rehydration [15]. This process is a result of a complex cascade of molecular events, which can be divided into signal precipitation, signal transduction, gene activation and biochemical changes leading to the acquisition of desiccation tolerance. It is proposed that proteins related to desiccation tolerance and involved in the metabolic changes, protection against oxidation and other putative protective molecules show a particular abundance during the desiccation. Small heat-shock proteins (sHSPs), also called molecular chaperons, cluster with enzymes and stabilize their conformation [1, 16]. Late embryogenesis abundant (LEA) proteins are among the molecules with increased abundance during drying of plants [3, 15]. Another important process that has been described in moss—*Tortula ruralis*—is the packing of mRNA transcripts into mRNPs (messenger ribonucleic protein complexes) with polysomes that allows a rapid availability of these transcripts upon rehydration [9]. The results of this study give evidence that also the MCF and black yeast analyzed here have some general response to desiccation: The formation of small sized proteins in

K. perforans and *E. jeanselmei* can possibly be interpreted as production of HSPs; bigger protein spots occurring after rehydration possibly are proteins the conformation of which is stabilized by molecular chaperons. However, there are two novel and

important findings that will have to be studied in more detail in order to understand the ecology and systems biology of the MCF:

(1) In *C. antarcticus,* there is an enormous loss of protein spots during desiccation indicating a down-regulation of the whole metabolism. The production of small proteins as reaction toward stress was not observed. Although the process of down-regulation—compared to production of HSPs—is less energy consuming and might reflect the oligotrophic environment of this fungus, it necessitates that the basic set of proteins is either highly specialized and thus not affected by desiccation or protected by other cell components which are not yet understood. Interestingly, Tesei et al. [33] demonstrated that *C. antarcticus* also does not produce any HSPs as a response to temperature stress. In order to understand the resistance of those proteins, identification by mass spectrometry will be carried out in the next study.
(2) When rehydrated in 98 % rH, none of the fungi tested was able to gain the fully hydrated state as on MEA. However, the protein pattern suggests a normalization of the metabolism after approximately 1 hour. For this reason, it will be necessary to carry out studies on the content of cellular water and water retaining abilities of these fungi using either in situ colonies from fresh environmental samples or—since samples are often difficult to obtain and the available biomass is extremely low—further experiments should be carried out with dehydrated laboratory grown cultures.

From here, we can conclude that *E. jeanselmei* and *K. perforans*—both being extremotolerant but mesophilic—show a clear response to desiccation. There is strong evidence that they react by the production of proteins which can be interpreted as small HSPs and chaperons as well as large protein clusters formed by protein plus chaperon. In *C. antarcticus*—the extremophilic fungus from Antarctica—very few large spots—indicating cluster formation—were detected, and there was no indication of small HSPs production. The most prominent reaction to dehydration, namely the loss of protein spots, indicates a down-regulation of the metabolism and related proteins. All fungi show a remarkable fast response to water availability. Moreover, the protein patterns indicate that the fungi are able to gain a full metabolic activity in a state of low cellular water content. The results of this study give a first impression of cellular reactions toward anhydrobiosis in black fungi. Of course, the nature of the proteins will have to be identified in further studies based on mass spectrometry.

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5.2 Protein patterns of black fungi under simulated Mars-like conditions

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Protein patterns of black fungi under simulated Mars-like conditions

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Two species of microcolonial fungi – *Cryomyces antarcticus* and *Knufia perforans* - and a species of black yeasts–*Exophiala jeanselmei* - were exposed to thermo-physical Mars-like conditions in the simulation chamber of the German Aerospace Center. In this study the alterations at the protein expression level from various fungi species under Mars-like conditions were analyzed for the first time using 2D gel electrophoresis. Despite of the expectations, the fungi did not express any additional proteins under Mars simulation that could be interpreted as stress induced HSPs. However, up-regulation of some proteins and significant decreasing of protein number were detected within the first 24 hours of the treatment. After 4 and 7 days of the experiment protein spot number was increased again and the protein patterns resemble the protein patterns of biomass from normal conditions. It indicates the recovery of the metabolic activity under Martian environmental conditions after one week of exposure.

Astrobiology is in the focus of many scientific investigations, trying to answer questions about the possible existence of life on other planets, survival in outer space and possible interplanetary transfers. Organisms which inhabit extreme environments – the so-called extremophiles - are generally considered to be the best model for exobiological studies[1]. Extremophiles can not only survive extreme thermo-physical conditions, but can also tolerate extreme saline, acidic, alkaline or other conditions which can induce DNA damage in less resistant organisms[2–5].

Earth's biosphere has evolved in more than 3 billion years while protected by the planet's magnetosphere and the atmosphere from the hostile environment of outer space. The conditions of outer space like space vacuum, thermal extremes, cold, solar UV radiation, ionizing radiation and galactic radiation are real challenges for any form of life[6]. Fungi in general and especially melanized ones showed high resistance when were exposed to ionizing radiation[7,8]. Large quantities of highly melanized spores have been found in early Cretaceous period deposits when many species of animals and plants become extinct. This period matches with Earth's crossing the 'magnetic zero event' when the planet lost its protection against cosmic radiation[9].

Martian climate conditions are too cold and the atmosphere too thin for allowing bulk water to be stable. However, at environmental conditions similar to Mars-like conditions the metabolic activity has been detected in presence of one or two monolayers of water, which was proved by laboratory experiments[10]. A precondition for habitability on Mars would be the water availability and interaction between regolith and near-surface atmosphere[11]. Some investigations were performed on organisms which might have a certain potential to live on Mars[1,4,12,13]. Albeit organisms need water activities of $a_w = 0.8–0.9$ for active metabolism, there are some special life forms which are also able to live in much drier conditions with a temporary saturation of atmospheric water vapor, for instance black microcolonial fungi (MCF) and some lichens. Such temporary saturation can occur on Mars[4,14,15] as it is known by recent studies some extremophilic organisms are able to cope successfully with Mars-like conditions. For instance, it was reported that the vitality and photosynthetic activity of lichens is maintained under Mars-like conditions[4,14].

Melanized fungi and lichens are colonizing extreme environments such as Arctic and Antarctic regions, including high altitude terrains. These specific regions are characterized by a combination of dry, cold, oligothophic extremes along with huge fluxes of UV radiation[16,17]. Therefore microcolonial fungi - and particularly *C. antarcticus* – have been suggested as eukaryotic models for studies on the habitability of Mars[14] and the biological exploration of Mars[1,18]. Both, Mars-simulation experiments and the exposition of the fungus to outer space have shown that *C. antarcticus* is able to survive under Mars and space conditions in a good manner[1,19]. Also the green alga *Stichococcus* sp. and the lichenized fungus *Acarospora* sp. were shown to be able to survive Mars and space conditions[20].

Until now the cellular mechanisms underlying the resistance of MCF against harsh space and Mars conditions are not yet understood. At the moment there is limited information existing on a genomic sequences of MCF due to some obstacles occurring during DNA extraction and purification. Proteomics approach leads to understanding of the expression and regulation of the entire set of proteins which are directly related to biological functions[21]. Therefore two dimensional gel electrophoresis (2D) for protein profiling is carried out in this study in order to get a more precise impression of cellular and metabolic activity of these fungi under simulated Mars-like conditions. Three model organisms were chosen for the experiments, that were carried out in the Mars-simulation chamber in the Mars Simulation Facility (MSF) of the German Aerospace Centre (DLR, Berlin): (1) *Cryomyces antarcticus* represents a group of extremophilic fungi occurring in Antarctic dry deserts; (2) *Knufia perforans* is a mesophilic but highly stress tolerant fungus found in hot and arid environments and (3) *Exophiala jeanselmei* represents a rock inhabiting black yeast closely related to opportunistic pathogens in humans. A realistic simulation of the Martian climate was based on data provided by Mars-related missions[4,14].

Results

The results of the experiments showed (Table 1) that the number of expressed proteins has changed during the experiment in all fungi tested. After exposure to Mars-like conditions for 24 hours (day simulation) *E. jeanselmei* reduced the number of protein spots from 473 to 237 (Fig. 2). Protein spots representing proteins with high molecular weight (<120 kDa) and basic pI-ranges of <7 were reduced in the number. From 24 hours to 4 days the number of proteins increased to 359 spots by expressing large proteins with basic pH. And after incubation for 7 days the number of protein spots reached nearly the initial spot number of 470. Within the protein profiles no significant changes of the patterns were observed; nearly all the spots were matched among the gels of different conditions, thus indicating there was no expression of new proteins after 24 hours (day simulation) or 4/7 days exposure. After 7 days the pattern and number of proteins resembles the cellular state at the starting point (the biomass from normal conditions) of the experiment, which indicated that no novel or stress-related proteins were expressed.

Also in *K. perforans* the number of proteins decreased after 24 hours before increasing during the following days of the experiment: after exposure for 24 hours (day simulation) the number of protein spots decreased significantly from 634 to 264 spots. The reduced proteins were of a large size (with the molecular weight of above 120 kDa and basic pI-ranges of 7 to 9). After 4 days of treatment 286 protein spots were detected and after 7 days the protein number increased to 328. The induction of new proteins was not detected in this fungus; however, many proteins were down-regulated and could not be visualized by silver-staining (Fig. 3). Protein extraction of *E. jeanselmei* and *K. perforans* for samples from night simulation was not successful and therefore the following 2D analysis could not be performed.

In *C. antarcticus* 406 spots were detected in the biomass from normal conditions before the start of the Mars-simulation treatment. After incubation for 24 hours at day and night simulation only 93 and 97 spots respectively were detected. There were no new protein spots appeared on the 2D gel patterns but over-expression of several proteins was observed (Fig. 4). Unfortunately, protein concentration of this fungus after 4 and 7 days of exposure to simulated Mars-like conditions was too low to perform further proteomic analyses.

After re-inoculation and incubation for 2 weeks at room temperature the increase of biomass (what is indicating growth of all the tested organisms) was observed. The biomass growth of all the tested strains manifested reproduction ability and survival for all fungal species after Mars-simulated treatments. The biomass, which used for the experiment was limited by the size of experimental chamber (Fig. 5). As it was mentioned in Materials and Methods section the extraction protocol was adapted to get sufficient protein amounts from those precious biomass samples. The analytical runs by 2DE was accomplished successfully for almost all the samples, however the amounts of the protein rather low to allow subsequent protein identification by proteomic approaches.

Table 1 | Number of protein spots detected in the 2D gels of the analyzed samples at each exposure condition. Opt-optimal laboratory conditions (normal conditions) at which fungi were grown for 30 days at temperature 20°C for *E. jeanselmei* and *K. perforans* and 15°C and for *C. antarcticus*. 24 hours-(D/N) - samples were exposed to Mars-simulated conditions for 24 hours with day/night simulation respectively. 4/7 days- samples were exposed to Mars-simulated conditions for 4 and 7 days respectively. Blank cells (−) indicate that the protein concentration was too low for 2DE analysis

	Number of spots at each exposure condition				
Strain	Opt	24h-D	24h-N	4days	7 days
E. jeanselmei	473	237	-	359	470
K. perforans	624	264	-	286	328
C. antarcticus	406	93	97	-	-

Discussion

The investigations of the microorganisms exposed to outer space and planetary simulated conditions support and enhance our understanding of basic biological mechanisms, such as biological effects caused by the radiation field in space and survival factors in the upper boundary of Earth's biosphere. Moreover the likelihood of interplanetary transport of microorganisms via meteorites, the use of microorganisms in bioregenerative life support systems, the monitoring, characterization and control of spacecraft microflora and associated microbial crew health are under concern[6]. Another aspect of space biological research is the investigation of the responses of microorganisms to simulated Mars-like conditions and their evaluation as potential forward contamination risks in the context of planetary protection[22].

The fungal abilities to withstand harsh outer space conditions and tendency to contaminate spacecrafts are of high importance due to: (1) some of the fungi are potential human pathogens and this could possess certain danger for the well-being of the crew in long-distant space journeys and (2) fungi have strong enzymatic systems and secrete various metabolites which can cause degradation of structural materials[8,23]. In their natural environment black microcolonial fungi from extreme environments are exposed to periods of temporary saturation with water vapor and temporary dryness. Although black microcolonial fungi, some lichens[4,14,19,23,24], bacteria and cyanobacteria[25] were proven to survive Martian and outer space conditions[1,19], it was never investigated on molecular level which cellular processes are responsible for this remarkable resistance.

The results of this study are the first approach to obtain a better understanding of survival mechanisms of MCF under Mars-like conditions. Although the experiments did not allow us to extract enough protein amounts for the identification of single protein spots (due to technical reasons allowing only very low amounts of the biomass to be placed inside the Mars simulation chamber), the results give some astonishing insights into ecology of MCF. The most important results of the study are:

The investigated fungi strains showed metabolic activity in close to anaerobic conditions during exposure to Mars-like conditions with a final exposure time of about 4 days in the first experiment and 7 days

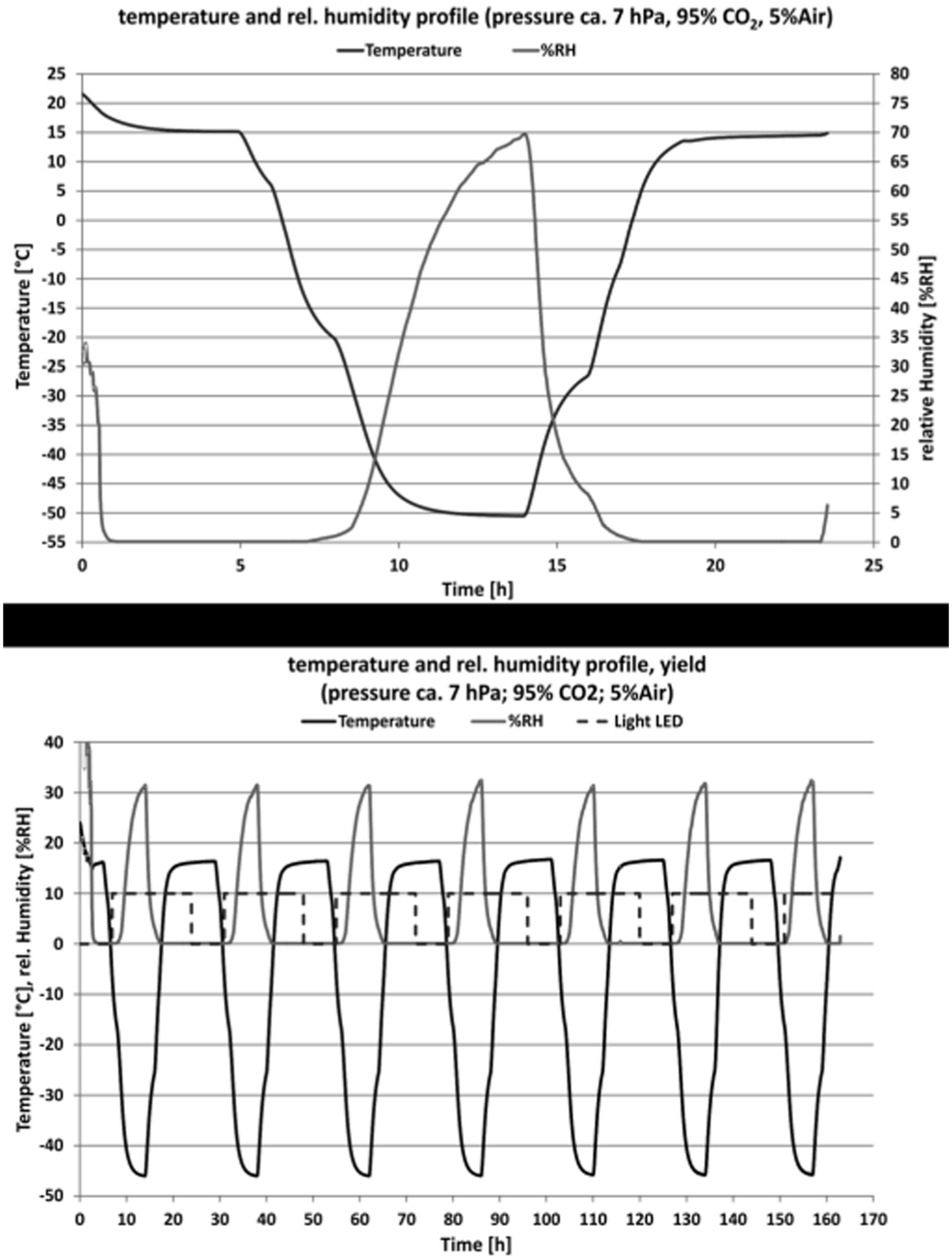

Figure 1 | An example over 24 hours and 7 days of the repetitive 'Mars-like' diurnal profile of temperature (circle curve) and humidity (smooth curve) in the experimental chamber. Temperature and relative humidity were measured above the sample (pressure approximately 7 hPa; 95% CO_2, 5% Air). (A): Results of 24 hours samples exposure to Mars-like conditions (night simulation). (B): Results of 7 day samples exposure to Mars-like conditions (day simulation).

in the second experiment. Although there was a significant decrease of the number of protein spots during the first 24 hours, the fungi seem to recover and gain the initial metabolic state after 7 days of the exposure. These results are in accordance with what was observed earlier by Zakharova and collegues[26], when fungi were desiccated to the constant weight and a decrease of proteins was observed in *C. antarcticus* whereas *K. perforans* and *E. jeanselmei* reacted by expression of new proteins, possibly representing protective proteins. During the following increase of relative humidity fungi did never gain a fully rehydrated state but they gained the original protein pattern after one hour of rehydration. The results of the Mars simulation experiments confirm the hypothesis that the fungi are able to be metabolic active with minimum amount of water and that their proteins have special conformations working with minimum water content[22,26].

The fungi do not show the signs of stress reaction during the experiment. Previous studies have also shown that *C. antarcticus* does not react to temperature stress or by desiccation through production of novel proteins[26,27]. However, for *E. jeanselmei* and for *K. perforans* it was shown that they express proteins which could be protein chaperons or the so-called heat shock proteins[26,27]. In case of the Mars simulation none of the fungi showed such signals of stress-response although they seem to be active when exposed to Mars-simulated conditions.

The results of our study suggest that MCF seem to be not significantly stressed by Mars-like conditions, which are a combination of

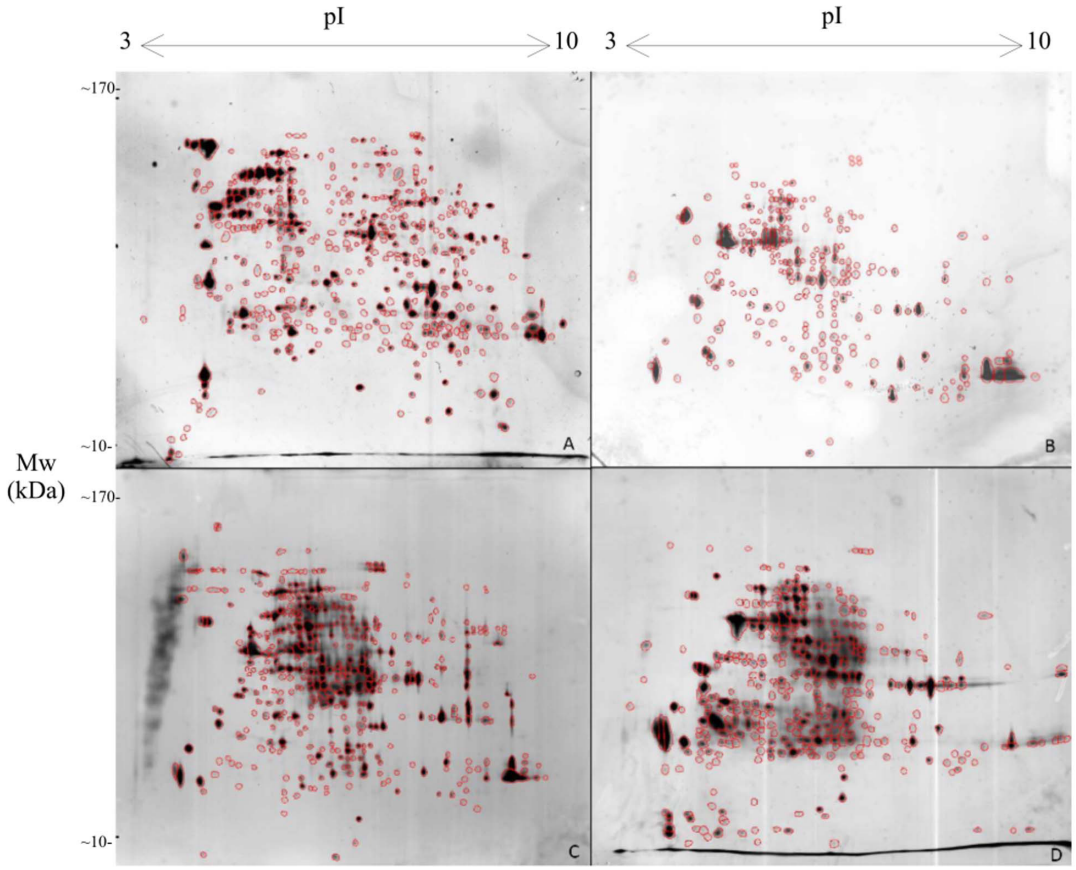

Figure 2 | 2D gel patterns obtained after exposure of *Exophiala jeanselmei*. (A): sample from optimal conditions. (B): sample was exposed to Mars-simulated conditions for 24 hours with day simulation. (C): sample was exposed to Mars-simulated conditions for 4 days with day and night simulation. (D): sample was exposed to Mars-simulated conditions for 7 days with day and night simulation.

temperature extremes, pressure, dryness and radiation. The reason for that could be that the fungi have a basic set of stress resistant proteins[21,26,27] and high doses of radiation enhance their growth capacity instead of damaging their cellular components. It was reported that the environments with high radiation, resulting from human activities, such as damaged reactor at Chernobyl and reactor cooling pool-water[28,29] are habitats for black yeasts and MCF and that "melanizes fungal cells manifested increased growth after exposure to ionizing radiation"[8]. Analyzing melanized fungal species which were found in Chernobyl reactor and in the reactor cooling pool water showed that high fluxes of radiation selected for highly radio-resistant types of microorganisms, showed increased catalase and nuclease activities[28,29].

The results achieved from our study led to the conclusion that black microcolonial fungi can survive in Mars environment.

Further investigations are needed to characterize the present proteins and their functions. In other distantly related fungi strains some proteins are discovered, which might play a key role in stress-resistance to different environmental stresses. For instance the results obtained by Gocheva and collegues[30] demonstrate that the growth at low temperatures does clearly induce oxidative stress events. Antarctic strains (*Penicillium* spp.) demonstrated a marked rise in activities of protective enzymes such as superoxide dismutase and catalase at decreasing temperatures. Low-temperature resistance is partially associated with enhanced scavenging systems[30]. Also it is known that protein carbonyls are biomarkers of protein oxidation followed by temperature stress[31]. The protein AoSO prevents excessive cytoplasmic leakage upon hyphal injury by accumulating at the septal pore, moreover in response to various stresses (low and high temperature; extreme acidic and alkaline pH; nitrogen and carbon deplation[32]). The protein Ipf2431 was discovered being important under several stress-induced proteins in fungus *Cryptoccocus neoformans*[33]. Heterotrimeric Gα protein Pga1 plays a central role in the regulation of the whole growth-developmental program of *Penicillium chrysogenum*. This protein also plays important role in germination (mediating carbon source sensing); absence of Pga1 increases resistance to thermal, oxidative and osmotic stress[34]. According to Deegenaars and collegues, a 110 kDa proteins play a role in stress tolerance in psychrophilic yeast, similar to that of HSP 104 in mesophilic species[35]. A described transcription factor PMsn2 in *Beauveria bassiana* and *Metarhizium robertsii* is important in tolerance to hyperosmolarity, oxidation, carbendazim, cell wall perturbing, high temperature and UV-B radiation[36]. The results of our study, however, suggest that stress-related protein machinery of MCF is more complex compared to other fungi strains and there are a number of highly abundant proteins needed for the survival in Mars-simulated conditions.

We can conclude that an unknown metabolic pathway might be discovered, which enables the fungi to live in a quasi-anaerobic Mars-like environment.

Methods

The biological samples as model organisms. The organisms used for this study were: (1) *Exophiala jeanselmei* MA 2853, a rock inhabiting black yeast closely related to opportunistic pathogens in humans; (2) *Knufia perforans* MA 1299, a mesophilic but highly stress tolerant fungus found in hot and dry environments, like the Mediterranean; (3) *Cryomyces antarcticus* MA 5682, an extremophilic fungus from Antarctica. The strains were obtained from the ACBR culture collection (Austrian Center of Biological Resources and Applied Mycology, www.acbr-database.at).

Inoculi were prepared as cell suspensions and drop-inoculated onto sterilized cellophane membranes (Model 583 gel dryer Backing. Catalog #1650963, Bio-Rad), which were placed on the surface of 2% malt-extract agar (MEA, Applichem GmbH,

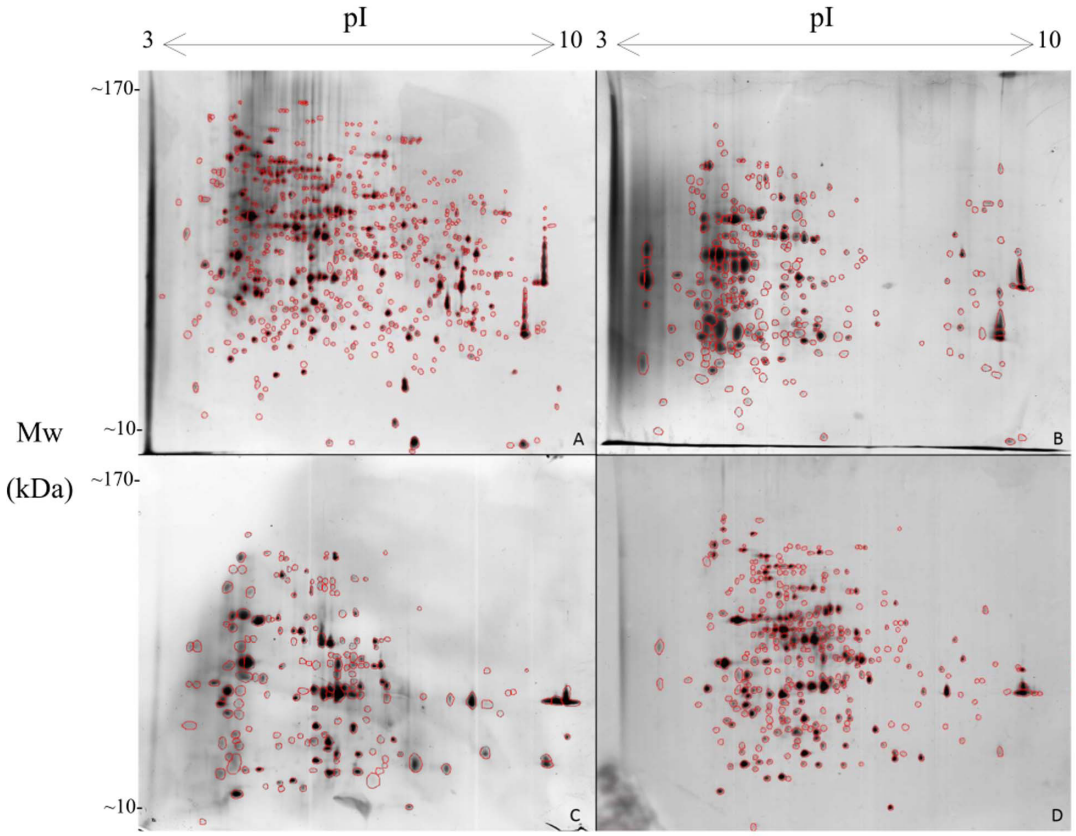

Figure 3 | 2D gel patterns obtained after exposure of *Knufia perforans*: (A): sample from optimal conditions. (B): sample was exposed to Mars-simulated conditions for 24 hours with day simulation. (C): sample was exposed to Mars-simulated conditions for 4 days with day and night simulation. (D): sample was exposed to Mars-simulated conditions for 7 days with day and night simulation.

Darmstadt, Germany). Fungi were grown at normal conditions: 20°C (293 K) for *E. jeanselmei* and *K. perforans* and 15°C (288 K) and for *C. antarcticus* for 30 days. For samples from normal conditions the biomass was harvested by scratching the material from the plates using a scalpel, transferred into a sterile tube, then immediately frozen in liquid nitrogen and stored at −80°C (193 K) for further analysis. Other samples were prepared as follows: round segments approximately three cm diameter from the cellophane membranes with well grown fungal colonies were cut out and transferred into empty three cm diameter dishes and then placed into the Mars simulation chamber.

Mars-simulation. The experiment was carried out in the Mars Simulation Facility (MSF) at the Institute of Planetary Research of the German Aerospace Center (DLR) Berlin. The MSF simulates Mars-like atmospheric conditions and is used to perform laboratory experiments with controlled time-profiles. The main part of the MSF is an "experimental chamber" (EC) located in a temperature test chamber. The experiments were performed in EC, which can be cooled, evacuated and filled with defined gas mixture and humidity in a controlled manner. The MSF, its performance and typical experiments are described by Lorek and Koncz[37].

For the experiment, two gases (CO_2 and air) were mixed and humidified. A gas volume flow of 10 liters per hour (approximately Standard Ambient Temperature 25°C (298 K) and Pressure 101325 Pa) of the resulting gas mixture (CO_2 95%/volume, air 5%/volume (N_2 4%/volume and O_2 1%/volume) was used to pass through the EC. Thus the humidity of the mixture inside the chamber, which was directly provided, corresponded to a partial water vapor pressure of about 3 Pa, which is the average closed to the 2.7 Pa vapor pressure in-situ measured in polar regions on Mars[4,38]. The pressure inside the experiment chamber was between 1000 Pa (at the starting time) and 700 Pa (main part until the end of the experiment). Complete Xenon-lamp radiation spectra between 200 nm and 2200 nm was used. The Xenon-lamp was switched on and off every day to stimulate the diurnal cycle of the sun. The day simulation was made by allowing light for 16 hours (for 8 hours light was off); night simulation for testing the effect of Mars-like atmospheric conditions without radiation was produced by covering the samples with aluminium foil. An applied

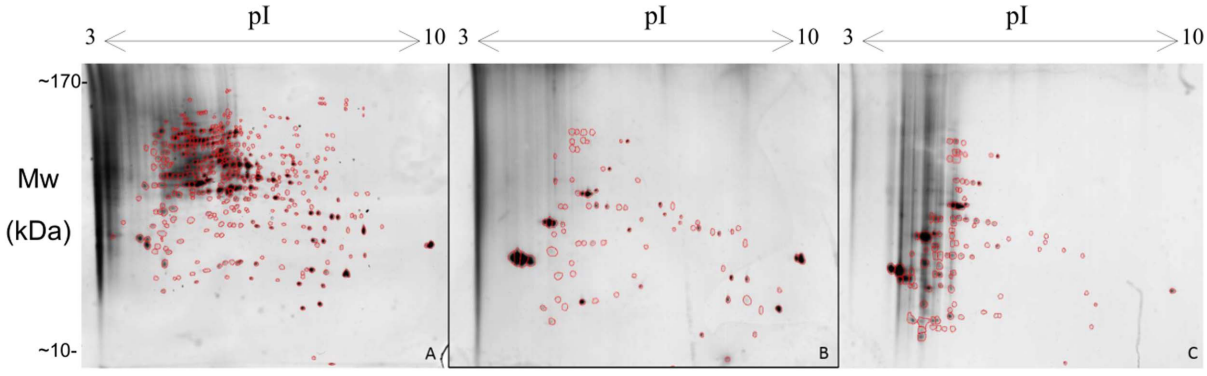

Figure 4 | 2D gel patterns obtained after exposure of *Cryomyces antarcticus*. (A): sample from optimal conditions. (B): sample was exposed to Mars-simulated conditions for 24 hours with day simulation. (C): sample was exposed to Mars-simulated conditions for 24 hours with night simulation.

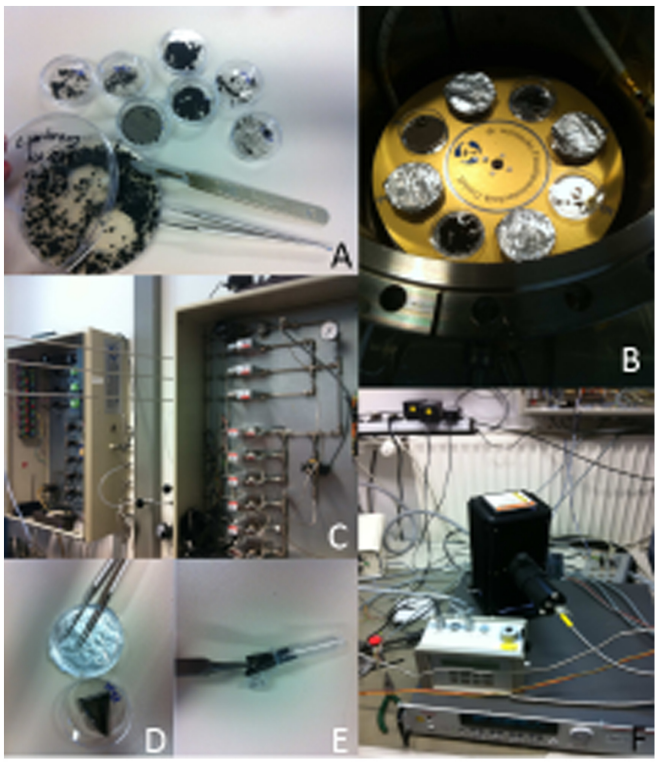

Figure 5 | Experimental flow. (A): Sample preparation for loading into experimental chamber. (B): samples prepared for the exposure with day and night (the lid of the plate is covered with aluminium foil, enabling prevention of UV radiation exposure). (C): gas-mixing system. (D, E): samples after exposure to Martian simulated conditions. (F): Mini-Pam (photosynthetic activity), Xenon-Lamp housing and measurement equipment.

simulation was used as follows: for samples treated for 24 hours (day and night simulation) was 153 kJ/m^2; for samples treated for 4 days 598.8 kJ/m^2 and for the samples treated for 7 days 1131 kJ/m^2. All the parameters are summarized in the Table 2.

All relevant experimental parameters and data were PC-controlled and logged in by software programs, which were LabView based.

For each experiment maximum eight of three cm diameter plates were placed on a rotation disc of the experimental chamber. Thus samples were placed on a sample holder which was exposed to the simulated Mars-like solar radiation provided by the Xenon lamp which is realizing the Mars-like surface conditions. The temperature varied in a diurnal cycle between −55°C (218°K) at night and 15°C (288°K) at daytime. The temperature and humidity inside the chamber were measured by three platinum resistance thermometers Pt100 (IST AG) which were arranged inside the chamber at the holder, two Pt100 temperature sensors and one capacitive humidity sensor. The humidity sensor and the Pt100 were fixed close to the biological samples at a distance between 1 and 2 cm. The second Pt100 was fixed in the middle of the chamber. Those controlled thermo-physical parameters such as humidity, gas mixture, temperature, pressure, and in addition the SOL-irradiation (including UV-irradiation with Xenon lamp via fiber inside the experimental chamber) allowed the simulation of Mars-like conditions (Fig. 1). Experimental scheme was as follows:

(1) samples were exposed for 24 hours with or without UV light (day/night simulation)as described above
(2) samples were exposed to treatments with a final exposure time of 4 days
(3) samples were exposed to treatments with a final exposure time of 7 days

After each experiment a small inoculum was transferred onto a Petri dish containing 2% MEA media to check the viability of the fungi. Remaining biomass was immediately frozen in liquid nitrogen for 2D gel electrophoresis.

Protein extraction and 2D gel protein profiling. Extraction of whole biomass proteins and 2D gel electrophoresis was based on a protocol, which was specially optimized for black fungi biomass[39] with some modifications. As it was mentioned before, the chamber allows using very limited amount of the samples; the obtained biomass was rare and precious therefore some modifications (described below) in the protocol were made which luckily allowed us to extract proteins from extreme low biomass quantities. Prior to the extraction the biomass was disrupted in liquid nitrogen by mortar. The powder-like biomass was washed in saline solution (NaCl 0.9%) and proceeded as given in the basic protocol[39]. After cell disruption the lysate was transferred into 15 ml polypropylene centrifuge tube and 3 ml of Tris-buffered phenol solution, pH 8.0 (Sigma-Aldrich, Steinheim, Germany) was added and mixed for 40 min at room temperature. The remaining steps were performed according to the basic protocol[39]. Protein determination was carried out using Bradford Protein Assay.

The Bradford protein Assay[40] was performed to determine the concentration of protein in fungal extracts. Reactions were carried out in microtiter plates according to the manufacturer instructions. A standard curve was established using serial dilutions from 0.8 μg ml^{-1} to 100 μg ml^{-1} of bovine serum albumin (BSA). The resulting optical density (OD) at 595 nm was analyzed with a plate reader (Magellan; Tecan Austria, Grödig, Austria). All experiments were carried out in triplicate. For each gel 20 μg of proteins were applied. IEF separation was performed using 13 cm strips pH 3–10NL. For each condition 2D gels were made in triplicate. Visualization of protein spots was made by a high sensitive mass spectrometric compatible silver staining[41]. The gels were fixed in 50% (v/v) methanol and 5% (v/v) acetic acid for 20 min, followed by washing in 50% (v/v) methanol for 10 min and then left in MilliQ water (Millipore, MA, USA) overnight at 4°C (277 K) with gentle agitation. Thereafter, the 2D gels were sensitized using a 0.02% (w/v) sodium thiosulphate solution for 1 min followed by incubation in 0.04% (v/v) formalin and 2% (w/v) sodium carbonate solution until the desirable intensity of staining was achieved. All the washing solutions were prepared in MilliQ water (Millipore, MA, USA).

After staining the gels were scanned in TIFF 16 bit format; for each sample (normal condition, 24 hours (day/night simulation) and exposure for 4/7 days); the 3 gels were matched by warping (Image Master 2D Platinum version 5.0, Amersham Biosciences, Swiss Institute of Bioinformatics, Geneva, Switzerland) and the sum of all spots which were present at least in two gels was taken into account for the protein pattern analysis.

Table 2 | Mars-simulated conditions used for the experiments

Parameter	Values
Gas mixing rates	CO_2 95%/volume, air 5%/volume (N_2 4%/volume and O_2 1%/volume)
Gas humidity	3 Pa
Volume flow	10 liters/hour*
Pressure inside the chamber	1000**–700*** Pa
Radiation spectra (Xenon-lamp)	200 nm and 2200 nm
Simulation	day simulation for 24 hours: light on for 16 hours and light off for 8 hours night simulation for 24 hours: light off
Temperature	Was varying in a diurnal cycle between: day simulation: 15°C (288°K) night simulation: −55°C (218°K)
Exposure time with corresponding dose	24 hours(day and night simulation): 153 kJ/m^2; 4 days simulation: 598.8 kJ/m^2 7 days simulation 1131 kJ/m^2

*at approximately Standard Ambient Temperature and Pressure 101325 Pa and 25°C.
**at the starting time and ***main part until the end of the experiment.

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Author contributions

K.S. conceived the project, she is the project-leader of the FWF project grant; she established the collaboration with J.-P.dV. K.S., J.-P.dV. and K.Z. made an experimental design. J.-P.dV. provided access to the facility of German Aerospace Center (DLR); J.-P.dV. and A.L. supervised the laboratory work at DLR; provided the data for 'Mars simulation' subunit in 'Materials and Methods' chapter; proof-read the manuscript. K.Z. performed the laboratory work (exposure of the samples to Mars-simulated conditions) at DLR; laboratory work and computer analysis (proteomics) at University of Natural Resources and Life Sciences, Vienna (BOKU); wrote the manuscript. G.M. and K.S. supervised the practical work in the laboratory of BOKU; proof-read the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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5.3 Global proteomics of the extremophile black fungus *Cryomyces antarcticus* using 2D-Electrophoresis

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Global Proteomics of the Extremophile Black Fungus *Cryomyces antarcticus* Using 2D-Electrophoresis

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Abstract

The microcolonial black fungus *Cryomyces antarcticus* is an extremophile organism growing on and in rock in the Antarctic desert. Ecological plasticity and stress tolerance make it a perfect model organism for astrobiology. 2D-gel electrophoresis and MALDI-TOF/TOF mass spectrometry were performed to explore the protein repertoire, which allows the fungus to survive in the harsh environment. Only a limited number of proteins could be identified by using sequence homologies in public databases. Due to the rather low identification rate by sequence homology, this study reveals that a major part of the proteome of *C. antarcticus* varies significantly from other fungal species.

Keywords

Microcolonial Fungi, Extremophiles, Proteomics, Protein Characterization

1. Introduction

The discovery of extreme earth's environments and the organisms that inhabit them has awakened curiosity of humans on the limits of life on and outside our planet and even through the universe. For a long time desert regions including the hot deserts of North America (Great Basin desert), Africa (Sahara desert, Kalahari desert),

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Asia (Gobi desert), the Middle East (Syrian desert), Australia (Great Victoria desert) as well as the permafrost environments up to the cold, dry valleys of Antarctica, Arctic and Patagonia were believed to be too hostile for any forms of life [1]. Today, we know that deserts are full of life forms with representatives of all domains and throughout all kingdoms of life [2]. Doubtlessly, deserts together with the deep biosphere provide the most extreme conditions for life on Earth and triggered manifold adaptation mechanisms in plants, animals and microorganisms [3] [4].

Antarctic ice-free deserts are among the closest analogues of Mars due to very cold and very dry conditions, extremely poor nutrient availability, and large fluxes of UV-light [5]. Despite of the hostile conditions, life is present in cryptic niches: microorganisms are organized in cryptoendolithic communities mostly dominated by lichens and by non-lichenized fungi and cyanobacteria [6]. Some of the components of those communities are black meristematic fungi, also known as microcolonial fungi (MCF) [7] [8].

The enormous stress resistance of MCF makes them important models for understanding stress resistance and adaptations in Eukaryont in general. Some special characteristics of the black fungi assumed to be involved in stress resistance are: 1) the cell composition of the fungi which consists mostly of very complex lipids (e.g. mono-, di- and tri-acylglycerols, phosphotidilcholine, phosphatidylethanolamines, sterols, sterol ethers, phosphatidilrthanolamines and free fatty acids); 2) pigments with their various protective effects as e.g. melanins, carotenoids in colourless and brown-red formations; and 3) the dense cell wall built of chitin, melanin and polysaccharides [9]. All those characteristics make the fungi able to survive diverse stress factors [10]-[12]; they are even known to be survivals of outer space and Mars like environments [13] [14].

At the same time the described characteristics of these fungi make it difficult to perform analysis on the cellular level. For instance the cell wall is the main obstacle in protein extraction from fungal biomass. Moreover, due to rigid cell wall and melanin content, also DNA extraction and purification have not been convenient to obtain genomic data of MCFs [15]. Up to now whole genome sequences of only two strains of black microclonial fungi—*Coniosporium apollinis* and *Cryomyces antarcticus*—exist in the public databases, however without annotation. In addition, the studied MCF strains have an extremely slow growth e.g. they need at least 30 days to reach the minimal quantity of biomass level for any type of experiment.

Up till now there are few proteomic data obtained from MCF showing protein patterns and their changes under varying conditions [12] [14] [16] [17]. However, no identification of proteins was done in any of the black MCF. Thus it was the aim of this study to perform the first protein identifications of the very particular fungal species *C. antarcticus* cultivated under laboratory conditions as a basis for a deeper understanding of the systems biology of this fungus and related extremophile genera.

2. Materials and Methods

2.1. Model Organisms

C. antarcticus, MA 5682, is one of the most extremophilic eukaryote on Earth was used as a model organism in this and previous studies. The strain was kindly provided by the Italian National Museum of Antarctica and was preserved in the ACBR culture collection (Austrian Centre of Biological Resources and Applied Mycology, http://www.acbr-database.at/). Inoculi were prepared as cell suspensions and drop-inoculated onto sterilized cellophane membranes (Model 583 gel dryer Backing; Bio-Rad, USA) placed on the surface of 2% malt-extract agar (MEA, Applichem, Germany). The fungus was grown at its optimal growth temperature: 15˚C (288 K) for 30 days, and the biomass was collected by scratching the material from the plates using a sterile scalpel, transferred into a sterile tube (Eppendorf, Germany), immediately frozen in liquid nitrogen and stored at −80˚C (193 K) for further analysis. All the experiments were performed in triplicate.

2.2. Proteomics

2.2.1. Protein Extraction

Protein extraction from biomass and the procedure of 2D gel electrophoresis was optimized for black fungi. Prior to the extraction, the biomass was disrupted in liquid nitrogen with mortar and pestle. Approximately 400 mg of the biomass were transferred into ice-cold 2 ml O-ring screw-capped microfuge tube (ribolyzer-tubes, Biozym Scientific GmbH, Germany). Subsequently the samples were washed two times with 500 µl of sterile saline solution (NaCl 0.9%, sodium chloride, Applichem, Germany), vortexed shortly and centrifuged for one minute after the first and 2 minutes after the second washing at maximal speed (200 ×g) at 4˚C and supernatant

was discarded. During the whole procedure, samples were kept on ice. Then approximately 400 mg of acid-washed 0.5 mm diameter glass beads (0.25 - 0.5 cm, Roth, Germany) were added. Prior to extraction, glass beads were washed as follows: approximately 100 g of glass beads were put into 200 ml distilled H_2O into a beaker and stirred using a glass rod. Then Triton X-100 (Merck, Germany) 100 ml was added under stirring until the detergent was completely dissolved. The Supernatant was carefully poured off and precipitate washed for 2 times 10 min each with distilled H_2O, followed by washing two times with 95% ethanol (Merck, Germany) under stirring for 2 minutes. Then beads were rinsed 2 times with distilled H_2O, then 2 times with 6 N HCl (hydrogen chloride, Merck, Germany), then rinsed 1 time with distilled H_2O and washed 2 times with 6 N HNO_3 (nitricacid, Merck, Germany). The cleaning of beads is completed after 3 times washing with distilled H_2O until the pH reached the value of approximately 6. Then the supernatant was discarded and the beads in the beaker were dried at 80˚C for 36 hours. The beads were added to the sample together with 1200 µl of Lysis Buffer (Tris 0.6 g (Invitrogen, USA), EDTA disodium salt 186 mg (Calbiochem, Germany), KCl 745.5 mg (potassium chloride, Merck, Germany), PVPP 1 g (polyvinylpolypyrrolidone, BioChemica, AppliChem, Germany), Sucrose 30 g (Merck, Germany)) in 100 ml MilliQ H_2O (Millipore, MA, USA). Lysis buffer should be stored at +4˚C for next use. The biomass was disrupted using Bead Beater/Rybolyzer (MP Bio, USA) and the tubes kept on ice between all the steps.

After disruption the lysate was transferred into 15 ml centrifugation tubes (Corning, Mexico) and 3 ml of tris-buffered phenol pH 8.0 (Sigma, USA) was added (mix the buffer before pipetting). Two ml of the buffer were added directly into the tube and with the rest the homogenized material was washed off the Rybolyzer tube. The tube was vortexed for 15 min at room temperature, followed by centrifugation for 10 min at 200 ×g at 4˚C. The phenol phase (upper phase) was transferred into a fresh pre-weighed tube (Corning, Mexico) and added 5 volumes of cold precipitation solution (ammonium acetate 7.7 g (AppliChem, Germany)) in 100% methanol (Merck, Germany). The precipitation was done overnight at −20˚C.

After the precipitation, samples were centrifuged for 30 min at 500 ×g at 4˚C. Supernatant was discarded and the pellet washed once with 2 ml cold 100% methanol (Merck, Germany) and then with cold 80% acetone (Merck, Germany). The pellet was air-dried at −20˚C overnight. Dried pellet was dissolved in Modified Sample Buffer (MSB, thiourea 6.09 g (Fluka, Germany), urea 16.9 g (AppliChem, Germany), CHAPS 1.6 g (Sigma, USA), DTT 400 mg (D, L-Dithiothreitol, Sigma, USA), carrier ampholytes pH 2 - 11 800 µl ((Servalyt® 2 - 11, Serva, Germany)), MilliQ water (Millipore, USA) was added to volume of 40 ml and stored at −20˚C in 1 ml aliquots). For each tube 400 µl of MSB was used.

2.2.2. Protein Determination

The Bradford [18] protein Assay (BioRad, U.S.A) was performed to determine the concentration of proteins in fungal extracts according to manufacturer's recommendation. Reactions were carried out in 96-welled microtiter plates (Nunclon Delta Surface, Denmark). A standard curve was established using serial dilutions from 0.8 $\mu g \cdot ml^{-1}$ to 100 $\mu g \cdot ml^{-1}$ of bovine serum albumin (BSA, Pierce, USA). The resulting optical density (OD) at 595 nm was measured with a plate reader (Tecan Austria, Austria). The protein concentration of three samples (experiment was performed in triplicate) was in a range 1010 - 1200 $\mu g \cdot ml^{-1}$.

2.2.3. 2D-Electrophoresis

All 2DE experiments were carried out in triplicates. For the analysis 13 cm strips non-linear pH 3 - 10 were used (GE-Healthcare Immobiline DryStrip pH 3 - 10 NL, 13 cm, Sweden). Using protein concentration value we calculated the sample amount which was loaded for the strip aiming to get the desirable amount of proteins which is normally 20 µg [12] [17]. However our pre-experiments showed that application of 20 µg of total protein to each gel was too low for the protein identification of this fungus using mass spectrometry. Therefore 200 µg of total protein were used to generate preparative 2D gels. The calculated sample amount and Reswelling solution (urea 6 g (AppliChem, Germany), CHAPS 0.5 g (Sigma, USA), DTT 37.5 mg (D, L-Dithiothreitol, Sigma, USA), bromphenol blue 1 ml (Serva, Germany), ampholytes 125 µl Servalyt® 2 - 11 (Serva, Germany) dissolved in 25 ml dH_2O) has to be stored at −20˚C) were mixed and applied to the gel in final volume 255 µl. Strip rehydration took approximately 20 hours.

Iso-electric focusing was performed using Protean IEF Cell (BioRad, USA) according to the manufacturer's instructions overnight. The re-equilibration was carried out prior to second dimension using an orbital shaker (Heidolph Unimax 1010, Germany,) at room temperature as follows. Two ml of equilibration Solution A (tris 10

ml (UltraPure™, Invitrogen, USA), urea 36 g (AppliChem, Germany), glycerol 30 ml (AppliChem, Germany/Sigma, USA), SDS 2 g (sodium dodecyl sulphate, Sigma, USA), DTT 2 g (D, L-Dithiothreitol, Sigma, USA) in dH_2O 100 ml) were added to each strip and incubated for 15 minutes. Then Solution A was replaced by Solution B (tris 10 ml (Sigma, USA), urea 36 g (AppliChem, Germany), glycerol 30 ml (AppliChem, Germany/Sigma, USA), SDS 2 g (sodium dodecyl sulphate, Sigma, USA), IAA 2.5 g (iodoacetamide, Sigma, USA), bromphenol blue 100 µ (Serva, Germany) in 100 ml dH_2O) and let incubate for 15 minutes. After the re-equilibration strips were applied to second dimension electrophoresis. Acrylamide solution (10%) (for 10 ml: dH_2O 4.8 ml, acrylamide 2.5 ml (ready to use solution 40%, Merck, Germany), tris 2.5 ml (UltraPure™, Invitrogen, USA), SDS 0.1 ml (sodium dodecyl sulphate, Sigma, USA), TEMED 0.004 ml (Serva, Germany), APS 0.1 ml (ammonium persulfate, AppliChem, Germany), for 1 gel approximately 30 ml of acrylamide solution is needed) was prepared in a beaker.

The gel chamber was thermostatized using a Heto water cooling (Heto CBN 8 - 30, Denmark). The second dimension was performed under following conditions: 160 V, 35 mA, 5 W for around 4 hours. Visualization of protein spots was performed by high sensitive mass spectrometric compatible silver staining [19]. Gels were washed for 1 min in MilliQ water at room temperature followed by incubation for 20 min in fixing solution (methanol 500 ml (Merck, Germany), acetic acid 50 ml (AppliChem, Germany), in MilliQ water to the volume of 1l, store at room temperature). Then gels were incubated at room temperature for 10 min in washing solution (methanol 500 ml (Merck, Germany) in MilliQ water to the volume of 1 L, store at room temperature). Then gels were washed overnight in MilliQ water at 4˚C with gentle agitation; incubated for 1 min in sensitizing solution (sodium thiosulfate 0.2 g (Merck, Germany) in MilliQ water to the volume of 1 L, has to be prepared fresh each time) followed by washing 2 times in MilliQ water 1 min each. Then gels were stained for 20 min at 4˚C in silver solution (silver nitrate 1 g (Merck, Germany) in MilliQ water to the volume of 1 L, has to be prepared fresh each time) followed by washing 2 times 1 min each in MilliQ water. Developing of the gels was performed in developing solution (sodium carbonate 20 g (Merck, Germany), formalin (35% formaldehyde, AppliChem, Germany) in MilliQ water to the volume of 1 L, has to be prepared fresh each time) and took 5 - 10 min. Then gels were washed 3 times 1 min each in stop solution (acetic acid 50 ml (AppliChem, Germany) in MilliQ water to the volume of 1 L, store at room temperature).

After staining, the gels were scanned as 16 bit TIFF images and stored at +4˚C in MilliQ water for further analysis. Gel images were matched by warping (Image Master 2D Platinum version 5.0, Amersham Biosciences, Swiss Institute of Bioinformatics, Geneva, Switzerland) and the sum of all spots which were present at least in two gels was taken into account for the protein pattern analyses as described in previous studies [12] [17]. The protein spots were excised from the gels by a manual spot-picker and transferred into a sterile tube (Eppendorf, Germany) containing 100 µl of MilliQ water (Millipore, MA, USA) and frozen at −80˚C for further MS analyses.

3. Protein Identification

3.1. Mass Spectrometry Using MALDI-TOF/TOF

Prominent 2-DE spots were excised by scalpel, washed, destained, reduced with DTT (D, L-Dithiothreitol, Sigma, USA) and alkylated with IAA (iodoacetamide, Sigma, USA). In-gel digestion was performed with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) [19]. Dried peptides were concentrated and de-salted using Zip-Tips C18 (microbed) (Millipore) according to the manufacturer's instructions.

3.2. Derivatisation for *de Novo* Sequencing

In order to enhance the quality of tandem mass spectrometry (MS/MS) spectra for de novo sequencing, N-terminal chemical modification using 4-sulfophenyl isothiocyanate (SPITC) was carried out with some modifications [20] [21]. The dried peptides were used for derivatisation with SPITC (4 µL of 10 mg/ml SPITC in 50 mM Tris-HCl buffer [pH 8.2]) for 30 min at 56˚C. Following acidification of the solution to stop the reaction with 6 µl 0.1% TFA, the peptides were de-salted using ZipTips C18 (microbed).

3.3. Spotting and Mass Spectrometry

Desalted peptides (0.5 µl) were spotted onto a disposable AnchorChip MALDI target plate pre-spotted with a-

cyano-4-hydroxycinnamic acid (PAC target, Bruker Daltonics). Data were acquired on a Matrix Assisted Laser Desorption Ionisation Tandem Time-of-Flight (MALDI-TOF/TOF) mass spectrometer (Ultraflex II, Bruker Daltonics) in MS and MS/MS modes. Spectra processing and peak annotation were carried out using FlexAnalysis and Biotools (Bruker Daltonics).

3.4. Data Analysis and *de Novo* Peptide Sequencing

Standard databases were used to search for processed spectra. Each spectrum was compared via an in-house Mascot server (Matrix Science) to the Swiss-Prot database, NCBInr and to a generic database-based on genomic data of the organism-using the search parameters like taxonomy all entries; global modifications carbamidomethylation on cysteine; variable modifications oxidation on methionine; MS tolerance 100 - 150 ppm; MS/MS tolerance 1 Da; one missed cleavage allowed. Identifications were considered statistically significant where $p < 0.05$. Peptide sequencing was carried out manually using FlexAnalysis. These sequences were then used for a homology search using MS-homology search (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology).

4. Results

In order to visualize and investigate the proteome of *C. antarcticus*, 2-DE was performed using an optimized protein extraction protocol for MCFs. The main aim of the experiment was to separate the total protein repertoire of *C. antarcticus*, which was grown under laboratory conditions as a major basis to study the adaptations of the fungus to the extreme environment of Antarctica. Due to the numerous obstacles caused by the dense and melanised cell wall the protein extraction was, therefore, adapted and optimized to bypass the complex matrix of MCFs [15]. In order to increase the success with protein identification, 10 times higher amounts of protein (200 µg of total protein instead of 20 µg) were loaded to each gel. Taking into consideration all possibilities and limitations, our results allowed a first insight into the protein expression pattern in *C. antarcticus.* The 2-DE electrophoresis delivered 200 well defined protein spots by the orthogonal properties of isoelectric point and molecular weight. However, the results showed that some of the spots include more than one protein. All spots were visualized using a MS compatible silver staining and are shown in **Figure 1**.

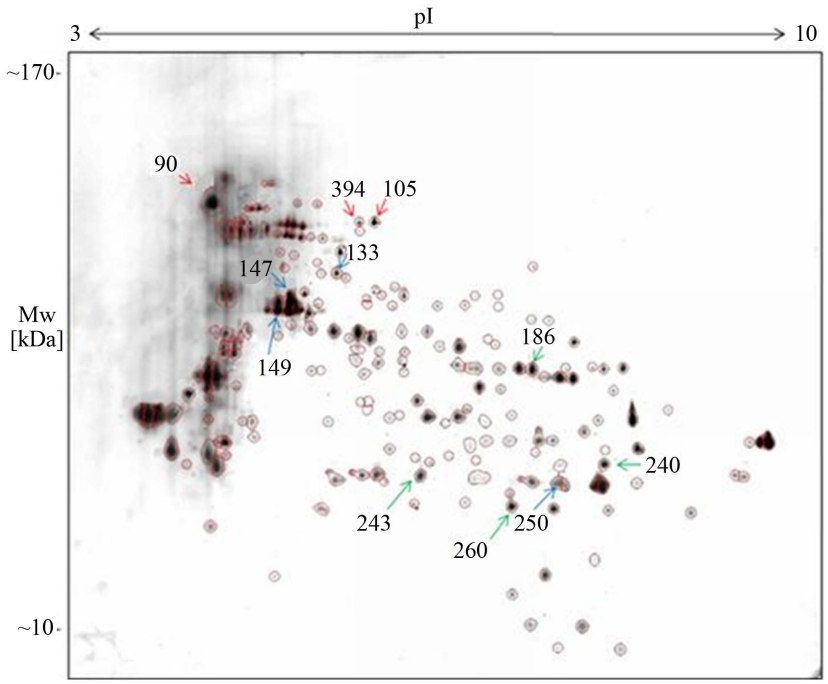

Figure 1. 2-DE pattern of *Cryomyces antarcticus* separated by two-dimensional electrophoresis. Red arrows: chaperons and heat shock proteins; blue arrows: enzymes and green arrows: other proteins.

The protein spots were distributed alongside the whole pI range of 3 - 10. A few high abundant and high molecular weight proteins were found in the acidic ranges between pI of 4 to 6.5. The resolution of the gel was very high and the spots could be excised without contamination. All protein spots were analyzed by MS-MALDI-TOF/TOF, however, only 11 proteins could be identified based on sequence homology with other species.

Although missing an annotated genome of *C. antarcticus* as a reference, some protein spots could be identified by *de novo* sequencing with high reliability (Mascot score 84% - 531%). The identified proteins are divided into three main categories: 1) heat shock proteins and 2) functional enzymes and 3) other proteins (**Tables 1-3**). Given the fact that *C. antarcticus* is an inhabitant of extreme environment it seems that its systems biology maintains the cell function by the expression of some prominent and conserved enzymes and commonly known heat shock genes even when growing under optimal temperature conditions.

Table 1. Chaperons and heat shock proteins identified in *Cryomyces antarcticus*. The scores of protein spots without MASCOT match are given only in homology score.

Spot ID	Entry	Description	MW [kDa]	pI	Mascot score	MS homology score	Peptides	SC [%]
90	F0UMD0	Hsp70-like protein	69.1	4.9	176.0		K.FELTGIPPAPR.G	7.8
		Ajellomyces capsulata (strain H88)					K.DAGLIAGLNVLR.I	
							K.SSVHEIVLVGGSTR.I	
							R.TTPSFVAFTDTER.L	
	A5DWD3	Heat shock protein SSA2	69.9	4.9	144.7		K.LVSDFFNGKEPNR.S[u]	6.2
		Lodderomyces elongisporus (strain ATCC 11503/CBS 2605/					K.SSVHEIVLVGGSTR.I	
		JCM 1781/NBRC 1676/NRRL YB-4239)					R.TTPSFVAFTDTER.L	
	H1V102	Heat shock protein	70.1	5.1	116.8		R.IINEPTAAAIAYGLDRK.N[u]	6.6
		Schizosaccharomyces japonicus (strain yFS275/FY16936)					K.DAGLIAGLNVLR.I	
							K.SSVHEIVLVGGSTR.I	
	M3C473	Heat shock 70 kDa protein	70.1	5.0		400	K.DAGLIAGLNVLR.I	
		Sphaerulina musiva (strain SO2202)					K.FELTGIPPAPR.G	
							R.IINEPTAAAIAYGLDKK.T	
							K.MVSDFFNGKEPNR.S	
							K.SSVHEIVLVGGSTR.I	
							R.TTPSFVAFTDTER.L	
394	F0XUL5	Mitochondrial hsp70 chaperone	73.1	5.7	171.5		K.EIQEVILVGGMTR.M	4.2
		Grosmannia clavigera (strain kw1407/UAMH 11150)					K.LLGNFQLVGIPPAHR.G	
105	F0XUL5	Mitochondrial hsp70 chaperone	73.1	5.7	111.7		K.EIQEVILVGGMTR.M	6.4
		Grosmannia clavigera (strain kw1407/UAMH 11150)					K.LLGNFQLVGIPPAHR.G	
							K.NAVVTVPAYFNDSQR.Q	

SC: sequence coverage; U: unique peptide; Red letters: exchanged amino acids in the homologue protein; Grey letters: homology searches in different species, or isoforms of the same protein, and peptides with an upper case u: are exceptional for *C. antarcticus*.

Table 2. Metabolic enzymes as identified by *de novo* sequencing and sequence homology to other species. The scores of protein spots without MASCOT match are given only in homology score.

Spot ID	Entry	Description	MW [kDa]	pI	Mascot score	MS homology score	Peptides	SC [%]
133	H6C9R1	V-type proton ATPase subunit B *Exophiala dermatitidis* (strain ATCC 34100/CBS 525.76/ NIH/UT8656)	56.6	5.7	350.8		K.GIYPPINVLPSLSR.L R.YNEIVSLTLPDGSER.S R.VTLFLNLANDPTIER.I R.GYPGYMYTDLSTIYER.A K.VLAEDYLDINGSPINPYSR.V K.IPIFSAAGLPHNEIAAQICR.Q	19.4
	R1GGL8	Putative vacuolar ATP synthase subunit *Botryosphaeria parva* (strain UCR-NP2)	57.4	5.2	283.7		K.TFIAQSAYESR.T[u] K.GIYPPINVLPSLSR.L R.VTLFLNLANDPTIER.I R.GYPGYMYTDLSTIYER.A K.IPIFSAAGLPHNEIAAQICR.Q	14.7
	F0XRR8	Vacuolar ATP synthase subunit b *Grosmannia clavigera* (strain kw1407/UAMH 11150)	56.0	5.2	265.9		K.IINEYYQR.T[u] R.GIYPPINVLPSLSR.L R.GFPGYMYTDLSTIYER.A K.VLAEDYLDINGSPINPYSR.V	11.3
	C4JV88	V-type ATPase, B subunit	56.4	5.9		600	K.GIYPPINVLPSLSR.L R.YNEIVSLTLPDGSER.S R.VTLFLNLANDPTIER.I R.GVPGYMYTDLSTIYER.A K.VLAEDYLDINGSPINPYSR.V K.IPIFSAAGLPHNEIAAQICR.Q K.TFISQSPYESR.T K.ILDEYYQR.T	
147	H0ELJ5	ATP synthase subunit beta *Glarea lozoyensis* (strain ATCC 74030/MF5533)	54.8	5.2	548.9		K.VVDLLAPYAR.G R.FTQAGSEVSALLGR.I K.VALVFGQMNEPPGAR.A K.LVLEVAQHLGENVVR.T R.IPSAVGYQPTLAVDMGLMQER.I	14.7
	B6QIQ4	ATP synthase subunit beta *Penicillium marneffei* (strain ATCC 18224/CBS 334.59/QM 7333)	55.6	5.2		53	T.LAVDMGIMQER.I	
	K2SN01	Peptidase M16 *Macrophomina phaseolina* (strain MS6)	53.3	5.5	197.4		R.IDDLVHFTLR.E R.SQSQLELEIENMGGHLNAYTSR.E	6.6
	A6QY85	Mitochondrial processing peptidase beta subunit *Ajellomyces capsulatus* (strain NAm1/WU24)	52.9	5.6		100	G.ITEKDVMSFAQ.R L.SLDGTTAVAEDIG.R	

Continued

149	L8G6D5	ATP synthase subunit beta *Geomyces destructans* (strain ATCC MYA-4855/20631-21)	55.4	5.4	559.8		K.VVDLLAPYAR.G R.IMNVTGDPIDER.G R.FTQAGSEVSALLGR.I K.VALVFGQMNEPPGAR.A R.LVLEVAQHLGENVVR.C R.TREGNDLYHEMQETK.V R.IPSAVGYQPTLAVDMGLMQER.I	19.8
	R1GS12	ATP synthase subunit beta *Botryosphaeria parva* (strain UCR-NP2)	136.1	5.5	531.8		K.LVDLKDTIR.S[u] K.VVDLLAPYAR.G R.FTQAGSEVSALLGR.I K.VALVFGQMNEPPGAR.A K.LVLEVAQHLGENVVR.C R.IPSAVGYQPTLAVDMGIMQER.I	6.9
	K2R9P7	ATP synthase subunit beta *Macrophomina phaseolina* (strain MS6)	55.5	5.4		467	K.AHGGFSVFTGVGE.R R.FTQAGSEVSALLGR.I R.IPSAVGYQPTLAVDMGAM.Q V.LEVAQHLGENVVR.C K.LVDLKDTIR.S V.TGDPIDER.G K.VALVFGQMNEPPGAR.A K.VVDLLAPYAR.G	
250	K1WPB3	Vacuolar aspartyl aminopeptidase Lap4 *Marssonina brunnea* f. sp. *multigermtubi* (strain MB_m1)	68.8	5.7		103	K.LQVFQIR.N K.LVGVFDDEEIGSQLR.Q	

SC: sequence coverage; U: unique peptide; Red letters: exchanged amino acids in the homologue protein; Grey letters: homology searches in different species, or isoforms of the same protein, and peptides with an upper case u: are exceptional for *C. antarcticus*.

4.1. Heat Shock Proteins

(HSP) are a group of proteins induced when cells are exposed to low or high temperature or other environmental stress. The most prominent members of this group are a class of functionally related proteins involved in the folding and unfolding of other proteins [22]. This increase in expression is regulated normally by gene transcription [23]. Expression of high levels of heat shock proteins are stimulated by different types of environmental conditions, like exposure of the cell to toxins (ethanol and trace metals) or to ultraviolet light, starvation, or water deprivation. As a result, the heat shock proteins are called stress proteins and their up-regulation sometimes described more generally as part of the stress response-network [24].

Molecular chaperones are originally defined as proteins that mediate the correct assembly of other proteins, but are not themselves components of the final structure. They bind to and stabilize an otherwise unstable conformation, and through a controlled cycle of binding and release facilitate the correct fate *in vivo* [25]. One major function of chaperones is to prevent both newly synthesized polypeptide chains and assembled subunits from aggregating into nonfunctional structures. For this reason, many chaperones are also known as heat shock proteins due to the increased protein aggregation by stresses [26].

Table 3. Other proteins, which are identified in *C. antarcticus*. The scores of protein spots without MASCOT match are given only in homology score.

Spot ID	Entry	Description	MW [kDa]	pI	Mascot score	MS homology score	Peptides	SC [%]
	F2Q1U3	Actin	32.2	5.0	160.5		R.AVFPSIVGRPR.H	13.1
		Trichophyton equinum (strain ATCC MYA-4606/CBS 127.97)					K.IWHHTFYNELR.V	
							K.SYELPDGQVITIGNER.F	
186	M2N0M3	Uncharacterized protein BLAST: Carboxypeptidase Y	60.9	5.1	102.0		K.YDSCNFDINR.N	4.4
		Baudoinia compniacensis (strain UAMH 10762)					R.NFLFQGDWMQPFHR.L	
	C9STI2	Pre mRNA splicing factor	33.2	9.6		107	S.LDVVYSETGR.D	
							R.DVVPAFHGSDFMGE.R	
240							R.LYLGNLPR.N	
	O74159	Brn1 (Fragment)	26.5	8.1		153	K.GAIETFVR.C	
		Curvularia intermedia					R.GQFFVAK.A	
260							R.VFNINTR.G	
							R.VGQPIDIAR.V	
243	L8FUI1	Uncharacterized protein *Geomyces destructans* (strain ATCC MYA-4855/20631-21)	32.1	4.7	176.0		R.YLEAGAPTGLTGLFTHPAPR.S	6.9
229	R1EAT2	Putative 14-3-3 family protein	29.6	4.6	157.0		K.DSTLIMQLLR.D	14.2
		Botryosphaeria parva (strain UCR-NP2)					R.YLAEFAIGDKR.K	
							K.AATEVAQTDLAPTHPIR.L	
	B6QL30	14-3-3 family protein ArtA, putative	29.7	4.8		227	K.AATEVAQTDLAPTHPIR.L	
		Penicillium marneffei (strain ATCC 18224/CBS 334.59/QM 7333)					K.DSTLIMQLLR.D	
							K.MKGDYHR.Y	
							R.YLAEFAIGDRR.K	

SC: sequence coverage; U: unique peptide; Red letters: exchanged amino acids in the homologue protein; Grey letters: homology searches in different species, or isoforms of the same protein, and peptides with an upper case u: are exceptional for *C. antarcticus*.

4.2. Heat Shock Protein 70

The *C. antarcticus* proteome contains the heat shock 70 kDa protein (spot 90), identified by several peptides and a Mascot score of 176.0. The expression of the HSP having a molecular mass of about 70 kDa, was observed in hyphomycetous fungi as e.g. *Fusarium oxysporum* f. sp. *niveum*, *Neurospora crassa* and *Saccharomyces cerevisiae* [27].

Proteins of HSPs 70 family are highly conserved in eukaryotic organisms. Their expression of HSP70 as a response to several stresses is universal. The respond was studied solely at the cytological level and several important observations were made: induces by other stress treatments; produced within a few minutes; associated with newly synthesized RNA, found in many different tissues, accompanied by the disappearance of previously active protein synthesis are suppressed. HSP70 chaperones make up a set of prominent cellular machines that assist with a wide range of protein folding processes in almost all cellular compartments. They are also involved in protein import and translocation processes, and in facilitating the proteolytic degradation of unstable proteins by targeting the proteins to lysosomes or proteasomes. HSP70 is also involved in the modulation of signal transducers such as protein kinase A, protein kinase C and protein phosphatise. In this respect, HSP70 chaperones might play a broad role by participating in modulating the expression of many downstream genes in signal

transduction pathways both during stress and under normal growth conditions [20].

Hsp70 shows essential functions in preventing aggregation and in assisting refolding of non-native proteins under both normal and stress conditions [20]. Under non-stressful conditions, HSP70 cognate functions in concert with a variety of co-chaperones facilitate folding of de novo synthesized proteins assisting the transport of precursor proteins into organelles and help to target damaged proteins for degradation. Stress-induced HSP70 functions to mitigate aggregation of stress-denatured proteins and to refold non-native proteins restoring their biological function through iterative cycles of adenine nucleotide hydrolysis-dependent peptide binding and release [28]. Molecular chaperones were defined as proteins that mediate the correct assembly of other proteins, but are not themselves components of the final structure. Those proteins bind and stabilize an unstable conformation, and through a controlled cycle of binding and release facilitate the correct fate *in vivo* [25].

Chaperones do not convey information directing a protein to correct molecular pathway. Instead, they reduce non-productive interactions. Both folding and aggregation can be very rapid, and thus the rates of chaperone recognition and binding must be high to intercept the de processes. Chaperones block entry into side reactions by their property of recognizing non-native polypeptide structures. Recognition must lead to tight binding and shielding of hydrophobic surfaces, so that aggregation of folding are prevented. In addition, binding must be more rapid than competing reactions that are essentially irreversible [25].

4.3. HSP 70 kDa Chaperone Precursor

Both HSP70 and HSP70 chaperon precursor could be identified in *C. antarcticus*. The HSP70 chaperones have implications on protein folding, assembly/disassembly of oligomeric complexes, protein synthesis, degradation, and the translation of polypeptides across cellular membranes [28]. As it was shown by Glick [28], ATP hydrolysis allows luminal HSP70 to bind strongly both to the precursor chain and to a membrane partner protein. HSP70 would then undergo a conformational change, thereby pulling a segment of the precursor chain through the translocation channel. Finally replacement of the bound ADP with ATP would release HSP70 from the precursor and from the membrane partner protein and the cycle could begin again. Translocation would be unidirectional because luminal HSP70 is anchored to the membrane in a defined orientation and because interactions of the precursor chain with the translocation channel would inhibit "backsliding" of the precursor between rounds of HSP70 binding. This mechanism for protein import is reminiscent of the ATP-driven translocation of actin filaments by myosin. Thus, even though the N-terminal domain of HSP70 is structurally related to actin, HSP70 proteins may be functionally analogous to myosin.

4.4. Chaperone Protein HSP 31

Under stress many cellular proteins lose their native configuration and tend to aggregate. This could pose a serious threat to the cellular functionality. Chaperones provide the essential assistance by promoting refolding of stress-denaturated proteins and preventing their aggregation. The predominant HSPs are classified by their molecular masses as HSP100, HSP90, HSP70, HSP60, HSP40 and small HSP (sHSPs) [29].

The functions of sHSPs, as ATP independent molecular chaperones, in protein folding and, as antistressors, in acquired thermotolerance have been well established [30]. Upon heat-shock or exposure to other stressors, sHSPs capture unfolded proteins and form high molecular weight multimeric complexes, thus prevent their irreversible aggregation by holding them in a soluble and folding competent state. Then, refolding of the captured proteins most likely occur with the assistance of the ATP dependent molecular chaperones, such as ClpB (or HSP104) and/or the DnaK/DnaJ/GrpE (or HSP70/HSP40) system [30].

4.5. Metabolic Enzymes

Most of the identified proteins of the *C. antarcticus* proteome belong to the category 2 and are essential metabolic enzymes. These enzymes catalyze reactions, which allow organisms to grow and reproduce, maintain their structures, and respond to their environments. The protein spots identified by de novo sequencing comprise a series of key enzymes, which are described in the following (**Table 2**).

4.6. V-Type Proton ATPas Subunit B

Spot number 133 contain at least two proteins, a V-type ATPase and with a lower rate of homology Vacuolar

ATP synthase subunit b. V-type ATPase is a multisubunit enzyme (approximately 500 kDA) composed of a water-soluble catalytic sector and an integral membrane proton channel complex [31]. V-ATPases have an important role in both endocytosis and intracellular transport [32]. The V-type H^+ ATPase comprises an ATP driven enzyme that transforms the energy of ATP hydrolysis to electrochemical potential differences of protons across diverse biological membranes via the primary active transport of H^+ [33].

The vacuolar (H^+) ATPase or V-ATPases are a family of ATP-driven proton pumps responsible for acidification of a variety of intracellular compartments in eukaryotic cells, including clathrin-coated vesicles, endosomes, lysosomes, Golgi, secretory vesicles. The V-ATPases function to acidify intracellular compartments in eukaryotic cells, playing an important role in such processes as receptor-mediated endocytosis, intracellular membrane traffic, protein degradation and coupled transport. V-ATPases in the plasma membrane of specialized cells also function in renal acidification, bone resorption and cytosolic pH maintenance. The V-ATPases are composed of two domains. The V1 domain is a 570 kDa peripheral complex composed of 8 subunits (A-H) of molecular weight 70 - 13 kDa which is responsible for ATP hydrolysis [34] [35]. The protein identified from the *C. antarcticus* belongs to subunit B.

V-ATPases play a role in energizing secondary transport and maintenance of solute homeostasis. Under stress conditions such as salinity, drought, cold, acid stress, anoxia, and others, survival of the cells depends strongly on maintaining or adjusting the activity of the V-ATPase. Regulation of gene expression and activity are involved in adapting the V-ATPase on long- and short-term bases [36].

4.7. ATP Synthase

Changes in metabolism under the influence of stress conditions include almost all the processes and pathways within the cells. The proper course of metabolic processes requires sufficient amount of biologically useful energy, usually in the form of high-energy phosphate bonds between residues in the ATP [37].

Adenosine triphosphate (ATP) synthase contains a rotary motor involved in biological energy conversion. ATP is the universal biological energy currency. ATP synthase produces ATP from adenosine diphosphate (ADP) and inorganic phosphate with the use of energy from a transmembrane proton-motive force generated by respiration or photosynthesis [38]. Recently two models are proposed to explain the requirement of ATP during protein import to mitochondria involving the action of HSP70 as the center of the protein translocase machinery. Both models agree that HSP70 proteins or homologues of them should bind to the pre-sequence of the pre-protein being imported with the binding cycle regulated by ATP hydrolysis [39]. Majority of experimental data support the proposal that ATP hydrolysis is essential for chaperone activity [39].

4.8. Carboxy Peptidase Y

The principle of self-assembly in protein structure states that the native three-dimensional structure of a protein are determined solely by the primary amino acid sequence and that folding of the peptide chain. This phenomenon is a spontaneous process which does not require input of energy or accessory factors. Some proteins can apparently not be re-natured once they have been fully denatured. Such proteins are frequently synthesized as zymogens that are later matured to active enzymes by proteolysis. The function of the function of the propeptides is not only to render the proenzymes catalytically inert but also to act as so-called co-translational chaperones, since they mediate correct folding without being components of the final three-dimensional structure. The folding is in some cases not dependent on the covalent linkage of the pro-peptide to the segment that becomes later a mature protein. Similar as described above, carboxypeptidase Y (CPY) is synthesized as a zymogen. CPY is a vacuolar protein that was extensively studied as a model for eukaryotic intracellular protein transport and targeting. The gene for CPY encodes a preproenzyme containing a signal peptide of 20 amino acid residues that directs the precursor across the endoplasmic reticulum membrane. In the endoplasmic reticulum the signal peptide is cleaved off and folding takes place. A 4-amino acid segment of the CPY propeptide serves as signal for vacuolar targeting. Upon arrival of pro-CPY in the vacuole it is activated by degradation of the propeptidein a process involving the vacuolar endopeptidase proteinase A [40] [41].

4.9. Tri-(Tetra-)Hydrohyl Naphthalene Reductase

It was reported that polyhydroxylnaphthalene reductase is described as an important element in melanin biosynthesis in pathogenic fungus *Magnaporthe grisea* [42]. Cell walls of *C. antarcticus* are incrusted with melanin

which gives it black colour. During the biosynthesis of fungal melanin, tetrahydroxylnaphthalene reductase catalyzes the NADPH-dependent reduction of 1, 3, 6, 8-tetrahydroxylnaphthalene (T4 HN) into (+)-scytalone and 1, 3, 8-trihydroxylnaphthalene into (−)-vermelone [43]. Melanin, a high-molecular mass black pigment, is synthesized in numerous pathogenic fungi, such as *Verticillium dahlia*, *Cochliobolus miyabeanus* and *Magnaporthe grisea* and others. Mutants of those pathogens lacking the capability to synthesize melanin lose their pathogenicity [43].

4.10. NADPH-Ubiqinone Oxidoreductase 29.9 kDa Subunit

NADPH oxidases (Nox), dedicated enzymes for reactive oxygen species (ROS) production, are widely distributed in eukaryotic cells [44]. In eukaryotic cells many Nox genes control different processes, including cell proliferation, apoptosis and hormone response in animals [44], and programmed cell death, hormone signalling and root hair tip growth in plants [45]. Nox can serve both defense and differentiation signalling roles [46].

The enzymes of this group in filamentous fungi play a key role in multi-cellular development and defense [46]. The study of Kayano and colleagues [47] suggests that NoxA and associated regulators are essential for polarized hyphal growth under conditions of nutrient starvation NADPH oxidase gene (NoxA) found in *Aspergillus nidulans* blocks differentiation of sexual fruiting bodies (cleistothecia) but has no effect on hyphal growth or asexual development [48]. Fungal NADPH oxidase catalysed production of ROS is also an important signalling mechanism for plant pathogens. Performed functional analysis emphasize the importance of ROS signalling for many different physiological and developmental processes in fungi [46].

4.11. Peptidase M18 Aminopeptidase I

Aminopeptidases catalyze the sequential removal of amino acids from unblocked N termini of peptides and proteins. These enzymes are widely distributed in eukaryotes and prokaryotes as either integral membrane or cytosolic proteins. Aminopeptidases are generally classified in terms of their substrate specificities-preference for a neutral, acidic, or basic amino acid in the P1 position. In addition to their role in general protein and peptide metabolism, aminopeptidases have more specific functions: activation and inactivation of biologically active peptides; removal of the N-terminal methionine of newly synthesized proteins; and possibly trimming of antigens for presentation by the major histocompatibility complex-1 system [49].

4.12. Other Proteins

Four proteins belong to different functional groups were identified by *de novo* sequencing and sequence homologies (**Table 3**). These proteins are shared among a wide variety of species from microorganisms to humans and are likely to be essential for surviving of any given organism on the earth.

4.13. Actin

Actin plays a crucial role in elementary eukaryotic cellular processes such as motility, cell growth regulation, cell differentiation and provides structural stability. Apparently these functions have not allowed actin of different species to diverge significantly, since the actin protein is highly conserved [50]. Plamann and colleagues reported that cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi [51]. There are some studies showing that actin is present in many filamentous fungi [52] [53].

4.14. Mitochondrial Processed Peptide

Mitochondrial processing peptidase (MPP) consists of α and β subunits that catalyze the cleavage of N-terminal mitochondrial-targeting sequence (N-MTSs) and deliver proproteins to the mitochondria [54]. The majority of mitochondrial proteins are synthesized as precursors on cytosolic ribosomes and transported post-translationally into the mitochondria. This process is facilitated by specific matrix-targeting signal sequences which are normally part of the N-termini of these proteins prior to their transportation. These proteins are unfolded and imported into the mitochondrial matrix across a double membrane through protein translocation machinery comprising translocases of the outer and inner mitochondrial membrane. After precursor translocation, the targeting signal is no longer necessary and is proteolitically removed. Although several mitochondrial peptidases partici-

pate in pre-protein processing, the most important role seems to belong to the Mitochondrial Processing Peptidase (MPP), since its deletion is lethal. Indeed, no inherited disorders have been linked with any mutants of MMP, indicating that its biological function is so vital that even relatively moderate disruption to its activity are also likely to produce non-viable organisms. This may be linked to its essential role in mitochondrial biogenesis [55].

The ability of the pre-sequence to adopt context-dependent conformations during different steps of MPP's action is a basic requirement for substrate recognition processing [55]. The mitochondrial precursors are translocated across the outer and the inner mitochondrial membrane via the Translocase of the Outer Membrane (TOM) and the Translocase of the Inner Membrane (TIM) machineries, respectively. Transport across the outer membrane does not require energy, whereas transport across the inner membrane is an energy-dependent process that requires a membrane potential across the inner membrane. Inside the mitochondrial matrix, the presequence of the imported precursor protein is cleaved off by the Mitochondrial Processing Peptidase (MPP). In yeast and mammals, MPP is localized to the matrix, whereas in plants MPP is integrated into the cytochrome bc1 complexof the respiratory chain as Core proteins of the complex facing the matrix. Cleavage by MPP may be followed by additional trimming of some precursors in the mitochondrial matrix by the Mitochondrial Intermediate Peptidase (MIP) or the Intermediate cleaving peptidase of 55 kDa, Icp55 [56].

4.15. 14-3-3 Protein

The cellular decision to live or die is finely controlled by tightly regulated signaling pathways. 14-3-3 proteins have a key role in this decision by controlling many of the signaling pathways that mediate this process. 14-3-3 proteins comprise a large family of acidic proteins that are expressed within all eukaryotic cells and function as homodimers and heterodimers. 14-3-3 dependent protein regulation occurs through phosphorylation-dependent binding which results in the release of survival signals in the cells [57].

14-3-3 proteins represent a family of highly homologous proteins that were described in all eukaryotic organisms from fungi to humans. These small acidic proteins have been implicated in a wide variety of cellular processes including signal transduction, cell cycle regulation and transcriptional regulation [58]. 14-3-3 monomers form cup-shaped homo- and hetero-dimers are able to bind protein ligands. Usually phosphorylated on serine or threonine residues or consensus binding motifs. 14-3-3 proteins are involved in altering the ability of the target protein to interact with other proteins; in modifying the subcellular portioning of a target, in enhancing/inhabiting the catalytic activity of the target; in protecting a target from dephosphorylation/proteolysis; and in acting as a scaffold. However, 14-3-3 proteins do not appear to be involved in the same process in different fungi [59].

The 14-3-3 protein family is shown to implicate in the dynamic nucleocytoplasmic transport of a number of proteins. They specifically recognize one or more short phosphoserine/theonine-containing sequence motifs on target proteins [60].

The members of this family interact with hundreds of protein partners, participate in regulation of many cellular processes like apoptosis, cell division, transcription, trafficking [61]. Moreover 14-3-3 proteins play an important role in regulation of signal transduction, apoptosis, cell cycle control, and nutrient-sensing pathways.

It was found that 14-3-3 itself can undergo different posttranslational modifications (including phosphorylation) strictly regulating its cellular functions. Phosphorylation seems to be the most important way of 14-3-3 regulation and can affect either direct interaction of 13-4-4 with its targets or induce dissociation of 14-3-3 dimers. As a rule, phosphorylation negatively affects interaction of 14-3-3 with its target [62].

The data of the study by Zakharova and colleagues showed that the basic level of sugars was high in fully hydrated *C. antarcticus* culture [12]. Trehalose is a naturally occurring non-reducing sugar found in a wide variety of organisms where it protects proteins and membranes from various stress conditions like dehydration, heat, cold, oxidation and desiccation and serves as a carbon and energy source. It was suggested that in yeast and plants it serves as a regulatory and signaling molecule to direct certain metabolic pathways or to affect growth. The neatral trehalase Nth1 of yeast is reported to be activated by 14-3-3 protein [63].

Among the many specialized cellular signal transduction models, the family of 14-3-3 protein occupies a remarkably ubiquitous role as downstream effectors of phosphorylation events. Essentially soluble dimers of single phosphor-Ser/Thr binding domains are reported to bind to this protein. The 14-3-3 proteins are additionally described to bind hundreds of different cellular protein, although there are also examples of binding to non-

phosphorylated target proteins. Upon binding to their phosphor-recognition site, they are reported to affect the Target Protein (TP) by modulation of its activity, interaction with other molecules, intracellular localization and stability [64].

4.16. Pre mRNA Splicing Factor

As it was shown by Keightley and colleagues the regulation of pre-mRNA splicing is critical for correct lineage specification in haematopoiesis [65]. Spliceosomes are composed of uridineuridine-rich small nuclear ribonuclearprotein particles (U-snRNPs) and multiple associated proteins. The vast majority of introns in pre-mRNA are excited by U2-dependent major spliceosomes, which contain U1, U2, U4/U6 and U5 snRNPs. Transcription and pre-mRNA splicing have emerged as highly coordinated processes. The close function coupling between the two processes is achieved through multiple mechanisms. A key role is played by the CTD domain of the largest subunit of RNA polymerase II, which both interacts with splicing factors and may function as a scaffold for co-transcriptional pre-mRNA splicing [66]. CPY is synthesized as a pre-enzyme containing an N-terminal 20-amino acid signal peptide, which directs pro-CPY to the lumen of the endoplasmic reticulum [66].

5. Discussion

C. antarcticus is an Antarctic rock-inhabiting microorganism, which lives at the absolute edge of life under the most extreme conditions known on the earth [67]. These fungi live between the limit of adaptability and near-death, barely surviving and rarely regenerating. They are assumed to be the most stress resistant eukaryotic organisms known on the earth [68]. In this context it is interesting that only a number of 200 different protein spots were exhibited and visualized on the gel although a high amount of total protein was loaded on the gel. This finding is in accordance with previous studies that have shown that a low number of proteins are expressed in Antarctic black fungi as *Cryomyces* and the second endemic genus *Friedmanniomyces*—also under optimal and eutrophic growth conditions. This can be interpreted as a high adaptation and narrow ecological amplitude in comparison to mesophilic but stress-tolerant meristematic fungi as e.g. fungi *Coniosporium* sp. [12] [16] [17] which might correspond to the number of essential proteins necessary for surveillance of this very particular microorganism. As a response to the short growth periods of the Antarctic summer and the extremely low nutrient availability these fungi express only those proteins and cellular functions that are absolutely necessary to survive. Recent studies on the whole genome of the fungus have shown that the genome size is approximately 28 kb and thus in average range for fungi (Sterflinger, unpublished data). The results of this study suggest that only a limited part of the genome is transcribed and most of it might have undergone genome silencing due to the "energy saving mode". Interestingly, HSPs and particular chaperons—which are expressed to compensate the risks of undesired protein denaturation and inactivations—were the most abundant proteins found although the fungus was grown under optimal temperature conditions (15˚C). This indicates that the fungus keeps up a basic level of these protective proteins also under non-stress conditions. On the one hand this could be explained by the fact that the fungus lost the ability to downscale these proteins due to a constant need in the natural environment. On the other hand a basic level of those proteins could enable the fungus to withstand rapid environmental changes without need for fast reactions on the cellular level. Previous studies showed that *C. antarcticus* reacts to rehydration after complete desiccation more slowly than other MCF [12]. This was explained by the fact that *C. antarcticus* has to respond do to long-yearly cycles of anhydrobiosis and its activity is depending on the Antarctic seasons whereas MCFs from more moderate climatic regions have much shorter cycles depending on weather and rain events [11].

After *de novo* sequencing and sequence homology searching in the public domain databases, only a minor portion of protein spots could be identified. The identified proteins belong mostly to very high conserved protein families, which are encoding key proteins guaranteeing the maintenance of the central metabolism for growth and regeneration after state of dormancy. However, the rest of the protein spots remained unidentified, since they show no similarities to the available data. This suggests that the proteome of *C. antarcticus* must comprise a high number of novel proteins with unknown functions. Also the phylogenetic position of the fungus confirms its special role within the fungal kingdom: together with *Cryomyces minteri*, *C. antarcticus* forms a distinct clade within the ascomycetes without any obvious direct ancestor [69].

The better characterization of the hitherto unidentified proteins will be possible after genome annotation of the whole genome and transcriptomic studies which are on the way (Sterflinger, paper in preparation). Since

black fungi have numerous outstanding abilities including the survival of radioactivity and the capacity to use radioactivity as an energy source for ATP generation—so-called "radiotropism" [70]—the whole protein pool might be a treasure box containing novel proteins with interesting and novel functions as well as cell protective agents. These proteins will be of high interest in view of pharmaceutical biotechnological applications and are in the focus of future studies.

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5.4 Fungi in hot and cold deserts with particular reference to microcolonial fungi

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Fungi in hot and cold deserts with particular reference to microcolonial fungi

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ABSTRACT

The occurrence of fungi in soils of the hot deserts and the dry areas of the Antarctic and Arctic are described. A number of filamentous fungi and yeasts have been documented from both – the hot and cold habitat – however, investigations on the abundance and activity of the filamentous hyphomycetes isolated are still missing. There is striking evidence that many ubiquitous species just survive in hot desert soil by their spores, however, without any physiological activity. There is also reasonable suspicion that man-made contamination of the Antarctic deserts might be the reason for finding a broad distribution of *Penicillium* and other ubiquitous fungi in soil and air. Basidiomycetous yeasts are reported to be endemic in the dry valley of Antarctica. Unrivaled conquerors of the desert environment are black, microcolonial fungi that are part of the epi- and endolithic community in hot and cold arid and semi-arid habitats. These fungi are one of the most stress-tolerant eukaryotic life forms on Earth.

Introduction

For a long time desert regions including the hot deserts of North America (Great Basin Desert), Africa (Sahara desert, Kalahari desert), Asia (Gobi desert), the Middle East (Syrian desert), Australia (Great Victoria desert) as well as the permafrost environments up to the cold, dry valleys of Antarctica, Arctic and Patagonia were believed to be too hostile for any forms of life (Cary *et al.* 2010). Today, we know that deserts are full of life with representatives of all domains and throughout all kingdoms of life (Cockell *et al.* 2001). Doubtlessly, deserts together with the deep biosphere provide the most extreme conditions for life on Earth and triggered manifold adaptation mechanisms in plants, animals and micro-organisms (Navarro-Gonzales *et al.* 2003; Direito *et al.* 2011).

The main and most important stress factors in desert regions are: (1) the constant or temporal matric stress due to the extremely limited availability of water; (2) the extreme temperatures and temperature changes; (3) the limited availability of organic carbon; (4) high ultraviolet and infrared irradiation; and (5) osmotic stress whenever raised amounts of salt are present in the soil or on rock surfaces. Characteristic parameters of some typical deserts are listed in Table 1.

Concerning stress tolerance, bacteria and archaea are regarded as the most extremophilic and extremotolerant organisms on Earth. The so called "Strain 121", growing at temperatures up to 121 °C (Kashefi & Lovley 2003) is regarded a marker for the upper limits of temperature for life; species of the genus "*Deinococcus*" are highly tolerant of radioactivity (Makarova & Daly 2011) and many chemolithotrophic bacteria tolerate pH values down to pH1 or up to pH13 (Huang *et al.* 2011; Sanchez *et al.* 2011). These findings led to the general view that prokaryotes – and especially those that are phylogenetically ancient – are much more extremotolerant than any eukaryotic

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Table 1 – Characteristic climate parameters of selected deserts[a]

Desert name	Location/ description	Precipitation [mm/year]	Temperature [°C]	Soil pH	Relative humidity
McMurdo	Antarctica, dry valley cold desert	Snow 3–50 mm (water equivalent)	Av winter −40 °C Av summer ±0 °C Min −49 °C Max 9 °C	Acidic to alkaline pH 4.1–8.4	58–68 %
Arctic desert	Arctic, cold desert	Snow and rain 150–250 mm	Av winter −34 °C Av summer 3–12 °C	Non acidic to acidic <pH 5.5 to >pH 6.5	50–60 %
Atacama	South America, Chile	0.6–2.1 mm some regions without rainfall for years	Av 22 °C (day) and 4 °C (night) not much variation between summer and winter	Acidic > pH 2.0	1.9–3.1 % during the day up to 27 % during the night
Negev	Middle East, Israel	31–200 mm	Av winter 14.2 °C Av summer 25.7 °C max 45 °C min −5 °C	7.6–8.0	<35–40 %
Mojave desert	North America	120 mm	Av winter 12 °C Av summer 30 °C Max 49–54 °C Min 8 °C	Alkaline > pH 8.0	<40 %, occasionally higher after rain events

a Sources: Fountain *et al.* 2009; Piacentini *et al.* 2003; Danmarks Meteorologiske Institut, Center for Ocean and Ice; Fricke *et al.* 2011; Dose *et al.* 2001; Wilhelm *et al.* 2011; Walker *et al.* 1998; Desert Studies Center, California State University.

life form known today. However, all these extremophilic bacteria and archaea live in the aqueous environments or in biofilms formed at the interface between a solid substratum and the aqueous phase, for example, thermal springs, deep sea sediments or alkaline lakes. Nearly all taxa of bacteria and archaea – with the exception of some cyanobacteria (Danin 1983; Buedel *et al.* 2009a, b) – need a relatively constant level of water potential beyond −14.5 MPa. The real challenge to survive in the desert environment is desiccation and neither bacteria nor archaea are specialists of survival under conditions of matric water stress. Some regions in one of the driest and Mars-like environments on Earth – the area around the Yungay station of the hyperarid Atacama desert (Chile) – are nearly free of any cultivable bacteria. In contrast several hyphomycetes survive in this region in a re-cultivable state by the formation thick-walled spores (Conley *et al.* 2006). Some fungi, thus, are really specialized to survive states of complete desiccation, by thick-walled spores, and also to thrive – albeit growing slowly – at extremely low levels of water availability.

Fungi existing in desert areas can be clustered in four main ecological groups: (1) terricolous, epi- and endolithic lichens with ascomycetous and – less frequent – basidiomycetous mycobionts in hot and cold deserts have been extensively studied and reported (Nienow & Friedmann 1993; Wirth 2010; Dojani *et al.* 2011); (2) fungi associated with plants as phylloplane fungi or in mycorrhizal associations – even truffles are able to grow in the Australian outback and the African Kalahari (Trappe *et al.* 2008, 2010); (3) free living hyphomycetes and yeasts living in the soil; and (4) microcolonial fungi (MCF) living as endo- and epilithic fungi. Lichenized and mycorrhizal fungi differ widely from free living hyphomycetes and MCF, both ecologically and in their phylogenetic position and will not be discussed here. The focus of the review is on fungi in groups (3) and (4), i.e. non-symbiotic fungi able to cope with desert conditions without the support of plant, algal or cyanobacterial partners.

Biodiversity of fungi in desert soil

The number of mycological studies on desert soil is rather limited. Several authors assume the diversity of microbes including fungi is low compared to soil in moderate or tropical regions. For this reason they suggest these extreme ecosystems as suitable *in-situ* models to study the relationship between phylogenetic biodiversity and function (Adams *et al.* 2006). Other taxonomic studies demonstrate that the fungal diversity in the soil is remarkably high (Mulder & El-Hendawy 1999; Ciccarone & Rambelli 2000). 185 species were found in the hot desert soil of Makhtesh Ramon desert (Israel) (Grishkan & Nevo 2010). The desert soil myco-biota was dominated by imperfect ascomycetes in all studies, with a clear dominance of the genus *Aspergillus* in many of them. In desert soils from Saudi Arabia and Libya *Aspergillus amstelodami*, *A. chevalieri*, *A. ruber*, *A. ochraceous*, *A. fumigatus*, *A. flavus*, *A. sydowii*, *A. terreus* and *A. ustus* were the most common species (Abdel-Hafez 1982, 1994). The teleomorph genera *Emericella* (*E. nidulans*) and *Eurotium* – with *E. amstelodami* and *E. chevalieri* – and *Chaetomium* are common in desert soils. Many so called "dematiaceous fungi" with darkly-pigmented and rigid-walled spores like *Cladosporium*, *Stachybotrys* and *Pleospora* are frequently found, although to a lesser extent than *Aspergillus* and *Eurotium* (El-Said & Saleem 2008). Species with large spores, like *Alternaria*, *Ulocladium* and *Drechslera* are also frequently found. Some, but not all of the fungi found in hot deserts, are thermophilic comprising ascomycetes and zygomycetous genera, including *Mucor* and *Rhizomucor* (e.g. *Mucor miehei*, *M. thermohyalospora*, *M. thermoaerospora*, *Rhizomucor tauricus* and *R. pusillus*) as well as perfect Ascomycota (*Talaromyces thermophilus*) and imperfect Ascomycota as *Remersonia thermophila* and *Stilbella thermophila* (Mouchacca 1997, 2007). Conley *et al.* (2006) reported the following 12 genera of fungi isolated from the driest locations on Earth – the Atacama

desert – where some regions have no recorded rainfall for decades: *Cladophialophora*, *Cladosporium*, *Leptosphaerulina*, *Alternaria*, *Ulocladium*, *Eupenicillium*, *Aspergillus*, *Penicillium*, *Ascobolus*, *Monodictys*, *Periconia* and *Giberella*. As in other studies, fungi with large, thick-walled and strongly melanized spores like *Cladosporium*, *Alternaria* and *Ulocladium* were clearly predominant. Species of the genus *Aspergillus*, being very abundant in Israel, Saudi Arabia and Libya, were missing in the Atacama soil.

Although myxomycetes are generally associated with moist habitats, Mandeel & Blackwell (2008) found *Comatricha mirabilis* as a frequent inhabitant in the desert of Bahrain. This myxomycete was not found free living in the soil but was growing on bark of large shrubs or trees. Other species, including *Protophysarium phleogenum*, *Badhamnia gracilis* and *Physarum straminipes*, occur in deserts of North America (Sonora and Mojave desert), and in dry regions of Europe and Russia. They are associated with bark of trees or with cortex tissues of the giant cactus (Moreno *et al.* 1998). Where the desert character of soil is combined with high salinity – as for example in saline soils around the Dead Sea, there is selection for halotolerant and halophilic fungi including *Aspergillus terreus*, *A. sydowii*, *A. versicolor*, *Eurotium herbariorum*, several *Penicillium* species and species from the *Cladosporium herbarum* complex as well as *C. sphaerospermum* (Kis-Papo *et al.* 2001).

Soil fungi in hot deserts (Negev) show assimilation patters different from the same species in Mediterranean soils: the utilization rates for plant polymers, like cellulose and xylan, were higher in desert fungi (Oren & Steinberger 2008). This indicates the need for a most effective utilization of each possible organic compound in an extremely oligotrophic environment, with a low abundance of plant material. Seasonal studies on community structure and function in the Chihiahuan desert grassland (a hot desert region in the Mexican Plateau) showed that fungal carbon utilization is more limited by (high) temperature – with higher utilization rates during the winter – than by water availability. Bacterial carbon use – in contrast – was higher in summer (Bell *et al.* 2009). Also the better carbon use efficiency of soil fungi compared to bacteria might support the survival of fungi in such oligotrophic habitats (Keiblinger *et al.* 2010).

There is strong evidence that *Penicillium chrysogenum*, *Phoma* sp. and *Trichoderma koningii* isolated from the desert in Mexico play an important role in breaking seed dormancy in the common desert plant *Opuntia streptacantha* (Cactaceae) (Delgado-Sanches *et al.* 2011). The funinculus of the plant seeds was eroded by the fungi thus decreasing resistance and increasing the ability of swelling and water uptake by the dormant seed.

In cold deserts, for example the dry valleys of the Antarctic, fungi are widely distributed in the soil but with low abundance (Vishniac 1996). Besides fungi that live in mycorrhizal symbiosis with liverworts and mosses (Tosi *et al.* 2002), 27 filamentous fungi in 17 genera were isolated from various locations around Antarctica (Sun *et al.* 1978). These were found particularly around camps, suggesting a man-made contamination of these sites rather than an established fungal community in these soils. Also Connell *et al.* (2006, 2008) state that human activities in the Arctic dry valley might be the reason for the broad distribution of some ubiquitous fungi in soil. The frequent finding of *Penicillium* species, for example, might be due to spores spread from contaminated food or from indoor environments of containers and Antarctic stations.

Endemic fungi were found associated with wood of historic expedition huts on Ross Island (Blanchette *et al.* 2004). In the dry polar desert soils only yeasts were found as endemic species (Vishniac 1993). A clear predominance of basidiomycetous yeasts of the genera *Cryptococcus*, *Trichosporon* and – albeit to a lesser extent – *Malassezia* was reported by Connell *et al.* (2006) and Fell *et al.* (2006). Arenz & Blachette (2011) isolated more that 57 different ascomycetes, 20 basidiomycetes and 16 different species of zygomycetes from soil samples in Antarctica. Their study confirmed the clear dominance of yeasts including the genera *Debariomyces*, *Candida* and *Cryptococcus*. These yeast genera are known to comprise a number of human associated species – either as opportunists or pathogens (de Hoog *et al.* 2000). This raises the question of whether adaptation to extreme environments – especially those with water matric and osmotic stress – is a prerequisite for the evolution of virulence factors in fungi.

It is important to mention that all studies cited above are based on cultivation of fungi onto different media. Today, it is well known that the plate count technique does not reflect the actual abundance of fungi in soil because of its selectivity, because heavily sporulating fungi necessarily dominate the plate counts. It is quite reasonable that most of the fungal cultures from desert soils form highly resistant spores and that the majority of cultivated fungi were from more fertile regions, deposited at the sample sites as wind-borne spores (Conley *et al.* 2006). To overcome the limitations of cultivation and cell counts, studies based on quantitative molecular methods (e.g. qPCR, RT qPCR, AFLP) have to be applied to samples from the desert areas and this could significantly improve the knowledge of biodiversity and abundance of fungi in desert soil. Techniques focussed on the proteome and transcriptome of desert soil samples could help us to understand not only the diversity but the function and metabolic rates of fungi in such habitats (Schneider *et al.*, in press).

Epi- and endolithic fungi: the real specialists of the desert environment

Whereas desert soil might still provide a basic level of organic carbon from primary production by plants and cyanobacteria, and can retain water for a short time after a rain event, exposed rock in hot and cold deserts is an even more extreme environment. In fact, rock in desert regions might be the most stressful environment on Earth. Surfaces of exposed rock reach temperatures from −45 °C up to 60 °C, infrared and ultraviolet radiation is high, organic carbon is rare and water might only be available as dewfall or in intercrystalline form under layers of snow and ice. Despite this, biofilms on rocks are complex communities, the evolution of which traces back to the early beginnings of life on earth (Gorbushina *et al.* 2002, 2007; Omelon 2008). Rock provides a suitable substratum for organisms because mechanical abrasion – as compared to soil and soil crusts – is slow and organisms attached to it stable.

Second, rock has crevices, cracks and pores that are suitable microclimatic caves for microbes. Together with lichen and cyanobacteria, a peculiar group of fungi are specialists for colonization of rock in hot and cold deserts (Fig 1A–C). Rock inhabiting fungi form black, clump-like colonies on the rock surface and inside of the pores and fissures of rock (Fig 1A, B). They are able to penetrate igneous hard rocks, like granite and basalt as well as sedimentary soft rock such as limestone, sandstone or marble (Sterflinger & Krumbein 1997; Sterflinger 2000). A typical example of a black fungus habitat is rock in the Arctic (Fig 1). Until the early 1980s the rock inhabiting fungi in desert and semi-arid areas were either misinterpreted as dirt or fly ash particles, or they were overlooked because of the striking similarity with cyanobacteria – nodules similar to meristematic fungi (MCF), for example, are formed by the genus *Nostoc* (Danin 1983, 1992).

First reports on black fungi with aggregated micro-colonies in deserts were published by Staley *et al.* (1982) for the hot deserts of Arizona, and by Friedmann for the cold deserts of Antarctica (Friedmann 1982; Friedmann *et al.* 1987). In the 1980s other scientists started to grow these fungi in the laboratory and opened a completely new and exciting field of mycological science: the taxonomic and phylogenetic characterization of black, epi- and endolithic MCF and black yeasts.

With a rapidly increasing scientific interest and effort to detect and isolate these organisms, black fungi emerged to have a worldwide distribution wherever environmental conditions are extreme due to extreme temperature, low nutrient availability, high radiation and lack of water: the deserts of Arizona and Negev, the semi-arid areas of the Mediterranean (Sterflinger & Gorbushina 1997; Blazquez *et al.* 1997; Sterflinger *et al.* 1997, 1999; Sterflinger 2000, 2005; Ruibal *et al.* 2005, 2008; Sert *et al.* 2007), the dry Antarctic valleys, the rocks and glaciers in the Arctic and high alpine regions. Isola (2010) isolated black fungi from samples collected by alpinists on the 8 611 m peak of the K2 (Himalaya). The temperature on this summit occasionally falls to −60 °C, the UV irradiation is very high and the oxygen is decreased to 50 mm Hg (as compared to 160 mm Hg on sea level). The most extensive work on diversity and taxonomy of MCF from the Antarctic environment was done by Onofri & Selbmann who isolated and described strains from the Antarctic desert and introduced the cryophilic fungal genera *Friedmanniomyces* with *F. simplex* and *F. endolithicus*, and *Cryomyces* with *C. minteri* and *C. antarcticus* (Zucconi *et al.* 1996; Onofri & Friedmann 1998; Onofri 1999; Onofri *et al.* 1999; Selbmann *et al.* 2005).

Black, rock inhabiting fungi – together with some lichens – are today assumed to be the most stress resistant eukaryotic organisms known on Earth (de Hoog & Grube 2008). Recent experiments showed that their stress resistance against solar radiation, radioactivity, desiccation and oligotrophic conditions even allows them to survive space and Martian conditions (Onofri *et al.* 2008). For this reason black fungi are now model organisms for Astrobiology (Onofri *et al.* 2004b) and for gamma radiation experiments. Black fungi are not only resistant to high levels of radioactivity, they even benefit from it, being able to use radioactivity as an energy source for ATP generation (Dadachova & Casadevall 2008).

From the taxonomic point of view, black fungi are a polyphyletic group of melanized, slow growing filamentous or yeast-like fungi also called black yeasts. The terms “meristematic fungi” and “microcolonial fungi” (MCF) are used with respect to their prominent morphological characteristics (Sterflinger 2005) (Fig 2). Black fungi share a number of universally present characters such as strong melanization, thick and even multi-layered cell walls (Fig 3), and exopolysaccharides production resulting in an extraordinary ability to tolerate chemical and physical stresses (Sterflinger 2005).

The molecular phylogeny and taxonomy of black fungi have been extensively studied since 1997 when some were described based on DNA sequencing data (Sterflinger *et al.* 1997; de Hoog *et al.* 1999). Although they have a very limited morphological diversity, DNA sequencing has shown that the genus and species diversity is high. The ability to form so called “meristematic” cells and to proliferate by budding is polyphyletic and occurs in several orders of the Euascomycetes (Fig 4) namely the *Chaetothyriales*, *Dothideales*, *Capnodiales*, *Pleosporales* and *Hysteriales* (Selbmann *et al.* 2005).

Ecology of microcolonial fungi

Survival limits have been investigated in black fungi. Various cultivation experiments have shown their enormous heat and

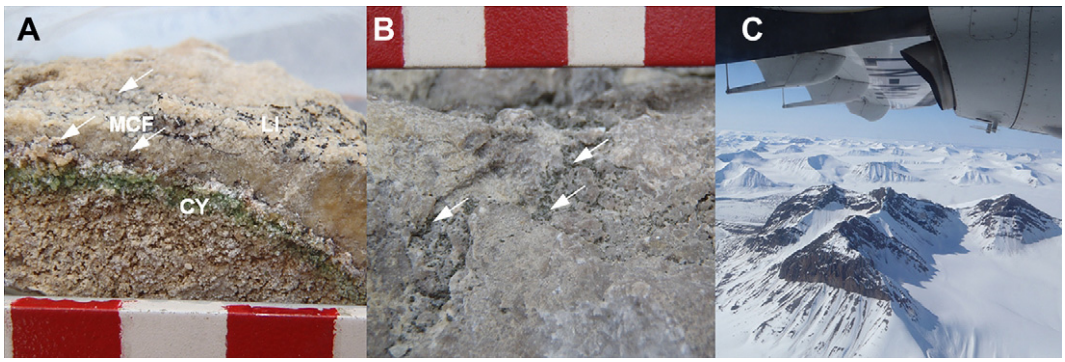

Fig 1 – (A) Epi- and endolithic rock community in the Arctic environment with black fungi (MCF) and lichen (LI) on the top and in the first mm of the rock, and cyanobacteria (CY) within the rock (bar 1 cm). (B) Rock surface in Svalbard (Arctic) with fungal colonies (MCF) located in depressions and fissures. (C) Arctic landscape seen from above; the mountains are typical habitat for black fungi (bar 1 cm). The arrows are indicating colonies of black fungi (photos: Sterflinger).

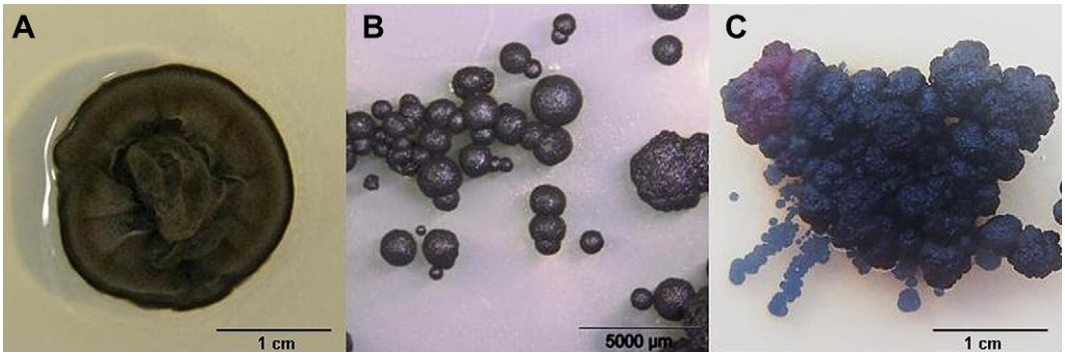

Fig 2 – Typical colony morphologies of MCF grown on agar plates: (A) *Coniosporium perforans*, (B) *Friedmanniomyces endolithicus*, (C) *Cryomyces minteri*.

acid tolerance, their ability to cope with high levels of UV radiation and even radioactivity as well as the halophilic ecology of some species (Urzì *et al.* 2000; Onofri *et al.* 2004a, b; Gorbushina *et al.* 2008). Species of *Capnobotryella*, *Coniosporium*, *Exophiala*, *Sarcinomyces*, *Hortaea*, *Taeniolella* and *Phaeotheca* survive 120 °C for at least 30 min when they are in a completely desiccated state. In a fully hydrated state the lethal temperature varies from 35 °C – for some strains of *Capnobotryella* – to 75 °C – for black yeasts (Sterflinger 1998). *Recurvomyces mirabilis*, *Elasticomyces elasticus* and *Hortaea acidophila* survive and grow at pH values down to 0 (Hölker *et al.* 2004; Selbmann *et al.* 2008). *Trimmatostroma salinum* was first isolated from salterns in Slovenia and it was the first fungal species that was shown to thrive in the low water potential environment created by pure salt (Zalar *et al.* 1999). Also species of *Wallemia* – a melanized fungus forming microcolonies – are representative of eukaryotic organisms that have successfully adapted to life in extremely saline environments (Kuncic *et al.* 2010). The reactor of the power plant in Chernobyl contained many black fungi the growth of which is enhanced by raised radioactivity (Dadachova & Casadevall 2008). Onofri *et al.* (2008) exposed strains of *Cryomyces* and *Friedmanniomyces* to simulated outer space conditions and these strains survived, and were able to grow after transfer to normal laboratory conditions.

Ecological and physiological data, indicate that MCF thrive in two main habitats which demand different degrees of specialization and allow different ecological amplitudes as follows:

(1) One habitat typical for black fungi is the rock surfaces in hot deserts and moderate climates. Here, meristematic fungi, many of which have the ability to form mycelium under laboratory conditions as well as to proliferate by budding, are predominant. They are thermotolerant but neither thermo- nor cryophilic and they thrive in moderate to dry environments, like low Alpine and Mediterranean areas, however, with high UV radiation due to sun exposure. The genera *Coniosporium*, *Sarcinomyces* and *Capnobotryella* are best known in this group. The group is hypothesized to have a fairly broad ecological plasticity reflected by their ability to withstand extreme conditions but to react to moderate conditions by faster growth and by morphological changes from meristematic towards mycelial growth (Tesei *et al.* under review).
(2) The second typical habitat is the cold deserts in Polar and high mountain regions. The Polar MCF are highly specialized to the cold and are real extremophiles amongst the black fungi. Species are cryophilic – with growth optima around 12 °C – and might have lost the ability to react to

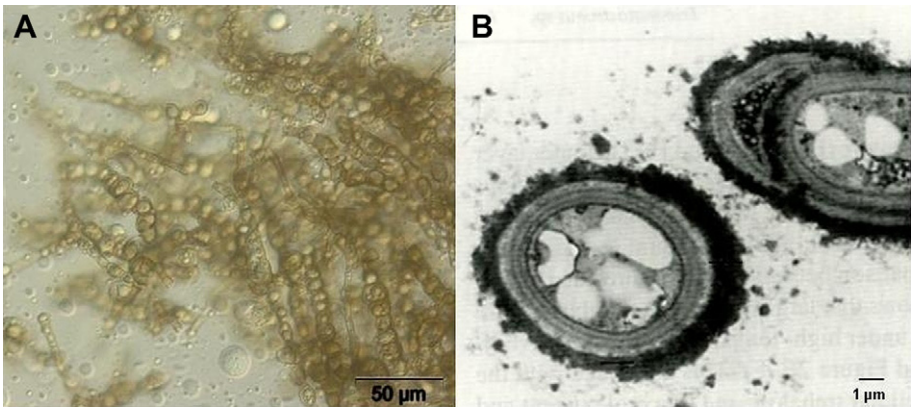

Fig 3 – (A) Meristematic growth and (B) TEM photograph of microcolonial fungi showing the multi-layered cell wall (photos: Sterflinger).

Fig 4 – Phylogenetic tree based on 1 700 positions of small subunit ribosomal DNA (tree modified from Sterflinger 2005). MCF growing on rock are highlighted. Bootstrap values were generated from 1 000 trees using the Felsenstein method.

moderate conditions and raised levels of nutrients by faster growth. Microcolonial growth seems to be obligate in this group since no change to mycelial growth has been observed. *Cryomyces* and *Friedmanniomyces* – both originally isolated from Antarctic rocks – are the largest genera representing this group (Selbmann *et al.* 2005; Isola 2010).

Some species of the first ecological group e.g. *Sarcinomyces petricola* and *Coniosporium perforans*, form yeast-like states during their life cycle and might turn into complete yeast-like growth after several transfers onto laboratory media. In this case MCF resemble a third group of black fungi only some members of which are found on rock surfaces: the black yeast in the genera *Exophiala*, *Fonsecea*, *Capronia*, *Phaeococcomyces* and *Cladophialophora*.

Since the publication of the first phylogeny of black fungi, where it was shown that the meristematic growth morphology is polyphyletic and that the MCF stains from different desert and semi-arid areas are members of different orders within the ascomycetes (Sterflinger *et al.* 1997, 1999), the taxonomy and phylogeny of MCF was extensively studied. As mentioned above, the extreme stress tolerance of the fungi was shown in experimental approaches simulating temperature stress, desiccation and even cosmic radiation. However, the cellular mechanisms underlying the extraordinarily high stress tolerance and adaptations of black fungi have not been investigated yet. The only factors that are known to be involved in the stress adaptation are:

(1) The strong melanization of the multi-layered cell walls (Figs 2 and 3). Melanin plays a very important role not only in UV-protection but also in desiccation and radiation tolerance. In *Aspergillus niger*, a common fungus also in dry soil, it was shown that melanin concentration differs in strains from different solar UV radiation pressure (Singaravelan *et al.* 2008). Seasonal patters from deserts in Israel showed that fungi with strongly melanized spores dominated the cell counts in summer, thus indicating the better survival of these fungi during the most extreme periods of the year (Grishkan & Nevo 2010).
(2) Carotenoids – β-carotene, ζ-carotene, phytoene, torularhodin and torulene – were stated to have some influence on stress tolerance of black fungi due to their ability to filter UV light, to be antioxidant and to stabilize membranes (Gorbushina *et al.* 2008).
(3) The production of polyols and trehalose under stress conditions. Sterflinger (1998) showed that the cellular level of glycerol inside of cells of *Coniosporium* sp., *Sarcinomyces* sp., *Capnobotryella* sp. and other strains of black fungi is increased with increasing salinity of the growth medium (nearly all black fungi tested tolerate 7 % NaCl, halophilic species, such as *Hortaea werneckii,* grow in saturated NaCl solutions). Polyols serve as intracellular osmolytes and thus stabilize the osmotic potential of the cells. Trehalose stabilizes enzymes and membranes and plays a major role in anhydrobiotic organisms that can survive complete desiccation (Crowe *et al.* 1984) under raised temperatures as well as in desiccated cells.
(4) The meristematic growth morphology through which an optimized surface-volume ratio can be achieved. The fungi form aggregated colonies without any hyphae. The cells in the colony are rather round to ovoid and build up colonies by isodiametric enlargement and subsequent division into separate cells (Wollenzien *et al.* 1995). Black fungi usually form colonies on the top of rock surfaces but they can also form extremely thin hyphae in order to penetrate fissures, crevices and pores of the rock. The penetration of the rock by these hyphae is then followed by growth of a new colony within rock.
(5) The ability to survive long periods of complete desiccation as prerequisite to withstand high temperatures (Sterflinger 1998).
(6) The absence of sexual reproduction. Until now no sexual reproduction has been seen in the life cycle of MCF. However, no research has been carried out to look for mating type genes in these fungi.
(7) The partial or complete absence of spores or conidia. Interestingly various studies on hyphomycetes in desert soils have shown that fungi with big, segmented and darkly-pigmented spores are found in desert soil – e.g. *Alternaria* and *Ulocladium* and *Cladosporium*. The similarity with the meristematic colonies of MCF is obvious. However, in MCF each single vegetative cell serves as a survival and dispersal state.

Until now, no studies have been carried out that analyze the ecological differences and analogies among the MCF from hot and cold deserts, nor to study stress resistance in relation to taxonomy, nor on the molecular level. Investigations of protein expression under stress conditions and on expression of heat shock proteins (HSPs) as protectants of enzymes, membranes or DNA are in progress (Tesei *et al.* under review), and a basic protocol for 2-dimensional gel electrophoresis in MCF was developed recently (Isola *et al.* 2011). Also, the existence of Mn-dependent antioxidant proteome shields, that were only recently described as main factors preventing protein damage in radiotolerant bacteria (*Deinococcus radiodurans*, Daly *et al.* 2004, 2010), might play a role for black fungi. This hypothesis is supported by the fact that desert fungi were associated with the formation of desert varnish, a dark crust rich in manganese and iron, and commonly found on desert rock (Krumbein & Jens 1981).

A second important question that has never been asked nor answered for the black fungi concerns metabolic activity versus dormancy and active growth phases in the natural environment. This is directly connected with the topic of ecological plasticity and adaptation to the extreme as described above. It is assumed, and supposed by field observations, that the fungi have slow growth rates in nature but it is not known under which conditions of humidity and temperature they are actually able to grow. In the polar environment it is likely that the fungi are in dormant states for most of the year and that they are only active during the short summer period when temperature rises and melting water is available. On the other hand, hot desert fungi might profit from dewfall that develops in deserts during a short time before sunrise (Ninari & Berliner 2002). This process would necessitate a very fast re-hydration and up-regulation of metabolic activity. For epi- and endolithic lichen species it was demonstrated that they can gain full metabolic activity

instantaneously (Liden et al. 2010) and that dewfall might be the only source of humidity, thus allowing the lichen a limited period of metabolic activity each day (Buedel et al. 2008). Moreover, a fast re-activation of metabolism could be one of the most important features to thrive and survive in desert environments (Weber et al. 2007). For rock inhabiting black fungi it is not known if they are able to gain full activity from dewfall, nor if their metabolic rhythm is daily, seasonally or both, and during which period net growth of the colonies is possible.

The ecological function of MCF in their environment is not yet understood. Certainly MCF are important agents of bio-weathering and mineral cycling in arid and semi-arid areas where less stress-tolerant biodeteriorative agents like bacteria and algae are missing (Sterflinger 2000; Fomina et al. 2006; Gadd 2007; Cutler & Viles 2010).

Conclusions

Fungi are common in desert soil and as epi- and endolithic organisms in both hot and cold environments. Hyphomycetes were detected in desert soil based on cultivation experiments and may be present in those soils as spores. However, information about their metabolic activity and about spore germination and mycelium formation is lacking. In future investigations it will be necessary to apply molecular methods in order to quantify fungal DNA – e.g. by qPCR – and to evaluate fungal activity by RNA analysis – e.g. using RT qPCR or micro-array-based expression profiling. There is also demand for studying ecophysiology in extremotolerant fungi. The stress resistance and adaptation mechanisms in the most extreme fungi – the rock inhabiting MCF – is almost unexplored. To understand the cellular stress response, fungi should be studied under simulated stress conditions and be investigated on the proteome, transcriptome and metabolome level. Due to their enormous stress tolerance, desert fungi could also be a promising source for new biotechnological and medical adaptations, as for example protective agents against oxidative stress.

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5.5 Alteration of protein patterns in black rock inhabiting fungi as a response to different temperatures

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Alteration of protein patterns in black rock inhabiting fungi as a response to different temperatures

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ABSTRACT

Rock inhabiting fungi are among the most stress tolerant organisms on Earth. They are able to cope with different stressors determined by the typical conditions of bare rocks in hot and cold extreme environments. In this study first results of a system biological approach based on two-dimensional protein profiles are presented. Protein patterns of extremotolerant black fungi – *Coniosporium perforans*, *Exophiala jeanselmei* – and of the extremophilic fungus – *Friedmanniomyces endolithicus* – were compared with the cosmopolitan and mesophilic hyphomycete *Penicillium chrysogenum* in order to follow and determine changes in the expression pattern under different temperatures. The 2D protein gels indicated a temperature dependent qualitative change in all the tested strains. Whereas the reference strain *P. chrysogenum* expressed the highest number of proteins at 40 °C, thus exhibiting real signs of temperature induced reaction, black fungi, when exposed to temperatures far above their growth optimum, decreased the number of proteins indicating a down-regulation of their metabolism. Temperature of 1 °C led to an increased number of proteins in all of the analysed strains, with the exception of *P. chrysogenum*. These first results on temperature dependent reactions in rock inhabiting black fungi indicate a rather different strategy to cope with non-optimal temperature than in the mesophilic hyphomycete *P. chrysogenum*.

Introduction

'Exposure of cells to suboptimal growth conditions or to any environment that reduces cell viability or fitness can be considered stresses' (de Nadal *et al.* 2011). Stress has been classified as either biotic or abiotic, these including thermal (hot or cold) and non-thermal stress, such as acid, water, or pressure (Mafart *et al.* 2001). Both the physiological state and the natural environment in which an organism has been evolutionarily selected, influence its adaptive responses and rapid adaptations are crucial to maximizing cell survival (de Nadal *et al.* 2011). Eukaryotic cells have evolved sophisticated cellular mechanisms in response to the stresses that regulate several aspects of cell physiology as e.g. gene expression,

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metabolism, cell cycle progression, cytoskeletal organization, protein expression and homeostasis, and modification of enzymatic activity. These stress tolerance responses can generate both immediate and long-term adaptations, which are especially crucial for the survival of organisms in environments with extreme physicochemical parameters. Within eukaryotes, a specialized group of fungi – the black yeasts and microcolonial fungi (MCF) – have been identified as conquerors of an extremely stressful habitat: the bare rock in hot and cold environments (Staley *et al.* 1982; de Hoog & Grube 2008; Sterflinger *et al.* 2012).

Due to their stress tolerance, MCF and black yeasts have a wide distribution that includes some of the most extreme environments of the Earth as well as extraterrestrial conditions (Onofri *et al.* 2012). Originally black fungi – also named dematiaceous fungi – were described as inhabitants of living and dead plant material. However, in the last 30 y they have been isolated from hypersaline waters (Gunde-Cimerman *et al.* 2000), acidic environments (Baker *et al.* 2004), radioactive areas (Dadachova *et al.* 2007), as human pathogens or opportunists (Matos *et al.* 2002) and as a dominant part of the epi- and endolithic microbial communities (Friedmann 1982; Sterflinger 2000; Burford *et al.* 2003; Ruibal *et al.* 2005; Sert *et al.* 2007; Selbmann *et al.* 2008). Together with cyanobacteria and lichens, they contribute to the global biogeochemical cycling by active weathering of natural rocks and stone monuments (Sterflinger & Krumbein 1997).

These habitats share some important characteristics: osmotic stress, UV and oxidative stress and rapid variation of temperature, water supply, and nutrient availability (Sterflinger *et al.* 1999; Vember & Zhdanova 2001; Sterflinger 2005). To withstand these changes, organisms living in such environments need either permanently existing or exceptionally fast adaptive cellular or metabolic responses. Although MCF and black yeasts are a diverse taxonomic group having polyphyletic origins within the Ascomycota, they have similar morphological and physiological characters. These similarities were interpreted as a 'principle of uniformity' by Urzí *et al.* (2000) being an obligate basis to tolerance of physical and chemical stress on rock and plant surfaces. Slow growth rates, an optimal surface/volume ratio of the cauliflower-like colonies, thick and strongly melanised cell walls, exopolysaccharides production, the high intracellular content of trehalose, and polyoles as well as lack of sexual reproductive structures, are considered as adaptations to the extreme environments (Sterflinger 1998; Selbmann *et al.* 2005; Onofri *et al.* 2007; Gostinčar *et al.* 2010).

Temperature is undoubtedly one of the major factors affecting the growth and survival of any microorganism (Deegenaars & Watson 1998): for this reason it is of great interest to investigate how MCF and black yeasts withstand temperatures that are significantly out of their growth range. Unlike in other Ascomycetes as *Neurospora crassa*, *Candida albicans*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Kraus & Heitman 2003; Bahn *et al.* 2007; Alonso-Monge *et al.* 2009), the stress-response mechanisms of MCF have not yet been investigated, either on the genomic or on the proteomic level. A very recent investigation has revealed the complexity of protein composition in cosmopolitan fungi as *Penicillium chrysogenum* and *Aspergillus* sp. (Jami *et al.* 2010a; Rizwan *et al.* 2010). Further studies that analyse the ecological differences and analogies among these fungi in a systematic approach and on the molecular level are missing. The production of molecular chaperons (MC), so called 'heat shock proteins' (HSPs), small HSPs, and also 'cold shock proteins' (CSPs) belong to the most important stress reactions of cells in general (Becker & Craig 1994; Albanese *et al.* 2006; Nakamoto & Vígh 2007; Nevarez *et al.* 2008) and are known to represent the main effect to temperature stress in mesophilic fungi such as *P. chrysogenum* (Raggam *et al.* 2011). Thus, protein expression profiling was chosen as the first tool to shed light on the biological response of MCF and black fungi towards suboptimal temperatures.

The main goal of the present paper was to reveal if black fungi and mesophilic hyphomycetes present a similar reaction to temperature stress, as reflected by the protein patterns. Three strains of black rock inhabiting fungi were chosen for this study: *Exophiala jeanselmei* MA 2853, *Coniosporium perforans* MA 1299 and *Friedmanniomyces endolithicus* CCFEE 5208. Fungi were grown at different temperatures and the protein profiles were analysed in comparison with each other and with *Penicillium chrysogenum* (strain MA 3995), as reference strain.

Materials and methods

Fungal strains

Three strains of black fungi, clustering within two different orders of Dothideomyceta (Chaetothyriales and Capnodiales), were used in the present study (Fig 1). The isolates were selected according to their bio-ecological characteristics. They all colonize rock epi- or endolithically but they have a diverse geographical distribution:

(1) *Exophiala jeanselmei* (MA 2853) is a mesophilic black yeast detected as a frequent colonizer of rock in moderate climates (Warscheid & Braams 2000; Sterflinger & Prillinger 2001). It has a close phylogenetic and physiological relation to human opportunists and pathogens (de Hoog 1993) which makes this strain a highly interesting model to study the evolution of virulence (Gostinčar *et al.* 2011).
(2) *Coniosporium perforans* (MA 1299) is a widely distributed microcolonial rock inhabitant fungus in both moderate and Mediterranean climates (Sterflinger *et al.* 1997; M. Owczarek, in preparation). Although it can be considered as mesophilic with respect to its growth optimum, this strain has a remarkable high temperature and desiccation tolerance (Sterflinger 1998).
(3) *Friedmanniomyces endolithicus* (CCFEE 5208) is a psychrophilic fungus with an outstanding and unique ecology and phylogeny. The considerable sequence deviation from known taxa, reflected by the phylogenetically isolated position, suggest *F. endolithicus* as an endemic species for the Antarctic (Selbmann *et al.* 2005), where it occurs cryptendolithically in rocks, having a strong degree of extremotolerant specialisation (Onofri *et al.* 1999).

The mesophilic hyphomycete *Penicillium chrysogenum* (MA 3995) was chosen as a reference strain since it is very well

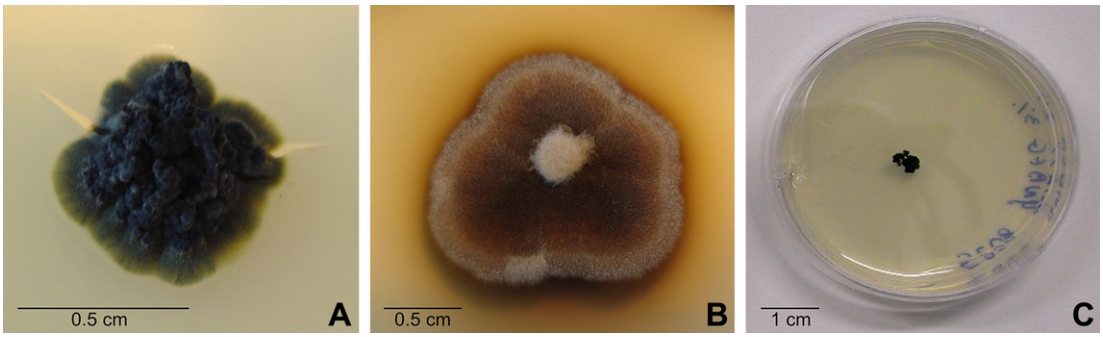

Fig 1 – Colony morphology of the analysed black fungi grown on MEA: (A) *Coniosporium perforans* MA 1299; (B) *Exophiala jeanselmei* MA 2853 (photos Tesei D); and (C) *Friedmanniomyces endolithicus* CCFEE 5208 (photo Selbmann L).

characterized under different growth conditions in nature and at laboratory scale. Further, the ecology and proteome of *P. chrysogenum* has been studied extensively (Tresner & Hayes 1971; Dantigny *et al.* 2007; Jami *et al.* 2010a, 2010b).

Exophiala jeanselmei, *C. perforans*, and *P. chrysogenum*, were obtained from the Austrian Center of Biological Resources and Applied Mycology culture collection (ACBR, Vienna, Austria; www.acbr-database.at). *Friedmanniomyces endolithicus* was provided by the Culture Collection of Fungi from Extreme Environments (CCFEE, Università della Tuscia, Viterbo, Italy; http://www.sma.unitus.it/index.php/museo-nazionale-del-lantartide-sezione-micologica.html).

Thermal preferences, cultivation, and exposure conditions

Temperature optima and growth rates were tested for all *Exophiala jeanselmei*, *Coniosporium perforans*, and *P. chrysogenum*. Data for *Friedmanniomyces endolithicus* were previously published by Selbmann *et al.* (2005). Strains were inoculated on malt extract agar (2 %, MEA (Malt Extract Agar)) and incubated for a maximum of 21 d at 0, 5, 10, 15, 20, 25, 28, 30, 35, 37 °C. The diameter of the colonies was recorded each day. All tests were performed in triplicate.

For further experiments, 28 °C was chosen as incubation temperature for *E. jeanselmei*, *C. perforans*, and *P. chrysogenum* mainly because this is standard incubation temperature in microbiology and it was still in the growth range of all three fungi. Since *F. endolithicus* does not grow above 15 °C and its growth optimum is within the range 10–15 °C (Selbmann *et al.* 2005), the incubation was performed at 15 °C. All the isolates were grown on 2 % MEA for 4 weeks in order to obtain enough biomass for protein extraction.

For stress simulation fungi were exposed to 1 °C and to 40 °C for 1 week; *F. endolithicus* was exposed to 1 °C and 28 °C. The viability of the colonies was evaluated after temperature treatment for 12, 24, 48, 72 h, and after 1 week. Biomass for protein profiling was harvested by scratching the material from the plates using a scalpel, immediately frozen and stored at −80 °C until protein extraction.

Protein extraction

Protein extracts were obtained as described by Isola *et al.* (2011). Briefly, cell disruption was performed by a mechanical method adding an EDTA based lysis buffer. Precipitation was based on phenol: 3 ml of Tris–buffered phenol solution pH 8.0 (Sigma–Aldrich, Steinheim, Germany) were added to every sample after the mechanical disruption, in a 15 ml polypropylene centrifuge tube. Centrifugation at 7834× *g* for 10 min at 4 °C was performed in order to separate the phenolic phase, subsequently transferred to a new pre-weighed tube. Five volumes of ice-cold 0.1 M ammonium acetate in methanol was added and after overnight precipitation (−20 °C), the protein pellet was obtained by centrifugation at 7834× *g* for 30 min at 4 °C. After washing it with ice-cold methanol (absolute) and then with ice-cold acetone (80 % v/v), the dried protein pellet (−20 °C) was suspended in Modified Sample Buffer (MSB) according to final pellet weight. The protein concentrations were determined by the Bio-Rad protein assay (BioRad Lab., Hartfordshire, USA) by establishing a standard curve using serial dilutions from 0.8–100 μg/ml^{-1} of bovine serum albumin (Thermo Scientific, Rockford, IL, USA).

2D gel electrophoresis

For each temperature tested (1, 15, 28, 40 °C) two technical replicates were performed. The 13 cm strips (IPG™ DryStrip 3-10 NL, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were rehydrated in a total volume of 255 μl rehydration buffer [8 M urea, 2 % (w/v) CHAPS, 10 mM dithiotreitol (DTT), 0.1 % bromophenol blue, and 0.5 % (v/v) Servalyte] including 20 μg of protein, at room temperature and for 16 h. Isoelectric focussing was carried out according to manufacturer instructions at 20 °C and a total of 14 kV h, using a Protean IEF cell system (Bio-Rad Hartfordshire, USA). The strips were re-equilibrated for 15 min under gentle shaking in 2 ml equilibration solution [50 mM Tris–HCl pH = 8.4, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) sodium dodecyl sulphate (SDS)], with 2 % (w/v) DTT and subsequently, for 15 min in 2 ml equilibration solution [50 mM Tris–HCl pH = 6.8, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS], with 2.5 % (w/v) iodoacetamide (IAA) and trace of bromophenol blue (Bjellqvist *et al.* 1993). Second dimension was performed in 10 % (w/v) SDS polyacrylamide gel electrophoresis (SDS-PAGE, 14 cm × 14 cm) with running buffer [24 mM Tris pH = 8.3, 192 mM glycine, 0.1 % (w/v) SDS]. For the electrophoretic run 160 V and variable mA were applied using the Perfect Blue Twin Gel System (PeqLab GmbH, Erlangen, Germany). The chamber was cooled at 4 °C (type CBN 8-30, Heto, Birkerød, Denmark).

Staining and analysis of 2D gels

Protein spots were visualized by a high sensitive mass spectrometric compatible silver staining (Shevchenko *et al.* 1996). The gels were fixed in 50 % (v/v) methanol and 5 % (v/v) acetic acid for 20 min, then washed in 50 % (v/v) methanol for 10 min and rinsed with MilliQ water (Millipore, MA, USA) overnight at 4 °C. Subsequently, the 2D gels were sensitized using a 0.02 % (w/v) sodium thiosulphate solution for 1 min and then incubated in 0.1 % (w/v) silver nitrate solution for 20 min at 4 °C, rinsing twice with MilliQ water for 1 min each, after incubation. The gel development was carried out by the incubation in 0.04 % (v/v) formalin and 2 % (w/v) sodium carbonate solution until the desired intensity of staining was achieved. Gels were washed with a 5 % (v/v) acetic acid. All the washing solutions used were prepared in MilliQ water (Millipore, MA, USA).

Stained gels were scanned in TIFF 16 bit format. Image Master 2D Platinum version 5.0 (Amersham Biosciences, Swiss Institute of Bioinformatics, Geneva, Switzerland) was used for spot-matching and image analysis. The spots intensity, densitometrically determined and expressed as spot volume, was evaluated (see Supplementary material for examples). Comparison reports of the qualitative differences of the samples were generated and served for the evaluation of the presence/absence of protein spots under the tested temperatures.

Results

Thermal preferences

As shown in Table 1, for *Exophiala jeanselmei*, *Coniosporium perforans*, and *Penicillium chrysogenum* the upper temperature limit was 30 °C, thus 40 °C can be considered as serious stress for these organisms. Since the *Friedmanniomyces endolithicus* growth limit is 15 °C (Selbmann *et al.* 2005), 28 °C, that were applied as uppermost temperature for the treatment of this fungus, can be also considered as serious stress.

Table 1 – Thermal preferences of model fungi. Thermal preferences have been reported as diameter of colonies (in cm) as the average of three different tests. MA Nr: strain number in the ACBR/BOKU Vienna culture collection.

Strain	Thermal preferences (°C)									
	0	5	10	15	20	25	28	30	35	37
P. chrysogenum MA 3995	0.50	0.93	1.50	1.90	2.80	2.95	1.30	0.75	_	_
E. jeanselmei MA 2853	_	0.40	0.75	1.20	1.40	1.70	1.60	1.25	_	_
C. perforans MA 1299	_	_	_	0.65	1.10	0.70	0.70	0.65	_	_

Thermal preferences have been reported as diameter of colonies (in cm) as the average of three different tests. MA Nr: strain number in the ACBR/BOKU Vienna culture collection.

Analysis of protein patterns

Each 2D protein gel – for each fungus and each of the temperatures tested – was carried out in duplicate resulting in a total of 24 gels. From the two technical replicates the gel exhibiting the highest number of spots was used for the following analysis: (1) to evaluate if the black fungi respond towards different temperatures by a change in the protein pattern; (2) to compare the changes of the protein patterns of the black fungi with *Penicillium chrysogenum* as reference; (3) to compare the changes of the protein patterns of the mesophilic fungi *Exophiala jeanselmei* and *Coniosporium perforans* with the extremophilic fungus *Friedmanniomyces endolithicus* as reference. For each comparison, after spot detection, the gels were aligned and matched to the reference gel. The analysis of groups of matching spots allowed the evaluation of changes and similarities in protein expression patterns.

2D protein patterns at different temperatures

At all conditions tested the protein pattern of the four fungal strains differed concerning the total number of spots (Table 2), their molecular weight as well as their isoelectric point [pI]-related distribution. At 28 °C the number of spots was 381 in *Penicillium chrysogenum*, 382 in *Exophiala jeanselmei*, and 325 in *Coniosporium perforans*; at 15 °C the number was 425 in *Friedmanniomyces endolithicus*. In *E. jeanselmei* the major protein spots had molecular weights within 70 and 170 kDa and pIs from 6 to 10 while *C. perforans* and *P. chrysogenum* expression profiles showed a high number of spots having molecular weight within 50 and 10 kDa. In *F. endolithicus* mainly protein spots with pI between 3 and 8 and molecular weights from 25 to 170 kDa, were detected.

The exposure of *P. chrysogenum* to different temperatures influenced both the expression pattern and the spots abundance (Fig 2, A–C). At 1 °C spots decreased from 381 to 358 and mainly proteins with basic pIs (8–10) were missing. At 40 °C, the number of detected protein spots increased to 601. Overlapping the gels obtained from three temperatures showed that 153 spots were matching at 28 °C and 1 °C while 211 spots were matching at 28 °C and 40 °C, thus indicating a greater similarity of proteins at the higher temperature range. A number of 100 protein spots was found to match at all temperature (Table 3).

Exophiala jeanselmei exposed to low temperature (Fig 2, D–F) showed no significant variations in the absolute number of

Table 2 – Number of protein spots detected in the 2-DE gels of the analysed strains at each exposure condition.

Strains	Number of spots at each exposure condition		
	28 °C	1 °C	40 °C
P. chrysogenum MA 3995	381	358	601
E. jeanselmei MA 2853	382	387	174
C. perforans MA 1299	325	494	255
	15 °C	1 °C	28 °C
F. endolithicus CCFEE 5208	425	466	284

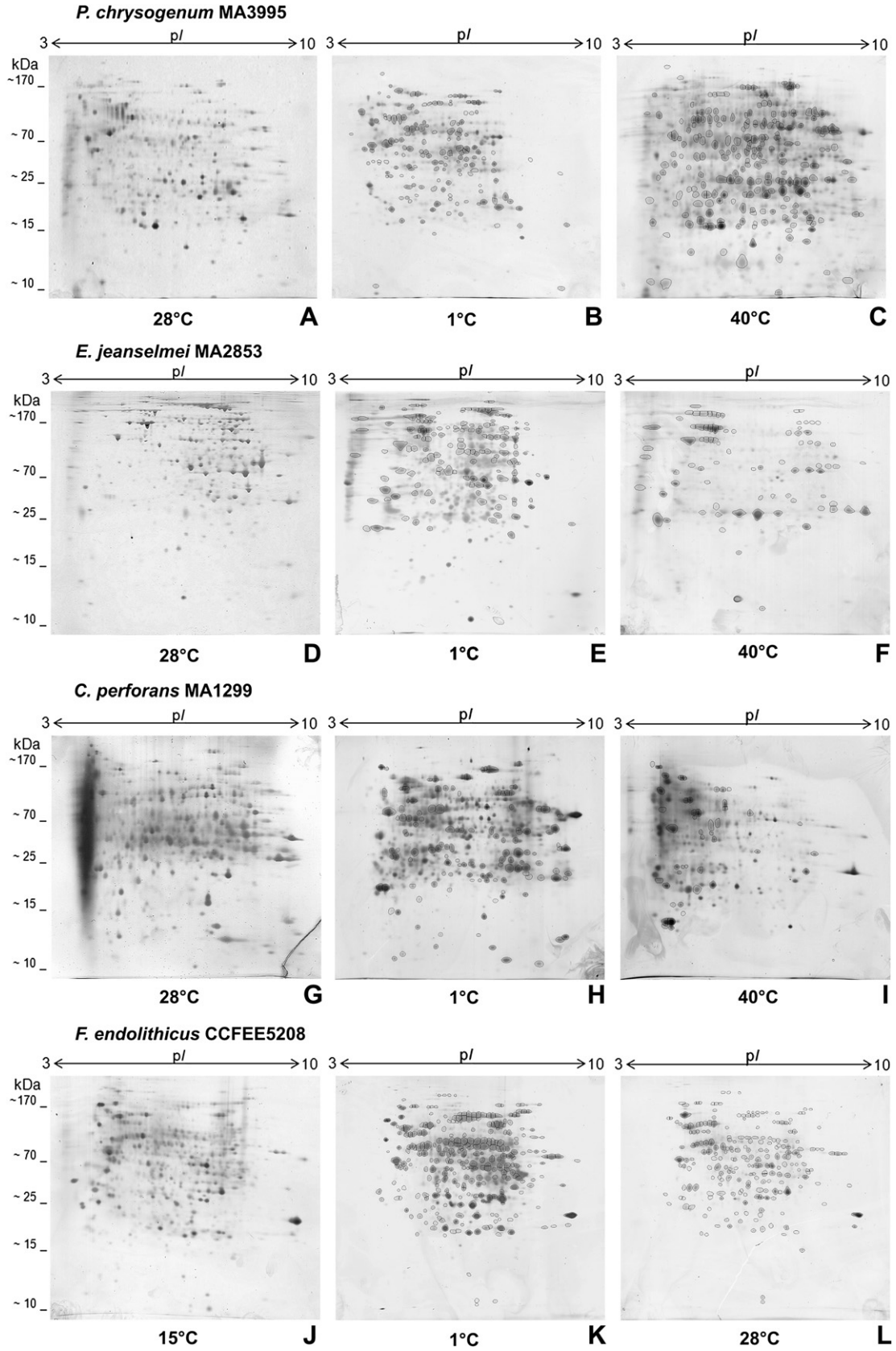

Fig 2 – 2D gel protein patterns obtained after exposure to different temperatures. *P. chrysogenum* MA 3995 (A–C), *E. jeanselmei* MA 2853 (D–F), *Coniosporium perforans* MA 1299 (G–I), and *F. endolithicus* CCFEE 5208 (J–L). Pairs of protein spots detected by overlapping are highlighted in black.

Table 3 – Number of matching protein spots detected. Gel matching was carried out selecting the temperatures 28 °C and 15 °C as reference.

Strain	Reference gel	Number of pairs at each exposure condition		
		1 °C	40 °C	All temperatures
P. chrysogenum MA 3995	**28** °C	153	211	100
E. jeanselmei MA 2853	**28** °C	147	81	46
C. perforans MA 1299	**28** °C	134	47	21
		1 °C	28 °C	All temperatures
F. endolithicus CFEE 5208	**15** °C	278	224	187

Table 4 – Number of matching protein spots detected. Gel matching was carried out selecting *P. chrysogenum* as reference strain.

Strain	Reference strain	Number of pairs at each exposure condition		
		28 °C	1 °C	40 °C
E. jeanselmei	*P. chrysogenum*	46	37	50
C. perforans	*P. chrysogenum*	43	59	62
		15 °C/28 °C	1 °C	28 °C/40 °C
F. endolithicus	*P. chrysogenum*	43	46	46
All strains	*P. chrysogenum*	3	6	2

spots; 387 spots were found at 1 °C whereas 382 spots were detected at 28 °C. However, a substantial change in the expression pattern, caused by the increase of proteins with acidic pIs (3–5) and molecular weight in the range of 25 and 100 kDa, was observed. The number of protein spots with a higher mol. wt – between 100 and 170 kDa – was instead reduced. A decrease to 174 spots occurred at 40 °C, mostly concerning spots within the pI range 5–7 and the mol. wt between 30 and 170 kDa. The number of spots commonly expressed at both 28 °C and 1 °C was 147 and thus it was significantly higher than the number of overlapping spots found both in the 28 °C and 40 °C gels (81). A total of 46 spots matched at all temperatures (Table 3).

In *C. perforans* (Fig 2, G–I), growth at 1 °C resulted in the expression of the highest number of protein spots (494 *vs* 325) which was especially related to an increase in the number of high molecular weight spots. Only 255 spots were observed as a consequence of the strain exposure to 40 °C; the reduction mostly concerned spots in the pIs range 5–9 and with molecular weight between 30 and 90 kDa. Common protein spots at 28 °C and 1 °C were 134 and only 47 at 28 °C and 40 °C. Altogether 21 protein spots were found to be expressed at all three temperatures (Table 3).

For *F. endolithicus* the analysis of the 2D gels (Fig 2, J–L) at 1 °C revealed an increase from 425 to 466 spots with an obvious change in the protein pattern. Mostly high molecular weight spots – within the range 70–170 kDa – and with pI values between 6 and 7, were observed. At 28 °C many proteins, respectively in the pI and mol. wt range 5–7 and 25–90 kDa, disappeared resulting in a total of 284 protein spots. When overlapping the gels, 278 spots were found at both 15 °C and 1 °C, while comparing the growth at 15 °C and 28 °C, 224 spots were matching. At all temperatures tested 187 common spots were detected (Table 3).

Protein pattern of black fungi as compared to Penicillium chrysogenum

The protein patterns of all the strains were analysed using *P. chrysogenum* as reference strain (Table 4). At standard incubation temperature (28 °C) *P. chrysogenum* shared 46 protein spots with *Exophiala jeanselmei* and 43 with *Coniosporium perforans*. An equal number of protein spots (43) was also shared by *P. chrysogenum* and *Friedmanniomyces endolithicus* – this incubated at 15 °C – while only 3 spots were detected as common among all samples. A similar result was obtained at 1 °C: 37 spots matched in *P. chrysogenum* and *E. jeanselmei*, while 59 spots and 46 in *P. chrysogenum* and, respectively, *C. perforans* and *F. endolithicus*. Only 6 protein spots were commonly found in all strains at 1 °C. The analysis of the samples exposed to 40 °C revealed the presence of 50 common protein spots in *P. chrysogenum* and *E. jeanselmei* and of 62 and 46 in *P. chrysogenum* and *C. perforans* and *F. endolithicus* respectively; merely 2 spots matched in all the strains. At 1 °C, but not at 28 °C and 40 °C, the matching spots were mainly represented by proteins with high molecular weight.

Common protein spots within black fungi as compared to Friedmanniomyces endolithicus

The comparison among the black fungal strains was carried out choosing the extremophilic fungus *Friedmanniomyces endolithicus* as reference strain (Table 5). At 15 °C and 28 °C respectively, 65 matching protein spots were detected in *F. endolithicus* and *Exophiala jeanselmei*, 68 in *F. endolithicus* and *Coniosporium perforans* and 17 spots were matching in all the samples. At 1 °C, 41 matching spots were observed as common between *F. endolithicus* and *E. jeanselmei* and 62 were common with *C. perforans*. Among the three fungi only 9 spots matched at 1 °C. The number of common spots decreased after exposure to 40 °C and 28 °C (the latter for *F. endolithicus*): While only 7 protein spots matched in all strains, 29 were detected as common in *F. endolithicus* and *E. jeanselmei* and 44 in *F. endolithicus* and *C. perforans*.

Discussion

This study is the first contribution on the response of extremotolerant and extremophilic black fungi towards suboptimal

Table 5 – Number of matching protein spots detected. Gel matching was carried out selecting *F. endolithicus* as reference strain.

Strain	Reference strain	Number of pairs at each exposure condition		
		28 °C/15 °C	1 °C	40 °C/28 °C
E. jeanselmei	*F. endolithicus*	65	41	29
C. perforans	*F. endolithicus*	68	62	44
All strains	*F. endolithicus*	17	9	7

temperatures, through the investigation of protein patterns. After exposure to different temperatures, qualitative changes – concerning the total number of spots, their molecular weight as well as their pI – suggested that the temperature response of black fungi differs considerably from mesophilic fungi and involves proteins that are hitherto unidentified and unexploited.

Generally, growth at low and high temperature requires diverse adaptations (Maheshwari *et al.* 2000; Margesin *et al.* 2007; Casanueva *et al.* 2010) and 'proteins are the main targets of these adaptations as they control the equilibrium between substrate and products, influx of nutrients, outflow of waste products, macromolecular assemblies, nucleic-acid dynamics and appropriate folding' (D'Amico *et al.* 2006). Also in mesophilic fungi stabilizing proteins as MC, HSPs, and CSPs are the most important effects to temperature stress (Haslbeck *et al.* 2005; Piette *et al.* 2010, 2011) but another consequence of non-optimal growth conditions can also be the downregulation of the metabolism and the proteins involved. Because the proteome of *Penicillium chrysogenum* has been widely characterized (Jami *et al.* 2010a, 2010b) it was used as a reference to the black fungi. In this study and in accordance with literature data (Raggam *et al.* 2011) *P. chrysogenum*, when exposed to 40 °C, exhibited a remarkable over-expression of proteins, which can clearly be interpreted as the synthesis of HSPs (Fig 2, A–C). The slight decrease in the number of spots exhibited at 1 °C indicates a downregulation of the metabolic activity.

The black fungi, when exposed to a temperature that is significantly above their growth regime, showed a reaction different from *P. chrysogenum*: All three stains responded to 40 °C and 28 °C – the latter for *Friedmanniomyces endolithicus* – with a reduction of the total number of protein spots (Fig 2, F, I, L) thus indicating a lack of a heat-shock response on the protein level. Interestingly, spots from the same pI and molecular weight range (respectively 5–7 and 30–90 kDa) were extinct after temperature increase thus suggesting that the strains probably downregulated similar sets of proteins. From this it can be concluded that the basic set of proteins necessary to survive high temperature is stable without the help of HSPs or that other, non-protein protective metabolites and molecules are involved. In *E. jeanselmei* and *Coniosporium perforans* the lack of a heat shock response might on the one hand reflect the necessity to survive temperatures up to 60 °C that are easily reached on the sun exposed rock surfaces inhabited by these fungi and moreover it helps to save energy – otherwise needed for the production of protective proteins – in an extremely oligotrophic habitat. Also in *F. endolithicus* an explanation for the lack of a heat shock response can be found in its ecology: The fungus is endemic in a permanently cold habitat where a heat-shock response was not developed during evolution (Hofmann *et al.* 2000). However, for non-endemic psychrophilic Antarctic yeasts a heat shock response was demonstrated (Deegenaars & Watson 1998).

In contrast to what was observed at high temperatures, the black fungi considerably increased the number of proteins at 1 °C (Fig 2, E, H, K). *Friedmanniomyces endolithicus* and *C. perforans* especially exhibited high molecular weight spots in the mol. wt range from 70 to 170 kDa. In *E. jeanselmei* the total number of spots did not change significantly but a remarkable modification of the expression pattern – mostly spots with a molecular weight between 25 and 100 kDa – was detected in response to the temperature decrease. The change of the protein patterns that occurred in the mesophilic fungi *C. perforans* and *E. jeanselmei* at 1 °C can be interpreted as a cold-shock response. Especially the significant increase of protein spots in *C. perforans* suggests the transient up-regulation of CSPs and HSPs, key proteins directly involved in the cell protection against the stress induced by temperature (Jones *et al.* 1987; Berry & Foegeding 1997; Phadtare & Inouye 2004). Also for psychrophilic organisms the production of CSPs is well known, however with the addition of special adaptations which are absent in mesophiles and also include the lack of repression of house-keeping protein synthesis (D'Amico *et al.* 2006). Antifreeze proteins (AFPs) and other cold-acclimation proteins (CAPs) have been demonstrated in some organisms (De Cross & Bidochka 2001; Jia & Davies 2002; Feller & Gerday 2003; Gocheva *et al.* 2006; Collins *et al.* 2007; Timperio *et al.* 2008). According to the results of this study, the production of CAPs can be hypothesized also for *F. endolithicus*.

The results of this study gave significant evidence that the temperature response – and possibly the general stress response in this special group of fungi – differs considerably from the response of mesophilic hyphomycetes as *P. chrysogenum*. Further it can be concluded that survival of cells without expression of protective proteins is either based on the thermostability of the basic sets of proteins present, on other protective molecules in the cell or on cellular mechanisms that are still unknown. The hypothesis that a special set of proteins is present in black fungi is supported by the fact that the maximum overlap between protein patters found in black fungi and in *P. chrysogenum* was 13 %. Thus, the results of this study give promising indications that the black fungi might be sources for a number of new proteins that do not commonly occur in mesophilic fungi and that could be of great biotechnological interest. This hypothesis, however, will have to be evaluated based on protein identification and de-novo sequencing. Also the influence of nutrient availability, water activity (a_w), osmotic stress, the solutes, and chemical composition of the rock as well as other biochemical and physical parameters on the stress tolerance and on the protein expression of these fungi, will be the focus of future investigations (Grant 2004; Chin *et al.* 2010). Currently, a unique climate chamber to simulate multiple stress factors on terrestrial organisms is constructed at BOKU University and will allow reproducing different environmental conditions separately or in combination with each other.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.funbio.2012.06.004.

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7 List of figures

Figure 9. Cells before and after desiccation for 1 week: a: *E. jeanselmei* (before); b: *E. jeanselmei* (after); c: *K. perforans* (before), d: *K. perforans* (after); e: *C. antarcticus* (before), f: *C. antarcticus* (after).

8 Outlook

Our work was a first attempt to study a particular group of microorganisms - black microcolonial fungi - on molecular level at extreme conditions which could occur on Earth and possibly on Mars.

Such analyses were performed for the first time, which is, considering all the obstacles and limitations, has opened a new horizon for future investigations.

The better characterization of the hitherto unidentified proteins will be possible after genome annotation of the whole genome and transcriptomic studies which are in progress.

Black microcolonial fungi and black yeast, which were in the focus of our work, have numerous outstanding abilities including the fact that they not only survive but also thrive from radioactivity.

The whole protein pool might be a treasure box containing proteins with interesting and novel functions as well as cell protective agents. These proteins could be in the focus of future studies and have a high interest in view of pharmaceutical biotechnological applications.

9 Curriculum Vitae

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Education:

01.09.1994-29.05.2004- Municipal Educational Institution Secondary School 80 of Samara City

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Participating in workshops during PhD study:

18-20 October 2011- CAREX Conference on Life in Extreme Environments, Dublin Ireland

19-24 September 2011- 15th International Biodeterioration and Biodegradation Symposium, Vienna, Austria

25-29 April 2011-“2D Gelektrophorese-Hands On Kurs” Vienna Austria

28 June-3 July 2010- CAREX Summer School on Life in Extreme Environments Pieve Tesino (Italian Alps) Italy

14-17 May 2010- 3rd Meeting of ISHAM Working Groups on Black Yeasts and chromoblastomycosis “Emerging Potential of Black Yeasts” Ljubljana, Slovenija

Internships:

1-31 May 2012 – German Aerospace Center (DLR), Berlin, Germany

1 April-30 September 2014- United Nations Office for Outer Space Affairs, Vienna, Austria

10 List of publications and presentations

Author/co-author of scientific papers:

Kristina Zakharova, Katja Sterflinger, Ebrahim Razzazi-Fazeli, Katharina Noebauer, Gorji Marzban (2014). Global proteomics of the extremophile black fungus Cryomyces antarcticus using 2D-Electrophoresis, *Natural Science*, In press

Kristina Zakharova, Gorji Marzban, Jean-Pierre de Vera, Andreas Lorek, Katja Sterflinger (2014). Protein patterns of black fungi under simulated Mars-like conditions, *Scientific Reports* **4**, Article number: 5114

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Katja Sterflinger, Donatella Tesei, **Kristina Zakharova** (2012). Fungi in hot and cold deserts with particular reference to microcolonial fungi, *Fungal Ecology* **5**: 453–462

Donatella Tesei, Gorji Marzban, **Kristina Zakharova,** Daniela Isola, Laura Selbmann, Katja Sterflinger (2012). Alteration of protein patterns in black rock inhabiting fungi as a response to different temperatures, *Fungal Biology,* **8**: 932–940

Author/co-author of conference presentations and abstracts:

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Kristina Zakharova, Gorji Marzban, Donatella Tesei, Katja Sterflinger

'Rock inhabiting microcolonial fungi: dormant states and active phases in extreme environments'.

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2010- CAREX summer school, Pieve Tesino, Italy

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'Taxonomy and ecology of rock inhabiting fungi: state of art and perspectives'.

2010- 9th International Mycological Congress, Edinburgh, UK

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'Simple living: survival strategies of rock inhabiting fungi in extreme environments'.