University of Natural Resources
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Department of Biotechnology
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PROTEOMICS OF BLACK FUNGI: A SYTEMS BIOLOGY APPROACH TO INVESTIGATE THE ADAPTATION TO EXTREME ENVIRONMENTS

Dissertation

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"The power of imagination
makes us infinite."
John Muir

Abstract

Black fungi are among the most stress tolerant organisms on Earth. They are able to withstand different stressors determined by the typical conditions of their habitats, such as bare rocks in extreme environments and animal or human hosts. In this study proteomics of black rock microcolonial fungi and pathogenic black yeasts was performed in order to evaluate the impact of temperature on the protein expression profile and ultimately detect temperature responsive proteins with a role in the stress tolerance. 2D-protein patterns of *Knufia perforans*, *Exophiala jeanselmei* and *Friedmanniomyces endolithicus* were compared with the mesophilic hyphomycete *Penicillium chrysogenum.* Whereas *P. chrysogenum* showed the highest number of protein spots at 40°C and the lowest at 1°C, thus exhibiting real signs of temperature induced reaction, black fungi — when exposed to temperatures far above their growth optimum — decreased the spots number indicating a down-regulation of the metabolism. At 1°C, an increase of protein spots occurred instead in all the black fungi strains. These results indicate a rather different strategy to cope with non-optimal temperature in rock inhabiting black fungi than in hyphomycetes as *P. chrysogenum.*

2D-DIGE was applied for characterization of temperature-related changes in total protein spot abundance in the black yeast *Exophiala dermatitidis,* an agent of primary and secondary diseases in both immunocompromised and healthy humans. Three incubation temperatures (37, 45, 1°C) and two time spans (1h, 1 week) were selected to simulate different environmental conditions and evaluate the effects of short- and long-term exposure. A total of thirty-two variable proteins were identified by mass spectrometry. Data about protein functions, localization and pathways were obtained. Interestingly, a typical stress response under non-optimal temperature was not observed at the proteome level, whereas a reduction of the metabolic activity was detected after exposure to cold. Our results suggests that under increased temperatures, a fine regulation of protein expression takes place, particularly concerning the protein sets which are involved in crucial biological processes, to guarantee the cell survival and maintenance. Similarly to the black fungi from rocks, the high rate of adaptation to altered growth conditions of *E. dermatitidis* is likely to be due to existing protein repertoire evolved to confer tolerance.

Kurzfassung

Schwarze Pilze gehören zu den stresstolerantesten Organismen auf der Erde. Sie sind durch die typische Beschaffenheit ihres Habitats gegen verschiedene Stressfaktoren resistent. In dieser Studie wurde Proteomik von mikrokolonialen schwarzen Pilzen und pathogenen schwarzen Hefen durchgeführt um die Auswirkung von Temperatur auf die Proteinexpression zu evaluieren und temperaturgesteuerte Proteine zu entschleiern. 2D-Proteinbandenmuster von *Knufia perforans*, *Exophiala jeanselmei* und von *Friedmanniomyces endolithicus* wurden mit dem mesophilen Hyphomycet *Penicillium chrysogenum* verglichen. Während *P. chrysogenum* die höchste Zahl an Proteinen bei 40°C und die niedrigste bei 1°C exprimierte und somit einen sichtbaren Hinweis auf Temperaturabhängigkeit gezeigt hat, verringerte sich die Anzahl der exprimierten Proteine bei schwarzen Pilzen, als dieser Temperaturen von weit über dem optimalen Wachstum ausgesetzt wurden. Dieses Phänomen weist wahrscheinlich auf eine Herabregulierung von metabolischen Prozessen hin. Bei 1°C stieg die Zahl der Proteine bei schwarzen Pilzstämmen an. Die Ergebnisse unserer Studie zeigen, dass schwarze Pilze eine andere Strategie verwenden als der Hyphomycet *P. chrysogenum* um die nicht optimalen Temperaturen zu bewältigen.

2D-DIGE wurde verwendet um die Charakterisierung der temperaturabhängigen Veränderungen in der Gesamtproteinexpression der schwarzen Hefe *Exophiala dermatitidis* ─ die Primär- und Sekundärinfektionen sowohl in immungeschwächten als auch in gesunden menschlichen Wirten verursacht ─ zu untersuchen. Drei Inkubationstemperaturen (37, 45, 1°C) und zwei Zeitspannen (1 Std., 1 Woche) wurden ausgewählt um unterschiedliche Umweltbedingungen zu simulieren und um die Auswirkungen der kurz- und langfristigen Expositionen zu bewerten. Zweiunddreißig unterschiedliche Proteine wurden mittels Massenspektrometrie identifiziert. Informationen über Proteinfunktionen, Lokalisierung und Metabolisierungswege wurden zugeordnet. Interessanterweise wurde bei 45°C keine signifikante Stressreaktion festgestellt, wodurch ─ bei 1°C ─ eine allgemeine Senkung des Metabolismus erreicht wurde. Unsere Ergebnisse lassen darauf schließen, dass unter erhöhten Temperaturen eine Feinregulation der Proteinexpression stattfindet, insbesondere bei Proteinsets, die in entscheidenden biologischen Prozessen involviert sind und das Zellüberleben und -erhaltung sicherstellen. Wie bei schwarzen Pilzen, die sich auf Steinen ansiedeln, könnte das hohe Anpassungsvermögen von *E. dermatitidis* gegenüber verändernden Wachstumsbedingungen an einem Proteinrepertoire liegen, das sich entwickelt hat um Toleranz zu erreichen.

Keywords

Fungal proteomics, Human pathogen, microcolonial fungi, protein pattern, DIGE, nLC-ESI-MS/MS

Table of abbreviations

2D-DIGE	Two Dimensional Difference Gel Electrophoresis
2D-E	Two Dimensional Electrophoresis
ACBR	Austrian Center of Biological resources and Applied Mycology
AFPs	Antifreeze Proteins
ANOVA	Analysis of Variance
Av. Ratio	Average Ratio
BLAST	Basic Local Alignment Search Tool
BLASTP	Protein-protein BLAST
BP	Band-Pass Filter
BVA	Biological Variation Analysis
CAPs	Cold-Acclimation Proteins
CBS-KNAW	Centraalbureau voor Schimmelcultures-Royal Netherlands Academy of Arts and Sciences
CCD	Charge Coupled Device
CCFEE	Culture Collection of Fungi from Extreme Environments
CH	Channel
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
circRNA	Circular RNA
CSP	Cold Shock Protein
C18	Resin for peptide desalting
DIA	Differential In-gel Analysis
DTT	Dithiothreitol
EDA	Extended Data Analysis
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polysaccharides
FDR	False Discovery Rate
GMS	Gomori methenamine silver
GO	Gene Ontology
HC	Hierarchical Clustering
HCT	High Capacity Ion Trap
HS	High Sensitivity
HSE	Heat Shock Elements
Hsf	Heat Shock Transcription Factor
HSP	Heat Shock Protein
HSR	Heat Shock Response
H&E	Hematoxylin and Eosin
IAA	Iodoacetamide

IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IS	Internal Standard
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOBAS	KEGG Orthology Based Annotation System
LPR	Light Pollution Reduction Filters
mRNA	Messenger RNA
miRNA	MicroRNA
MASCOT	Software search engine for protein identification using mass spectrometry
MEA	Malt Extract Agar
MilliQ	Trademark created by Millipore to describe ultrapure water of Type 1
MS	Mass Spectrometry
Mw	Molecular Weight
NCBI	National Center for Biotechnology Information
NCBInr	NCBI non-redundant protein database
ncRNA	Non-coding RNA
PCA	Principal Component Analysis
PVPP	Polyvinylpolypyrrolidone
REVIGO	Reduce + Visualize Gene Ontology
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
SERVALYT	Carrier Ampholytes from SERVA
SFA	Saturated Fatty Acid
sHSP	Small Heat Shock Protein
TCA	Tricarboxylic Acid
TEM	Transmission electron microscopy
TIFF	Tagged Image File Format
TRIS	Tris(hydroxymethyl) aminomethane
UFA	Unsaturated fatty acid
UHQ	Ultra High Quality water of Type 1
UV	Ultraviolet

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1 Introduction

1.1 Black fungi as model organism for stress tolerance

Eukaryotes such as yeasts and fungi represent interesting model organisms for several studies spanning from physiology to genetics and proteomics. Unlike superior eukaryotes, fungi can in fact be easily cultivated and are characterized by short generation time (van der Klei and Veenhuis, 2006). Furthermore, since the first whole genome sequencing (*Saccaromyces cerevisiae*) in 1996, the number of genomic and transcriptomic studies has significantly increased.

With more than 100000 known species (http://www.catalogueoflife.org/col/info/totals), fungi managed to occupy several niche typologies, developing symbiotic relationships with plants (mycorrhiza) and algae (lichens) but also causing diseases in humans and other organisms.

Within fungi, black yeasts and microcolonial fungi (MCF) — generally named black fungi — have been identified as particularly stress tolerant organisms, presenting a wide distribution that includes some of the most extreme environments of the Earth as well as space and Martian 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 years they have been identified as inhabitants of the bare rock in both hot and cold environments (de Hoog and Grube, 2008; Staley et al., 1982; Sterflinger et al., 2012) along with being isolated from hypersaline waters (Gunde-Cimerman et al., 2000), acidic environments (Baker et al., 2004) and radioactive areas (Dadachova et al., 2007). Moreover, their role as human pathogens or opportunists (Matos et al., 2002), as degraders of volatile aromatic hydrocarbons (Gostinčar et al., 2011a) and as a dominant part of the epi- and endolithic microbial communities has emerged (Friedmann, 1982; Ruibal et al., 2005; Selbmann et al., 2008; Sert et al., 2007). Together with cyanobacteria and lichens, they contribute to the global biogeochemical cycling by active weathering of natural rocks and stone monuments (Sterflinger and Krumbein, 1997).

Such wide diversity in distribution and habitat (Fig. 1) is achieved due to a high spectrum of adaptation to a multitude of biotic and a-biotic factors like osmotic, UV and oxidative stress, desiccation, temperature fluctuation, water supply and nutrient availability (Vember and Zhdanova, 2001). Survival experiments also showed the resistance of black fungi against high levels of radioactivity, which they are even able to convert into chemical energy for growth (Dadachova and Casadevall, 2008). Moreover, preliminary

results of an ongoing study on a natural mutant demonstrated the fungus ability to cope with high ozone concentration (i.e. 11 ppm), in presence of which the organism growth is actually enhanced (Tesei and Sterflinger, unpublished data).

To withstand these kind of stressors, organisms need either permanently existing or exceptionally fast adaptive cellular or metabolic responses.

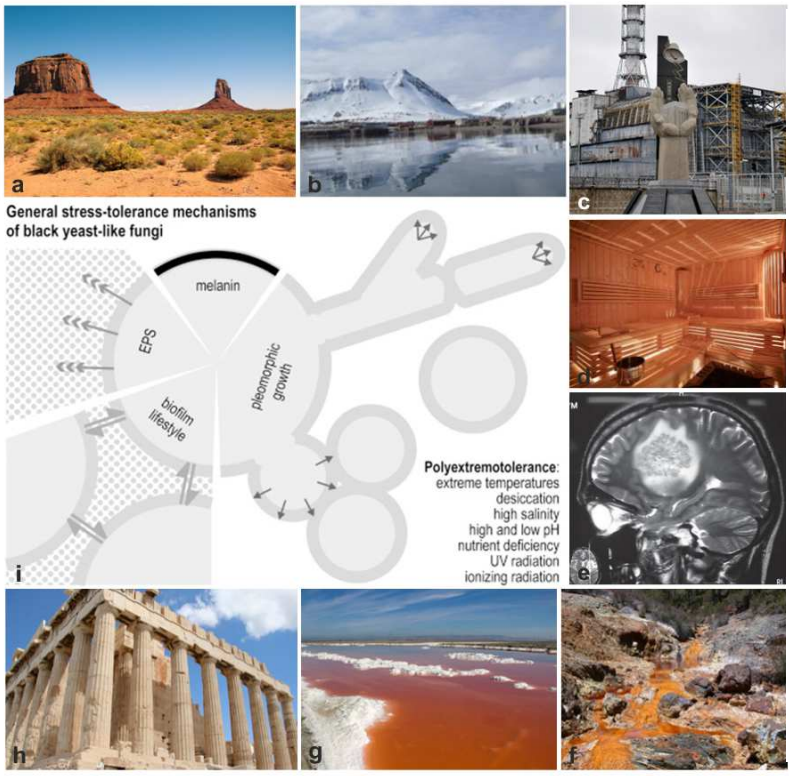

Figure 1. Black Fungi habitat and adaptations. (a-h) Habitat were the presence of black fungi has been detected. (a) hot deserts (Monument valley; photo: www.beheld.wordpress.com); (b) Artic regions (photo: Sterflinger K.); (c) radioactive areas (Chernobyl, reactor 4; photo: Kötter M.); (d) sauna facilities; (e) Magnetic Resonance Imaging (MRI) brain showing lesion in the left front-parietal region due to the black yeast *E. dermatitidis* (Sood et al., 2014); (f) Acid-rock drainage (photo: sosbluewaters.com); (g) salt ponds (photo: www.naturetime.wordpress.com); (h) stone monuments (Parthenon, photo: www.smithsonianmag.com); (i) Stress-tolerance mechanisms in black fungi (Gostinčar et al., 2011b)

MCF and black yeasts are a diverse taxonomic group having polyphyletic origins within several orders of Ascomycota, being *Chaetothyriales*, *Dothideales*, *Capnodiales, Pleosporales, Xylariales, Myriangiales, Mycocaliciales* and *Hysteriales* the most prominent ones (Selbmann et al., 2008). Recent phylogenetic studies showed that black fungi originated during periods of dry climate in the late Devonian and middle Triassic, also revealing the earlier origin of Dothideomycetes compared with the other classes (Gueidan et al., 2011). These data and the paleoclimate provide a good explanation for the diversification of fungi subjected to abiotic stress and adapted to life in oligotrophic conditions.

Despite representing a diverse taxonomic group, MCF and black yeasts share a number of morpho- and physiological characters, which underpins their wide stress tolerance (Selbmann et al., 2005). The microcolonial growth morphology – which results in the so

called cauliflower-like colonies and allows to achieve an optimized surface-volume ratio – the strong melanization of the multilayered cell walls, the synthesis of carotenoids, exopolysaccharides (EPS), trehalose and polyoles as well as lack of sexual reproductive structures, are considered as adaptations to the extreme environments (Gostincar et al., 2010; Onofri et al., 2007; Selbmann et al., 2005; Sterflinger, 1998; Fig. 1). Besides playing a role in UV-protection, melanin improves the desiccation and radiation tolerance; carotenoids shelter the cell from UV radiation along with having a function as antioxidant and in stabilization of membranes. Polyols and trehalose act as osmoprotectants and as stabilizer of enzymes and membranes, respectively. The absence of spores or conidia results in the use of each single vegetative cell as both a survival and a dispersal state (Sterflinger et al., 2012).

Their ecological plasticity makes black fungi and MCF, highly interesting organisms and good candidates for studies of the extremophile nature of fungi as well as of the stress tolerance. Since temperature is undoubtedly one of the major factors affecting the growth and survival of any microorganism (Deegenaars and Watson, 1998), it is of great interest to investigate how MCF and black yeasts withstand temperatures that are significantly out of their growth range.

1.1.1 Microcolonial fungi

The term “microcolonial fungi” (MCF) is used with respect to the prominent morphological characteristics of these fungi, namely the formation of compact and cerebriform or cauliflower-like colonies (Sterflinger et al., 1999).

Besides being highly distributed in temperate environments, MCF have been often isolated from habitats characterized by more extreme physico-chemical parameters such as exposed rock in hot and cold deserts, which they colonize both epi- and endolithically. Rock in desert regions might be considered as one of the most stressful environment on Earth: extreme temperature values can be reached (e.g. -45-60°C), the irradiation rate is high, organic nutrient’s availability is minimal and water might only be present as dewfall or in intercrystalline form (Sterflinger et al., 2012). Nevertheless, the rock represents a suitable substratum due to its porosity and the presence of cracks that offer microclimatic caves for microbes. Rock inhabiting fungi form black and clump-like colonies on the surface and inside the fissures of hard rocks like granite and basalt as well as soft rocks as limestone, sandstone or marble.

Frequent habitats of rock black fungi are the Arctic, the cold deserts and dry valleys in Antarctica (Friedmann et al., 1987), high alpine regions, the hot deserts of Arizona

(Staley et al., 1982) but also the semi-arid areas of the Mediterranean (Ruibal et al., 2008, 2005; Sert et al., 2007; Sterflinger et al., 1997; Sterflinger, 2005; Sterflinger et al., 1999). Despite their limited morphological diversity, DNA sequencing studies have shown that the genus and species diversity of microcolonial fungi is high (de Hoog GS et al., 1999; Sterflinger et al., 1997). According to their prevalent habitats, their degree of specialization to extreme environments and their ecological plasticity, microcolonial fungi can be distinguished into two main groups or ecotypes (Fig. 2). The first group includes fungi, many of which form mycelium under laboratory conditions but are also able to proliferate by budding (Fig. 2 a-e). They are thermo-tolerant and therefore thrive in moderate to dry environments like lower Alpine and Mediterranean areas, however with high UV radiation due to sun exposure.

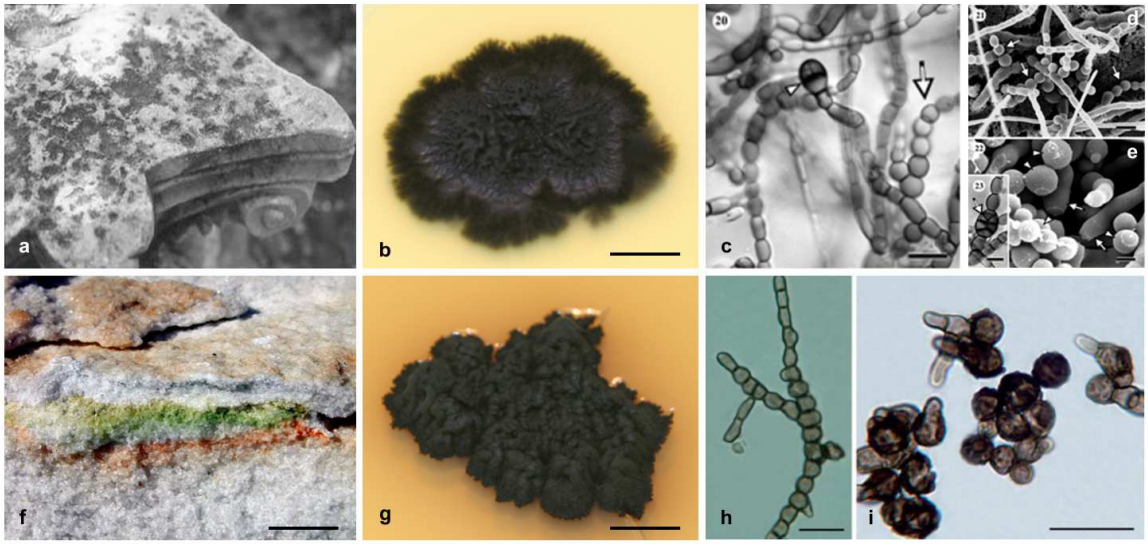

Figure 2. Examples of the two main ecotypes of microcolonial fungi and corresponding habitats. (a-e) First ecotype: thermo-tolerant microcolonial fungi from lower Alpine and Mediterranean areas. (a) Fungal colonization on the surface of a column from the ruins of Perge (Sert et al., 2007); (b) *Knufia perforans* MA 1299 (foto Tesei D.; Scale bar = 10 mm); (c-e) *K. perforans* (CBS 885.95), (c) light microscopy; (d-e) scanning electron microscopy; (c-d) Acropetal, catenate, subglobose, blastic conidia (arrows) and a terminal, multicellular body (arrowhead). Scale bars = 8µm; (e) Detached blastic (arrowheads) and thallic conidia (arrows). Scale bar = 3µm. Intercalary, multicellular body (in the small box). Scale bar = 5µm (Tsuneda et al., 2011); (f-i) Second ecotype: microcolonial fungi from extreme environments. (f) Cryptoendolithic lichen-dominated community colonizing sandstone in the McMurdo Dry Valleys, Antarctica (Scale bar = 10 mm) (Onofri et al., 2006); (g) *Friedmanniomyces endolithicus* CCFFE 5208 (photo Tesei D.; Scale bar = 10 mm); (h-i) (photo Selbmann L.; Ruibal et al., 2009) (h) Melanized hypha of *F. endolithicus;* (i) Meristematic growth of *Cryomyces antarcticus* (Scale bars = 10 µm).

These fungi are highly resistant against desiccation and withstand high temperatures when in the dehydrated stage, nevertheless their growth optimum is below 30°C, thus they must be regarded as thermo-tolerant but mesophilic. The group is hypothesised to have a quite broad ecological plasticity reflected by their ability to tolerate extreme conditions but to react to moderate conditions by faster growth and by morphological

changes from meristematic towards mycelial growth. The best-known genera in this group are *Knufia* (prev. *Coniosporium:* order *Chaetotyriales)*, *Sarcinomyces* (order not assigned) and *Capnobotryella (*order *Capnodiales)*.

The other group is characterized by microcolonial fungi which, despite resembling morphologically the fungi from the first group, are highly specialized to extreme environments and can thus be considered as the real extremophiles amongst the black fungi (Fig. 2 f-i). All species are cryophilic - with growth optima around 12°C - and might have lost the ability to react to moderate conditions and raised levels of nutrients by faster growth (Sterflinger, personal communication). Microcolonial growth seems to be obligate in this group since no change to mycelial growth has been observed under laboratory conditions. *Cryomyces* (order not assigned) and *Friedmanniomyces (*order *Capnodiales)* - both originally isolated from Antarctic rocks - are the largest genera representing this group (Onofri et al., 1999; Selbmann et al., 2005). Since microcolonial fungi were reported also from hot deserts like Arizona, we can assume that also some real thermophilic MCF exist (Staley et al, 1982).

1.1.2 Black fungi as pathogens

Several black fungi species rarely occur on bare rock in desert environments and do not form cauliflower-like colonies. Nevertheless, they have been found in habitats characterized by very different climate and temperature conditions. Many species are associated with animals such as fish - e.g. *Exophiala salmonis* - with humans – e.g. *Exophiala dermatitidis* and *Cladophialophora bantiana* - or human environments as bathrooms, sauna facilities or dishwashers, being agents of phaeohyphomycosis, chromoblastomycosis and mycetoma in both immunocompromised and healthy hosts (Fig. 3). Phaeohyphomycoses range from superficial or subcutaneous to systemic infections and occur through the colonization of the host tissues by hyphal elements or yeast-like cells. Chromoblastomycosis and mycetoma are instead characterized by spherical, muriform cells exhibiting meristematic growth. While phaeohyphomycosis and mycetoma result in tissue necrosis, chromoblastomycosis leads to excessive proliferation of host tissue (Matsumoto et al., 1987). Moreover, asymptomatic colonization of the skin and lungs can also occur.

These species actually form a third ecotype of black fungi that includes the typical black yeasts belonging to the genera *Exophiala, Fonsecea, Capronia, Phaeococcomyces* and *Cladophialophora* of the order *Chaetothyriales.* They are mostly mesophilic but with a tendency to be moderate termophilic as a prerequisite for pathogenicity. Mesophilic

species with maximum growth temperatures of 27-33°C are restricted to cold-blooded animals (de Hoog et al., 2011). Species that are able to grow at temperatures equal or above 37°C may cause systemic infections in mammals; those with maximum growth temperatures of around 35-37°C cause instead sub-cutaneous, cutaneous and superficial infections in humans and systemic infections in cold-blooded animals. Maximum growth temperatures within the range 42-45°C characterize species having a natural habitat in association with birds and bats, whose body temperature is above that of humans (Sudhadham et al., 2008).

Beside thermo-tolerance, the deposition of melanin in the cell walls, the presence of a yeast-like phase, adhesion, osmotolerance and hydrophobicity are at the base of pathogenicity (Seyedmousavi et al., 2014).

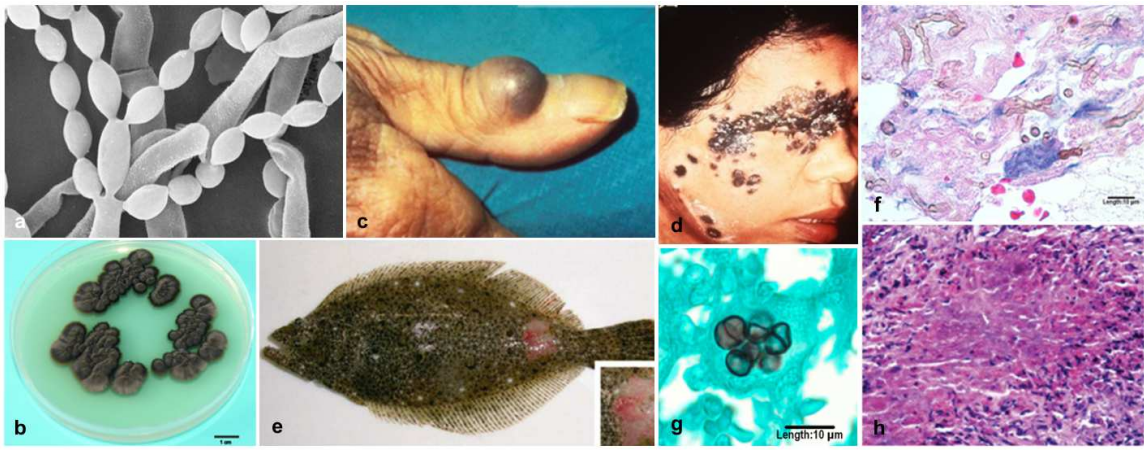

Figure 3. Pathogenic species of black fungi, their hosts and host tissues invasion. (a-b); (a) *Cladophialophora carrionii* SEM, Acropetal chains of smooth, lemon-shaped conidia (photo: Nishimura K); (b) *E. dermatitidis* CBS 525.76 on 2% MEA (photo: Tesei D.); (c-d) (sub) cutaneous phaeohyphomycosis caused by species of the *Exophiala* genus in human hosts; (c) (photo: Rippon JW, Doctorfungus Corporation); (d) (de Hoog and Hermanides-Nijhof, 1977); (e) Skin of a fish (Japanese flounder) with cutaneous phaeohyphomycosis due to an *Exophiala* sp (Seyedmousavi et al., 2013); (f-h) fungal colonization of host tissues; (f) Hematoxylin-and-eosin (H&E) stain of melanized, moniliform hyphal elements of *Cladiophialophora bantiana* from a brain abscess (Revankar and Sutton, 2010) (g) Gomori methenamine silver (GMS) stain of sclerotic bodies produced by *Fonsecaea pedrosoi* (Revankar and Sutton, 2010); (h) High power view demonstrated multiple pigmented fungal hyphae with marked de-granulated eosinophils (H&E, X400; Patra et al., 2014).

Melanin plays a major role in pathogenesis by reducing the susceptibility to the host immune system (Langfelder et al., 2003) and in addition it has an essential biomechanical function, which enables the hyphae to penetrate the host tissues (Brush and Money, 1999). The yeast-like phase might enable the fungus to disseminate hematogenously in the host, whereas the hyphal growth alone usually results only in localized infections (Seyedmousavi et al., 2014). The presence of an EPS capsule promotes adhesion to substrates as well as to the host's epithelial cells (Hayakawa et al., 2006). Numerous species of black yeasts of the *Chaetothyriales* are also highly resistant against toxic hy-

drocarbons, volatile organic compounds and mineral oil or surfactants, being in some cases - e.g. *Exophiala xenobiotica* and *Cladophialophora psammophila* - even able to degrade those substances (Badali et al., 2010; De Hoog et al., 2006). Due to the structural similarity of these compounds and neurotransmitters, a potential role in virulence of this assimilative ability has been proposed by Zhao et al. (2010).

1.2 General response to stress

Exposure of organisms to sub-optimal growth conditions or to any environment that reduces cell viability or fitness can represent stress (de Nadal et al., 2011). Stress has been classified as either biotic or abiotic, the latter separated into thermal and non-thermal stress (Mafart et al., 2001). Thermal stress involves heat, cold and freezing; non-thermal stress are instead represented by altered levels of acidity, water availability (flooding or drought), salinity, pressure and nutrients (excess or deficit). Different stress elicit diverse symptoms, in addition multiple stress factors can occur simultaneously, thus potentially affecting the activity and functionality of molecules, the membrane's integrity as well as many cellular processes. Consequently, the organism's growth and development result to be delayed and the organism survival might also be compromised (Krasensky and Jonak, 2012; Russell, 2008).

Rapid adaptations to stress are essential to maximize cell survival and represent the result of an evolutionary process, which led to a certain level of stress tolerance and fitness of the species in its own habitat. Both the physiological state and the natural environment in which an organism has been evolutionarily selected, influence indeed its adaptive responses (de Nadal et al., 2011).

Eukaryotic cells have evolved sophisticated cellular mechanisms in response to stress that allow them to adapt to certain ranges of temperature, light, water requirements and nutrients by regulating several aspects of cell physiology as e.g., gene expression, cell cycle progression, cytoskeletal organization, protein homeostasis, modification of enzymatic activity and metabolism.

1.3 Temperature stress

Temperature is undoubtedly one of the major factors affecting the growth and survival of any organism (Deegenaars and Watson, 1998) either directly, by its influence on the growth rate, enzyme activity and cell composition, or indirectly by its effects on the solu-

bility of solute molecules, ion transport and diffusion, osmotic effects on membranes, surface tension and density (Herbert, 1986).

Temperature stress generally implies the organism exposure to temperatures, which are far above or below the temperature optimum. Since heat and cold stress have the potential to affect the cell in multiple ways, almost all living organisms have evolved signaling pathways to sense minor changes in environmental temperature (Bahn et al., 2007a).

Heat stress compromises the stability of proteins, membranes, RNA species and cytoskeleton structures and alters the efficiency of enzymatic reactions in the cell, eventually causing a state of metabolic imbalance (McClung and Davis, 2010; Richter et al., 2010; Fig. 4). As a consequence toxic by-products such as reactive oxygen species (ROS) can accumulate in the cell, thus establishing a direct connection between oxidative stress and heat stress (Suzuki et al., 2012). Cold stress also has a major impact on the cell biochemical processes, mostly acting by slowing down the reaction rate and often leading to the inhibition of DNA, RNA and protein synthesis. Low temperatures strengthen in fact the interaction between DNA strands in the double helix thus hampering the RNA polymerase's access. Furthermore, at low temperatures the formation of RNA secondary structures often interfering with translation, is promoted (Feller and Gerday, 2003).

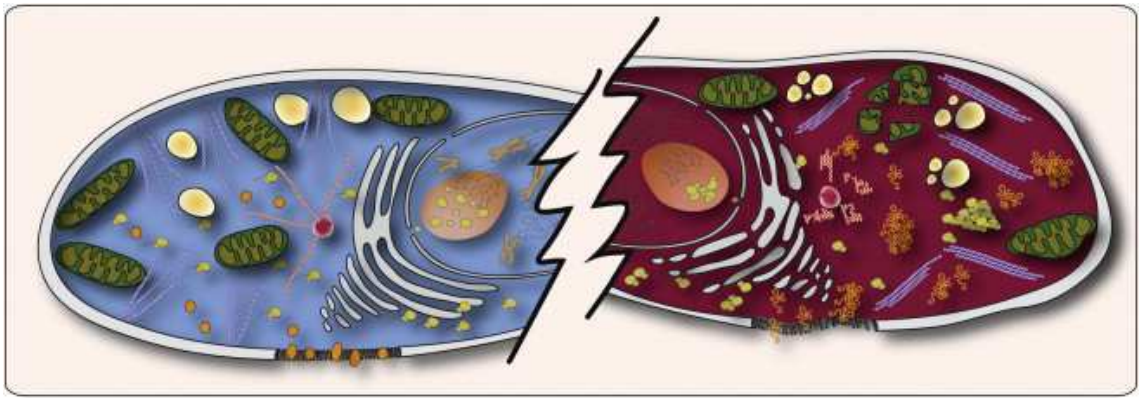

Figure 4. Effects of Heat Shock on the Organization of the Eukaryotic Cell. An unstressed eukaryotic cell (left) as compared to a cell under heat stress (right). Cytoskeleton damage as well as the reorganization of actin filaments (blue) into stress fibers and the aggregation of other filaments (microtubuli, red) are visible. The Golgi and the endoplasmic reticulum (white) disassemble, mitochondria (green) and lysosomes (yellow-white gradient) are reduced in number. The nucleoli, sites of ribosome (yellow) assembly swell. Large granular depositions – the stress granula (yellow), formed by the aggregation of proteins and RNA – accumulate in the cytosol along with protein aggregates (hexagonal versus spaghetti style, orange). Changes also occur at the membrane level: the fluidity increases and membrane proteins aggregate. Together, all these effects stop growth and lead to cell-cycle arrest as indicated by the non-condensed chromosomes in the nucleus (Richter et al., 2010).

Under both heat and cold stress, protein's conformation and membrane's properties are altered, thus resulting in compromised functions (Fig. 5). While high temperatures lead to protein denaturation and to enhanced membrane fluidity, some of the normally fluid components of the membrane become gel-like when the temperature lowers (Turk et al., 2007). A further aspect connected to temperature downshift is the temperature-related change in dissolved oxygen concentration — the solubility of oxygen and other gases will indeed increase as temperature decreases — which represents an additional stress for the cell and makes the protection against ROS a paramount aspect (Ohsaka et al., 2002; Gocheva et al., 2006).

To counteract the effects of temperature stress, effective environmental response strategies have been elaborated over evolutionary time. Such strategies involve the modulation of the transcriptome, proteome, metabolome and lipidome and therefore enable the organisms to prevent heat- and cold-related damages by adjusting both metabolism and cell functions (Mittler et al., 2012). This dynamic process helps maintaining the protein and metabolic homeostasis under variable environmental conditions. However, a reverse process of deprogramming takes place as soon as the temperature returns to normal levels (Mittler et al., 2012). Since energy from other cellular functions is diverted into the stress response, adaptive responses have indeed to be limited in time, as their constitutive induction negatively affects the cell growth. (de Nadal et al., 2011).

An abrupt increase in ambient temperature initiates a stress response in the organism, commonly known as heat-shock. Many aspects of the heat shock response (HSR) are universally conserved among both prokaryotes and eukaryotes. Protein denaturation, which is triggered by an abrupt rise in temperature, lies behind this phenomenon.

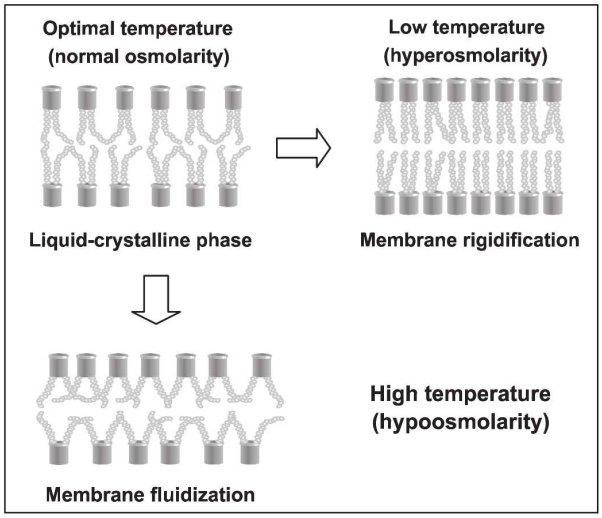

Figure 5. Schematic representation of changes in membrane structure and the behavior of lipid bilayers under low- and high-temperature stress. While low temperatures cause "rigidification" of membranes, high temperatures cause their "fluidization" (Los and Murata, 2004).

As shown in Fig. 6, a common response occurs through the induction of a set of proteins important for high-temperature growth called heat shock proteins (HSPs), which, in eukaryotes, are dependent on the heat-shock transcription factor (Hsf1) (Albanèse et al., 2006; Leach and Cowen, 2013; Nakamoto and Vígh, 2007; Nevarez et al., 2008). Also known as molecular chaperons, HSPs are divided into different classes according to their molecular mass — Hsp100s, Hsp90s, Hsp70s, Hsp60s, Hsp40s and small HSPs (sHSPs: 15-30kDa) — and their function primarily deals with protein folding. HSPs assist the macromolecules during their normal biological functions, however have a crucial role in the folding and re-folding of polypeptides as they are unfolded following the stress (Arndt et al., 2007; Tkáčavá and Angelovičova, 2012). Along with protein denaturation, an increase in the membrane's fluidity also prompt an increase in heat-shock gene transcription. On this base, the presence and accumulation of both damaged proteins and membranes represent the primary sensor of heat and a signal for the initiation of the heat shock response (Bohnert, 2007).

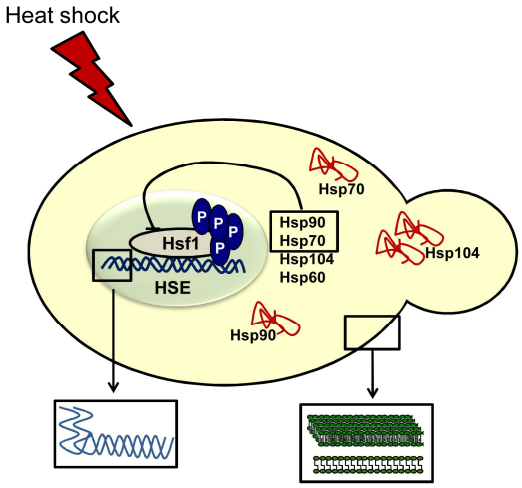

Figure 6. Mechanisms of temperature sensing in fungi. Upon a thermal insult, cells utilize different mechanisms to sense changes in the surrounding temperature for adaptation and survival. Hsf1 becomes phosphorylated, binding to heat-shock elements (HSEs) in target promoters, leading to the up-regulation of chaperone proteins. These aid in the stabilization and refolding of denatured proteins, and Hsp90 and Hsp70 assist in the down-regulation of Hsf1. The cellular plasma membrane becomes more fluid in response to thermal insults (right inset box). Moreover, although little is known about RNA thermometers in fungi, there is evidence suggesting they are involved in RNA decay during heat shock (left inset box) (Leach and Cowen, 2013).

Signal transduction events that involve protein kinases, phosphatases and hormones among others, are subsequently activated. The induction of HSPs to mitigate the effects of heat stress is in fact not the only way through which the HSR occurs, but the modulation of several pathways mediated by regulatory proteins also takes place. All cellular processes, which are affected by the heat stress — e.g. altered protein-DNA interaction,

translation or degradation of proteins, changes in the chromatin, histone displacements in the nucleus, RNA unfolding, the accumulation of ROS — can in turn trigger cascades of signal transduction resulting in the alteration of the cell metabolism.

The use of phosphorylation cascades represents a conserved mechanism to achieve the activation of signal transduction processes within seconds of exposure to stress (Alonso-Monge et al., 2009; Bahn et al., 2007b). The speed of activation and the duration of the stress-response are in fact paramount for the organism survival to the stress.

Responses to a significant reduction in temperature involve instead a pattern of gene expression, termed the “cold shock response”, which takes place through the synthesis of cold shock proteins (CSPs). Studies to date have demonstrated that, both in prokaryotes and in eukaryotes cells generally suppress transcription and translation in response to cold stress, with the only exception of a selected number of CSPs whose synthesis continues or is up-regulated during cold shock (Ermolenko and Makhatadze, 2002). However, CSPs do not appear to be as conserved as HSPs between prokaryotic and eukaryotic systems (Al-Fageeh and Smales, 2006). CSPs have fundamental functions such as DNA replication, transcription, translation, RNA stabilization, ribosome assembly and protein folding (Gualerzi et al., 2003). As these proteins can stabilize mRNA, they contribute to overcome partial blocks in protein synthesis that appears to be one direct consequence of temperature downshifts (Beales, 2004). mRNAs tend in fact to form stable secondary structures upon cold stress, thereby masking the Shine–Dalgarno sequence of mRNAs from the ribosome or interfering with translation elongation steps (Ermolenko and Makhatadze, 2002).

Other CSPs — such as inducible desaturases — are dedicated to the maintenance of membrane fluidity, which occurs by increasing the proportion of unsaturated fatty acids as the growth temperature decreases. Although studies have shown that mRNA translation appears to be a key control point in the cold-shock response, the overall mechanism of cold-shock- mediated inhibition of translation is yet to be fully elucidated.

1.3.1 Mechanisms of tolerance in heat-and cold-adapted organisms

Beside sudden changes of temperature, some organisms have the necessity to adapt to constantly high or low temperature values, which do endanger life. Due to their ability to tolerate severe environmental conditions, heat- and cold-adapted organisms are also defined as thermophilic (heat-loving) or thermotolerant (heat-tolerant) and psychrophilic (cold-loving) or psychrotolerant (cold-tolerant) respectively, to distinguish them from the mesophiles, whose temperature optima are between 24 and 28°C. While thermo- and

psychrotolerant grow over a wide temperature range, thermo- and psychrophiles persist in permanently hot and cold habitats. To grow successfully in their habitats heat- and cold-adapted organisms have evolved adaptations at the cellular level, which concern membranes, proteins, energy-generating systems, along with components responsible for nutrient up-take and the synthesis of compounds conferring heat and cold tolerance (Margesin and Miteva, 2011). Nevertheless, genome analysis — e.g. of the Antarctic endemic black fungus *Cryomyces antarcticus* — hitherto suggested no significant deviations from genomes of mesophilic species and therefore excluded the presence of unique sets of genes specifically conferring the ability to withstand sub-optimal temperature (Sterflinger et al., 2014). Synergistic changes in overall genome content — such as genetic redundancy and duplications — and in amino acid compositions of proteins, the latter conferring stress resistance to proteins, would rather play a major role in tolerance (Methé et al., 2005; Kondrashov, 2012; Plemenitaš et al., 2014).

Mechanisms of both heat- and cold-adaptation in Eukaryotes involve — as mentioned above — the maintenance of membrane fluidity for the optimal functioning of membrane-localized proteins and membrane-associated cellular processes while reducing proton loss, a phenomenon called homeoviscous adaptation (Sinensky, 1974). In Fungi and in several other organisms an increase in temperature results in an increase in the proportion of the saturated fatty acids (SFAs) incorporated into phospholipids. By contrast, the proportion of unsaturated fatty acids (UFAs) generally increases in response to lower temperatures (Maheshwari et al., 2000; Turk et al., 2011). A higher content of UFAs — generally up to 90% of the total fatty acids, with oleic and linoleic acids predominating — than those observed in mesophilic or psychrotolerant species, characterizes the membranes of psychrophilic black yeasts and seems to be the crucial factor allowing proper functioning of metabolic processes at low and even sub-zero temperature (Gunde-Cimerman et al., 2014). Shortening the length of the membrane fatty acid chains represents an additional strategy to counteract the effect of cold stress (Al-Fageeh and Smales, 2006). Moreover, a key factor in the preservation of the membrane's physical characteristics and functionality is the relative proportions of total sterols and phospholipids, which seem to prevent the formation of non-bilayer phases during cold exposure (Russell, 2008).

Modifications in the molecular structure of proteins are also important to ensure functionality and flexibility at high and low temperatures. Cold-active enzymes have been reported in cold-adapted organisms as showing high catalytic efficiency at low temperatures as well as a shift in their maximal activity towards low temperatures (Collins et al., 2008). Similarly, heat-adapted proteins have optimal conformational flexibility at high

temperatures, this involving a repertoire of mechanisms of protein stabilization such as intrinsic thermo-stability and stabilization by ions (Maheshwari et al., 2000). Moreover, an increased number of hydrogen bonds and salt bridges and a higher proportion of thermophilic amino acids — e.g. proline residues with fewer degreed of freedom — have been generally reported for thermophilic proteins, while a higher content of arginine and lower content of lysine seem to characterize thermostable proteins (Satyanarayana et al., 2005). In cold-adapted organisms, flexibility is obtained by changes in the frequency of molecular bonds, in the amino acids side chains and by reducing hydrophobic interactions between protein sub-units. Being essential for catalysis, flexibility of the protein structure is thought to compensate for the lower thermal energy provided by the low temperature habitat and thus to provide high efficiency at low temperature (Methé et al., 2005). Since transcription and translation are temperature-sensitive steps, in addition to structural-related changes of proteins, the process of protein synthesis itself requires an adaptation to constantly high and low environmental temperatures in thermophiles and psychrophiles respectively.

Special classes of proteins have also been isolated from heat- and cold-adapted microorganisms. Generally, HSPs are involved in stabilization and refolding of proteins as they begin to denature in response to heat stress. Studies on the HSR of thermophilic and thermotolerant fungi actually show parallelisms with the mesophilic response regarding the transient nature of the HSR, which involves both the induction of HSPs and the repression of housekeeping proteins (Oberson et al., 1999). Mostly high and medium molecular weight (Mw) HSPs (e.g. Hsp60) are reported as being expressed in relation to a mild temperature stress, whereas lower molecular weight proteins are synthesized as a result of a further increase in temperature (Chen and Chen, 2004). However, some species of thermophilic and thermotolerant fungi do not synthesize heat shock proteins following an abrupt increase in temperature. Besides HSPs, histone-like proteins that bind DNA with a function in DNA protection have been identified in thermophiles (Satyanarayana et al., 2005).

Due to the adverse effects of low temperatures on DNA synthesis and on transcription, nucleic-acid-binding proteins are expected to have a central role in the adaption to cold. Cold-acclimation proteins (CAPs), which have a role in promoting protein synthesis, additionally seem to be essential for the maintenance of the cell cycle and for the organism growth at low temperatures. These proteins are permanently expressed in bacteria during constant growth at low temperatures (D'Amico et al., 2006). Antifreeze proteins (AFPs) have also been detected in cold-adapted organisms — and in snow-mould fungi among them — in form of peptides and glycopeptides of various sizes acting as cellular

protectants against freezing. AFPs manage indeed to decrease the freezing point of cellular water, by binding to ice crystals during formation and thereby promoting fungal survival through freeze-thaw cycles (Feller and Gerday, 2003).

The prevention of cell freezing is also accomplished through the synthesis of intracellular compatible solutes such as polyols and sugars. In fungi, polyols as glycerol and mannitol are thought to play a role in maintaining a suitable milieu for enzyme activity by acting as physiological buffering agents (Jennings, 1985). Mannitol is additionally known to have cryoprotectant properties, since it is produced at high levels during the growth at low temperatures (Robinson, 2001). Along with polyols, fungi do recur to trehalose in response to high and low temperatures.

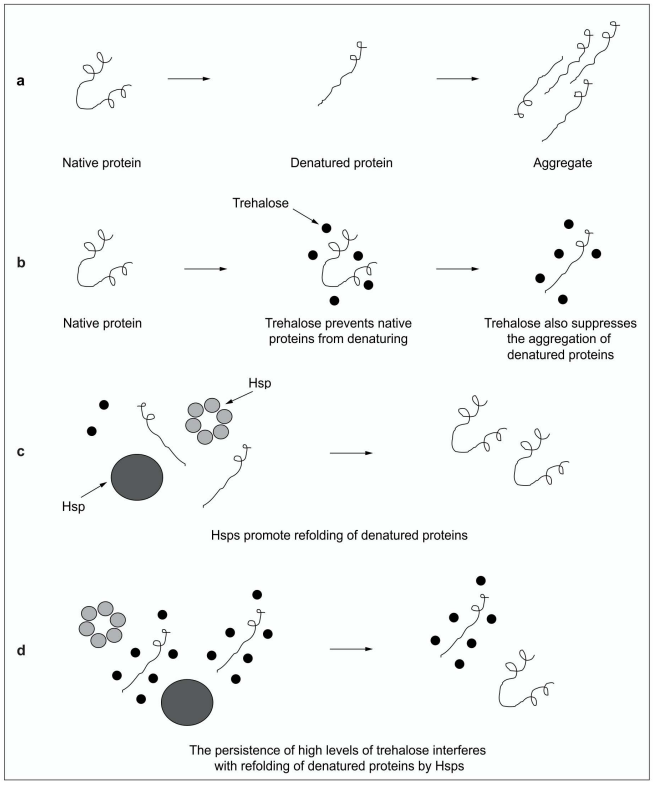

Figure 7. Trehalose and HSPs prevent protein denaturation and aggregation and assist protein refolding (**a**) High-temperature induced protein denaturation. Unfolded proteins can associate to form aggregates. (**b**) Large quantities of the disaccharide trehalose are synthesized during heat shock to stabilize proteins in their native state and also to suppress aggregation of denatured proteins. (**c**) Trehalose is rapidly degraded after heat shock. During recovery, molecular chaperones promote the reactivation of denatured substrates that have been prevented from aggregating. (**d**) The persistence of high levels of trehalose interferes with the reactivation of denatured substrates, explaining both the need to degrade trehalose during recovery and the thermosensitivity of mutants unable to do so (Singer and Lindquist, 1998).

Trehalose acts as a nuclear and cytosolic stress protectant in many organisms across kingdoms with the only exception of mammals (Fig. 7). Substantial accumulation of trehalose in fungal hyphae has been detected indeed in arctic and antarctic fungi when

grown at low temperature (Ruisi et al., 2007; Weinstein et al., 2000). Similarly, trehalose concentrations increase as part of the cellular response to elevated temperatures, thus resulting in an increased tolerance of the organism to adverse environmental conditions (Van Laere, 1989). On this base, a number of studies on yeasts and fungi indicate that trehalose promotes survival under heat, by stabilizing membranes, enabling proteins to keep their native conformation and even suppressing the aggregation of the denatured ones, the latter capacity being previously thought to characterize only HSPs (Jain and Roy, 2009; Singer and Lindquist, 1998). As the most abundant disaccharide, trehalose functions in fungi — and also in black fungi (Zakharova et al., 2013) — in tolerance towards both desiccation and osmotic stress and has a role in pathogenicity, besides being widely employed as storage compound (Goodrich et al., 1988; Iturriaga et al., 2009; Tournu et al., 2013).

Prolonged exposure to non-optimal conditions ultimately leads to acclimation, which represents a survival strategy enabling the organism to start a quick response in case of repeated and recurrent stress. Acclimated organisms display high level of stabilizing and temperature- responsive proteins, thus being able to start a quick response if exposed to even more extreme temperatures (Cabané et al., 1993). Nevertheless, by promoting metabolic homeostasis despite environmental changes, adaptation carries energetic costs (Johnston and Bennett, 2008). Consequently, stress-adapted species usually exhibit lower growth rate in their natural environments than they would potentially show under optimal conditions.

1.4 Elucidation of the fungal temperature stress response by proteomics

Proteins such as HSPs, CAPs and AFPs certainly have a crucial role in the stress tolerance by acting as cellular protectants and by stabilizing denaturing proteins, thus guaranteeing the organism's survival. On this base, studies of the proteome — as the global set of expressed proteins (Wasinger et al., 1995) — are important in order to detect the presence of ongoing stress reactions and to ultimately understand the organism behavior under stress. The term proteomics accordingly indicates large-scale studies, whose aim is to examine the entire complement of proteins expressed by an organelle, cell, tissue or organism at a specific time and under a given set of conditions (Calvete et al., 2014).

The proteome represents the result of transcription and translation, protein processing and turnover (Burg et al., 2011) and therefore contains information about the proteins

that are synthesized, their abundance, variations and modifications as well as their interactions and networks (Geisow, 1998; Pandey et al., 2000, Aebersold and Mann, 2003). Therefore, as compared to transcriptomic, proteomics leads to more reliable conclusions, since proteins are directly related to biological functions and phenotypes (Bhadauria et al., 2009). By recreating environmental conditions, proteomics can then be used to deepen the knowledge on the ecology and physiology of an organism and to shed light on its adaptive responses toward different kinds of stress.

As mentioned before, black fungi are able to cope with unfavorable life conditions. Among them, the prolonged exposure to non-optimal temperatures undoubtedly represents an environmental challenge. Temperature has in fact a major impact on cellular processes and functions and therefore on the organisms' survival. Despite the black fungi extremophilic character and pathogenicity are known, there is very little information regarding the molecular mechanisms, which are at the base of their stress resistance. Although a number of studies focusing on the changes of the transcriptome in response to stress have been recently performed, knowledge mainly at the functional proteomic level is unfortunately still lacking.

As extremophiles, black fungi offer potential as a natural resource of novel proteins and compounds, which possibly play a role in the stress resistance. It follows that, investigating the alteration of the protein pattern after exposure to temperatures that are significantly out of their growth range or beyond those considered optimal for human life can be of great interest, besides improving the understanding of the black fungi system biology.

However, the application of protein techniques to extremophiles can be challenging, being the sample preparation the main bottleneck. The lack of proteomic data for black fungi indeed indicates major analytical and methodological challenges (Marzban et al., 2013). As a consequence of the adaptation to harsh environmental conditions, proteins have evolved a range of specific properties (Siddiqui and Thomas, 2008), which indeed hamper the extraction and separation procedures. Generally, thermo-stable and chemical-denaturants resistant proteins hardly undergo denaturation, while proteins with altered surface charges ─ e.g. from acidophiles or alkaliphiles ─ usually have migration issues during the isoelectric focusing (IEF) (Burg et al., 2011). Similarly, less water-soluble proteins ─ from organisms inhabiting dry environments ─ are susceptible to irreversible precipitation. In black fungi, the rigid cell wall and the high content of melanin, pigments, polysaccharides and lipids represent an obstacle to cell disruption and to protein determination and separation, respectively. Moreover, the fungal protein solubility is quite low and tends towards spontaneous precipitation (Marzban et al., 2013). There-

fore, the very low protein yield and the high content of sample impurities represent the major obstacles to the proteomics of black fungi. The analysis of their protein profiles thereby requires optimization and a case-by-case assessment of the protein preparation and separation procedures.

2 Aims of the study

Black fungi are specialized in the colonization of habitats characterized by stressful conditions such as acidic and polluted environments, salt ponds and bare rocks in hot and cold dry areas (Gunde-Cimerman et al., 2000; Selbmann et al., 2014, 2005; Sterflinger et al., 2012). Moreover, several species ─ including the pathogenic ones ─ successfully conquered a number of human habitats, which range from saunas and steam baths to tap water and dishwashers (Gunde-Cimerman and Zalar, 2011; Zalar et al., 2011).

The incredible tolerance to a combination of stressors displayed by black fungi makes these microorganisms excellent candidates for studies of the extremophile nature of fungi and potential keepers of novel enzymes being key determinants of stress resistance. The pathogenic species can be further employed as model organism for studies about virulence. Since the ability to cope with temperature stress enables the persistence of black fungi in their habitat and has a role in virulence, it is of great interest to investigate the impact of temperature on their physiology and cellular processes.

The target of the present study was to get a deeper understanding of the ecology of black fungi and to analyze the cellular mechanisms that are responsible for their temperature stress tolerance. To this aim, a system biology approach was used for the first time in black fungi research. Because of the direct connection between proteins and biological functions, proteomics was applied to determine changes in the expression profile under sub- and supra optimal temperatures and to detect protein species and pathways that are involved in the response to temperature stress. However, due to the absence of previous knowledge at the functional proteomic level in black fungi, our investigations could be carried out only after accurate optimization of the whole proteomic workflow. Thus, the establishment of a protocol spanning from extraction to identification of proteins from black fungi represented an additional goal of our study (Marzban et al., 2013). Two different proteomic experiments were carried out, to analyze the response of different species of black fungi towards different temperature conditions. For all experiments, incubation temperatures were assessed based on the results of tests for thermal preferences and viability. In order to simulate temperature stress, temperatures well above or below the growth optimum of each of the strains were selected.

The first temperature experiment (Tesei et al., 2012) had the aim to elucidate if MCF from hot and cold deserts and black fungi from more temperate environments share common sets of adaptations towards temperature, on the proteome level. It was indeed our interest to verify the hypothesis on the existence of three ecotypes within back fungi ─ moderate and human-associated; moderate-extremotolerant; obligate-extremophilic ─ on the basis of comparative proteome profiling. According to their bio-ecological charac-

teristics, *Friedmanniomyces endoliticus* and *Knufia perforans,* isolated from rocks in Polar regions and from dry Mediterranean areas respectively and the black yeast with phylogenetic relation to important human pathogens *Exophiala jeanselmi,* were selected. In order to look for similarities in the reaction to temperature stress, protein patterns from these species and from the cosmopolitan mesophilic hyphomycetes *Penicillium chrysogenum* were also compared.

In Tesei et al. (2015) the poly-extremophilic black yeast and human pathogen *E. dermatitidis* was selected as model organism. As the fungus can cause infections even in healthy hosts, its presence in man-made environments generates alarm, besides raising questions about the infection route. As recently suggested, the black yeasts' transition from natural habitat to humans might be achieved using natural spring water as a vector (Novak Babič et al., 2014). Despite the strains extremophilic character and pathogenicity are known, there is very little information regarding the molecular mechanisms, which are at the base of its stress resistance and virulence. Our work therefore aimed to be the first contribution on the proteome of *E. dermatitidis*, through the evaluation of the effect of different temperature conditions on the protein pattern and a comprehensive identification of the temperature responsive proteins and pathways. TEM observations were performed ─ with a special focus on melanin ─ in order to detect alterations of the cell structures due to temperature stress.

While to the first experiments classical two-dimensional electrophoresis (2D-E) was applied, two-dimensional difference gel electrophoresis (2D-DIGE) was used for detection and quantification of proteins from *E. dermatitidis*. The identification of proteins significantly modulated in response to temperature and thus involved in stress tolerance and adaptations of MCF and black fungi, was the ultimate goal of both projects.

3 Materials and Methods

3.1 Species object of the study

In Tesei et al. (2012) three strains of black fungi, clustering within two different orders of *Dothideomyceta* (*Chaetothyriales* and *Capnodiales*) were used. The isolates were selected according to their bio-ecological characteristics. Despite they all colonize rock epi- or endolithically the strains have a diverse geographical distribution: *E. jeanselmei* (MA 2853) is a mesophilic black yeast detected as a frequent colonizer of rock in moderate climates (Sterflinger and Prillinger, 2001; Warscheid and Braams, 2001) with a close phylogenetic and physiological relation to human opportunists and pathogens (de Hoog, 1993); *K. perforans* (MA 1299) is a rock inhabitant MCF with a wide distribution in both moderate and Mediterranean climates (Sterflinger et al., 1997) and a remarkable high temperature and desiccation tolerance (Sterflinger, 1998); *F. endolithicus* (CCFEE 5208) is a psychrophilic MCF probably endemic species for the Antarctic (Selbmann et al., 2005), which occurs cryptoendolithically in rocks, and has a strong degree of extremotolerant specialisation (Onofri et al., 1999). Based on the extensive study of its ecology and proteome, the mesophilic hyphomycete *P. chrysogenum* (MA 3995) was chosen as a reference strain for the proteomic experiments (Dantigny et al., 2007; Jami et al., 2010a, 2010b; Tresner and Hayes, 1971).

The black yeast and human pathogen *E. dermatitidis* was selected as model organism in Tesei et al. (2015). The fungus — belonging to the *Ascomycete* order *Chaetothyriales* — is an agent of primary and secondary diseases in both healthy and immune-compromised hosts (Li et al., 2011) and has a very wide geographical distribution, which includes man-made habitats besides natural niches. Based on the incredible tolerance to a combination of stressors displayed by the black yeast and its pathogenicity, *E. dermatitidis* can be employed as model organism for studies of the extremophile nature of fungi as well as of virulence of human pathogen species (Abramczyk et al., 2009).

3.2 Thermal preferences, cultivation and exposure conditions

Temperature optima were tested prior to setting up the experimental conditions for all strains with the only exception of *F. endolithicus,* for which data were already available in literature (Selbmann et al., 2005). Temperature tests were performed on 2% malt extract agar (MEA) plates at 1, 10, 15, 20, 25, 28, 30, 35, 37 °C, for up to three weeks. Additionally, the viability of each strain was evaluated after exposure to temperatures

well above and below the growth optimum – 1 and 45°C for *E. dermatitidis* and 1 and 40°C for all other strains – 12, 24, 48, 72 hours and 1 week. According to the results of the preliminary tests, 28°C was chosen as incubation temperature prior to temperature treatment – and thus as control temperature – for *E. jeanselmei, K. perforans*, and *P. chrysogenum,* because this is in the growth range of all three fungi and also a standard incubation temperature in microbiology. *F. endolithicus* was incubated at 15°C*,* while *E. dermatitidis* at 37°C, which were detected as the strains' temperature optima, respectively. The isolates were grown on 2% MEA for up to seven weeks. Subsequently *E. jeanselmei, K. perforans*, and *P. chrysogenum* were exposed to 1 and 40°C, *F. endolithicus* to 1 and 28°C and *E. dermatitidis* to 1 and 45°C, for 1 week (Fig. 8). In the case of *E. dermatitidis,* 45°C indeed represents a temperature above the growth regime but also the temperature of warm tap water as well as being in the range of temperatures recorded in steam bath and sauna facilities. In addition, 45°C it's representative of the body temperature of frugivorous animals having a natural habitat in association with the fungus in the Tropics. The exposure to 1°C simulates instead the cold natural habitat and putative reservoir (e.g. glaciers) from which the species might have access to the human environment.

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.

3.3 Electron microscopy

A 1 mm^3 portion of fungal colony from each experimental condition (Tesei et al., 2015) was fixed overnight in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 (v/v). The following steps were carried out according to Arcalis et al. (2004) with a few modifications. The samples were washed with 0.1 M phosphate buffer and subsequently fixed using 1% osmium tetroxide in phosphate buffer (0.1 M, pH 7.4, v/v) for 3 hours on ice and in the dark. After an additional washing steps in phosphate buffer (0.1 M, pH 7.4), first dehydration (50, 70, 90, 96, 100% acetone in MilliQ water, v/v) and then infiltration (15, 30, 45, 60, 75, 90, 100% resin/acetone solution, w/v) in low viscosity epoxy resin (Agar Scientific) were performed. Polymerization was carried out at 60°C for 48 hours. Ultrathin 70 nm sections were mounted on gold grids, stained in 2% (w/v) aqueous uranyl acetate followed by Reynold's lead citrate and inspected in both a Morgagni 268D transmission electron microscope (FEI) and in a Tecnai $G^2$20 (FEI) operated at 80kV. Images were acquired using an 11 megapixel Morada CCD camera (Olympus-SIS) and an Eagle 4k HS CCD Camera (FEI), respectively.

3.4 Proteomics

3.4.1 Sample preparation and labelling

Protein extraction was carried out according to a previously described protocol (Isola et al., 2011). However, some modifications were applied in order to increase the protein yield in *E. dermatitidis* (Marzban et al., 2013; Tesei et al., 2015): the biomass was disrupted using mortar and pestle under liquid nitrogen and further processed using a beating mill. In addition, 2% SDS and 0.1 M NaOH in a 1:1 ratio (biomass/NaOH, w/v) have been added to the homogenization buffer (50 mM Tris- HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose) to enhance the disintegration of the cell walls. Phenol precipitation was performed for all samples as well as the overnight precipitation of proteins (-20°C) by addition of 5 volumes of 0.1 M ammonium acetate in methanol (w/v). After a few washing steps with ice-cold methanol (absolute) and with ice-cold acetone (80%, v/v), the dried pellet was re-solubilized in 7 M Urea, 2 M Thiourea, 4% CHAPS, 1% DTT and 2% Servalyt - in the case of protein extracts to be processed through classical 2-DE - whereas samples intended for DIGE analysis were re-suspended using 7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris HCl pH 8.5. Protein concentration was determined using the Bradford assay (BioRad). When testing the response to temperature of *E. dermatitidis,* four individuals — i.e. inoculated petri plates — were used as biological replicates at each temperature condition, therefore a total of 20 protein extracts were obtained. For the study on *E. jeanselmei, K. perforans*, and *F. endolithicus* technical replicates were instead used.

Prior to 2D-DIGE analysis, protein extracts were labelled using Refraction-2D™ minimal dyes (NH DyeAGNOSTICS). An experimental plan was designed, according to which all biological replicates of control and temperature stressed samples were labelled with either G-Dye200 or G-dye300 (Table 1; Tesei et al., 2015). A dye swap was also applied to minimize the effect of preferential labelling. To ensure normalization of spot intensities for protein abundance differences analysis, an internal standard (IS) was generated by pooling aliquots of equal amounts of all samples and incorporated within each gel after labelling it with G-Dye100. G-Dye 100, G-Dye 200 and G-Dye 300 labelled samples were combined to be separated on each gel. As a result 20 protein extracts and 10 IS were applied to 10 gels (Fig. 8).

Aliquots of 50 µg protein were separately labelled with 400 pM minimal dye according to company's recommendations. Samples and IS were subsequently pooled together and incubated with 2× Lysis Buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.1% bromophenol

blue, 130 mM DTT, 1% Servalyt™ 2-11 (Serva) in a 1:1 ratio (protein extract/2× Lysis Buffer, v/v) on ice in the dark.

3.4.2 Protein separation

The 13 cm non-linear IPG dry strip (pH 3-10, GE Healthcare) were loaded with a total volume of 255 µl rehydration buffer containing 8 M urea, 2% CHAPS, 0.1% bromophenol blue, 10 mM DTT and 0,5% Servalyt™ 2-11 (Serva) including 20 µg of protein. Two technical replicates were carried out for each of the model fungi and of the temperatures tested.

Combined samples of *E. dermatitidis* including IS and 2× Lysis Buffer were instead added with rehydration buffer [8 M urea, 2% CHAPS, 0.1% bromophenol blue, 13 mM DTT and 0,5% Servalyt™ 2-11 (Serva)] to a final volume of 450 µl for loading on a 24 cm non -linear IPG dry strip (pH 3-10, GE Healthcare). The 13 and 24 cm strips were rehydrated at room temperature for 16 hours and consequently focused at 20°C for 14 kVh and 60.0 kVh respectively, using a Protean IEF cell system (BioRad). The current was limited to 50 µA per strip in both cases.

Strip equilibration was performed for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris HCl pH 8.4) with 2% DTT and subsequently for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris HCl pH 6.8) containing 0.1 % bromophenol blue and 2.5% IAA. The volume of buffer used was 2 ml for the smaller strips and 6 ml for the bigger ones.

Classical 2-DE was performed using 10 % (w/v) SDS-PAGE gels (14x14 cm) and running buffer containing 24 mM Tris pH 8.3, 192 mM glycine, 0.1% (w/v) SDS. For the electropho retic 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).

Fluorescence labeled proteins from *E. dermatitidis* were instead resolved in the second dimension on 12% SDS-PAGE gel (20x26x0.15 cm) using the Ettan DALT six system (GE Healthcare) at 1 W/gel for 1 h, then at 15 W/gel, until the bromophenol blue line reached the bottom of the gels. Lower and upper running buffer were prepared by diluting 1:10 and 1:5 the 10× Running buffer (240 mM Tris, 1.92 M Glycine, 1% SDS), respectively. The chamber was cooled at 4°C (Multi temp III, GE Healthcare).

3.4.3 Staining and analysis of 2D-gels

Following 2-DE, protein spots were visualized by a mass spectrometry (MS) 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 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 finally washed with a 5 % (v/v) acetic acid. 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. From the two technical replicates, the gel exhibiting the highest number of spots was aligned and matched to the reference gel. The spots intensity, densitometrically determined and expressed as spot volume, was evaluated. 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.

DIGE gels did not undergo post-staining, since the proteins were labeled prior to separation. However, preparative gels – obtained in the same way as the analytical gels but using a higher protein amount (400 µg) – were stained with a MS compatible silver staining as previously described (Blum H et al., 1989). Briefly, gels were fixed in 30% ethanol and 10% acetic acid for 1 h and then sensitized in a solution containing 0.5 M sodium acetate, 8 mM sodium thiosulfate pentahydrate and 30% of ethanol, at 4°C overnight. Subsequently three washing steps with MilliQ water (Millipore) were performed, then the gels were incubated in a 0.2% silver nitrate solution for 30 min at 4°C and further rinsed after the incubation. A 2.5% sodium carbonate and 0.01% formalin solution was applied for gel development until the desired intensity of staining was achieved. The staining process was stopped by incubating the gels in a 1% glycine solution, followed by a final washing step with MilliQ water.

The DIGE analytical gels were scanned in a Typhoon™ FLA 9500 (GE Healthcare) at a resolution of 100 µm using the 473 nm laser for G-Dye 100, the 532 nm for G-Dye 200 and the 635 nm for G-Dye 300 and the BPB1 530 DF 20, BPG1 570 DF 20 and LPR (CH.2) R665 filters, respectively. Images were acquired using ImageQuant v8.1 (GE Healthcare) and analysed by DeCyder™ 2D v7.0 (GE Healthcare) for spot count, gel

matching and detection of changes in protein abundance. Protein spots were automatically detected and matched intra-gel using the batch processor module. The estimated number of spots was set to 10000, spot slope and volume were set to 1.12 and 50000 respectively. During the following stage of gel processing, spots intensities were normalized to the IS and inter-gel spot matching and quantification were performed using the BVA module (biological variation analysis). Matches were evaluated and improved by visual inspection. Experimental sets were then created in the EDA (extended data analysis) to compare all experimental conditions with each other and with the control. The percentage of spot maps where proteins had to be present was set to >75%. The results of differential abundances of spots were validated through statistical analysis. An independent Student's t-test and the one-way ANOVA were performed and the data were corrected applying the FDR (false discovery rate). Principal component analysis (PCA) and pattern analysis — i.e. hierarchical clustering (HC) — were further performed to search for outliers and for groups of co-varying spots. Protein spots with an average ratio increasing or decreasing more than 1.5–fold and with a p-value ≤ 0.05 were considered as significantly modulated and assigned to a pick list.

After formaldehyde free silver staining, preparative gels were compared with the analytical gels in order to match and localize the spots of interest. Protein spots were excised manually, using a 1.5 mm diameter one-touch spot picker (Gel Company) and subjected to digestion prior to MS analysis.

3.4.4 In gel tryptic digestion and nLC-ESI-MS/MS analysis

In gel tryptic digestion of the DIGE separated proteins (Tesei et al., 2015) was carried out according to Shevchenko (1996), with a few modifications. Briefly, spots of interest were manually excised from the gels and subsequently de-stained with 100mM sodium thiosulfate/ 30mM Potassium hexacyanoferrate (III) (1:1, v/v). The gel particles were then treated with acetonitrile and rehydrated by adding an equal volume of 100 mM ammonium bicarbonate buffer pH 8.5. After replacing the buffer with acetonitrile, the gel pieces were incubated in a 100 mM ammonium bicarbonate pH 8.5/acetonitrile (1:1, v/v) solution and ultimately dried in a vacuum centrifuge (Unicryo MC2L-60, UniEquip). Gel particles were then incubated at 56°C in a solution containing 10 mM DTT and 100 mM ammonium bicarbonate, for protein reduction and thereafter in an alkylation solution (54 mM IAA, 100 mM ammonium bicarbonate) at room temperature and in the dark. A further incubation step with 100 mM ammonium bicarbonate and then with an equal amount of acetonitrile were performed prior to drying the spots in a vacuum centrifuge.

50 ng trypsin (proteomics grade, Roche Diagnostics) in 50 mM ammonium bicarbonate and 5% acetonitrile were added. Digestion was carried out over-night at 37°C, 350 rpm. Peptides were extracted with 50 mM ammonium bicarbonate, then acetonitrile and finally two times with 1% formic acid/acetonitrile solution (1:1, v/v). All extracts from one spot were combined and subsequently evaporated to complete dryness. Peptides were redissolved in 10 µl 0.1% formic acid solution and subjected to C18 Zip-Tip® purification (Merk Millipore). Briefly, the tip was washed with acetonitrile and then equilibrated with 0.1% formic acid. After evaporation in a vacuum centrifuge, the peptides were resuspended in 10 µl 5% acetonitrile/0.05% formic acid solution.

The tryptic peptides were subjected to nLC-ESI-MS/MS analysis performed on a nano-LC system (Ultimate 3000, Dionex) coupled with an HCT^{plus} (High Capacity Ion Trap) mass spectrometer (Bruker Daltonics). The peptides were separated on an Acclaim PepMap 100 C18 reverse phase column (75 µm i.d. x 15 cm, 3 µm, 100 Å, Thermo Scientific). The mobile phase consisted of acetonitrile and UHQ (Ultra High Quality) water, both containing 0.05% formic acid. A 90 min chromatography run was performed on each sample (0-10 min 5% acetonitrile; 10-60 min linear gradient from 5% to 50% acetonitrile; 60-61 min increase to 70% acetonitrile; 61-71 min 70% acetonitrile; 71-72 min decrease to 5% acetonitrile, 72-90 min column re-equilibration with 5% acetonitrile). Flow rate was set to 250 nL/min.

Mass Spectra were acquired in the positive-ion mode by using the enhanced scanning mode (m/z 300–1500) with a capillary temperature of 200°C and dry gas equal to 0.5 l/min. The maximum accumulation time was 200 ms and the smart target, the averages and the rolling averaging were set to 100000, 3 and 2, respectively. The parameter for fragmentation was set to one precursor ion using active exclusion (excluded after two spectra and released after 0.25 min). Time frame of repeated precursor ions was 25 sec and the relative threshold was set to 5%.

3.4.5 Protein identification and bioinformatics analysis

The compound list, containing peak lists of all acquired MS/MS spectra were extracted using Data Analysis v3.2 (Bruker Daltonics) and submitted to the latest publically available version of MASCOT v2.5.1. (Matrix Science, www.matrixscience.com), through Biotools v3.2 (Bruker), for searches against the NCBI database (NCBInr 20140906; 48573147 sequences; 17378694133 residues). The following parameters were set prior to database search: one missed cleavage site by trypsin (porcine, Roche), MS tolerance of 0.5Da, MS/MS tolerance of 0.3Da, peptide charge of 1+, 2+, 3, carbamidomethylation

of cysteine and oxidation of methionine as fixed and variable modification. Searches were performed using a taxonomy parameter set to Fungi (2952989 sequences).

The search results were evaluated on the base of the MASCOT score cut-off calculated for a significance threshold set to 0.05, number of matching peptides, sequence coverage, p*I* and Mw. Particular attention was dedicated to those proteins whose identification was based on one or two peptide sequences, by verifying the MS/MS spectra manually. BLASTP algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied for protein sequences identified as hypothetical or with uncharacterized function, in order to search for homologues. Knowledge about the single protein's function was gained by inputting each protein sequence into the Uniprot database (http://www.uniprot.org/blast/) and detecting the respective Gene Ontology (GO) terms and annotations (Dimmer et al., 2012, 2008). When terms were not assigned to a protein, the most closely related protein sequence from a different organism, whose GO terms were available, was used. In the case GO terms were not accessible even for homologous proteins, the protein sequence was submitted as query to InterProScan 5 (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) to scan for matches against the InterPro collection of protein signature databases using applications as PANTHER v9.0 (http://www.pantherdb.org) or SUPERFAMILY v1.75 (http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html). In the latter case, only terms with FDR <0.001 were selected.

GOstats (Falcon and Gentleman, 2014) and KOBAS v2.0 (http://kobas.cbi.pku.edu.cn) were used to search for overrepresented GO terms in the group of increased and decreased proteins. GO terms with an uncorrected p-value < 0.05 were considered significantly enriched. The resulting lists of GO terms, i.e. one list per conditions' comparison, were summarized with REVIGO (http://revigo.irb.hr) (Supek et al., 2011) by clustering semantically close GO terms and presented as pie chart with custom R scripts. The threshold for the categorization was set to 0.5.

KOBAS was used to look for enriched pathways in the KEGG, BioCyc and Reactome (Caspi et al., 2008; Joshi-Tope et al., 2005; Ogata et al., 1998) databases based on the set of up- and down-regulated genes. A pathway was considered significantly enriched when its uncorrected p-value was smaller than 0.05 (Xie et al., 2011). The available graphical representations of the overrepresented pathways were downloaded and annotated from the corresponding website.

Information about the sub-cellular localization of the differentially abundant proteins was gained by using the YLoc prediction system based on the YLoc+ model for fungal proteins (www.multiloc.org/YLoc, Briesemeister et al., 2010; Fig. 8)

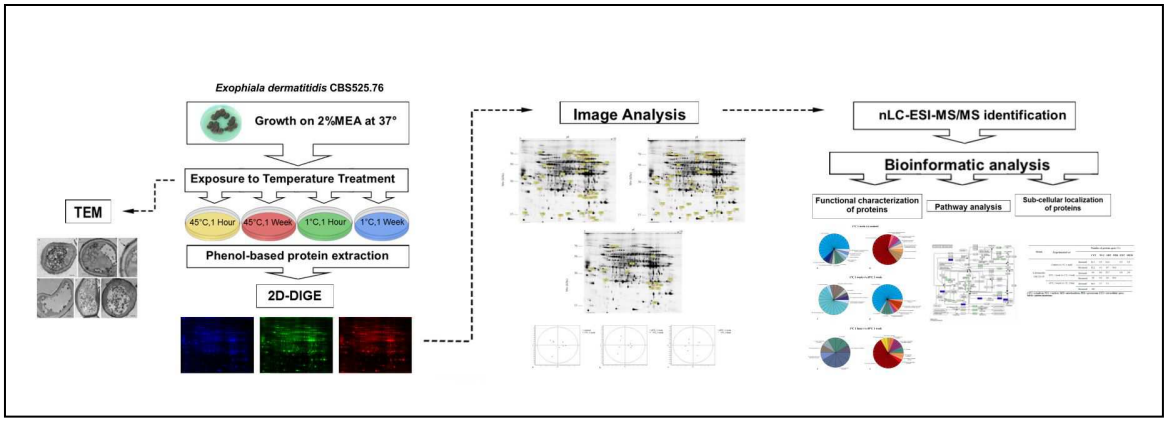

Figure 8. **Workflow of the *E. dermatitidis* proteomic experiment from temperature treatment to bioinformatics analysis** (Tesei et al., 2015)**.**

4 Results and discussion

4.1 Thermal preferences and temperature treatments

As shown in Table 1, for *E. jeanselmei*, *K. perforans*, and *P. chrysogenum* the upper temperature detect limit was 30°C. Tests for thermal preferences further indicated the range from 25 to 37°C as optimal growth temperatures for *E. dermatitidis*.

Fungal growth was still detected after temperature treatment at 28°C, 40°C and 45°C for up to one week in *F. endolithicus*, in *E. jeanselmei* and *K. perforans* and in *E. dermatitidis*, respectively. Growth was also observed after a one week exposure to 1°C, thus ensuring the strains' viability at the chosen experimental conditions. The incubation temperatures and the respective timespans were selected to simulate different environmental conditions and evaluate the effects of short- and long-term exposure on the proteome level.

Table 1_ Thermal preferences of model fungi.

Strain	Thermal preferences (°C)									
	1	**5**	**10**	**15**	**20**	**25**	**28**	**30**	**35**	**37**
P. chrysogenum MA 3995	0.33	6.33	11.67	15.67	26.00	27.50	10.67	5.33	-	-
E. jeanselmei MA 2853	-	0.5	4.00	8.33	10.00	13.33	13.00	9.00	-	-
K. perforans MA 1299	-	-	-	3.00	5.83	4.25	2.83	1.50	-	-
F. endolithicus CCFEE 5208	0.20	0.35	0.45	0.60	-	-	-	-	-	-
E. dermatitidis CBS 525.76	0.17	0.33	2.33	5.50	13.33	14.33	16.50	16.33	13.83	16.67

Thermal preferences are reported as difference in size (mm) between inocula and colonies, three weeks after the beginning of the experiment. The values represent the average of three different tests. All tests were performed on 2% MEA. MA No.: strain number in the ACBR/BOKU culture collection; CBS No.: strain number in the CBS-KNAV culture collection; CCFEE No.: strain number in the CCFEE culture collection of the Tuscia University.

4.2 Temperature-related changes in 2D protein patterns from three black fungi strains

In Tesei et al. (2012), two technical replicates were carried out for each of the fungi and of the temperatures tested, resulting in a total of 24 gels. The analysis of groups of matching spots allowed the evaluation of changes and similarities in protein expression patterns. At all conditions tested the protein pattern of the four fungal strains differed concerning the total number of spots, their molecular weight as well as their isoelectric

point p*I*-related distribution (Table 2). In *E. jeanselmei* the major protein spots had molecular weights above 70 and pIs from 6 to 10, while *K. 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 number of spots found to match at the different temperatures conditions are shown in Table 3.

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

Strains	**Exposure conditions (°C)**			
	28°C	**1°C**	**40°C**	
P. chrysogenum MA3995	381	358	601	**Spots No.**
E. jeanselmei MA2853	382	387	174	
K. perforans MA1299	325	494	255	
	15°C	**1°C**	**28°C**	
F. endolithicus CCFEE5208	425	466	284	**Spots No.**

In accordance with literature data, the exposure of *P. chrysogenum* — used as a reference to the black fungi in the study — to different temperatures influenced both the expression pattern and the spots abundance. While at 40°C, the number of detected protein spots increased to 601, thus resembling a HSR and the related synthesis of stabilizing proteins, the slight decrease in the spots number occurring at 1°C, which mainly affected proteins with basic pIs (8-10), indicated a down-regulation of the metabolic activity. 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).

When exposed to temperatures above their growth regime, the black fungi showed a reaction different from *P. chrysogenum.* All three stains responded to 40°C and 28°C - the latter for *F. endolithicus* - with a reduction of the total number of protein spots, thus indicating a lack of a heat-shock response on the protein level. In *E. jeanselmei* the number of spots switched from 387 to 174, mostly concerning spots with pI in the range 5-7 and Mw between 30 and 170 kDa. In *K. perforans* 255 spots — 70 less than the ones detect-

ed at 28°C — were observed as a consequence of the exposure to 40°C, the reduction mostly concerning spots in the pIs range 5-9 and with Mw between 30 and 90 kDa. From 425 spots detected at 15°C, 284 could be detected in *F. endolithicus* after exposure to 28°C, the loss mostly involving spots in the pI and Mw range 5-7 and 25-90 kDa, respectively.

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 matching protein spots		
		1°C	**40°C**	**All temperatures**
P. chrysogenum MA3995	28°C	153	211	100
E. jeanselmei MA2853	28°C	147	81	46
K. perforans MA1299	28°C	134	47	21
		1°C	**28°C**	**All temperatures**
F. endolithicus CFEE5208	15°C	278	224	187

Since spots from the same pI and molecular weight range were extinct in the protein patterns after temperature increase, it is suggested that the black fungi probably down-regulate similar sets of proteins. The comparison among protein patterns from the three black fungal strains indeed showed a maximum overlap (i.e. 16%), which is higher than the one found among the protein profiles of black fungi and of *P. chrysogenum* (i.e. 13%). 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 *K. perforans* the lack of a heat shock response might reflect the necessity to survive temperatures up to 60°C that are easily reached on the sun exposed rock surfaces inhabited by these fungi. Moreover, this might represent a mechanism 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: as previously reported in Antarctic endemic fish, a HSR might not have been developed during evolution because not necessary for life in a permanently cold habitat (Hofmann

et al., 2000). However, for non-endemic psychrophilic Antarctic yeasts a heat shock response was demonstrated (Deegenaars and Watson, 1998).

In contrast with what observed at high temperatures, the black fungi increased the number of proteins once exposed to 1° C. In *K. perforans*, growth at 1°C resulted in the expression of the highest number of protein spots (494), mostly high Mw spots (higher than 70 kDa). The analysis of gels from *F. endolithicus* revealed a change in the protein pattern also due to an increase in the number of high Mw spots and of spots with pI between 6 and 7 to a total of 466 spots. 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 and acidic pIs (3-5) — was detected in response to the temperature decrease. The change of the protein patterns that occurred in the mesophilic fungi *K. perforans* and *E. jeanselmei* at 1°C can be interpreted as a cold-shock response. The significant increase of protein spots in *K. perforans* especially suggests the transient up-regulation of CSPs and HSPs, key proteins directly involved in the cell protection against the stress induced by temperature (Berry and Foegeding, 1997; Jones et al., 1987; Phadtare and 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). The expression of AFPs and CAPs has been also demonstrated in bacteria, pathogenic fungi, and plants (Collins et al., 2008; De Croos and Bidochka, 2001; Feller and Gerday, 2003; Gocheva et al., 2006; Jia and Davies, 2002; Timperio et al., 2008). According to the results of this study, the production of CAPs can be hypothesized also for *F. endolithicus*.

4.3 Morphology of *E. dermatitidis* under diverse temperature conditions

TEM images of osmium tetroxide-fixed specimens of *E. dermatitidis* control sample reveal cells with a big vacuole filled with abundant floccular material of high electron-density (Fig. 9). Three layers could be distinguished in the cell wall: an inner and scarcely pigmented layer, a middle electron-opaque layer and an outer electron-dense layer, the latter most likely encrusted with melanin. When comparing temperature stressed samples with the control, no dramatic alteration of the cell morphology could be observed. However, an increase in melanization was detected at 1°C and 45°C compared to the optimal growth temperature (37°C). In addition, the long-term exposure to low temperature (1°C, 1 week) seemed to have a greater impact on the cell morphology:

under this condition, a thickening of the outermost cell wall layer was observed, which acquires a fibrillar-floccular consistency. Such floccular layer could not be seen at 45°C 1 week experimental condition, albeit melanization persists in the outer cell wall layer (Fig. 9d). The amount of floccular material appears thereby to be related to the strain prolonged exposure to low temperature and might act as a barrier between the microorganism and the external environment, protecting the cell from the possible damages caused by exposure to sub-optimal temperature or to freezing (Durán and Nombela, 2004). The presence of a floccular layer in the cell wall has been previously described in pathogenic fungi as *Candida albicans* as a crucial element mediating the attachment to the host tissues during the initial steps of infection (Centeno et al., 1983; Montes and Wilborn, 1968). Unlike *C. albicans*, in *E. dermatitidis* such a floccular layer was not detected in the present study at 37°C or at higher temperature, therefore its correlation with the pathogenic activity can possibly be excluded.

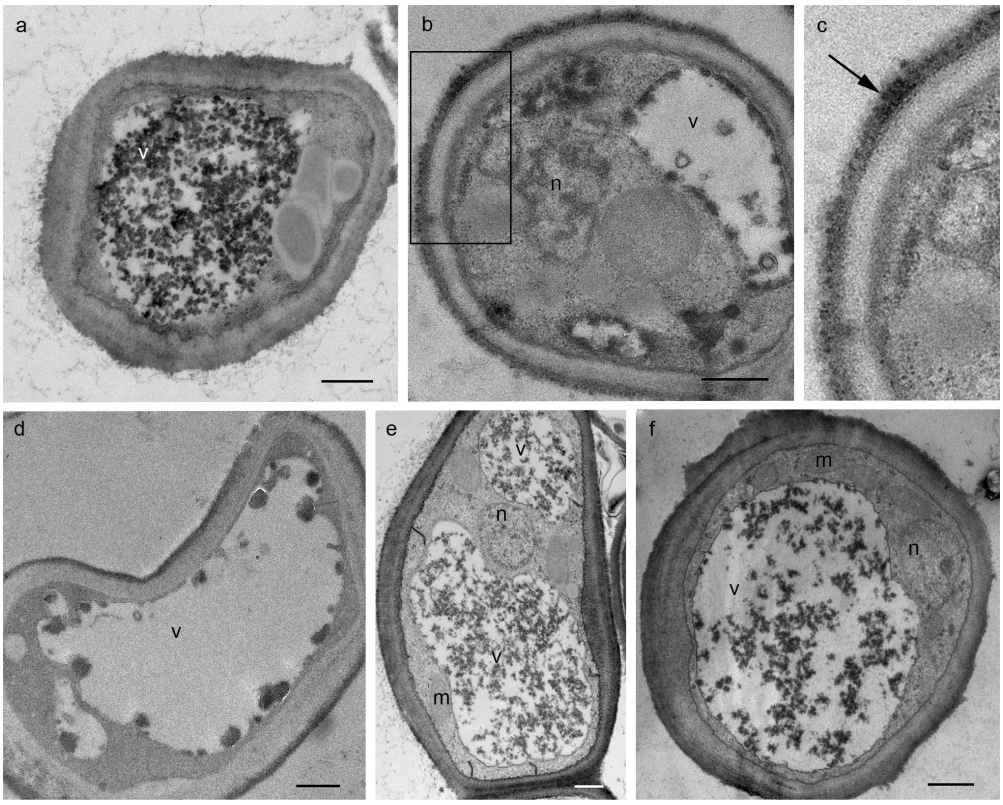

Figure 9_Morphological analysis of *E. dermatitidis* CBS 525.76 under temperature treatments. a. Control condition (37°C); b-d. 1 week exposure at 1°C (b) or at 45°C (d). c. Enlargement of the inset in b. e-f. 1 hour exposure at 1°C (e) or at 45°C (f). Cells show a prominent vacuole (v) filled with floccular electron-dense material. The nucleus (n) and some mitochondria (m) are also visible. The cell wall consist of three layers, the outermost of which is pigmented due to melanization and exhibits a floccular-like consistency in relation to the prolonged exposure to low temperature (c). Bars = 0.5 µm

It can further be hypothesized that the fibrillar-floccular texture of the outermost layer of the wall is the result of the deposition of extracellular polysaccharides (EPS), which might also act as sheltering molecules and prevent cell freezing by slowing the accumulation of ice crystals at the cell wall. The attempt to increase the degree of freezing tolerance in response to low non-freezing temperatures was described in many species as part of the cold acclimation (Theocharis et al., 2012).

The accumulation of melanin in the cell wall also plays a shielding role for the cell. Along with being a virulence factor in pathogenic species, melanin has a remarkable antioxidant activity, protecting the cells from damage by ROS, which are both part of the host immune response and are accumulated during temperature stress (Schnitzler et al., 1999). As previously demonstrated, ROS scavenging and tolerance to temperature stress are directly linked (Suzuki and Mittler, 2006) and it is not surprising that a high proportion of melanin-producing fungi are isolated from environmentally stressed habitat in hot and cold deserts, alpine and Antarctic regions, where the exposure to extreme temperatures is prolonged in time (Eisenman and Casadevall, 2012; Nosanchuk et al., 2012; Zhan et al., 2011). It follows that, in the present study, a higher abundance of melanin undoubtedly reflects the necessity to tolerate sub- and supra-optimal temperatures (i.e. 45 and 1°C).

4.4 Analysis of 2D-DIGE gels and identification of the temperature responsive proteins

2D-DIGE analysis of *E. dermatitidis* total protein extract was performed using four biological replicates — biomass from four petri dishes — for each of the four temperature treatments and for the control condition (Tesei et al., 2015). The results of gel imaging on a total of 30 spot maps showed an average of 1700 protein spots being detected on each map. After comparing all experimental conditions with each other by creating experimental sets, protein significant modulation ($p \leq 0.05$, Av.Ratio > 1.5 fold) was observed only in (a) control condition as compared with 1°C 1 week, (b) 45°C 1 week as compared with 1°C 1 week and (c) 45°C 1 week as compared to 1°C 1 hour. The number of protein spots detected as differentially abundant in the three experimental sets are shown in Table 4. Since some of the protein spots were found to be increased or decreased in more than one of the experimental sets, the total number of spots detected as differentially abundant was 75. From these 75 spots, 54 were successfully analyzed and a total of 32 different proteins identified (Table 3; Tesei et al., 2015). The table also

shows the alterations in spot abundance (as average ratios) for the three experimental sets where protein significant modulation was detected.

Out of the 54 identified protein, 5 spots most likely represent fragments of full-length proteins (e.g. spot N° 1507, 1582 1787, 943 and 947, Table 3; Tesei et al., 2015) since only C- or N-terminal peptides were identified. Furthermore, a few spots with different p*I* but identical Mw are found to match the same particular protein (e.g. spot n° 1125 and 1896), this representing a quite common phenomenon in 2D electrophoresis, possibly involving posttranslational modifications. In some cases (e.g. spot N° 1582, 1799) from a single spot, peptides matching two or more different proteins were detected, thus not allowing unambiguous identifications.

The identification of the remaining spots failed due to several reasons: 9 protein spots could not be localized and excised from the preparative gels (spot N° 1575, 1712, 1741, 1788, 1792, 1903, 1904, 1906, 1941), 12 spots could instead not be identified due to their very low abundance. The lack of sequence similarity in the database also represented an obstacle to protein successful identification. In a few cases the presence, in a single spot, of peptides matching two or more different proteins did not allow unambiguous identification.

To elucidate the identity of the 5 observed hypothetical proteins, a search for homology was performed. In all cases the highest rate of sequence similarity is found with proteins belonging to the fungal species *Capronia coronata* and *C. epimyces,* both closely related to *E. dermatitidis* and forming *Exophiala*-like anamorphs in cultures.

Table 4. Number of differentially abundant protein spots detected at the 1°C, 1 week and 1°C, 1 hour experimental condition, in the different experimental sets.

Strain	Experimental set	Number of protein spots		
		Total	Increased	Decreased
E. dermatitidis CBS 525.76	Control v/s 1°C, 1 week	47	26	21
	45°C, 1 week v/s 1°C, 1 week	50	33	17
	45°C, 1 week v/s 1°C, 1 hour	15	13	2

However, since the identity of the proteins from *Capronia* is also not known, next closely related proteins — whose identity is instead assigned — have been considered (Table 4; Tesei et al., 2015).

The major part of the matching proteins are stress response proteins, however in some cases — i.e. spots 1290 and 1197 — the BLAST results did not allow to unambiguously establish homology between identified sequences and characterized proteins included in the NCBInr database.

PCA analysis was additionally performed on all matched spots across the gels to test the reproducibility of the four biological replicates and to verify the clustering of protein profiles according to presence or absence of similarity. As shown in Fig. 4a (Tesei et al., 2015), all four 1°C 1 week protein profiles cluster together, relative to the PC1 calculation. The same clustering pattern was observed for the control protein profiles. The PCA results demonstrated that, in all experimental sets, protein profiles of the four biological replicates from the same experimental condition group together while profiles from different experimental conditions are instead distinct. Component 1 explains 83% (4a), 90.6% (4b) and 93.4% (4c) of the variance, while component 2 explains 6.5%, 2.9% and 2.6%, respectively.

HC analysis based on similarities of abundance patterns in the spot maps reveals clusters of co-varying spots graphically represented as dendrograms, for a standardized logarithmic abundance scale ranging from — 1.0 to + 1.0. As shown in Fig. 5 (Tesei et al., 2015), proteins with increased and with decreased abundance levels are visualized as large spots clusters in each of the experimental sets. Moreover, changes in the protein abundance are consistent within the four biological replicates of each experimental condition. In agreement with the PCA results, this demonstrates that the biological samples share a very similar spot map. Since the statistical analysis was performed only on proteins present in at least 75% of the spot maps, in a few cases data for a specific protein spot were not available. The absence of a protein spot in a spot map is shown as a grey box in the dendrogram.

4.5 Temperature-related alterations in the proteome of *E. dermatitidis*

The analysis of protein profiles from *E. dermatitidis* revealed changes in the protein abundance — beyond the established cut-off value — only in response to the prolonged exposure to low temperature. The protein pattern does not undergo any remarkable increase or decrease in the number of protein spots as well as any statistically significant changes in protein abundance under the other temperature treatments. Even when lowering the average ratio to >1, protein modulation is not detected. This indicates that the

short-term exposure to both high and low temperature does not trigger a rearrangement in the *E. dermatitidis* proteome.

This is in complete contrast to what was hitherto found in other fungi and in other pathogenic species (Enjalbert and Whiteway, 2003). According to the ecology of a species, the over-expression of chaperones, HSPs and CSPs, or on the contrary the down-regulation of the protein expression, represent the most important effects to temperature stress (Piette et al., 2010; Tesei et al., 2012). In pathogenic species, generally a temperature increase to 37°C is the signal for expression of virulence genes. Conversely this temperature might contribute to protein denaturation especially in less adapted species, thus resulting in the consequent induction of HSPs as stabilizers (Leach and Cowen, 2013). Previous studies conducted on human pathogens such as *C. albicans* and *Aspergillus fumigatus* demonstrated re-arrangements of the protein pattern — including the over-expression of HSPs among other proteins — after a shift from 37 to supra-optima temperatures (i.e. 45 and 48°C) (Albrecht et al., 2010; Zeuthen and Howard, 1989).

Less is known about the reaction of fungal pathogens to low temperature. However, a correlation between cold and the over-expression of temperature responsive proteins — defined as cold-stress markers — has been previously described in the yeast *S. cerevisiae* (Aguilera et al., 2007). The induction of stabilizing proteins is usually time-dependent and changes in the protein expression are usually detectable already after 15-30 minutes from the beginning of the stress. The abundance of the induced proteins normally peaks and thereafter returns on the basal level by a time range which depends on the intensity and duration of the stress (Kniemeyer, 2011).

Although the synthesis of stabilizing proteins represents a conserved and universal response to temperature stress, the results of the present study show a different trend in *E. dermatitidis*. The absence of a shock response under temperature stress in the black fungus could possibly find an explanation in the basic set of proteins expressed at 37°C. The combination of housekeeping, stabilizing and putative virulence proteins might per se confer temperature tolerance and thus guarantee the organism's survival once exposed to temperature stress, without requiring additional protective proteins.

The results clearly show that the short-term exposure to sub- and supra-optimal temperatures (i.e. 45°C and 1°C) is not perceived as a stress by the fungus. Interestingly, this also applies to the long-term exposure to high temperature (45°C, 1 week). In contrast, temperature-responsive and stabilizing proteins normally serve as survival strategy in case of prolonged exposure to non-optimal temperatures, which leads to acclimation. Acclimated organisms display high level of those proteins, thus being able to start a

quick response if exposed to even more extreme temperatures (Cabané M et al., 1993). Nevertheless, by promoting metabolic homeostasis despite environmental changes, acclimation requires energetic costs (Johnston and Bennett, 2008). Costs that might become too high to sustain for organisms having to cope with long lasting unfavorable life conditions. On this basis, we state that in the case of *E. dermatitidis* — where the costs may outweigh the benefits — a lack of response represents the best adaptation. This idea finds support in the fact that the recourse of black fungi to energy saving mechanisms, in both changing and constant environments, was already demonstrated to be crucial for the organism survival (Tesei et al., 2012; Zakharova et al., 2014, 2013).

It can therefore be speculated that *E. dermatitidis* manages to adapt to temperature thanks to the thermo-stability of the basic set of proteins which, by remaining functional under temperature shifts, allows the organism to save precious energy. In addition, protective molecules or still unknown cellular mechanisms may also have a role in the tolerance and adaptation. Recently, the fusion of distinct mRNAs to produce the necessary proteins once in need has been proposed as a solution for thriving in the natural and in the human-made environment as well as in the human host (Blasi et al., 2015).

4.6 Functional analysis of the identified proteins

In order to clarify the putative biological function of the identified proteins and their involvement in particular cellular pathways, protein functional analysis was carried out on the base of cellular process GO terms. This approach is generally used in transcriptomics, however, it could indicate the importance of the identified proteins for the cellular processes as well.

In the cases where the MS analyses of a single protein spot resulted in multiple protein identification, only the proteins characterized by the highest MASCOT score were considered for the analysis. If no term was available for a single protein, GO terms assigned to the most closely related protein sequence from a different organism, were used. No GO term could be assigned to roughly 5% of the identified proteins, namely to the hypothetical proteins gi|378733644 and gi|378726833, whose putative identity could be established through homology search.

Pie charts showing the over-represented GO terms for the proteins were obtained for each condition comparison using REVIGO, with the aim to elucidate which cellular processes are mostly affected by temperature treatment. In each pie chart, semantically close terms are clustered into categories of cellular processes, being each category represented by a different color (Fig. 6, Table 5; Tesei et al., 2015). The complete and

summarized lists of over-represented cellular process GO terms (uncorrected p-value < 0.05) per condition comparison are shown in Supplementary Table1 (Appendix A and B).

Knowledge about the functional characterization of the identified proteins was integrated with data about biological pathways. The identification of differentially abundant proteins indeed enabled us to discover biological pathways, which might accordingly be modulated in response to temperature exposure. KOBAS v2.0 was used to look for over-represented categories of pathways on the base of the sets of genes encoding for the increased and decreased proteins. A complete list of significantly enriched GO pathways (uncorrected p-value < 0.05) and links to the respective annotated graphical representations are shown in Supplementary Table 2 (Appendix C). In each pathway the significantly regulated proteins are highlighted in blue and red according to decrease and increase in abundance, respectively. Green is used for the rest of the genes characterizing the pathway.

Both analysis were performed on the base of set of identified proteins with changed abundance. Further, for each condition comparison, the two sets of proteins — with increased and with reduced abundance — were processed separately. In a number of cases assessing the up- or down-regulation of a single protein was critical, especially when the protein actually represented a fragment of the whole sequence based on the identification of only N- or C-terminal peptides. However, the sets of data from each condition were directly submitted to proteins' functional characterization and pathway analysis, without prior rearranging them.

In a few cases the same cellular process and/or pathway are detected as both up- and down-regulated within the same experimental set. This concerns all fundamental cellular processes and pathways involving Hsp70-like proteins, acetyl-CoA synthetases, aldehyde dehydrogenases and glyceraldehyde 3-P dehydrogenase (indicated by their correspondent genes HMPREF1120_02626, HMPREF1120_06094, HMPREF1120_01109 and HMPREF1120_01270, respectively in Supplemental Table 1 and 2, Appendix A-C).
In the case of Hsp70-like proteins (gene HMPREF1120_02626) three protein spots, probably corresponding to as many protein species and exhibiting different p*I*s, are found. Two of the spots show increased abundance at low temperature while the third one is decreased. Differences due to alternative splicing can be speculated, however it is not possible to exclude the possibility that the three proteins are encoded by different genes, some of which have not been yet annotated. Post-translational modifications, phosphorylation or methylation, can be further hypothesized.

In the case of the acetyl-CoA synthetases, the only protein spot detected (i.e. 1507) presents increased abundance at low temperature. Aldehyde dehydrogenase is instead detected in 5 protein spots from different condition comparisons. While 2 spots resulted to have increased abundance (i.e. 943 and 947), three spots were detected as less abundant (i.e. 466, 649 and 1899). Nevertheless, all spots with increased abundance from both proteins show a lower Mw than expected. Since the masses do not match — although the protein identity match is high confidence — in this case a cleavage event can be hypothesized, which may be of evidence of protein degradation or truncation. Indeed, for spot 1507 only C-terminal peptides were identified in the mass spectra. Peptides spanning only the central part of the sequence were instead found in the mass spectra of protein spots 943 and 947, thus also indicating protein fragmentation. Since — as expected in such cases — a decrease in abundance concerning the proteins whose experimental Mw matches the expected Mw value was also observed, the decrease of the full-variant and the increase of the fragmented counterpart suggest the down-regulation of these proteins. Similarly, the glyceraldehyde 3-P dehydrogenase resembles a degradation product although an increase in its abundance is observed at low temperature. Hence, the respective proteins were considered as down-regulated.

In addition to protein functional characterization, the sub-cellular localization of all identified temperature responsive proteins was predicted. By using YLoc+ interpretable server, the prediction was carried out on the base of each protein biological properties and performed into 10 different locations — also searching for multiple locations — taking into account GO terms transferred from close homologous proteins. Protein localizations for each condition comparison are showed in Table 5. The complete list of all identified proteins and respective sub-cellular localization is presented in Supplementary Table 3 (Appendix D).

The results of protein functional characterization and of the prediction of protein pathways and sub-cellular localization — according to the identified proteins — are presented and commented as follows: since 27% of the differentially abundant protein spots could not be identified, additional protein functions and pathways are expected to play a role in *E. dermatitidis* response to temperature treatment. The same applies to the identified proteins that have been excluded from the functional analyses in order to avoid ambiguities in the prediction of up- and down-regulated cellular processes.

4.7 Proteins involved in the response to low temperature

In *E. dermatitidis* the greatest impact on the cellular level was observed under the 1°C 1 week incubation condition. In accordance with the findings of a recent study on the transcriptome of this fungus (Blasi et al., 2015), the highest number of cellular processes was altered by the exposure to low temperature, thus indicating the strain's effort to cope with the cold.

The major part of proteins whose abundance changed significantly under low temperature were cytoplasmic and mitochondrial, however peroxisomal proteins were also detected as being only down-regulated at 1°C 1 week (Table 5). The performed proteome analysis enabled to highlight those biological processes and metabolic pathways mostly affected by low temperature, therefore providing first indicators ─ on the proteome level ─ for regulated pathways during temperature stress.

A large number of the temperature-modulated proteins were found to be associated with the carbohydrate synthesis and metabolism. The result of both functional characterization and pathways analysis showed that especially proteins involved in the pyruvate (p=8.55e-05), acetate (p=1.1e-04) and carbon metabolism (p=0.002) exhibit lower levels at 1°C. This is in agreement with what was previously reported in yeasts following the incubation to low temperature (Dragosits et al., 2009) and suggests a reduced metabolism. Further components of basic energy metabolism such as malate synthase and malate dehydrogenase ─ which are responsible for the formation of malate from acetyl-CoA and of oxaloacetate from malate respectively in the glyoxylate shunt ─ showed lower levels too at 1°C. The glyoxylate shunt is an ancillary cycle to the TCA cycle and is essential for growth on two-carbon compounds, along with being required for fungal virulence (Lorenz and Fink, 2001). It is not surprising that also the ethanol oxidation, whose product acetyl-coA can be metabolized by reacting with the intermediates of the glyoxylate cycle, resulted to be down-regulated (p=6.71 e-05). Moreover, as expected in response to the thermal downshift, proteins involved in the response to heat stress such as Hsp70s, elongation factor 1-alpha and the hypothetical protein gi|378729668 identified as Hsp30 through homology search, also showed decreased levels (de Nadal et al., 2011). Similarly, the down-regulation of cell aging at low temperature can be interpreted as direct consequence of the slowing of the metabolic rate, which is linked to a reduction of energy consumption and of ROS (Lorin et al., 2006). As already shown in the methylotrophic yeast *Pichia pastoris*, at low temperature and on non-methanolic growth substrates (Dragosits et al., 2009), also in *E. dermatitidis* a decrease in the abundance of alcohol oxidases was detected. Multiple spots with different electrophoretic mobility

were actually identified as alcohol oxidase, thus suggesting the presence of isoforms (Kemptner et al., 2010).

Table 5. Predicted sub-cellular localization of the differentially abundant protein spots detected at the 1°C, 1 week and 1°C, 1 hour experimental condition, in the different experimental sets.

Strain	Experimental set		Number of protein spots (%)					
			CYT	NUC	MIT	PER	EXT	MEM
E. dermatitidis CBS 525.76	Control v/s 1°C, 1 week	Increased	61.1	5.5	22.2	-	5.5	5.5
		Decreased	52.2	4.3	8.7	34.8	-	-
	45°C, 1 week v/s 1°C, 1 week	Increased	60	8.6	25.7	-	2.8	2.8
		Decreased	40	6.6	6.6	46.6	-	-
	45°C, 1 week v/s 1°C, 1 h	Increased	84.6	7.7	7.7	-	-	-
		Decreased	100	-	-	-	-	-

CYT = cytoplasm; NUC = nucleus; MIT= mitochondrion; PER = peroxisome; EXT = extracellular space; MEM = plasma membrane.

Moreover, the spots show different ratios of abundance, the ones with most basic p*I* resulting to be more decreased. Although this study did not focus on the regulation of post-translational modifications of single proteins, the observation of concerted regulations of protein species ─ for e.g. the above mentioned alcohol oxidases isoforms ─ corroborates the finding that the pathways they are involved to, are also modulated. Depending on the species, the alcohol oxidases' function can be related to methanol metabolism, however a methylotrophic activity is not known for *E. dermatitidis.*

Interestingly, a few proteins involved in carbohydrate metabolism (i.e. glycolysis/gluconeogenesis, carbon and pyruvate metabolism) showed a higher abundance at 1°C. In accordance with the morphological changes in the cell wall observed at low temperature condition ─ thickening and floccular-like aspect ─ and with some of the enriched biological process GO terms (i.e. Table 5, XV k, l, n; Tesei et al., 2015), the induction of these proteins might have the aim to enhance the synthesis of cell wall polysaccharides which have a physical protective function for the wall.

However, the presence of truncated and/or degraded proteins interferes with the above mentioned categories of biological processes and pathways, thus their evaluation as of generally actually up-regulated processes is critical. The examination of the MS/MS spectra showed in fact that peptides with higher identification scores covered only parts of the total protein sequence. This phenomenon concerned other up-regulated biological processes and pathways as well. Protein fragments can be the product of both truncation and degradation, two phenomena that have different impact on the protein function.

Unlike degradation, truncation can represent transcript splicing or functional cleavage (Rogowska-Wrzesinska et al., 2013). Furthermore, it should be considered that the procedure for protein extraction can also have an impact. In the current proteomic study, down-regulation was suggested in all cases where the proteins with increased abundance resembled a degradation product and the full-length protein counterpart was found to be decreased. The Hsp30 (spot N° 1787) represented a good example: one protein spot identified as a fragment of Hsp30 with experimental Mw of approximately 10 kDa and p*I* around 9.0 showed higher abundance at 1°C. A second spot identified as the *E. dermatitidis* hypothetical protein gi|378729668 and homologous of a 30 kDa Hsp of *Fusarium oxysporum,* exhibited instead a decreased level. This spot had experimental Mw and p*I* values of approximately 30 kDa and 5.9 respectively, which indeed match the theoretical values of the full-length Hsp30. Hsp30 was accordingly deemed down-regulated at low temperature. Similarly, the glycolysis/gluconeogenesis pathway and the methane metabolism are not to be considered as up-regulated.

An additional pathway to be up-regulated was the regulation of ligase activity, which based on the associated GO terms, is involved in the cell cycle, filamentous growth and the negative regulation of apoptotic process.

4.8 High and low temperatures comparison

Significant re-arrangements of the proteome were also detected between the low temperature and the high temperature incubation conditions. Similarly to what observed when comparing the low temperature incubation condition with the control, the processes to be down-regulated are primarily related to carbohydrate metabolism. By comparison with 45°C 1 week condition, at 1°C 1 hour the down-regulation was limited to the TCA cycle (p=0.004) and the glyoxylate metabolism. One of the enzymes of the glyoxylate shunt – the malate dehydrogenase – that showed lower levels at 1°C at all condition comparisons represented an interesting case of protein regulation. Two different protein spots (spot n°1896 and 1125, Table 3; Tesei et al., 2015) having diverse Mw and p*I* were identified as the enzyme, thus denoting the presence of post translational modified forms of the same protein being modulated at different growth conditions (Gygi et al., 2000). After the incubation to 1°C for a week, pentose-phosphate shunt (p=9.95e-05), carbon and pyruvate metabolism were down-regulated. Enzymes of the pentose phosphate pathway are actually known to have a heat shock-dependent regulation in yeasts, thus suggesting their role in the depletion of ROS and in balancing the organism redox state (Albrecht et al., 2010; Eastmond and Nelson, 2006). Cellular processes in-

volving aldehydes, other components of basic energy metabolism which are responsible for interconversion of acetaldehyde and acetate (Dragosits et al., 2009), also showed lower levels at 1°C 1 week. Similarly, at 1°C 1 hour proteins involved in the aspartate degradation exhibited lower abundance. Since aspartate can be converted to intermediates of multiple pathways and can additionally act as precursor for the biosynthesis of many other compounds such as lysine and NAD^+, it actually represents a link between amino acid and carbohydrate metabolism (Voet and Voet, 2004).

Proteins involved in the beta-oxidation of fatty acids ($p=0.0007$) and peroxisomal lipid metabolism ($p=0.006$) showed instead higher levels at 1°C 1 week. This may be explained by the fact that modifications in membrane lipid composition are a typical response to temperature changes in both uni- and multicellular organisms (Gunde-Cimerman et al., 2014). With the lowering of growth temperature also the fluidity of the cell membrane decreases, thus affecting membrane-associated cellular functions and ultimately compromising the organism survival (Russell, 2008). Shortening the length of the membrane fatty acid chains represents a strategy to counteract the effect of cold stress by maintaining a constant degree of membrane fluidity (Al-Fageeh and Smales, 2006). No evidence of modulation of lipid-related pathways was observed at 45°C, thus indicating that this temperature has no impact on the membrane status. By having a role in the breakdown of storage lipids, β-oxidation additionally represents an advantageous mechanism to produce energy by the use of existing molecules instead of investing energy in the synthesis. This aspect becomes more relevant under non-optimal conditions of growth.

The up-regulation of the ubiquitin-protein ligase activity at low temperature can actually denote the degradation of target proteins, which enables the organism to degrade unnecessary proteins and maximize the survival under different conditions, such as heat and oxidative stress (Dreher and Callis, 2007). Since poly- and mono ubiquitination — as one of the fundamental post-translational modifications for regulating protein function — indeed control many aspects of cellular function in yeasts and in all eukaryotes (Sadowski et al., 2012), it is not possible to exclude that other cellular processes, such as membrane transport and transcriptional regulation (Hicke, 2001) are also involved.

The abundance of proteins related to the cell cycle control was increased after both short- and long-term exposure to 1°C. Proteins like glutathione S-transferase and the 14-3-3 family proteins play a regulatory role in all eukaryotic cells, having important cell signaling implications (Fu et al., 2000; Laborde, 2010). The up-regulation of 14-3-3 may also result in in the alteration of target proteins' function as well as in protein sequestering. Spatial sequestration of misfolded and stress-denatured proteins aiming to protein

degradation, may actually enhance cellular fitness during stress (Escusa-Toret et al., 2013). Moreover, according to the nature of the protein ligand, the 14-3-3 binding may lead to cell cycle arrest (Hermeking and Benzinger, 2006). Both in prokaryotes and in eukaryotes, the slowdown of the cell cycle is known to be induced by cold stress (Al-Fageeh and Smales, 2006), being triggered in yeasts by near-freezing temperatures [83].

Similarly, at both 1°C 1 week and 1°C 1 hour condition as compared to 45°C 1 week, proteins involved in carbohydrate metabolism (e.g. acetate and pyruvate metabolism) showed higher abundance. The synthesis of cell wall polysaccharides can, in this case, be speculated. However, due to the fragmentation of the involved proteins, these processes can hardly be considered as actually up-regulated. The same applies to the up-regulated categories of cell aging.

A small number of proteins involved in the cellular responses to stress — mostly superoxide dismutases, isoform of Hsp70s and 14-3-3 family proteins were also recorded as up-regulated at 1°C — at all condition comparisons. In combination with the down-regulation of the metabolism and the re-arrangements at the cell membrane level, this is a clear hint that low temperature is perceived by the fungus as non-optimal condition.

5 Conclusions and outlook

The results of our studies, which are the first contribution to the investigation of the effects of temperature on the proteomes of black fungi, 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 other fungi.

Despite their ecology and habitat, black fungi show a similar reaction to temperature treatment, which concerns the lack of a heat shock response following exposure to high temperature, a response that is generally observed in yeast and fungi spanning from mesophilic to pathogenic species. By contrast, lower temperatures lead to the synthesis of additional proteins even in Antarctica-endemic species. In the latter case the expression of CSPs, AFPs and CAPs can be hypothesized, while the survival of cells despite heat seems to take place without expression of protective proteins. A similar trend was observed in the human pathogen *E. dermatitidis:* tests for thermal preferences and viability after heat and cold treatment confirmed that the black yeast is capable to thrive at a wide range of temperatures. Whereas 25-37°C supports fast growth, the fungus is able to survive and to grow — albeit with slow growth rates — at temperatures far from the optimum (1 and 45°C). This is in accordance with the morphological, ultrastructural observations as well as with the proteomic analysis.

While the exposure to 1°C led to a metabolic down-regulation and re-arrangements at the cell membrane and cell-wall level (i.e. increase in pigmentation and floccular-like appearance of the outer layer), no HSR was recorded at high temperature.

Such ecological plasticity and ability to grow at different temperature conditions also characterizes the other black fungi species analyzed in the present study, however to a less extent *F. endolithicus*, due to its nature of endemic species in a permanently cold habitat. Based on our studies, we suggest that the ability to withstand long lasting unfavorable temperature conditions can possibly rely on a fine-tuning regulation of the protein expression, mostly involving housekeeping proteins such as components of the major metabolic pathways. Together with the thermo-stability of the basic set of proteins and the morpho-physiological adaptations, this is what probably makes black fungi fully successful organisms. Other protective molecules in the cell and cellular mechanisms that are still unknown might additionally play a role.

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 only 13 % (Tesei et al., 2012). 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.

The finding that *E. dermatitidis* response to temperature treatment differs from the response of other human fungal pathogens is also important in view of the fungus distribution, transmission and infection route. A transition from glaciers, as a natural reservoir, to the water reservoirs and from there to the human environment is likely.

A fine-tuning regulation of the protein expression and the down-regulation of cellular metabolism can ensure survival under low temperature condition. From glaciers and natural springs, the strain can possibly access the tap water system. Due to its low metabolic activity and without the need to produce stress related proteins or other stress related compounds the fungus can easily survive the oligotrophic conditions in the water. Despite the switch from low to high temperature, the colonization of human habitats as sauna facilities and dishwashers takes place without issues. This transition is made possible thanks to morpho- physiological evolutionary adaptations that involve a basic set of stable proteins.

Besides the ecological meaning of the results, an in depth analysis of single proteins could have important implications not only in view of virulence but also for biotechnological applications especially regarding thermo-stable proteins and functions. Proteins involved in the glyoxylate shunt like formate and malate dehydrogenase, or alcohol oxidases and 14-3-3 family proteins, which participate in the basic cellular metabolism and whose levels are down-regulated by lowered temperature, represent good candidates for knock-out experiments. Further, the information gained about the sub-cellular localization of the identified proteins can be verified by immunocytochemistry aiming at target proteins. Improving the understanding of the identified “key metabolic proteins” involved in tolerance fine-tuning can be implemented into biotechnological applications, regarding recombinant product formation. Additionally, the investigation of identified metabolic processes ultimately supports the development of antimycotic strategies.

Our results strongly suggest that the black fungi whole proteome might represent a treasure box, containing novel proteins with interesting properties and functionalities. On this basis, it will be of great interest to characterize the hitherto unidentified set of non-temperature responsive proteins from *E. dermatitidis,* in order to search for biomarkers of virulence and for proteins with pharmaceutical and biotechnological applications, such as enzymes active at wide temperature ranges. Similarly, the identification of proteins from the remaining black fungi species — i.e. *Knufia perforans, Cryomyces antarcticus* and *Exophiala jeanselmei* — will be possible in the next future thanks to the annotated

full genomes and transcriptomes sequences which became recently available (Sterflinger et al., 2014b) or are now ongoing (Sterflinger, unpublished data).

Studies on *E. dermatitidis* involving three dimensional human skin models will be of major interest in order to investigate the mechanism responsible for the fungus pathogenicity (Poyntner et al, unpublished data). A comparative analysis of proteome, transcriptome and metabolome profiling will help elucidating pathways involved in fungal infection, skin responses and their mutual interactions. In this direction, a study on the exosomes released by *E. dermatitidis* during infection, aiming to detect mRNA and miRNA (as described in fungal pathogens as *Paracoccidioides brasiliensis*), would help elucidating the role of these vesicles in the fungus virulence and in the modulation of the host immunity.

The potential of black fungi doesn't in fact solely lie in the protein pool. Recent transcriptomics studies carried on in our research group show that ─ beside the thermostability of the basic set of proteins elucidated in the present work ─ black fungi recur to further mechanisms of tolerance involving diverse RNA species. CircRNAs, ncRNAs as well as fusion transcripts seem to enable *E. dermatitidis* to synthesize proteins on-the-fly under temperature stress, thus supporting the adaptation to different environmental conditions (Blasi et al., 2015; Poyntner et al, upublished). Further, RNA-seq of transcriptoms from *E. dermatitidis* under different kinds of stress ─ e.g. UV, Ozone, or a combination of both ─ could improve the knowledge on stress resistance in the fungus and in fungi in general.

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7 List of tables

8 List of figures

to an *Exophiala sp* (Seyedmousavi et al., 2013); (f-h) fungal colonization of host tissues; (f) H&E stain of melanized, moniliform hyphal elements of *Cladiophialophora bantiana* from a brain abscess (Revankar and Sutton, 2010) (g) GMS stain of sclerotic bodies produced by *Fonsecaea pedrosoi* (Revankar and Sutton, 2010); (h) High power view demonstrated multiple pigmented fungal hyphae with marked de-granulated eosinophils (H&E, X400; Patra et al., 2014).

Figure 4: Effects of Heat Shock on the Organization of the Eukaryotic Cell. An unstressed eukaryotic cell (left) as compared to a cell under heat stress (right). Cytoskeleton damage as well as the reorganization of actin filaments (blue) into stress fibers and the aggregation of other filaments (microtubuli, red) are visible. The Golgi and the endoplasmic reticulum (white) disassemble, mitochondria (green) and lysosomes (yellow-white gradient) are reduced in number. The nucleoli, sites of ribosome (yellow) assembly swell. Large granular depositions – the stress granula (yellow), formed by the aggregation of proteins and RNA – accumulate in the cytosol along with protein aggregates (hexagonal versus spaghetti style, orange). Changes also occur at the membrane level: the fluidity increases and membrane proteins aggregate. Together, all these effects stop growth and lead to cell-cycle arrest as indicated by the non-condensed chromosomes in the nucleus (Richter et al., 2010).

Figure 5: Schematic representation of changes in membrane structure and the behavior of lipid bilayers under low- and high-temperature stress. While low temperatures cause “rigidification” of membranes, high temperatures cause their “fluidization” (Los and Murata, 2004).

Figure 6: Mechanisms of temperature sensing in fungi. Upon a thermal insult, cells utilize different mechanisms to sense changes in the surrounding temperature for adaptation and survival. Hsf1 becomes phosphorylated, binding to heat-shock elements (HSEs) in target promoters, leading to the up-regulation of chaperone proteins. These aid in the stabilization and refolding of denatured proteins, and Hsp90 and Hsp70 assist in the down-regulation of Hsf1. The cellular plasma membrane becomes more fluid in response to thermal insults (right inset box). Moreover, although little is known about RNA thermometers in fungi, there is evidence suggesting they are involved in RNA decay during heat shock (left inset box) (Leach and Cowen, 2013).

Figure 7: Trehalose and HSPs prevent protein denaturation and aggregation and assist protein refolding (a) High-temperature induced protein denaturation. Unfolded proteins can associate to form aggregates. (b) Large quantities of the disaccharide trehalose are synthesized during heat shock to stabilize proteins in their native state and also to suppress aggregation of denatured proteins. (c) Trehalose is rapidly degraded after heat shock. During recovery, molecular chaperones promote the reactivation of denatured substrates that have been prevented from aggregating. (d) The persistence of high levels of trehalose interferes with the reactivation of denatured substrates, explaining both the need to degrade trehalose during recovery and the thermosensitivity of mutants unable to do so (Singer and Lindquist, 1998).

Figure 8: Workflow of the *E. dermatitidis* proteomic experiment from temperature treatment to bioinformatics analysis (Tesei et al., 2015).

Figure 9: Morphological analysis of *E. dermatitidis* CBS 525.76 under temperature treatments. a. Control condition (37°C); b-d. 1 week exposure at 1°C (b) or at 45°C (d). c. Enlargement of the inset in b. e-f. 1 hour exposure at 1°C (e) or at 45°C (f). Cells show a prominent vacuole (v) filled with floccular electron-dense material. The nucleus (n) and some mitochondria (m) are also visible. The cell wall consist of three layers, the outermost of which is pigmented due to melanization and exhibits a floccular-like consistency in relation to the prolonged exposure to low temperature (c). Bars = 0.5 µm.

9 Enclosed Publications

Tesei, D., Marzban, G., Marchetti-Deschmann, M., Tafer, H., Arcalis, E., Sterflinger, K., 2015. Proteome of tolerance fine-tuning in the human pathogen black yeast *Exophiala dermatitidis*. J. Proteomics 128, 39-57. doi:10.1016/j.jprot.2015.07.007

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10 Curriculum Vitae

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1998-2003_ Classical gymnasium “Galileo Galilei”, Tarquinia, Italy.

2003-2007_ Faculty of Mathematics, Physics and Natural Sciences, Tuscia University, Viterbo, Italy. Bachelor’s degree in Biological Sciences.

2008-2010_ Faculty of Mathematics, Physics and Natural Sciences, Tuscia University, Viterbo, Italy. Master’s degree in Diversity of Biological Systems.

Jul-Dec 2010_ University of Natural Resources and Life Sciences Vienna, Austria. Postgraduate short term fellowship.

Since April 2011_ University of Natural Resources and Life Sciences Vienna, Austria. Doctoral studies of Natural Resources and Applied Life Sciences. Diploma program: Food chemistry and Biotechnology.

Participation to workshops and conferences

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

4-8 September 2011_The 4th International Conference on Polar and Alpine Microbiology, Ljubljana, Slovenia.

19-24 September 2011_The 15th International Biodeterioration and Biodegradation Symposium, Vienna, Austria. Organizing komittee.

30 June - 4 July 2014_8th Central and Eastern European Proteomics Conference (CEEPC), Vienna, Austria.

3-8 August 2014_The 10th International Mycological Congress, Bangkok, Thailand.

31 August - 4 Sept 2014_10th Siena Meeting from Genome to Proteome, Siena, Italy.

20-21 April, 2015_Genomics of Neglected Pathogens, Utrecht, The Netherlands.

11 List of publications, posters and presentations

Author/co-author of scientific papers:

Tesei, D., Marzban, G., Marchetti-Deschmann, M., Tafer, H., Arcalis, E., Sterflinger, K., 2015. Proteome of tolerance fine-tuning in the human pathogen black yeast Exophiala dermatitidis. J. Proteomics 128, 39–57.

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Author/co-author of conference posters and presentations

Posters

Tesei D.; Marzban G.; Tafer H.; Marchetti-Deschmann M.; Sterflinger K. Proteome fine-tuning in the black yeast human pathogen *Exophiala dermatitidis*. *10th Siena Meeting from Genome to Proteome,* August 31- September 4, 2014, Siena, Italy.

Tesei D.; Marzban G.; Tafer H.; Marchetti-Deschmann M.; Sterflinger K. Proteome fine-tuning in the black yeast human pathogen *Exophiala dermatitidis*. *The 10th International Mycological Congress,* August 3-8, 2014, Bangkok, Thailand.

Presentations

Tesei D., Marzban G., Marzban G.; Marchetti-Deschmann M.; Sterflinger K. Stress resistance of black yeasts and microcolonial fungi: a matter of fine-tuning at the proteome level. *Seminar of the Institute of Molecular Biology, Slovak Academy of Science,* June 9, 2015, Bratislava, Slovakia.

Tafer H., **Tesei D**., Blasi B., Poyntner C., Lopandic K., Sterflinger K. Transcriptome and proteome fine-tuning in *Exophiala dermatitidis* under different stress conditions. Genomics of neglected pathogens, *Centraalbureau voor Schimmelcultures, CBS Symposium Week,* April 20, 2015*,* Utrecht, The Netherlands.

Sterflinger K., **Tesei D.**, Blasi B., Poyntner C., Marchetti-Deschmann M., Tafer H. Proteome and transcriptome fine tuning of the polyextremophilioc fungus *Exophiala dermatitidis*. Invited lecture. *Fungal Genetics Conference*, March 17-22, 2015, Pacific Grove CA, USA.

Tesei D.; Marzban G.; Marchetti-Deschmann M.; Sterflinger K. Proteome fine-tuning in the black yeast human pathogen Exophiala dermatitidis.*8th Central and Eastern European Proteomics Conference (CEEPC),* June 30th - July 4th*,* 2014, Vienna University of Technology, Vienna, Austria.

Sterflinger K., Marzban G., **Tesei D**., Zakharova K., Isola D., Selbmann L., Onofri S. Microcolonial fungi and extreme environmental conditions: cellular response at the proteome level. *Centraalbureau voor Schimmelcultures, CBS-lecture series,* February 20, 2012*,* Utrecht, The Netherlands.

Sterflinger K., Marzban G., Selbmann L., Isola D., Zakharova, K., **Tesei D**. Microcolonial fungi and extreme environmental conditions: cellular response on the proteome level. *CAREX conference on life in extreme environments,* October 18-21, 2011, Dublin, Ireland.

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12 Appendix

A_Supplementary Table 1. GO Terms, Raw Data

B_Supplementary Table 1. GO Terms, Summarized Data

C_Supplementary Table 2. Predicted pathways

D_Supplementary Table 3. Sub-cellular localization of all identified proteins, as predicted by YLoc.

A_Supplementary Table 1_GO Terms, Raw Data

EXPERIMENTAL SET: control (37°C) v/s 1°C1Week_UP

Term	Database	ID	Input Number	Background Number P-Value	p-value	Corrected p-value	Genes	Hyperlink
pyruvate_metabolic_process	Gene_Ontology	GO:0006090	3	46	0.0004616	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006090
oxidation-reduction_process	Gene_Ontology	GO:0055114	6	437	0.0025464	0.2678100	HMPREF1120_01270T0|HMPREF1120_08836T0|HMPREF1120_03849T0|HMPREF1120_05220T0|HMPREF1120_00315T0|HMPREF1120_02805T0|HMPREF1120_03849T0|HMPREF1120_00576T0|HMPREF1120_00315T0|HM	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0055114
generation_of_precursor_metabolites_and_energy	Gene_Ontology	GO:0006091	4	189	0.0029953	0.2678100	PREF1120_01270T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006091
carbohydrate_biosynthetic_process	Gene_Ontology	GO:0016051	3	97	0.0036129	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0|HMPREF1120_00315T0|HMPREF1120_03849T0|HMPREF1120_05220T0|HMPREF1120_08836T0|HM	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016051
oxidoreductase_activity	Gene_Ontology	GO:0016491	5	339	0.0042633	0.2678100	PREF1120_02805T0|HMPREF1120_01270T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016491
glycolytic_process	Gene_Ontology	GO:0006096	2	32	0.0048317	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006096
gluconeogenesis	Gene_Ontology	GO:0006094	2	32	0.0048317	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006094
hexose_biosynthetic_process	Gene_Ontology	GO:0019319	2	33	0.0051142	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019319
monosaccharide_biosynthetic_process	Gene_Ontology	GO:0046364	2	34	0.0054042	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046364
transporter_complex	Gene_Ontology	GO:1990351	2	37	0.0063183	0.2678100	HMPREF1120_03849T0|HMPREF1120_06254T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1990351
replicative_cell_aging	Gene_Ontology	GO:0001302	2	47	0.0098290	0.2678100	HMPREF1120_08836T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001302
monocarboxylic_acid_metabolic_process	Gene_Ontology	GO:0032787	3	155	0.0126643	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032787
purine_ribonucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009205	2	58	0.0144728	0.2678100	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009205
purine_nucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009144	2	60	0.0154008	0.2678100	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009144
mitochondrial_intermembrane_space	Gene_Ontology	GO:0005758	2	61	0.0158741	0.2678100	HMPREF1120_03444T0|HMPREF1120_06254T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005758
ribonucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009199	2	62	0.0163537	0.2678100	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009199
acid-thiol_ligase_activity	Gene_Ontology	GO:0016878	1	5	0.0180546	0.2678100	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016878
phosphopyruvate_hydratase_complex	Gene_Ontology	GO:0000015	1	5	0.0180546	0.2678100	HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000015
pyrimidine_ribonucleoside_triphosphate_biosynthetic_process	Gene_Ontology	GO:0009209	1	5	0.0180546	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009209
CTP_metabolic_process	Gene_Ontology	GO:0046036	1	5	0.0180546	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046036
phosphopyruvate_hydratase_activity	Gene_Ontology	GO:0004634	1	5	0.0180546	0.2678100	HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004634
CTP_biosynthetic_process	Gene_Ontology	GO:0006241	1	5	0.0180546	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006241
pyrimidine_ribonucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009208	1	5	0.0180546	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009208
organelle_envelope_lumen	Gene_Ontology	GO:0031970	2	66	0.0183327	0.2678100	HMPREF1120_03444T0|HMPREF1120_06254T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0031970
glucose_metabolic_process	Gene_Ontology	GO:0006006	2	67	0.0188425	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006006
nucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009141	2	70	0.0204072	0.2678100	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009141
cell_aging	Gene_Ontology	GO:0007569	2	71	0.0209405	0.2678100	HMPREF1120_08836T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007569
negative_regulation_of_cell_death	Gene_Ontology	GO:0060548	1	6	0.0210319	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0060548
regulation_of_ubiquitin-protein_ligase_activity_involved_in_mitotic_cell_cycle	Gene_Ontology	GO:0051439	1	6	0.0210319	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051439
superoxide_dismutase_activity	Gene_Ontology	GO:0004784	1	6	0.0210319	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004784
negative_regulation_of_programmed_cell_death	Gene_Ontology	GO:0043069	1	6	0.0210319	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043069
acetyl-CoA_biosynthetic_process	Gene_Ontology	GO:0006085	1	6	0.0210319	0.2678100	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006085
age-dependent_general_metabolic_decline_involved_in_chronological_cell_aging	Gene_Ontology	GO:0001323	1	6	0.0210319	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001323
negative_regulation_of_apoptotic_process	Gene_Ontology	GO:0043066	1	6	0.0210319	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043066
age-dependent_response_to_oxidative_stress_involved_in_chronological_cell_aging	Gene_Ontology	GO:0001324	1	6	0.0210319	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001324
guanosine-containing_compound_biosynthetic_process	Gene_Ontology	GO:1901070	1	6	0.0210319	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901070
oxidoreductase_activity;_acting_on_superoxide_radicals_as_acceptor	Gene_Ontology	GO:0016721	1	6	0.0210319	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016721
aging	Gene_Ontology	GO:0007568	2	72	0.0214795	0.2678100	HMPREF1120_08836T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007568
nucleotide_phosphorylation	Gene_Ontology	GO:0046939	1	7	0.0240001	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046939
pyrimidine_nucleoside_triphosphate_biosynthetic_process	Gene_Ontology	GO:0009148	1	7	0.0240001	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009148
proteasome_core_complex;_alpha-subunit_complex	Gene_Ontology	GO:0019773	1	7	0.0240001	0.2678100	HMPREF1120_07169T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019773
nucleoside_diphosphate_metabolic_process	Gene_Ontology	GO:0009132	1	7	0.0240001	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009132
mitochondrial_respiratory_chain_complex_III_assembly	Gene_Ontology	GO:0034551	1	7	0.0240001	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0034551
respiratory_chain_complex_III_assembly	Gene_Ontology	GO:0017062	1	7	0.0240001	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0017062
regulation_of_ubiquitin-protein_transferase_activity	Gene_Ontology	GO:0051438	1	8	0.0269594	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051438
negative_regulation_of_protein_modification_by_small_protein_conjugation_or_removal	Gene_Ontology	GO:1903321	1	8	0.0269594	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1903321
protein_phosphorylated_amino_acid_binding	Gene_Ontology	GO:0045309	1	8	0.0269594	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0045309
phosphoserine_binding	Gene_Ontology	GO:0050815	1	8	0.0269594	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0050815
age-dependent_response_to_oxidative_stress	Gene_Ontology	GO:0001306	1	8	0.0269594	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001306
age-dependent_general_metabolic_decline	Gene_Ontology	GO:0007571	1	8	0.0269594	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007571

thioester_biosynthetic_process	Gene_Ontology	GO:0035384	1	8	0.0269594	0.2678100	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0035384
negative_regulation_of_protein_ubiquitination	Gene_Ontology	GO:0031397	1	8	0.0269594	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0031397
acyl-CoA_biosynthetic_process	Gene_Ontology	GO:0071616	1	8	0.0269594	0.2678100	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071616
acetate_metabolic_process	Gene_Ontology	GO:0006083	1	8	0.0269594	0.2678100	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006083
single-organism_carbohydrate_catabolic_process	Gene_Ontology	GO:0044724	2	82	0.0271785	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044724
hexose_metabolic_process	Gene_Ontology	GO:0019318	2	84	0.0283836	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019318
cellular_response_to_oxygen_radical	Gene_Ontology	GO:0071450	1	9	0.0299097	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071450
regulation_of_apoptotic_process	Gene_Ontology	GO:0042981	1	9	0.0299097	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042981
superoxide_metabolic_process	Gene_Ontology	GO:0006801	1	9	0.0299097	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006801
phosphoprotein_binding	Gene_Ontology	GO:0051219	1	9	0.0299097	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051219
oxidoreductase_activity;_acting_on_diphenols_and_related_substances_as_donors;_cytochron	Gene_Ontology	GO:0016681	1	9	0.0299097	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016681
cellular_response_to_superoxide	Gene_Ontology	GO:0071451	1	9	0.0299097	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071451
regulation_of_ligase_activity	Gene_Ontology	GO:0051340	1	9	0.0299097	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051340
response_to_superoxide	Gene_Ontology	GO:0000303	1	9	0.0299097	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000303
pyrimidine_nucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009147	1	9	0.0299097	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009147
oxidoreductase_activity;_acting_on_diphenols_and_related_substances_as_donors	Gene_Ontology	GO:0016679	1	9	0.0299097	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016679
removal_of_superoxide_radicals	Gene_Ontology	GO:0019430	1	9	0.0299097	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019430
regulation_of_programmed_cell_death	Gene_Ontology	GO:0043067	1	9	0.0299097	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043067
ubiquinol-cytochrome-c_reductase_activity	Gene_Ontology	GO:0008121	1	9	0.0299097	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0008121
response_to_oxygen_radical	Gene_Ontology	GO:0000305	1	9	0.0299097	0.2678100	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000305
regulation_of_cell_death	Gene_Ontology	GO:0010941	1	9	0.0299097	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0010941
monosaccharide_metabolic_process	Gene_Ontology	GO:0005996	2	90	0.0321242	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005996
respiratory_chain_complex_III	Gene_Ontology	GO:0045275	1	10	0.0328510	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0045275
mitochondrial_respiratory_chain_complex_III	Gene_Ontology	GO:0005750	1	10	0.0328510	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005750
guanosine-containing_compound_metabolic_process	Gene_Ontology	GO:1901068	1	10	0.0328510	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901068
carbohydrate_catabolic_process	Gene_Ontology	GO:0016052	2	95	0.0353804	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016052
mitochondrial_respiratory_chain_complex_III_biogenesis	Gene_Ontology	GO:0097033	1	11	0.0357835	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0097033
transmembrane_transporter_complex	Gene_Ontology	GO:1902495	1	11	0.0357835	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1902495
mitochondrial_electron_transport;_ubiquinol_to_cytochrome_c	Gene_Ontology	GO:0006122	1	11	0.0357835	0.2678100	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006122
protein_import_into_mitochondrial_inner_membrane	Gene_Ontology	GO:0045039	1	11	0.0357835	0.2678100	HMPREF1120_06254T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0045039
mitotic_spindle_orientation_checkpoint	Gene_Ontology	GO:0031578	1	11	0.0357835	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0031578
purine_ribonucleotide_metabolic_process	Gene_Ontology	GO:0009150	2	96	0.0360464	0.2678100	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009150
fungal-type_cell_wall_chitin_biosynthetic_process	Gene_Ontology	GO:0034221	1	12	0.0387070	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0034221
signal_transduction_involved_in_filamentous_growth	Gene_Ontology	GO:0001402	1	12	0.0387070	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001402
protein_domain_specific_binding	Gene_Ontology	GO:0019904	1	12	0.0387070	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019904
purine_nucleotide_metabolic_process	Gene_Ontology	GO:0006163	2	100	0.0387582	0.2678100	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006163
purine_nucleoside_metabolic_process	Gene_Ontology	GO:0042278	2	101	0.0394480	0.2678100	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042278
coenzyme_binding	Gene_Ontology	GO:0050662	2	101	0.0394480	0.2678100	HMPREF1120_01270T0|HMPREF1120_02805T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0050662
purine_ribonucleoside_metabolic_process	Gene_Ontology	GO:0046128	2	101	0.0394480	0.2678100	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046128
acetyl-CoA_metabolic_process	Gene_Ontology	GO:0006084	1	13	0.0416217	0.2678100	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006084
pyrimidine_ribonucleotide_metabolic_process	Gene_Ontology	GO:0009218	1	13	0.0416217	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009218
pyrimidine_nucleoside_biosynthetic_process	Gene_Ontology	GO:0046134	1	13	0.0416217	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046134
pyrimidine_ribonucleoside_biosynthetic_process	Gene_Ontology	GO:0046132	1	13	0.0416217	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046132
cell_wall_chitin_biosynthetic_process	Gene_Ontology	GO:0006038	1	13	0.0416217	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006038
pyrimidine_ribonucleotide_biosynthetic_process	Gene_Ontology	GO:0009220	1	13	0.0416217	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009220
nucleobase-containing_compound_kinase_activity	Gene_Ontology	GO:0019205	1	14	0.0445276	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019205
fungal-type_cell_wall_polysaccharide_biosynthetic_process	Gene_Ontology	GO:0051278	1	14	0.0445276	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051278
proteasomal_ubiquitin-independent_protein_catabolic_process	Gene_Ontology	GO:0010499	1	14	0.0445276	0.2678100	HMPREF1120_07169T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0010499
threonine-type_endopeptidase_activity	Gene_Ontology	GO:0004298	1	14	0.0445276	0.2678100	HMPREF1120_07169T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004298
regulation_of_vacuole_fusion;_non-autophagic	Gene_Ontology	GO:0032889	1	14	0.0445276	0.2678100	HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032889
threonine-type_peptidase_activity	Gene_Ontology	GO:0070003	1	14	0.0445276	0.2678100	HMPREF1120_07169T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0070003
fungal-type_cell_wall_polysaccharide_metabolic_process	Gene_Ontology	GO:0071966	1	14	0.0445276	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071966
ribonucleotide_metabolic_process	Gene_Ontology	GO:0009259	2	109	0.0451307	0.2678100	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009259
proteasome_core_complex	Gene_Ontology	GO:0005839	1	15	0.0474247	0.2678100	HMPREF1120_07169T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005839
pyrimidine_ribonucleoside_metabolic_process	Gene_Ontology	GO:0046131	1	15	0.0474247	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046131
cell_wall_polysaccharide_biosynthetic_process	Gene_Ontology	GO:0070592	1	15	0.0474247	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0070592
pyrimidine_nucleoside_metabolic_process	Gene_Ontology	GO:0006213	1	15	0.0474247	0.2678100	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006213
amino_acid_binding	Gene_Ontology	GO:0016597	1	15	0.0474247	0.2678100	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016597
ribonucleoside_metabolic_process	Gene_Ontology	GO:0009119	2	115	0.0495778	0.2740452	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009119

EXPERIMENTAL SET: control (37°C) v/s 1°C 1 Week_ DOWN

Term	Database	ID	Input Number	Background Number	p-value	Corrected p-value	Genes	Hyperlink
acetate_metab+115:187olic_process	Gene_Ontology	GO:0006083	2	8	0.0001090	0.0161728	HMPREF1120_01109T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006083
monocarboxylic_acid_metabolic_process	Gene_Ontology	GO:0032787	4	155	0.0001254	0.0161728	HMPREF1120_05043T0|HMPREF1120_01109T0|HMPREF1120_05378T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032787
oxidoreductase_activity#_acting_on_the_aldehyde_or_oxo_group_of_donors#_NAD_or_NADP_	Gene_Ontology	GO:0016620	2	18	0.0004554	0.0356777	HMPREF1120_05043T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016620
carboxylic_acid_metabolic_process	Gene_Ontology	GO:0019752	5	409	0.0005387	0.0356777	HMPREF1120_05043T0|HMPREF1120_01109T0|HMPREF1120_05378T0|HMPREF1120_05340T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019752
oxoacid_metabolic_process	Gene_Ontology	GO:0043436	5	425	0.0006387	0.0356777	HMPREF1120_05043T0|HMPREF1120_01109T0|HMPREF1120_05378T0|HMPREF1120_05340T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043436
organic_acid_metabolic_process	Gene_Ontology	GO:0006082	5	426	0.0006453	0.0356777	HMPREF1120_05043T0|HMPREF1120_01109T0|HMPREF1120_05378T0|HMPREF1120_05340T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006082
oxidoreductase_activity#_acting_on_the_aldehyde_or_oxo_group_of_donors	Gene_Ontology	GO:0016903	2	25	0.0008352	0.0387654	HMPREF1120_05043T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016903
citrate_metabolic_process	Gene_Ontology	GO:0006101	2	29	0.0011019	0.0387654	HMPREF1120_05340T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006101
tricarboxylic_acid_cycle	Gene_Ontology	GO:0006099	2	29	0.0011019	0.0387654	HMPREF1120_05340T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006099
tricarboxylic_acid_metabolic_process	Gene_Ontology	GO:0072350	2	29	0.0011019	0.0387654	HMPREF1120_05340T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072350
small_molecule_metabolic_process	Gene_Ontology	GO:0044281	6	783	0.0016783	0.0541257	HMPREF1120_05340T0|HMPREF1120_01109T0|HMPREF1120_06094T0|HMPREF1120_05043T0|HMPREF1120_07361T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044281
replicative_cell_aging	Gene_Ontology	GO:0001302	2	47	0.0027352	0.0705684	HMPREF1120_05340T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001302
cellular_aldehyde_metabolic_process	Gene_Ontology	GO:0006081	2	52	0.0033112	0.0800885	HMPREF1120_05378T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006081
oxidation-reduction_process	Gene_Ontology	GO:0055114	4	437	0.0053820	0.1076941	HMPREF1120_05043T0|HMPREF1120_01109T0|HMPREF1120_05378T0|HMPREF1120_05340T0|HMPREF1120_02626T0|HMPREF1120_01109T0|HMPREF1120_07361T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0055114
organic_substance_catabolic_process	Gene_Ontology	GO:1901575	5	707	0.0056142	0.1076941	PREF1120_05378T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901575
cell_aging	Gene_Ontology	GO:0007569	2	71	0.0059628	0.1076941	HMPREF1120_05340T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007569
aging	Gene_Ontology	GO:0007568	2	72	0.0061221	0.1076941	HMPREF1120_05340T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007568
aerobic_respiration	Gene_Ontology	GO:0009060	2	81	0.0076422	0.1183007	HMPREF1120_05340T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009060
oxidoreductase_activity#_acting_on_the_CH-OH_group_of_donors#_NAD_or_NADP_as_acce	Gene_Ontology	GO:0016616	2	81	0.0076422	0.1183007	HMPREF1120_05043T0|HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016616
oxidoreductase_activity#_acting_on_CH-OH_group_of_donors	Gene_Ontology	GO:0016614	2	84	0.0081828	0.1217977	HMPREF1120_05043T0|HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016614
acid-thiol_ligase_activity	Gene_Ontology	GO:0016878	1	5	0.0093470	0.1277505	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016878
catabolic_process	Gene_Ontology	GO:0009056	5	823	0.0103433	0.1277505	HMPREF1120_02626T0|HMPREF1120_01109T0|HMPREF1120_07361T0|HMPREF1120_05378T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009056
aldehyde_dehydrogenase_(NAD)_activity	Gene_Ontology	GO:0004029	1	6	0.0108963	0.1277505	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004029
acetyl-CoA_biosynthetic_process	Gene_Ontology	GO:0006085	1	6	0.0108963	0.1277505	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006085
transferase_activity#_transferring_aldehyde_or_ketonic_groups	Gene_Ontology	GO:0016744	1	6	0.0108963	0.1277505	HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016744
cytosol	Gene_Ontology	GO:0005829	4	540	0.0110283	0.1277505	HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005829
allantoin_metabolic_process	Gene_Ontology	GO:0000255	1	7	0.0124432	0.1277505	HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000255
allantoin_catabolic_process	Gene_Ontology	GO:0000256	1	7	0.0124432	0.1277505	HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000256
cellular_respiration	Gene_Ontology	GO:0045333	2	106	0.0126467	0.1277505	HMPREF1120_05340T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0045333
catalytic_activity	Gene_Ontology	GO:0003824	9	2337	0.0127169	0.1277505	HMPREF1120_05340T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HMPREF1120_02657T0|HMPREF1120_05043T0|HMPREF1120_07361T0|HMPREF1120_01109T0|HMPREF1120_02626T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0003824
small_molecule_catabolic_process	Gene_Ontology	GO:0044282	2	110	0.0135497	0.1277505	HMPREF1120_05043T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044282
thioester_biosynthetic_process	Gene_Ontology	GO:0035384	1	8	0.0139877	0.1277505	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0035384
acyl-CoA_biosynthetic_process	Gene_Ontology	GO:0071616	1	8	0.0139877	0.1277505	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071616
cellular_amide_catabolic_process	Gene_Ontology	GO:0043605	1	9	0.0155298	0.1277505	HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043605
SRP-dependent_cotranslational_protein_targeting_to_membrane#_translocation	Gene_Ontology	GO:0006616	1	9	0.0155298	0.1277505	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006616
nucleotide_binding	Gene_Ontology	GO:0000166	5	933	0.0168451	0.1277505	HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HMPREF1120_02657T0|HMPREF1120_05043T0|HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HMPREF1120_02657T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000166
nucleoside_phosphate_binding	Gene_Ontology	GO:1901265	5	933	0.0168451	0.1277505	HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901265
oxidoreductase_activity	Gene_Ontology	GO:0016491	3	339	0.0170217	0.1277505	HMPREF1120_05043T0|HMPREF1120_01109T0|HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016491
glyoxylate_cycle	Gene_Ontology	GO:0006097	1	10	0.0170695	0.1277505	HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006097
carbohydrate_metabolic_process	Gene_Ontology	GO:0005975	3	340	0.0171524	0.1277505	HMPREF1120_05340T0|HMPREF1120_06041T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005975
glyoxylate_metabolic_process	Gene_Ontology	GO:0046487	1	11	0.0186068	0.1277505	HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046487
transferase_activity#_transferring_acyl_groups#_acyl_groups_converted_into_alkyl_on_transfe	Gene_Ontology	GO:0046912	1	11	0.0186068	0.1277505	HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046912

							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
small_molecule_binding	Gene_Ontology	GO:0036094	5	968	0.0193763	0.1277505	PREF1120_02657T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0036094
acetyl-CoA_metabolic_process	Gene_Ontology	GO:0006084	1	13	0.0216741	0.1277505	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006084
translation_elongation_factor_activity	Gene_Ontology	GO:0003746	1	14	0.0232042	0.1277505	HMPREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0003746
coenzyme_metabolic_process	Gene_Ontology	GO:0006732	2	152	0.0246012	0.1277505	HMPREF1120_06094T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006732
primary_alcohol_metabolic_process	Gene_Ontology	GO:0034308	1	15	0.0247319	0.1277505	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0034308
ethanol_metabolic_process	Gene_Ontology	GO:0006067	1	15	0.0247319	0.1277505	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006067
pentose-phosphate_shunt	Gene_Ontology	GO:0006098	1	15	0.0247319	0.1277505	HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006098
glyceraldehyde-3-phosphate_metabolic_process	Gene_Ontology	GO:0019682	1	15	0.0247319	0.1277505	HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019682
energy_derivation_by_oxidation_of_organic_compounds	Gene_Ontology	GO:0015980	2	155	0.0254941	0.1277505	HMPREF1120_05340T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0015980
alcohol_catabolic_process	Gene_Ontology	GO:0046164	1	16	0.0262572	0.1277505	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046164
tRNA_export_from_nucleus	Gene_Ontology	GO:0006409	1	16	0.0262572	0.1277505	HMPREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006409
single-organism_catabolic_process	Gene_Ontology	GO:0044712	3	409	0.0275307	0.1277505	HMPREF1120_05043T0|HMPREF1120_01109T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044712
ligase_activity#_forming_carbon-sulfur_bonds	Gene_Ontology	GO:0016877	1	17	0.0277801	0.1277505	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016877
organic_hydroxy_compound_catabolic_process	Gene_Ontology	GO:1901616	1	17	0.0277801	0.1277505	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901616
purine_nucleobase_metabolic_process	Gene_Ontology	GO:0006144	1	19	0.0308188	0.1277505	HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006144
glucose_6-phosphate_metabolic_process	Gene_Ontology	GO:0051156	1	19	0.0308188	0.1277505	HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051156
tRNA_transport	Gene_Ontology	GO:0051031	1	19	0.0308188	0.1277505	HMPREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051031
cellular_carbohydrate_metabolic_process	Gene_Ontology	GO:0044262	2	176	0.0321018	0.1277505	HMPREF1120_05340T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044262
cofactor_metabolic_process	Gene_Ontology	GO:0051186	2	176	0.0321018	0.1277505	HMPREF1120_06094T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051186
acyl-CoA_metabolic_process	Gene_Ontology	GO:0006637	1	20	0.0323346	0.1277505	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006637
SRP-dependent_cotranslational_protein_targeting_to_membrane	Gene_Ontology	GO:0006614	1	20	0.0323346	0.1277505	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006614
thioester_metabolic_process	Gene_Ontology	GO:0035383	1	20	0.0323346	0.1277505	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0035383
							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
purine_nucleoside_binding	Gene_Ontology	GO:0001883	4	761	0.0331890	0.1277505	PREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001883
							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
purine_ribonucleoside_binding	Gene_Ontology	GO:0032550	4	761	0.0331890	0.1277505	PREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032550
							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
purine_ribonucleoside_triphosphate_binding	Gene_Ontology	GO:0035639	4	761	0.0331890	0.1277505	PREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0035639
							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
purine_ribonucleotide_binding	Gene_Ontology	GO:0032555	4	762	0.0333235	0.1277505	PREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032555
							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
ribonucleoside_binding	Gene_Ontology	GO:0032549	4	765	0.0337289	0.1277505	PREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032549
misfolded_or_incompletely_synthesized_protein_catabolic_process	Gene_Ontology	GO:0006515	1	21	0.0338481	0.1277505	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006515
cotranslational_protein_targeting_to_membrane	Gene_Ontology	GO:0006613	1	21	0.0338481	0.1277505	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006613
							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
nucleoside_binding	Gene_Ontology	GO:0001882	4	766	0.0338647	0.1277505	PREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001882
							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
purine_nucleotide_binding	Gene_Ontology	GO:0017076	4	767	0.0340008	0.1277505	PREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0017076
NADP_metabolic_process	Gene_Ontology	GO:0006739	1	22	0.0353591	0.1314278	HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006739
							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
ribonucleotide_binding	Gene_Ontology	GO:0032553	4	779	0.0356587	0.1314278	PREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032553
generation_of_precursor_metabolites_and_energy	Gene_Ontology	GO:0006091	2	189	0.0364915	0.1332284	HMPREF1120_05340T0|HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006091
							HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
carbohydrate_derivative_binding	Gene_Ontology	GO:0097367	4	791	0.0373626	0.1351340	PREF1120_02657T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0097367
carboxy-lyase_activity	Gene_Ontology	GO:0016831	1	24	0.0383742	0.1375075	HMPREF1120_06041T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016831
NAD_binding	Gene_Ontology	GO:0051287	1	27	0.0428792	0.1522407	HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051287
monocarboxylic_acid_catabolic_process	Gene_Ontology	GO:0072329	1	28	0.0443761	0.1542797	HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072329
chronological_cell_aging	Gene_Ontology	GO:0001300	1	28	0.0443761	0.1542797	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001300
							HMPREF1120_05340T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HM	
							PREF1120_05043T0|HMPREF1120_07361T0|HMPREF1120_01109T0|HMPR	
single-organism_metabolic_process	Gene_Ontology	GO:0044710	7	2047	0.0448021	0.1542797	EF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044710
mitochondrial_matrix	Gene_Ontology	GO:0005759	2	215	0.0459078	0.1558449	HMPREF1120_05340T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005759
nucleobase_metabolic_process	Gene_Ontology	GO:0009112	1	31	0.0488530	0.1644010	HMPREF1120_05378T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009112

EXPERIMENTAL SET: 45°C1W eek v/s 1°C1W eek_UP

Term	Database	ID	Input Number	Background Number	p-value	Corrected p-value	Genes	Hyperlink
				P-Value				

oxidoreductase_activity	Gene_Ontology	GO:0016491	10	339	0.0000023	0.0014259	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_08836T0|HMPREF1120_04591T0|HMPREF1120_03849T0|HMPREF1120_05220T0|HMPREF1120_08713T0|HMPREF1120_01097T0|HMPREF1120_02805T0|HMPREF1120_02451T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016491
oxidation-reduction_process	Gene_Ontology	GO:0055114	11	437	0.0000032	0.0014259	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_08836T0|HMPREF1120_04591T0|HMPREF1120_03849T0|HMPREF1120_05220T0|HMPREF1120_08713T0|HMPREF1120_01097T0|HMPREF1120_00315T0|HMPREF1120_02805T0|HMPREF1120_02451T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0055114
monocarboxylic_acid_metabolic_process	Gene_Ontology	GO:0032787	7	155	0.0000054	0.0016141	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_00576T0|HMPREF1120_06094T0|HMPREF1120_02451T0|HMPREF1120_08713T0|HMPREF1120_01097T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032787
hydro-lyase_activity	Gene_Ontology	GO:0016836	3	31	0.0003674	0.0709999	HMPREF1120_00576T0|HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016836
oxoacid_metabolic_process	Gene_Ontology	GO:0043436	8	425	0.0004689	0.0709999	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_00576T0|HMPREF1120_06094T0|HMPREF1120_04591T0|HMPREF1120_02451T0|HMPREF1120_08713T0|HMPREF1120_01097T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043436
organic_acid_metabolic_process	Gene_Ontology	GO:0006082	8	426	0.0004760	0.0709999	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_00576T0|HMPREF1120_06094T0|HMPREF1120_04591T0|HMPREF1120_02451T0|HMPREF1120_08713T0|HMPREF1120_01097T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006082
acetate_metabolic_process	Gene_Ontology	GO:0006083	2	8	0.0007303	0.0879559	HMPREF1120_01109T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006083
carbon-oxygen_lyase_activity	Gene_Ontology	GO:0016835	3	42	0.0008427	0.0879559	HMPREF1120_00576T0|HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016835
phosphoprotein_binding	Gene_Ontology	GO:0051219	2	9	0.0008902	0.0879559	HMPREF1120_00315T0|HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051219
pyruvate_metabolic_process	Gene_Ontology	GO:0006090	3	46	0.0010810	0.0879559	HMPREF1120_01270T0|HMPREF1120_00576T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006090
fatty_acid_beta-oxidation	Gene_Ontology	GO:0006635	2	12	0.0014609	0.0901945	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006635
fatty_acid_oxidation	Gene_Ontology	GO:0019395	2	13	0.0016812	0.0901945	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019395
lipid_oxidation	Gene_Ontology	GO:0034440	2	13	0.0016812	0.0901945	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0034440
carboxylic_acid_metabolic_process	Gene_Ontology	GO:0019752	7	409	0.0017770	0.0901945	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_00576T0|HMPREF1120_06094T0|HMPREF1120_02451T0|HMPREF1120_08713T0|HMPREF1120_01097T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019752
small_molecule_metabolic_process	Gene_Ontology	GO:0044281	10	783	0.0018140	0.0901945	HMPREF1120_03444T0|HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_06094T0|HMPREF1120_04591T0|HMPREF1120_03849T0|HMPREF1120_02451T0|HMPREF1120_01097T0|HMPREF1120_00576T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044281
fatty_acid_metabolic_process	Gene_Ontology	GO:0006631	3	65	0.0027766	0.1220966	HMPREF1120_01097T0|HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006631
oxidoreductase_activity;_acting_on_the_aldehyde_or_oxo_group_of_donors;_NAD_or_NADP_	Gene_Ontology	GO:0016620	2	18	0.0030013	0.1220966	HMPREF1120_01270T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016620
fatty_acid_catabolic_process	Gene_Ontology	GO:0009062	2	18	0.0030013	0.1220966	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009062
oxidoreductase_activity;_acting_on_the_aldehyde_or_oxo_group_of_donors	Gene_Ontology	GO:0016903	2	25	0.0054407	0.1838485	HMPREF1120_01270T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016903
oxidoreductase_activity;_acting_on_CH-OH_group_of_donors	Gene_Ontology	GO:0016614	3	84	0.0055463	0.1838485	HMPREF1120_01097T0|HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016614
monocarboxylic_acid_catabolic_process	Gene_Ontology	GO:0072329	2	28	0.0066886	0.2064229	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072329
single-organism_catabolic_process	Gene_Ontology	GO:0044712	6	409	0.0076199	0.2178567	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_02451T0|HMPREF1120_00576T0|HMPREF1120_00315T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044712
carbohydrate_biosynthetic_process	Gene_Ontology	GO:0016051	3	97	0.0081377	0.2178567	HMPREF1120_01270T0|HMPREF1120_00576T0|HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016051
generation_of_precursor_metabolites_and_energy	Gene_Ontology	GO:0006091	4	189	0.0083765	0.2178567	HMPREF1120_01270T0|HMPREF1120_00576T0|HMPREF1120_00315T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006091
gluconeogenesis	Gene_Ontology	GO:0006094	2	32	0.0085337	0.2178567	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006094
glycolytic_process	Gene_Ontology	GO:0006096	2	32	0.0085337	0.2178567	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006096
hexose_biosynthetic_process	Gene_Ontology	GO:0019319	2	33	0.0090266	0.2178567	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019319
antioxidant_activity	Gene_Ontology	GO:0016209	2	33	0.0090266	0.2178567	HMPREF1120_08836T0|HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016209
monosaccharide_biosynthetic_process	Gene_Ontology	GO:0046364	2	34	0.0095319	0.2187451	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046364
organic_substance_catabolic_process	Gene_Ontology	GO:1901575	8	707	0.0098986	0.2214814	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_07169T0|HMPREF1120_02626T0|HMPREF1120_02451T0|HMPREF1120_00576T0|HMPREF1120_00315T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901575
lyase_activity	Gene_Ontology	GO:0016829	3	107	0.0105415	0.2301133	HMPREF1120_00576T0|HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016829
small_molecule_catabolic_process	Gene_Ontology	GO:0044282	3	110	0.0113342	0.2415258	HMPREF1120_01109T0|HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044282
cellular_lipid_catabolic_process	Gene_Ontology	GO:0044242	2	40	0.0128190	0.2668134	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044242
lipid_modification	Gene_Ontology	GO:0030258	2	45	0.0158804	0.2775122	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0030258
monocarboxylic_acid_biosynthetic_process	Gene_Ontology	GO:0072330	2	45	0.0158804	0.2775122	HMPREF1120_01109T0|HMPREF1120_01097T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072330
replicative_cell_aging	Gene_Ontology	GO:0001302	2	47	0.0171839	0.2775122	HMPREF1120_08836T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001302
catabolic_process	Gene_Ontology	GO:0009056	8	823	0.0221876	0.2775122	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_07169T0|HMPREF1120_02626T0|HMPREF1120_02451T0|HMPREF1120_00576T0|HMPREF1120_00315T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009056

lipid_catabolic_process	Gene_Ontology	GO:0016042	2	55	0.0228283	0.2775122	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016042
CTP_metabolic_process	Gene_Ontology	GO:0046036	1	5	0.0241949	0.2775122	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046036
acid-thiol_ligase_activity	Gene_Ontology	GO:0016878	1	5	0.0241949	0.2775122	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016878
phosphopyruvate_hydratase_complex	Gene_Ontology	GO:0000015	1	5	0.0241949	0.2775122	HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000015
pyrimidine_ribonucleoside_triphosphate_biosynthetic_process	Gene_Ontology	GO:0009209	1	5	0.0241949	0.2775122	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009209
phosphopyruvate_hydratase_activity	Gene_Ontology	GO:0004634	1	5	0.0241949	0.2775122	HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004634
CTP_biosynthetic_process	Gene_Ontology	GO:0006241	1	5	0.0241949	0.2775122	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006241
pyrimidine_ribonucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009208	1	5	0.0241949	0.2775122	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009208
fatty_acid_elongation	Gene_Ontology	GO:0030497	1	5	0.0241949	0.2775122	HMPREF1120_01097T0|HMPREF1120_03444T0|HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_06094T0|HMPREF1120_08075T0|HMPREF1120_08836T0|HMPREF1120_04591T0|HMPREF1120_03849T0|HMPREF1120_05220T0|HMPREF1120_08713T0|HMPREF1120_01097T0|HMPREF1120_00576T0|HMPREF112	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0030497
single-organism_metabolic_process	Gene_Ontology	GO:0044710	15	2047	0.0245304	0.2775122	0_00315T0|HMPREF1120_02805T0|HMPREF1120_02451T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044710
purine_ribonucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009205	2	58	0.0251156	0.2775122	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009205
cytosol	Gene_Ontology	GO:0005829	6	540	0.0257132	0.2775122	HMPREF1120_03444T0|HMPREF1120_06094T0|HMPREF1120_07169T0|HMPREF1120_02626T0|HMPREF1120_04591T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005829
response_to_inorganic_substance	Gene_Ontology	GO:0010035	2	59	0.0258979	0.2775122	HMPREF1120_08836T0|HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0010035
purine_nucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009144	2	60	0.0266900	0.2775122	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009144
regulation_of_ubiquitin-protein_ligase_activity_involved_in_mitotic_cell_cycle	Gene_Ontology	GO:0051439	1	6	0.0281701	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051439
negative_regulation_of_cell_death	Gene_Ontology	GO:0060548	1	6	0.0281701	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0060548
superoxide_dismutase_activity	Gene_Ontology	GO:0004784	1	6	0.0281701	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004784
aldehyde_dehydrogenase_(NAD)_activity	Gene_Ontology	GO:0004029	1	6	0.0281701	0.2775122	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004029
negative_regulation_of_programmed_cell_death	Gene_Ontology	GO:0043069	1	6	0.0281701	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043069
acetyl-CoA_biosynthetic_process	Gene_Ontology	GO:0006085	1	6	0.0281701	0.2775122	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006085
age-dependent_general_metabolic_decline_involved_in_chronological_cell_aging	Gene_Ontology	GO:0001323	1	6	0.0281701	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001323
negative_regulation_of_apoptotic_process	Gene_Ontology	GO:0043066	1	6	0.0281701	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043066
age-dependent_response_to_oxidative_stress_involved_in_chronological_cell_aging	Gene_Ontology	GO:0001324	1	6	0.0281701	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001324
guanosine-containing_compound_biosynthetic_process	Gene_Ontology	GO:1901070	1	6	0.0281701	0.2775122	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901070
oxidoreductase_activity;_acting_on_superoxide_radicals_as_acceptor	Gene_Ontology	GO:0016721	1	6	0.0281701	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016721
ribonucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009199	2	62	0.0283034	0.2775122	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009199
nucleotide_phosphorylation	Gene_Ontology	GO:0046939	1	7	0.0321292	0.2775122	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046939
glutathione_peroxidase_activity	Gene_Ontology	GO:0004602	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004602
negative_regulation_of_nucleocytoplasmic_transport	Gene_Ontology	GO:0046823	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046823
negative_regulation_of_protein_import_into_nucleus	Gene_Ontology	GO:0042308	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042308
negative_regulation_of_intracellular_transport	Gene_Ontology	GO:0032387	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032387
protein_urmylation	Gene_Ontology	GO:0032447	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032447
negative_regulation_of_protein_localization_to_nucleus	Gene_Ontology	GO:1900181	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1900181
pyrimidine_nucleoside_triphosphate_biosynthetic_process	Gene_Ontology	GO:0009148	1	7	0.0321292	0.2775122	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009148
proteasome_core_complex;_alpha-subunit_complex	Gene_Ontology	GO:0019773	1	7	0.0321292	0.2775122	HMPREF1120_07169T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019773
negative_regulation_of_cellular_protein_localization	Gene_Ontology	GO:1903828	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1903828
nucleoside_diphosphate_metabolic_process	Gene_Ontology	GO:0009132	1	7	0.0321292	0.2775122	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009132
mitochondrial_respiratory_chain_complex_III_assembly	Gene_Ontology	GO:0034551	1	7	0.0321292	0.2775122	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0034551
respiratory_chain_complex_III_assembly	Gene_Ontology	GO:0017062	1	7	0.0321292	0.2775122	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0017062
negative_regulation_of_cytoplasmic_transport	Gene_Ontology	GO:1903650	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1903650
negative_regulation_of_intracellular_protein_transport	Gene_Ontology	GO:0090317	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0090317
nitrogen_cycle_metabolic_process	Gene_Ontology	GO:0071941	1	7	0.0321292	0.2775122	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071941
glucose_metabolic_process	Gene_Ontology	GO:0006006	2	67	0.0325018	0.2775122	HMPREF1120_01270T0|HMPREF1120_00576T0|HMPREF1120_03444T0|HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_06094T0|HMPREF1120_08075T0|HMPREF1120_03849T0|HMPREF1120_08836T0|HMPREF1120_04591T0|HMPREF1120_02626T0|HMPREF1120_05220T0|HMPREF1120_08713T0|HMPREF1120_07169T0|HMPREF112	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006006
catalytic_activity	Gene_Ontology	GO:0003824	16	2337	0.0350538	0.2775122	0_01097T0|HMPREF1120_00576T0|HMPREF1120_02805T0|HMPREF1120_0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0003824
nucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009141	2	70	0.0351305	0.2775122	HMPREF1120_03444T0|HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009141
cell_aging	Gene_Ontology	GO:0007569	2	71	0.0360245	0.2775122	HMPREF1120_08836T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007569
regulation_of_ubiquitin-protein_transferase_activity	Gene_Ontology	GO:0051438	1	8	0.0360721	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051438
protein_phosphorylated_amino_acid_binding	Gene_Ontology	GO:0045309	1	8	0.0360721	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0045309
negative_regulation_of_protein_ubiquitination	Gene_Ontology	GO:0031397	1	8	0.0360721	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0031397
phosphoserine_binding	Gene_Ontology	GO:0050815	1	8	0.0360721	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0050815
age-dependent_response_to_oxidative_stress	Gene_Ontology	GO:0001306	1	8	0.0360721	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001306

age-dependent_general_metabolic_decline	Gene_Ontology	GO:0007571	1	8	0.0360721	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007571
thioester_biosynthetic_process	Gene_Ontology	GO:0035384	1	8	0.0360721	0.2775122	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0035384
negative_regulation_of_protein_modification_by_small_protein_conjugation_or_removal	Gene_Ontology	GO:1903321	1	8	0.0360721	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1903321
acyl-CoA_biosynthetic_process	Gene_Ontology	GO:0071616	1	8	0.0360721	0.2775122	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071616
aging	Gene_Ontology	GO:0007568	2	72	0.0369272	0.2775122	HMPREF1120_08836T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007568
isomerase_activity	Gene_Ontology	GO:0016853	2	73	0.0378385	0.2775122	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016853
microbody	Gene_Ontology	GO:0042579	2	74	0.0387584	0.2775122	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042579
peroxisome	Gene_Ontology	GO:0005777	2	74	0.0387584	0.2775122	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005777
regulation_of_apoptotic_process	Gene_Ontology	GO:0042981	1	9	0.0399990	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042981
cellular_response_to_oxygen_radical	Gene_Ontology	GO:0071450	1	9	0.0399990	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071450
superoxide_metabolic_process	Gene_Ontology	GO:0006801	1	9	0.0399990	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006801
oxidoreductase_activity;_acting_on_diphenols_and_related_substances_as_donors;_cytochron	Gene_Ontology	GO:0016681	1	9	0.0399990	0.2775122	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016681
cellular_response_to_superoxide	Gene_Ontology	GO:0071451	1	9	0.0399990	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071451
SRP-dependent_cotranslational_protein_targeting_to_membrane;_translocation	Gene_Ontology	GO:0006616	1	9	0.0399990	0.2775122	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006616
removal_of_superoxide_radicals	Gene_Ontology	GO:0019430	1	9	0.0399990	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019430
pyrimidine_nucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009147	1	9	0.0399990	0.2775122	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009147
oxidoreductase_activity;_acting_on_diphenols_and_related_substances_as_donors	Gene_Ontology	GO:0016679	1	9	0.0399990	0.2775122	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016679
response_to_oxygen_radical	Gene_Ontology	GO:0000305	1	9	0.0399990	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000305
response_to_superoxide	Gene_Ontology	GO:0000303	1	9	0.0399990	0.2775122	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000303
regulation_of_ligase_activity	Gene_Ontology	GO:0051340	1	9	0.0399990	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051340
regulation_of_programmed_cell_death	Gene_Ontology	GO:0043067	1	9	0.0399990	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043067
ubiquinol-cytochrome-c_reductase_activity	Gene_Ontology	GO:0008121	1	9	0.0399990	0.2775122	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0008121
regulation_of_cell_death	Gene_Ontology	GO:0010941	1	9	0.0399990	0.2775122	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0010941
unfolded_protein_binding	Gene_Ontology	GO:0051082	2	78	0.0425219	0.2830353	HMPREF1120_02626T0|HSP30	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051082
regulation_of_protein_import_into_nucleus	Gene_Ontology	GO:0042306	1	10	0.0439098	0.2830353	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042306
regulation_of_protein_targeting	Gene_Ontology	GO:1903533	1	10	0.0439098	0.2830353	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1903533
guanosine-containing_compound_metabolic_process	Gene_Ontology	GO:1901068	1	10	0.0439098	0.2830353	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901068
respiratory_chain_complex_III	Gene_Ontology	GO:0045275	1	10	0.0439098	0.2830353	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0045275
mitochondrial_respiratory_chain_complex_III	Gene_Ontology	GO:0005750	1	10	0.0439098	0.2830353	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005750
transcription_factor_import_into_nucleus	Gene_Ontology	GO:0042991	1	10	0.0439098	0.2830353	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042991
oxidoreductase_activity;_acting_on_the_CH-OH_group_of_donors;_NAD_or_NADP_as_accep	Gene_Ontology	GO:0016616	2	81	0.0454303	0.2830353	HMPREF1120_02451T0|HMPREF1120_08713T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016616
single-organism_carbohydrate_catabolic_process	Gene_Ontology	GO:0044724	2	82	0.0464156	0.2830353	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044724
transmembrane_transporter_complex	Gene_Ontology	GO:1902495	1	11	0.0478048	0.2830353	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1902495
mitochondrial_respiratory_chain_complex_III_biogenesis	Gene_Ontology	GO:0097033	1	11	0.0478048	0.2830353	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0097033
cytoplasmic_sequestering_of_protein	Gene_Ontology	GO:0051220	1	11	0.0478048	0.2830353	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051220
mitochondrial_electron_transport;_ubiquinol_to_cytochrome_c	Gene_Ontology	GO:0006122	1	11	0.0478048	0.2830353	HMPREF1120_03849T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006122
mitotic_spindle_orientation_checkpoint	Gene_Ontology	GO:0031578	1	11	0.0478048	0.2830353	HMPREF1120_00315T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0031578
hexose_metabolic_process	Gene_Ontology	GO:0019318	2	84	0.0484097	0.2830353	HMPREF1120_01270T0|HMPREF1120_00576T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019318

EXPERIMENTAL SET: 45°C1W eek v/s 1°C1W eek_DOW N

Term	Database	ID	Input Number	Background Number P-Value	p-value	Corrected p-value	Genes	Hyperlink
transferase_activity;_transferring_aldehyde_or_ketonic_groups	Gene_Ontology	GO:0016744	2	6	0.0000206	0.0048404	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016744
pentose-phosphate_shunt	Gene_Ontology	GO:0006098	2	15	0.0000995	0.0072071	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006098
glyceraldehyde-3-phosphate_metabolic_process	Gene_Ontology	GO:0019682	2	15	0.0000995	0.0072071	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019682
oxidoreductase_activity;_acting_on_the_aldehyde_or_oxo_group_of_donors;_NAD_or_NADP_	Gene_Ontology	GO:0016620	2	18	0.0001388	0.0072071	HMPREF1120_05043T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016620
glucose_6-phosphate_metabolic_process	Gene_Ontology	GO:0051156	2	19	0.0001533	0.0072071	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051156
NADP_metabolic_process	Gene_Ontology	GO:0006739	2	22	0.0002012	0.0078800	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006739
oxidoreductase_activity;_acting_on_the_aldehyde_or_oxo_group_of_donors	Gene_Ontology	GO:0016903	2	25	0.0002554	0.0085750	HMPREF1120_05043T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016903
organic_substance_catabolic_process	Gene_Ontology	GO:1901575	5	707	0.0004192	0.0104228	HMPREF1120_02626T0|HMPREF1120_01109T0|HMPREF1120_07361T0|HM PREF1120_08453T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901575
carbohydrate_derivative_catabolic_process	Gene_Ontology	GO:1901136	2	34	0.0004561	0.0104228	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901136
organophosphate_catabolic_process	Gene_Ontology	GO:0046434	2	35	0.0004819	0.0104228	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046434
single-organism_catabolic_process	Gene_Ontology	GO:0044712	4	409	0.0004879	0.0104228	HMPREF1120_02626T0|HMPREF1120_01109T0|HMPREF1120_07361T0|HM PREF1120_08453T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044712
catabolic_process	Gene_Ontology	GO:0009056	5	823	0.0008241	0.0160658	HMPREF1120_02626T0|HMPREF1120_01109T0|HMPREF1120_07361T0|HM PREF1120_08453T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009056
nicotinamide_nucleotide_metabolic_process	Gene_Ontology	GO:0046496	2	51	0.0009881	0.0160658	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046496

pyridine_nucleotide_metabolic_process	Gene_Ontology	GO:0019362	2	52	0.0010255	0.0160658	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019362
cellular_aldehyde_metabolic_process	Gene_Ontology	GO:0006081	2	52	0.0010255	0.0160658	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006081
pyridine-containing_compound_metabolic_process	Gene_Ontology	GO:0072524	2	67	0.0016669	0.0217625	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072524
oxidoreduction_coenzyme_metabolic_process	Gene_Ontology	GO:0006733	2	67	0.0016669	0.0217625	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006733
small_molecule_catabolic_process	Gene_Ontology	GO:0044282	2	110	0.0043103	0.0506461	HMPREF1120_05043T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044282
small_molecule_metabolic_process	Gene_Ontology	GO:0044281	4	783	0.0050430	0.0564335	HMPREF1120_05043T0|HMPREF1120_01109T0|HMPREF1120_07361T0|HMPREF1120_08453T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044281
aldehyde_dehydrogenase_(NAD)_activity	Gene_Ontology	GO:0004029	1	6	0.0060073	0.0614573	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004029
ribose_phosphate_metabolic_process	Gene_Ontology	GO:0019693	2	131	0.0060150	0.0614573	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019693
acetate_metabolic_process	Gene_Ontology	GO:0006083	1	8	0.0077170	0.0745977	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006083
coenzyme_metabolic_process	Gene_Ontology	GO:0006732	2	152	0.0079775	0.0745977	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006732
monocarboxylic_acid_metabolic_process	Gene_Ontology	GO:0032787	2	155	0.0082784	0.0745977	HMPREF1120_05043T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032787
SRP-dependent_cotranslational_protein_targeting_to_membrane;_translocation	Gene_Ontology	GO:0006616	1	9	0.0085708	0.0745977	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006616
cofactor_metabolic_process	Gene_Ontology	GO:0051186	2	176	0.0105238	0.0852790	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051186
cytosol	Gene_Ontology	GO:0005829	3	540	0.0119070	0.0932715	HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005829
ethanol_metabolic_process	Gene_Ontology	GO:0006067	1	15	0.0136781	0.0958278	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006067
primary_alcohol_metabolic_process	Gene_Ontology	GO:0034308	1	15	0.0136781	0.0958278	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0034308
alcohol_catabolic_process	Gene_Ontology	GO:0046164	1	16	0.0145267	0.0958278	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046164
nucleotide_metabolic_process	Gene_Ontology	GO:0009117	2	210	0.0146584	0.0958278	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009117
nucleoside_phosphate_metabolic_process	Gene_Ontology	GO:0006753	2	214	0.0151840	0.0958278	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006753
organic_hydroxy_compound_catabolic_process	Gene_Ontology	GO:1901616	1	17	0.0153747	0.0958278	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901616
catalytic_activity	Gene_Ontology	GO:0003824	6	2337	0.0167783	0.1011001	HMPREF1120_08453T0|HMPREF1120_06041T0|HMPREF1120_05043T0|HMPREF1120_07361T0|HMPREF1120_01109T0|HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0003824
SRP-dependent_cotranslational_protein_targeting_to_membrane	Gene_Ontology	GO:0006614	1	20	0.0179141	0.1027300	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006614
misfolded_or_incompletely_synthesized_protein_catabolic_process	Gene_Ontology	GO:0006515	1	21	0.0187591	0.1027300	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006515
cotranslational_protein_targeting_to_membrane	Gene_Ontology	GO:0006613	1	21	0.0187591	0.1027300	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006613
nucleobase-containing_small_molecule_metabolic_process	Gene_Ontology	GO:0055086	2	246	0.0196717	0.1027300	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0055086
carboxy-lyase_activity	Gene_Ontology	GO:0016831	1	24	0.0212898	0.1087633	HMPREF1120_06041T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016831
NAD_binding	Gene_Ontology	GO:0051287	1	27	0.0238141	0.1185252	HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051287
monocarboxylic_acid_catabolic_process	Gene_Ontology	GO:0072329	1	28	0.0246540	0.1185252	HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072329
gluconeogenesis	Gene_Ontology	GO:0006094	1	32	0.0280067	0.1316314	HMPREF1120_06041T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006094
hexose_biosynthetic_process	Gene_Ontology	GO:0019319	1	33	0.0288430	0.1329042	HMPREF1120_06041T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019319
monosaccharide_biosynthetic_process	Gene_Ontology	GO:0046364	1	34	0.0296787	0.1341248	HMPREF1120_06041T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046364
carbon-carbon_lyase_activity	Gene_Ontology	GO:0016830	1	35	0.0305136	0.1352962	HMPREF1120_06041T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016830
single-organism_metabolic_process	Gene_Ontology	GO:0044710	5	2047	0.0335072	0.1391823	HMPREF1120_05043T0|HMPREF1120_06041T0|HMPREF1120_07361T0|HMPREF1120_01109T0|HMPREF1120_08453T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044710
protein_targeting_to_ER	Gene_Ontology	GO:0045047	1	39	0.0338462	0.1391823	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0045047
carbohydrate_derivative_metabolic_process	Gene_Ontology	GO:1901135	2	335	0.0345991	0.1391823	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901135
establishment_of_protein_localization_to_endoplasmic_reticulum	Gene_Ontology	GO:0072599	1	40	0.0346775	0.1391823	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072599
oxidoreductase_activity	Gene_Ontology	GO:0016491	2	339	0.0353478	0.1391823	HMPREF1120_05043T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016491
carbohydrate_metabolic_process	Gene_Ontology	GO:0005975	2	340	0.0355359	0.1391823	HMPREF1120_08453T0|HMPREF1120_06041T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005975
monocarboxylic_acid_biosynthetic_process	Gene_Ontology	GO:0072330	1	45	0.0388236	0.1471540	HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072330
protein_localization_to_endoplasmic_reticulum	Gene_Ontology	GO:0070972	1	47	0.0404771	0.1486268	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0070972
organophosphate_metabolic_process	Gene_Ontology	GO:0019637	2	389	0.0452171	0.1634771	HMPREF1120_08453T0|HMPREF1120_07361T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019637
nucleotide_binding	Gene_Ontology	GO:0000166	3	933	0.0479166	0.1680658	HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000166
nucleoside_phosphate_binding	Gene_Ontology	GO:1901265	3	933	0.0479166	0.1680658	HMPREF1120_02626T0|HMPREF1120_06041T0|HMPREF1120_05043T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901265
carboxylic_acid_metabolic_process	Gene_Ontology	GO:0019752	2	409	0.0494165	0.1686569	HMPREF1120_05043T0|HMPREF1120_01109T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019752
protein_targeting_to_membrane	Gene_Ontology	GO:0006612	1	58	0.0495205	0.1686569	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006612

EXPERIMENTAL SET: 45°C1W eek v/s 1°C1Hour_UP

Term	Database	ID	Input Number	Background Number P-Value	p-value	Corrected p-value	Genes	Hyperlink
antioxidant_activity	Gene_Ontology	GO:0016209	2	33	0.0016771	0.1535609	HMPREF1120_08836T0|HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016209
oxidoreductase_activity	Gene_Ontology	GO:0016491	4	339	0.0030537	0.1535609	HMPREF1120_01270T0|HMPREF1120_05220T0|HMPREF1120_08836T0|HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016491
pyruvate_metabolic_process	Gene_Ontology	GO:0006090	2	46	0.0031331	0.1535609	HMPREF1120_01270T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006090
replicative_cell_aging	Gene_Ontology	GO:0001302	2	47	0.0032627	0.1535609	HMPREF1120_08836T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001302
response_to_inorganic_substance	Gene_Ontology	GO:0010035	2	59	0.0050088	0.1535609	HMPREF1120_08836T0|HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0010035

cell_aging	Gene_Ontology	GO:0007569	2	71	0.0070962	0.1535609	HMPREF1120_08836T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007569
aging	Gene_Ontology	GO:0007568	2	72	0.0072851	0.1535609	HMPREF1120_08836T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007568
							HMPREF1120_01270T0|HMPREF1120_05220T0|HMPREF1120_08836T0|HM	
oxidation-reduction_process	Gene_Ontology	GO:0055114	4	437	0.0073522	0.1535609	PREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0055114
acid-thiol_ligase_activity	Gene_Ontology	GO:0016878	1	5	0.0102277	0.1535609	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016878
CTP_metabolic_process	Gene_Ontology	GO:0046036	1	5	0.0102277	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046036
pyrimidine_ribonucleoside_triphosphate_biosynthetic_process	Gene_Ontology	GO:0009209	1	5	0.0102277	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009209
CTP_biosynthetic_process	Gene_Ontology	GO:0006241	1	5	0.0102277	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006241
pyrimidine_ribonucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009208	1	5	0.0102277	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009208
superoxide_dismutase_activity	Gene_Ontology	GO:0004784	1	6	0.0119221	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004784
acetyl-CoA_biosynthetic_process	Gene_Ontology	GO:0006085	1	6	0.0119221	0.1535609	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006085
age-dependent_general_metabolic_decline_involved_in_chronological_cell_aging	Gene_Ontology	GO:0001323	1	6	0.0119221	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001323
age-dependent_response_to_oxidative_stress_involved_in_chronological_cell_aging	Gene_Ontology	GO:0001324	1	6	0.0119221	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001324
guanosine-containing_compound_biosynthetic_process	Gene_Ontology	GO:1901070	1	6	0.0119221	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901070
oxidoreductase_activity#_acting_on_superoxide_radicals_as_acceptor	Gene_Ontology	GO:0016721	1	6	0.0119221	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016721
nucleotide_phosphorylation	Gene_Ontology	GO:0046939	1	7	0.0136136	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046939
glutathione_peroxidase_activity	Gene_Ontology	GO:0004602	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004602
negative_regulation_of_nucleocytoplasmic_transport	Gene_Ontology	GO:0046823	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046823
negative_regulation_of_protein_import_into_nucleus	Gene_Ontology	GO:0042308	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042308
negative_regulation_of_intracellular_transport	Gene_Ontology	GO:0032387	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032387
protein_urmylation	Gene_Ontology	GO:0032447	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032447
negative_regulation_of_protein_localization_to_nucleus	Gene_Ontology	GO:1900181	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1900181
pyrimidine_nucleoside_triphosphate_biosynthetic_process	Gene_Ontology	GO:0009148	1	7	0.0136136	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009148
negative_regulation_of_cellular_protein_localization	Gene_Ontology	GO:1903828	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1903828
nucleoside_diphosphate_metabolic_process	Gene_Ontology	GO:0009132	1	7	0.0136136	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009132
negative_regulation_of_cytoplasmic_transport	Gene_Ontology	GO:1903650	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1903650
negative_regulation_of_intracellular_protein_transport	Gene_Ontology	GO:0090317	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0090317
nitrogen_cycle_metabolic_process	Gene_Ontology	GO:0071941	1	7	0.0136136	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071941
							HMPREF1120_03444T0|HMPREF1120_02626T0|HMPREF1120_06094T0|HM	
cytosol	Gene_Ontology	GO:0005829	4	540	0.0148933	0.1535609	PREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005829
age-dependent_response_to_oxidative_stress	Gene_Ontology	GO:0001306	1	8	0.0153023	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001306
age-dependent_general_metabolic_decline	Gene_Ontology	GO:0007571	1	8	0.0153023	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007571
thioester_biosynthetic_process	Gene_Ontology	GO:0035384	1	8	0.0153023	0.1535609	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0035384
acyl-CoA_biosynthetic_process	Gene_Ontology	GO:0071616	1	8	0.0153023	0.1535609	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071616
acetate_metabolic_process	Gene_Ontology	GO:0006083	1	8	0.0153023	0.1535609	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006083
cellular_response_to_oxygen_radical	Gene_Ontology	GO:0071450	1	9	0.0169880	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071450
superoxide_metabolic_process	Gene_Ontology	GO:0006801	1	9	0.0169880	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006801
phosphoprotein_binding	Gene_Ontology	GO:0051219	1	9	0.0169880	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051219
cellular_response_to_superoxide	Gene_Ontology	GO:0071451	1	9	0.0169880	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0071451
SRP-dependent_cotranslational_protein_targeting_to_membrane#_translocation	Gene_Ontology	GO:0006616	1	9	0.0169880	0.1535609	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006616
pyrimidine_nucleoside_triphosphate_metabolic_process	Gene_Ontology	GO:0009147	1	9	0.0169880	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009147
removal_of_superoxide_radicals	Gene_Ontology	GO:0019430	1	9	0.0169880	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019430
response_to_superoxide	Gene_Ontology	GO:0000303	1	9	0.0169880	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000303
response_to_oxygen_radical	Gene_Ontology	GO:0000305	1	9	0.0169880	0.1535609	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000305
regulation_of_protein_targeting	Gene_Ontology	GO:1903533	1	10	0.0186709	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1903533
regulation_of_protein_import_into_nucleus	Gene_Ontology	GO:0042306	1	10	0.0186709	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042306
guanosine-containing_compound_metabolic_process	Gene_Ontology	GO:1901068	1	10	0.0186709	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1901068
transcription_factor_import_into_nucleus	Gene_Ontology	GO:0042991	1	10	0.0186709	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0042991
cytoplasmic_sequestering_of_protein	Gene_Ontology	GO:0051220	1	11	0.0203509	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051220
regulation_of_intracellular_protein_transport	Gene_Ontology	GO:0033157	1	13	0.0237022	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0033157
pyrimidine_ribonucleotide_metabolic_process	Gene_Ontology	GO:0009218	1	13	0.0237022	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009218
acetyl-CoA_metabolic_process	Gene_Ontology	GO:0006084	1	13	0.0237022	0.1535609	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006084
pyrimidine_nucleoside_biosynthetic_process	Gene_Ontology	GO:0046134	1	13	0.0237022	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046134
pyrimidine_ribonucleoside_biosynthetic_process	Gene_Ontology	GO:0046132	1	13	0.0237022	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046132
pyrimidine_ribonucleotide_biosynthetic_process	Gene_Ontology	GO:0009220	1	13	0.0237022	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009220
nucleobase-containing_compound_kinase_activity	Gene_Ontology	GO:0019205	1	14	0.0253736	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019205
pyrimidine_ribonucleoside_metabolic_process	Gene_Ontology	GO:0046131	1	15	0.0270422	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046131
regulation_of_nitrogen_utilization	Gene_Ontology	GO:0006808	1	15	0.0270422	0.1535609	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006808
pyrimidine_nucleoside_metabolic_process	Gene_Ontology	GO:0006213	1	15	0.0270422	0.1535609	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006213
regulation_of_protein_localization_to_nucleus	Gene_Ontology	GO:1900180	1	16	0.0287079	0.1556096	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1900180

negative_regulation_of_protein_transport	Gene_Ontology	GO:0051224	1	16	0.0287079	0.1556096	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051224
phosphotransferase_activity#_phosphate_group_as_acceptor	Gene_Ontology	GO:0016776	1	16	0.0287079	0.1556096	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016776
transcription_corepressor_activity	Gene_Ontology	GO:0003714	1	16	0.0287079	0.1556096	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0003714
monocarboxylic_acid_metabolic_process	Gene_Ontology	GO:0032787	2	155	0.0300969	0.1557722	HMPREF1120_01270T0|HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032787
ligase_activity#_forming_carbon-sulfur_bonds	Gene_Ontology	GO:0016877	1	17	0.0303707	0.1557722	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016877
peroxidase_activity	Gene_Ontology	GO:0004601	1	17	0.0303707	0.1557722	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0004601
regulation_of_nucleocytoplasmic_transport	Gene_Ontology	GO:0046822	1	17	0.0303707	0.1557722	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0046822
reactive_oxygen_species_metabolic_process	Gene_Ontology	GO:0072593	1	17	0.0303707	0.1557722	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072593
oxidoreductase_activity#_acting_on_the_aldehyde_or_oxo_group_of_donors#_NAD_or_NADP_	Gene_Ontology	GO:0016620	1	18	0.0320307	0.1591524	HMPREF1120_01270T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016620
oxidoreductase_activity#_acting_on_peroxide_as_acceptor	Gene_Ontology	GO:0016684	1	18	0.0320307	0.1591524	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016684
pyrimidine_nucleotide_biosynthetic_process	Gene_Ontology	GO:0006221	1	19	0.0336878	0.1639704	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006221
pyrimidine_nucleotide_metabolic_process	Gene_Ontology	GO:0006220	1	19	0.0336878	0.1639704	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006220
SRP-dependent_cotranslational_protein_targeting_to_membrane	Gene_Ontology	GO:0006614	1	20	0.0353422	0.1652766	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006614
acyl-CoA_metabolic_process	Gene_Ontology	GO:0006637	1	20	0.0353422	0.1652766	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006637
cellular_response_to_reactive_oxygen_species	Gene_Ontology	GO:0034614	1	20	0.0353422	0.1652766	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0034614
thioester_metabolic_process	Gene_Ontology	GO:0035383	1	20	0.0353422	0.1652766	HMPREF1120_06094T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0035383
misfolded_or_incompletely_synthesized_protein_catabolic_process	Gene_Ontology	GO:0006515	1	21	0.0369936	0.1660575	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006515
nitrogen_utilization	Gene_Ontology	GO:0019740	1	21	0.0369936	0.1660575	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0019740
cotranslational_protein_targeting_to_membrane	Gene_Ontology	GO:0006613	1	21	0.0369936	0.1660575	HMPREF1120_02626T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006613
oxoacid_metabolic_process	Gene_Ontology	GO:0043436	3	425	0.0380135	0.1660575	HMPREF1120_01270T0|HMPREF1120_06094T0|HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043436
organic_acid_metabolic_process	Gene_Ontology	GO:0006082	3	426	0.0382341	0.1660575	HMPREF1120_01270T0|HMPREF1120_06094T0|HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006082
purine_nucleoside_triphosphate_biosynthetic_process	Gene_Ontology	GO:0009145	1	22	0.0386423	0.1660575	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009145
NADP_binding	Gene_Ontology	GO:0050661	1	22	0.0386423	0.1660575	HMPREF1120_01270T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0050661
negative_regulation_of_transport	Gene_Ontology	GO:0051051	1	22	0.0386423	0.1660575	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051051
purine_ribonucleoside_triphosphate_biosynthetic_process	Gene_Ontology	GO:0009206	1	22	0.0386423	0.1660575	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009206
regulation_of_protein_transport	Gene_Ontology	GO:0051223	1	24	0.0419312	0.1754490	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051223
regulation_of_cytoplasmic_transport	Gene_Ontology	GO:1903649	1	24	0.0419312	0.1754490	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:1903649
regulation_of_establishment_of_protein_localization	Gene_Ontology	GO:0070201	1	24	0.0419312	0.1754490	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0070201
oxidoreductase_activity#_acting_on_the_aldehyde_or_oxo_group_of_donors	Gene_Ontology	GO:0016903	1	25	0.0435714	0.1807267	HMPREF1120_01270T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016903
regulation_of_intracellular_transport	Gene_Ontology	GO:0032386	1	26	0.0452089	0.1843131	HMPREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032386
ribonucleoside_triphosphate_biosynthetic_process	Gene_Ontology	GO:0009201	1	26	0.0452089	0.1843131	HMPREF1120_03444T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009201
response_to_reactive_oxygen_species	Gene_Ontology	GO:0000302	1	27	0.0468435	0.1877676	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0000302
NAD_binding	Gene_Ontology	GO:0051287	1	27	0.0468435	0.1877676	HMPREF1120_01270T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0051287
							HMPREF1120_03444T0|HMPREF1120_01270T0|HMPREF1120_06094T0|HM	
small_molecule_metabolic_process	Gene_Ontology	GO:0044281	4	783	0.0476383	0.1893623	PREF1120_04591T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044281
oxidoreductase_activity#_acting_on_NAD(P)H	Gene_Ontology	GO:0016651	1	28	0.0484753	0.1895305	HMPREF1120_05220T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016651
chronological_cell_aging	Gene_Ontology	GO:0001300	1	28	0.0484753	0.1895305	HMPREF1120_08836T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001300

EXPERIMENTAL SET: 45°C1W eek v/s 1°C1Hour_DOW N

Term	Database	ID	Input Number	Backgroun d Number P-Value	p-v alue	Corrected p-v alue	Genes	Hyperlink
chronological_cell_aging	Gene_Ontology	GO:0001300	1	28	0.0041533	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001300
tricarboxylic_acid_cycle	Gene_Ontology	GO:0006099	1	29	0.0042963	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006099
citrate_metabolic_process	Gene_Ontology	GO:0006101	1	29	0.0042963	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006101
tricarboxylic_acid_metabolic_process	Gene_Ontology	GO:0072350	1	29	0.0042963	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0072350
dicarboxylic_acid_metabolic_process	Gene_Ontology	GO:0043648	1	44	0.0064376	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0043648
replicative_cell_aging	Gene_Ontology	GO:0001302	1	47	0.0068653	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0001302
cell_aging	Gene_Ontology	GO:0007569	1	71	0.0102806	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007569
aging	Gene_Ontology	GO:0007568	1	72	0.0104226	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0007568
aerobic_respiration	Gene_Ontology	GO:0009060	1	81	0.0117002	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0009060
oxidoreductase_activity;_acting_on_the_CH-OH_group_of_donors;_NAD_or_NADP_as_accep	Gene_Ontology	GO:0016616	1	81	0.0117002	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016616
oxidoreductase_activity;_acting_on_CH-OH_group_of_donors	Gene_Ontology	GO:0016614	1	84	0.0121257	0.0778065	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016614
cellular_respiration	Gene_Ontology	GO:0045333	1	106	0.0152405	0.0902706	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0045333
energy_derivation_by_oxidation_of_organic_compounds	Gene_Ontology	GO:0015980	1	155	0.0221433	0.1016689	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0015980
mRNA_binding	Gene_Ontology	GO:0003729	1	172	0.0245271	0.1016689	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0003729
poly(A)_RNA_binding	Gene_Ontology	GO:0044822	1	173	0.0246671	0.1016689	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044822
cellular_carbohydrate_metabolic_process	Gene_Ontology	GO:0044262	1	176	0.0250871	0.1016689	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044262
generation_of_precursor_metabolites_and_energy	Gene_Ontology	GO:0006091	1	189	0.0269051	0.1035847	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0006091

mitochondrial_matrix	Gene_Ontology	GO:0005759	1	215	0.0305311	0.1048832	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005759
single-organism_developmental_process	Gene_Ontology	GO:0044767	1	309	0.0435299	0.1208404	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0044767
developmental_process	Gene_Ontology	GO:0032502	1	312	0.0439420	0.1208404	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0032502
oxidoreductase_activity	Gene_Ontology	GO:0016491	1	339	0.0476423	0.1226330	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0016491
carbohydrate_metabolic_process	Gene_Ontology	GO:0005975	1	340	0.0477791	0.1226330	HMPREF1120_05340T0	http://amigo.geneontology.org/cgi-bin/amigo/term_details?term=GO:0005975

B_Supplementary Table 1_GO Terms, Summarized Data

EXPERIMENTAL SET: control (37°C) v/s 1°C1Week_UP

Term_ID	Description	FreqInDb Percent	Abslog10p value	Uniqueness	Dispensability	Representative
GO:0006090	pyruvate metabolic process	1.8820000	3.3357000	0.7210000	0.0000000	pyruvate metabolism
GO:0032787	monocarboxylic acid metabolic process	6.5860000	1.8974000	0.6820000	0.5510000	pyruvate metabolism
GO:0009205	purine ribonucleoside triphosphate metabolic process	15.8600000	1.8394000	0.4950000	0.4950000	pyruvate metabolism
GO:0046939	nucleotide phosphorylation	0.1340000	1.6198000	0.7040000	0.2710000	pyruvate metabolism
GO:0071966	fungal-type cell wall polysaccharide metabolic process	0.1340000	1.3514000	0.7700000	0.3040000	pyruvate metabolism
GO:0009132	nucleoside diphosphate metabolic process	0.1340000	1.6198000	0.7100000	0.2710000	pyruvate metabolism
GO:0016052	carbohydrate catabolic process	2.2850000	1.4512000	0.8230000	0.4180000	pyruvate metabolism
GO:0016051	carbohydrate biosynthetic process	2.2850000	2.4421000	0.6440000	0.1160000	pyruvate metabolism
GO:0009148	pyrimidine nucleoside triphosphate biosynthetic process	4.1820000	1.6198000	0.4730000	0.6580000	pyruvate metabolism
GO:0005996	monosaccharide metabolic process	0.2690000	1.4932000	0.7630000	0.4550000	pyruvate metabolism
GO:0006122	mitochondrial electron transport	0.4030000	1.4463000	0.6550000	0.5480000	pyruvate metabolism
GO:1901070	guanosine-containing compound biosynthetic process	3.9250000	1.6771000	0.4710000	0.6010000	pyruvate metabolism
GO:0006094	gluconeogenesis	0.1340000	2.3159000	0.6550000	0.4250000	pyruvate metabolism
GO:0006096	glycolytic process	1.8820000	2.3159000	0.6030000	0.6400000	pyruvate metabolism
GO:0055114	oxidation-reduction process	12.6340000	2.5941000	0.7800000	0.1540000	pyruvate metabolism
GO:0006085	acetyl-CoA biosynthetic process	0.9410000	1.6771000	0.7060000	0.2870000	pyruvate metabolism
GO:0046036	CTP metabolic process	1.2100000	1.7434000	0.4580000	0.1880000	pyruvate metabolism
GO:0006083	acetate metabolic process	1.7810000	1.5693000	0.7220000	0.6350000	pyruvate metabolism
GO:0051340	regulation of ligase activity	0.2690000	1.5242000	0.8220000	0.0080000	regulation of ligase activity
GO:0043066	negative regulation of apoptotic process	11.8280000	1.6771000	0.5910000	0.6070000	regulation of ligase activity
GO:0001402	signal transduction involved in filamentous growth	1.7140000	1.4122000	0.6750000	0.3740000	regulation of ligase activity
GO:0034551	mitochondrial respiratory chain complex III assembly	0.1340000	1.6198000	0.7850000	0.0130000	mitochondrial respiratory chain complex III assembly

GO:0045039	protein import into mitochondrial inner membrane	0.1340000	1.4463000	0.7990000	0.5320000	mitochondrial respiratory chain complex III assembly
GO:0006801	superoxide metabolic process	0.1340000	1.5242000	0.9030000	0.0380000	superoxide metabolism
GO:0010499	proteasomal ubiquitin-independent protein catabolic process	3.2260000	1.3514000	0.8090000	0.0540000	proteasomal ubiquitin-independent protein catabolism
GO:0006091	generation of precursor metabolites and energy	7.6610000	2.5236000	0.8940000	0.0670000	generation of precursor metabolites and energy
GO:0001323	age-dependent general metabolic decline involved in chronological cell aging	2.9470000	1.6771000	0.7380000	0.0860000	age-dependent general metabolic decline involved in chronological cell aging
GO:0007568	aging	2.9470000	1.6680000	0.8390000	0.3980000	age-dependent general metabolic decline involved in chronological cell aging

EXPERIMENTAL SET: control (37°C) v/s 1°C1Week_DOWN

Term_ID	Description	FreqInDb Percent	Abslog10p value	Uniqueness	Dispensability	Representative
GO:0006083	acetate metabolic process	1.7810000	3.9627000	0.6550000	0.0000000	acetate metabolism
GO:0006067	ethanol metabolic process	0.4030000	1.6067000	0.7420000	0.1650000	acetate metabolism
GO:0032787	monocarboxylic acid metabolic process	6.5860000	3.9018000	0.6150000	0.6230000	acetate metabolism
GO:1901575	organic substance catabolic process	19.4890000	2.2507000	0.7680000	0.5190000	acetate metabolism
GO:0009112	nucleobase metabolic process	6.3520000	1.3111000	0.6620000	0.6070000	acetate metabolism
GO:0006144	purine nucleobase metabolic process	11.8620000	1.5112000	0.6210000	0.3730000	acetate metabolism
GO:1901616	organic hydroxy compound catabolic process	0.2690000	1.5563000	0.7930000	0.6690000	acetate metabolism
GO:0044281	small molecule metabolic process	31.5860000	2.7751000	0.7420000	0.2760000	acetate metabolism
GO:0044282	small molecule catabolic process	0.2690000	1.8681000	0.7460000	0.1590000	acetate metabolism
GO:0072350	tricarboxylic acid metabolic process	4.0660000	2.9579000	0.6380000	0.5100000	acetate metabolism
GO:0006099	tricarboxylic acid cycle	1.6130000	2.9579000	0.7290000	0.1100000	acetate metabolism
GO:0046164	alcohol catabolic process	0.2690000	1.5808000	0.6680000	0.6690000	acetate metabolism
GO:0006515	misfolded or incompletely synthesized protein catabolic process	3.2260000	1.4705000	0.7420000	0.2820000	acetate metabolism
GO:0055114	oxidation-reduction process	12.6340000	2.2691000	0.7770000	0.2790000	acetate metabolism
GO:0006081	cellular aldehyde metabolic process	4.1670000	2.4800000	0.7750000	0.1250000	acetate metabolism
GO:0006082	organic acid metabolic process	12.2310000	3.1902000	0.6770000	0.2570000	acetate metabolism
GO:0034308	primary alcohol metabolic process	0.4030000	1.6067000	0.7420000	0.6940000	acetate metabolism
GO:0015980	energy derivation by oxidation of organic compounds	3.4950000	1.5936000	0.7230000	0.6340000	acetate metabolism
GO:0009056	catabolic process	19.4890000	1.9853000	0.9420000	0.0140000	catabolism
GO:0005975	carbohydrate metabolic process	15.4570000	1.7657000	0.9030000	0.0400000	carbohydrate metabolism
GO:0006085	acetyl-CoA biosynthetic process	0.9410000	1.9627000	0.6390000	0.0460000	acetyl-CoA biosynthesis
GO:0051156	glucose 6-phosphate metabolic process	8.3310000	1.5112000	0.7890000	0.3640000	acetyl-CoA biosynthesis

GO:0051186	cofactor metabolic process	2.1510000	1.4935000	0.8880000	0.0510000	cofactor metabolism
GO:0006616	SRP-dependent cotranslational protein targeting to membrane	0.1340000	1.8088000	0.7620000	0.0610000	SRP-dependent cotranslational protein targeting to membrane
GO:0044262	cellular carbohydrate metabolic process	8.3330000	1.4935000	0.8390000	0.0710000	cellular carbohydrate metabolism
GO:0006091	generation of precursor metabolites and energy	7.6610000	1.4378000	0.8720000	0.0800000	generation of precursor metabolites and energy
GO:0007569	cell aging	2.9470000	2.2245000	0.7410000	0.0850000	cell aging
GO:0044710	single-organism metabolic process	44.4890000	1.3487000	0.8210000	0.1010000	cell aging
GO:0007568	aging	2.9470000	2.2131000	0.8340000	0.3980000	cell aging

EXPERIMENTAL SET: 45°C1W v/s 1°C1Week_UP

Term_ID	Description	FreqInDb Percent	Abslog10p value	Uniqueness	Dispensability	Representative
GO:0006083	acetate metabolic process	1.7810000	3.1365000	0.6870000	0.0000000	acetate metabolism
GO:0032787	monocarboxylic acid metabolic process	6.5860000	5.2668000	0.6640000	0.5470000	acetate metabolism
GO:0072329	monocarboxylic acid catabolic process	1.7350000	2.1747000	0.6240000	0.6290000	acetate metabolism
GO:0072330	monocarboxylic acid biosynthetic process	0.5380000	1.7991000	0.6710000	0.5490000	acetate metabolism
GO:0009208	pyrimidine ribonucleoside triphosphate metabolic process	10.1250000	1.6163000	0.6230000	0.6980000	acetate metabolism
GO:0046939	nucleotide phosphorylation	0.1340000	1.4931000	0.7670000	0.2710000	acetate metabolism
GO:0044712	single-organism catabolic process	15.5910000	2.1181000	0.7270000	0.3390000	acetate metabolism
GO:0044281	small molecule metabolic process	31.5860000	2.7414000	0.7570000	0.2790000	acetate metabolism
GO:0044282	small molecule catabolic process	0.2690000	1.9456000	0.7880000	0.1590000	acetate metabolism
GO:0009132	nucleoside diphosphate metabolic process	0.1340000	1.4931000	0.7730000	0.2710000	acetate metabolism
GO:0016051	carbohydrate biosynthetic process	2.2850000	2.0895000	0.7240000	0.1150000	acetate metabolism
GO:0009148	pyrimidine nucleoside triphosphate biosynthetic process	4.1820000	1.4931000	0.5940000	0.6580000	acetate metabolism
GO:0006122	mitochondrial electron transport	0.4030000	1.3205000	0.7230000	0.5480000	acetate metabolism
GO:1901070	guanosine-containing compound biosynthetic process	3.9250000	1.5502000	0.6200000	0.6010000	acetate metabolism
GO:0006094	gluconeogenesis	0.1340000	2.0689000	0.7400000	0.4250000	acetate metabolism
GO:0006096	glycolytic process	1.8820000	2.0689000	0.5990000	0.6400000	acetate metabolism
GO:0006090	pyruvate metabolic process	1.8820000	2.9662000	0.6860000	0.6350000	acetate metabolism
GO:0034440	lipid oxidation	1.5120000	2.7744000	0.6040000	0.1090000	acetate metabolism
GO:0055114	oxidation-reduction process	12.6340000	5.4967000	0.7920000	0.1540000	acetate metabolism
GO:0006085	acetyl-CoA biosynthetic process	0.9410000	1.5502000	0.7880000	0.2870000	acetate metabolism
GO:1901068	guanosine-containing compound metabolic process	9.1400000	1.3574000	0.6490000	0.4520000	acetate metabolism
GO:0046036	CTP metabolic process	1.2100000	1.6163000	0.6840000	0.1870000	acetate metabolism

GO:0006082	organic acid metabolic process	12.2310000	3.3224000	0.7050000	0.2570000	acetate metabolism
GO:0010035	response to inorganic substance	0.1340000	1.5867000	0.8980000	0.0000000	response to inorganic substance
GO:0034551	mitochondrial respiratory chain complex III assembly	0.1340000	1.4931000	0.9000000	0.0130000	mitochondrial respiratory chain complex III assembly
GO:0009056	catabolic process	19.4890000	1.6539000	0.9620000	0.0140000	catabolism
GO:0071941	nitrogen cycle metabolic process	24.1040000	1.4931000	0.9020000	0.0270000	nitrogen cycle metabolism
GO:0051439	regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	0.1340000	1.5502000	0.6940000	0.0610000	regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle
GO:0043066	negative regulation of apoptotic process	11.8280000	1.5502000	0.5750000	0.4670000	regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle
GO:0032387	negative regulation of intracellular transport	0.2690000	1.4931000	0.7030000	0.3000000	regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle
GO:0006091	generation of precursor metabolites and energy	7.6610000	2.0769000	0.9070000	0.0610000	generation of precursor metabolites and energy
GO:0007569	cell aging	2.9470000	1.4434000	0.7380000	0.0850000	cell aging
GO:0044710	single-organism metabolic process	44.4890000	1.6103000	0.8320000	0.1010000	cell aging
GO:0007568	aging	2.9470000	1.4327000	0.8400000	0.3980000	cell aging
GO:0001324	age-dependent response to oxidative stress involved in chronological cell aging	0.1340000	1.5502000	0.6930000	0.6750000	cell aging

EXPERIMENTAL SET: 45°C1W v/s 1°C1Week_DOWN

Term_ID	Description	FreqInDb Percent	Abslog10p value	Uniqueness	Dispensability	Representative
GO:0006098	pentose-phosphate shunt	0.1340000	4.0020000	0.4660000	0.0000000	pentose-phosphate shunt
GO:0019637	organophosphate metabolic process	17.0700000	1.3447000	0.6850000	0.6120000	pentose-phosphate shunt
GO:0032787	monocarboxylic acid metabolic process	6.5860000	2.0821000	0.6740000	0.5470000	pentose-phosphate shunt
GO:0006067	ethanol metabolic process	0.4030000	1.8640000	0.7320000	0.1650000	pentose-phosphate shunt
GO:1901575	organic substance catabolic process	19.4890000	3.3776000	0.7280000	0.5190000	pentose-phosphate shunt
GO:0072330	monocarboxylic acid biosynthetic process	0.5380000	1.4109000	0.7020000	0.5490000	pentose-phosphate shunt
GO:0051156	glucose 6-phosphate metabolic process	8.3310000	3.8143000	0.6720000	0.5930000	pentose-phosphate shunt
GO:0046164	alcohol catabolic process	0.2690000	1.8378000	0.6400000	0.6690000	pentose-phosphate shunt
GO:0019682	glyceraldehyde-3-phosphate metabolic process	8.0330000	4.0020000	0.5950000	0.3210000	pentose-phosphate shunt
GO:0006515	misfolded or incompletely synthesized protein catabolic process	3.2260000	1.7268000	0.7180000	0.2820000	pentose-phosphate shunt
GO:1901616	organic hydroxy compound catabolic process	0.2690000	1.8132000	0.7550000	0.6690000	pentose-phosphate shunt
GO:0006083	acetate metabolic process	1.7810000	2.1126000	0.7040000	0.1480000	pentose-phosphate shunt
GO:0044282	small molecule catabolic process	0.2690000	2.3655000	0.7310000	0.2500000	pentose-phosphate shunt

GO:0034308	primary alcohol metabolic process	0.4030000	1.8640000	0.7320000	0.6940000	pentose-phosphate shunt
GO:0072524	pyridine-containing compound metabolic process	0.2690000	2.7781000	0.8650000	0.1780000	pentose-phosphate shunt
GO:0009056	catabolic process	19.4890000	3.0840000	0.9360000	0.0100000	catabolism
GO:1901135	carbohydrate derivative metabolic process	16.8010000	1.4609000	0.8970000	0.0280000	carbohydrate derivative metabolism
GO:0051186	cofactor metabolic process	2.1510000	1.9778000	0.8950000	0.0390000	cofactor metabolism
GO:0006616	SRP-dependent cotranslational protein targeting to membrane	0.1340000	2.0670000	0.6630000	0.0490000	SRP-dependent cotranslational protein targeting to membrane
GO:0005975	carbohydrate metabolic process	15.4570000	1.4493000	0.8920000	0.0650000	carbohydrate metabolism
GO:0006081	cellular aldehyde metabolic process	4.1670000	2.9891000	0.7920000	0.0920000	cellular aldehyde metabolism
GO:0055086	nucleobase-containing small molecule metabolic process	16.8010000	1.7062000	0.6850000	0.3060000	cellular aldehyde metabolism
GO:0044710	single-organism metabolic process	44.4890000	1.4749000	0.8480000	0.2220000	cellular aldehyde metabolism
GO:0044281	small molecule metabolic process	31.5860000	2.2973000	0.7690000	0.2080000	cellular aldehyde metabolism

EXPERIMENTAL SET: 45°C1W v/s 1°C1Hour_UP

Term_ID	Description	FreqInDb Percent	Abslog10p value	Uniqueness	Dispensability	Representative
GO:0006090	pyruvate metabolic process	1.8820000	2.5040000	0.7660000	0.0000000	pyruvate metabolism
GO:0009208	pyrimidine ribonucleoside triphosphate metabolic process	10.1250000	1.9902000	0.6020000	0.6980000	pyruvate metabolism
GO:0046939	nucleotide phosphorylation	0.1340000	1.8660000	0.7520000	0.2710000	pyruvate metabolism
GO:0044281	small molecule metabolic process	31.5860000	1.3220000	0.8050000	0.2790000	pyruvate metabolism
GO:0009132	nucleoside diphosphate metabolic process	0.1340000	1.8660000	0.7520000	0.2710000	pyruvate metabolism
GO:0009148	pyrimidine nucleoside triphosphate biosynthetic process	4.1820000	1.8660000	0.5290000	0.6580000	pyruvate metabolism
GO:0009147	pyrimidine nucleoside triphosphate metabolic process	11.5590000	1.7699000	0.6040000	0.5500000	pyruvate metabolism
GO:0043436	oxoacid metabolic process	12.2310000	1.4201000	0.7120000	0.6070000	pyruvate metabolism
GO:1901070	guanosine-containing compound biosynthetic process	3.9250000	1.9236000	0.5860000	0.4020000	pyruvate metabolism
GO:0006085	acetyl-CoA biosynthetic process	0.9410000	1.9236000	0.7020000	0.2870000	pyruvate metabolism
GO:0055114	oxidation-reduction process	12.6340000	2.1336000	0.8290000	0.1490000	pyruvate metabolism
GO:1901068	guanosine-containing compound metabolic process	9.1400000	1.7288000	0.6440000	0.6520000	pyruvate metabolism
GO:0046036	CTP metabolic process	1.2100000	1.9902000	0.4630000	0.1880000	pyruvate metabolism
GO:0006082	organic acid metabolic process	12.2310000	1.4175000	0.7380000	0.2600000	pyruvate metabolism
GO:0006083	acetate metabolic process	1.7810000	1.8152000	0.7670000	0.6350000	pyruvate metabolism
GO:0010035	response to inorganic substance	0.1340000	2.3003000	0.8560000	0.0000000	response to inorganic substance

GO:0032387	negative regulation of intracellular transport	0.2690000	1.8660000	0.5890000	0.0140000	negative regulation of intracellular transport
GO:0006808	regulation of nitrogen utilization	0.4030000	1.5680000	0.8000000	0.3270000	negative regulation of intracellular transport
GO:0070201	regulation of establishment of protein localization	0.3140000	1.3775000	0.6600000	0.4630000	negative regulation of intracellular transport
GO:0071941	nitrogen cycle metabolic process	24.1040000	1.8660000	0.8780000	0.0150000	nitrogen cycle metabolism
GO:0006801	superoxide metabolic process	0.1340000	1.7699000	0.9060000	0.0380000	superoxide metabolism
GO:0032447	protein urmylation	0.1340000	1.8660000	0.9220000	0.0380000	protein urmylation
GO:0006515	misfolded or incompletely synthesized protein catabolic process	3.2260000	1.4319000	0.8800000	0.3990000	protein urmylation
GO:0072593	reactive oxygen species metabolic process	0.1340000	1.5175000	0.9310000	0.0380000	reactive oxygen species metabolism
GO:0019740	nitrogen utilization	0.4030000	1.4319000	0.9200000	0.0460000	nitrogen utilization
GO:0007569	cell aging	2.9470000	2.1490000	0.7190000	0.0860000	cell aging
GO:0007568	aging	2.9470000	2.1376000	0.8390000	0.3980000	cell aging

EXPERIMENTAL SET: 45°C1W v/s 1C1Hour_DOWN

Term_ID	Description	FreqInDb Percent	Abslog10p value	Uniqueness	Dispensability	Representative
GO:0032502	developmental process	22.2500000	1.3571000	0.8990000	0.0000000	developmental process
GO:0072350	tricarboxylic acid metabolic process	4.0660000	2.3669000	0.6060000	0.0000000	tricarboxylic acid metabolism
GO:0006099	tricarboxylic acid cycle	1.6130000	2.3669000	0.5090000	0.1230000	tricarboxylic acid metabolism
GO:0043648	dicarboxylic acid metabolic process	0.1340000	2.1913000	0.6830000	0.3760000	tricarboxylic acid metabolism
GO:0015980	energy derivation by oxidation of organic compounds	3.4950000	1.6548000	0.5310000	0.6340000	tricarboxylic acid metabolism
GO:0006101	citrate metabolic process	12.2310000	2.3669000	0.5170000	0.6950000	tricarboxylic acid metabolism
GO:0005975	carbohydrate metabolic process	15.4570000	1.3208000	0.8510000	0.0470000	carbohydrate metabolism
GO:0044262	cellular carbohydrate metabolic process	8.3330000	1.6005000	0.7930000	0.0710000	cellular carbohydrate metabolism
GO:0006091	generation of precursor metabolites and energy	7.6610000	1.5702000	0.7990000	0.0800000	generation of precursor metabolites and energy
GO:0007569	cell aging	2.9470000	1.9880000	0.3800000	0.0960000	cell aging
GO:0044767	single-organism developmental process	18.0300000	1.3612000	0.5130000	0.4460000	cell aging
GO:0007568	aging	2.9470000	1.9820000	0.5790000	0.3980000	cell aging

C_Supplementary Table 2_Predicted Pathways

Pathway	Database	Pathway Id	Genes Regulated in Pathway	Genes in Pathway	p VALUE	Corrected p-value	Genes	Link to pathways
EXPERIMENTAL SET: WT37CWT1C1W_DOWN								
Pyruvate_metabolism	KEGG_PATHWAY	sce00620	5	36	0.0000855	0.0161728	HMPREF1120_06094T0|HMPREF1120_06041T0|HMPREF1120_05378T0|HMPREF1120_01109T0|HMPREF1120_05340T0	http://www.genome.jp/kegg-bin/show_pathway?sce00620/sce:YKL085W%09blue/sce:YER073W%09blue/sce:YIR031C%09blue/sce:YLR153C%09blue/sce:YKR097W%09blue
Carbon_metabolism	KEGG_PATHWAY	sce01200	6	110	0.0018269	0.0543848	HMPREF1120_05340T0|HMPREF1120_06041T0|HMPREF1120_06094T0|HMPREF1120_05043T0|HMPREF1120_07361T0|HMPREF1120_05378T0	http://www.genome.jp/kegg-bin/show_pathway?sce01200/sce:YPR074C%09blue/sce:YKL085W%09blue/sce:YIR031C%09blue/sce:YKR097W%09blue/sce:YOR388C%09blue/sce:YLR153C%09blue
Glyoxylate_and_dicarboxylate metabolism	KEGG_PATHWAY	sce00630	3	22	0.0026704	0.0705684	HMPREF1120_05043T0|HMPREF1120_05378T0|HMPREF1120_05340T0	http://www.genome.jp/kegg-bin/show_pathway?sce00630/sce:YOR388C%09blue/sce:YKL085W%09blue/sce:YIR031C%09blue
Ethanol_degradation_IV	BioCyc	PWY66-162	2	8	0.0044621	0.1015773	HMPREF1120_01109T0|HMPREF1120_06094T0	http://biocyc.org/YEAST/NEW-IMAGE?type=NIL&object=PWY66-162
Cellular_response_to_heat_stress	Reactome	REACT_313907	2	24	0.0055635	0.1076941	HMPREF1120_02626T0|HMPREF1120_02657T0	http://www.reactome.org/cgi-bin/eventbrowser_st_id?ST_ID=REACT_313907
EXPERIMENTAL SET: WT37CWT1C1W_UP								
Glycolysis_/_Gluconeogenesis	KEGG_PATHWAY	sce00010	3	56	0.0060108	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0|HMPREF1120_06094T0	http://www.genome.jp/kegg-bin/show_pathway?sce00010/sce:YGR254W%09red/sce:YJL052W%09red/sce:YLR153C%09red
Carbon_metabolism	KEGG_PATHWAY	sce01200	3	110	0.0344052	0.2678100	HMPREF1120_01270T0|HMPREF1120_00576T0|HMPREF1120_06094T0	http://www.genome.jp/kegg-bin/show_pathway?sce01200/sce:YGR254W%09red/sce:YJL052W%09red/sce:YLR153C%09red
Cellular_responses_to_stress	Reactome	REACT_336855	2	42	0.0360122	0.2678100	HMPREF1120_08836T0|HMPREF1120_00315T0	http://www.reactome.org/cgi-bin/eventbrowser_st_id?ST_ID=REACT_336855
EXPERIMENTAL SET: WT45C1WT1C1H_DOWN								
aspartate_degradation_II	BioCyc	MALATE-ASPARTATE-SHUTTLE-PWY	1	5	0.0098643	0.0778065	HMPREF1120_05340T0	http://biocyc.org/YEAST/NEW-IMAGE?type=NIL&object=MALATE-ASPARTATE-SHUTTLE-PWY
Citric_acid_cycle_(TCA_cycle)	Reactome	REACT_278278	1	24	0.0214731	0.1016689	HMPREF1120_05340T0	http://www.reactome.org/cgi-bin/eventbrowser_st_id?ST_ID=REACT_278278
Glyoxylate_and_dicarboxylate metabolism	KEGG_PATHWAY	sce00630	1	22	0.0364296	0.1122031	HMPREF1120_05340T0	http://www.genome.jp/kegg-bin/show_pathway?sce00630/sce:YKL085W%09red
EXPERIMENTAL SET: WT45C1WT1C1H_UP								
Cellular_responses_to_stress	Reactome	REACT_336855	2	42	0.0126175	0.1535609	HMPREF1120_02626T0|HMPREF1120_08836T0	http://www.reactome.org/cgi-bin/eventbrowser_st_id?ST_ID=REACT_336855
Glycolysis_/_Gluconeogenesis	KEGG_PATHWAY	sce00010	2	56	0.0309719	0.1571658	HMPREF1120_01270T0|HMPREF1120_06094T0	http://www.genome.jp/kegg-bin/show_pathway?sce00010/sce:YJL052W%09red/sce:YLR153C%09red
EXPERIMENTAL SET: WT45C1WT1C1W_DOWN								
pentose_phosphate_pathway_(non-oxidative_branch)	BioCyc	NONOXIPENT-PWY	2	6	0.0013226	0.0194252	HMPREF1120_08453T0|HMPREF1120_07361T0	http://biocyc.org/YEAST/NEW-IMAGE?type=NIL&object=NONOXIPENT-PWY
Carbon_metabolism	KEGG_PATHWAY	sce01200	4	110	0.0134752	0.0958278	HMPREF1120_05043T0|HMPREF1120_06041T0|HMPREF1120_07361T0|HMPREF1120_08453T0	http://www.genome.jp/kegg-bin/show_pathway?sce01200/sce:YPR074C%09blue/sce:YOR388C%09blue/sce:YLR354C%09blue/sce:YKR097W%09blue
Pyruvate_metabolism	KEGG_PATHWAY	sce00620	2	36	0.0383408	0.1471540	HMPREF1120_06041T0|HMPREF1120_01109T0	http://www.genome.jp/kegg-bin/show_pathway?sce00620/sce:YER073W%09blue/sce:YKR097W%09blue
EXPERIMENTAL SET: WT45C1WT1C1W_UP								
Beta-oxidation_of_very_long_chain fatty_acids	Reactome	REACT_301991	2	6	0.0022644	0.1066633	HMPREF1120_02451T0|HMPREF1120_08713T0	http://www.reactome.org/cgi-bin/eventbrowser_st_id?ST_ID=REACT_301991

Glycolysis_/_Gluconeogenesis	KEGG_PATHWAY	sce00010	4	56	0.0045221	0.1618911	HMPREF1120_01270T0|HMPREF1120_01109T0|HMPREF1120_00576T0|HMPREF1120_06094T0	http://www.genome.jp/kegg-bin/show_pathway?sce00010/sce:YGR254W%09red/sce:YJL052W%09red/sce:YER073W%09red/sce:YLR153C%09red
Peroxisomal_lipid_metabolism	Reactome	REACT_32655 7	2	11	0.0061213	0.1956636	HMPREF1120_02451T0|HMPREF1120_08713T0	http://www.reactome.org/cgi-bin/eventbrowser_st_id?ST_ID=REACT_326557
Cellular_responses_to_stress	Reactome	REACT_33685 5	3	42	0.0081161	0.2178567	HMPREF1120_02626T0|HMPREF1120_08836T0|HMPREF1120_00315T0	http://www.reactome.org/cgi-bin/eventbrowser_st_id?ST_ID=REACT_336855

D_Supplementary Table 3_ Sub-cellular localization of all identified proteins, as predicted by YLoc.

NCBI accession number	ORF	Protein name	Predicted Location[a]	Probability[b] (%)	Confidence[c]
gi|378725637	HMPREF1120_00315	14-3-3 family protein	cytoplasm	91.34	small (0.29)
gi|378725903	HMPREF1120_00576	enolase	cytoplasm	99.29	very strong (0.96)
gi|378726433	HMPREF1120_01097	7-alpha-hydroxysteroid dehydrogenase	mitochondrion and cytoplasm	45.68	normal (0.35)
gi|378726445	HMPREF1120_01109	aldehyde dehydrogenase	cytoplasm/peroxisome	60.71	normal (0.49)
gi|378726610	HMPREF1120_01270	glyceraldehyde-3-phosphate dehydrogenase	cytoplasm	91.20	normal (0.49)
gi|378726833	HMPREF1120_01486	hypoth. protein HMPREF1120_01486	mitochondrion	92.97	normal (0.77)
gi|378727822	HMPREF1120_02451	oxidoreductase, short-chain dehydrogenase/reductase	mitochondrion and cytoplasm	40.48	normal (0.46)
gi|378727999	HMPREF1120_02626	hsp70-like protein	cytoplasm	90.47	strong (0.90)
gi|378728030	HMPREF1120_02657	elongation factor 1-alpha	cytoplasm	90.31	strong (0.92)
gi|378728179	HMPREF1120_02805	minor allergen Alt a 7	extracellular space, cytoplasm, plasma membrane and nucleus	20.76	small (0.00)
gi|378728843	HMPREF1120_03444	nucleoside diphosphate kinase	cytoplasm	95.76	strong (0.87)
gi|378729152	HMPREF1120_03741	alcohol oxidase	peroxisome/cytoplasm	85.63	strong (0.84)
gi|378729265	HMPREF1120_03849	ubiquinol-cytochrome c reductase sub. 7	mitochondrion	99.79	very strong (1.00)
gi|19073387	HMPREF1120_04221	HSP30	cytoplasm/nucleus	73.58	strong (0.86)
gi|378729668	HMPREF1120_04224	hypothetical protein HMPREF1120_04224	cytoplasm and nucleus	47.17	small (0.19)
gi|378729910	HMPREF1120_04451	haloalkanoic acid dehalogenase	cytoplasm/nucleus	74.32	strong (0.82)
gi|378730051	HMPREF1120_04591	Glutathione S-transferase	cytoplasm/nucleus	64.28	normal (0.52)
gi|378730392	HMPREF1120_04915	beta-lactamase	cytoplasm	80.81	normal (0.68)
gi|378730528	HMPREF1120_05043	formate dehydrogenase	mitochondrion/cytoplasm	67.52	normal (0.52)
gi|378732712	HMPREF1120_07169	proteosom component PUP2	cytoplasm	96.99	strong (0.93)
gi|378730713	HMPREF1120_05220	hypothetical protein HMPREF1120_05220	cytoplasm/nucleus	46.58	normal (0.50)
gi|378730838	HMPREF1120_05340	malate dehydrogenase	cytoplasm/mitochondrion	61.81	small (0.18)

gi|378730878	HMPREF1120_05378	malate synthase, glyoxysomal	peroxisome	98.33	very strong (0.98)
gi|378731564	HMPREF1120_06041	Phosphoenol pyruvate carboxykinase	cytoplasm/nucleus	73.20	strong (0.90)
gi|378731617	HMPREF1120_06094	acetyl-coenzyme A synthetase	cytoplasm/nucleu/peroxisome	57.92	normal (0.70)
gi|378731783	HMPREF1120_06254	mitochondrial protein-transporting ATPase	cytoplasm/nucleus	85.12	normal (0.47)
gi|378731870	HMPREF1120_06341	hypoth. protein HMPREF1120_06341	cytoplasm/peroxisome	70.12	normal (0.31)
gi|378732911	HMPREF1120_07361	transketolase	cytoplasm, nucleus and peroxisome	31.28	small (0.19)
gi|378733644	HMPREF1120_08075	hypoth. protein HMPREF1120_08075	nucleus and cytoplasm	49.62	strong (0.86)
gi|378734310	HMPREF1120_08713	tetrahydroxynaphthalene reductase	cytoplasm, nucleus and mitochondrion	32.50	small (0.21)
gi|378734433	HMPREF1120_08836	Fe-Mn family superoxide dismutase	mitochondrion	99.53	very strong (0.99)
gi|378734037	HMPREF1120_08453	transaldolase	cytoplasm/nucleus	72.67	normal (0.72)

[a] sub-cellular localization of the protein as predicted by YLoc+ according to the biological properties of the protein sequence

[b] probability of the sub-cellular location

[c] confidence that the prediction is reliable

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Proteome of tolerance fine-tuning in the human pathogen black yeast *Exophiala dermatitidis*

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ABSTRACT

The black yeast *Exophiala dermatitidis* is a worldwide distributed agent of primary and secondary diseases in both immunocompromised and healthy humans, with a high prevalence in human-made environments. Since thermo-tolerance has a crucial role in the fungus persistence in man-dominated habitat and in its pathogenicity, three incubation temperatures (37, 45, 1 °C) and two time spans (1 h, 1 week) were selected to simulate different environmental conditions and to investigate the effect of temperature on the proteome of *E. dermatitidis* CBS 525.76. Using a novel protocol for protein extraction from black yeasts, 2-D DIGE could be applied for characterization of changes in total protein spot abundance among the experimental conditions. A total of 32 variable proteins were identified by mass spectrometry. Data about protein functions, localization and pathways were also obtained. A typical stress response under non-optimal temperature could not be observed at the proteome level, whereas a reduction of the metabolic activity, mostly concerning processes as the general carbon metabolism, was detected after exposure to cold. These results suggest that a fine protein modulation takes place following temperature treatment and a repertoire of stable protein might be at the base of *E. dermatitidis* adaptation to altered growth conditions.

Significance: E. dermatitidis is a pathogenic black yeast causing neurotropic infections, systemic and subcutaneous disease in a wide range of hosts, including humans. The discovery of the fungus high prevalence in man-made habitats, including sauna facilities, drinking water and dishwashers, generated concern and raised questions about the infection route. In the present work — which is the first contribution on *E. dermatitidis* proteome — the effect of different temperature conditions on the fungus protein pattern have been analyzed by using a gel-based approach and the temperature responsive proteins have been identified.

The absence of a typical stress response following the exposure to non-optimal temperature was detected at the proteome level, along with a general reduction of the metabolic activity after exposure to cold. These results suggest that a very fine regulation of the protein expression as well as adaptations involving a basic set of stable proteins may be at the base of *E. dermatitidis* enormous ecological plasticity, which plays a role in the fungus distribution, also enabling the transition from natural to human habitat and to the human host.

1. Introduction

The opportunistic black yeast *Exophiala [Wangiella] dermatitidis* (Kano, de Hoog 1977), belonging to the *Ascomycete* order *Chaetothyriales,* is an agent of primary and secondary diseases in both healthy and immune-compromised hosts, including humans [1]. Clinical forms of the infection range from localized cutaneous and subcutaneous phaeohyphomycosis to more severe systemic forms such as neurotropic infections, whose prevalence is effectively growing [2,3]. In addition, this species is often found as pulmonary colonizer in patients affected by cystic fibrosis [4].

Although there is increasing evidence supporting the tropical rain forest as the strain's natural reservoir — where its life cycle involves fruit-eating animals [5] — *E. dermatitidis* has a much wider distribution as assumed earlier. Due to its extremophilic character, the fungus manages to thrive in a broad variety of environments whether natural or not [6]. Along with the isolation from the tropical and sub-tropical areas, strains of *E. dermatitidis* have been found in habitat characterized by very different climate and temperature conditions such as deep-sea hydrothermal systems and hot water from natural springs [7,8], the Antarctic continent and Apennine glaciers [9,10]. Interestingly, this

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clinically relevant species shows high prevalence in man-made habitats, being frequently detected in steam bath and sauna facilities [11], sink and drainpipes, drinking water and dishwashers [12–14], which are colonized as artificial equivalents to the natural niches [15].

Such wide diversity in distribution and habitat is achieved due to a high spectrum of adaptation to a multitude of biotic and a-biotic factors. Independently on the origin, whether polar or Arctic regions, tropics, steam baths, dishwashers or the human body, *E. dermatitidis* strains share a remarkable thermo-, halo- and pH-tolerance together with an extraordinary ability to withstand oligotrophism and osmotic stress [16,17]. As recently demonstrated, *E. dermatitidis* can also resist ionizing radiations and furthermore reacts to them by enhancing the cell growth [18].

This combination of properties arises from a number of morpho- and physiological characters such as thick and multilayered cell walls, extracellular polysaccharides (EPS) capsule [19], melanization, intracellular accumulation of polyols and trehalose, that are universally present in black yeasts [20,21]. Most importantly melanin, whose deposition in the cell walls is responsible for *E. dermatitidis* dark pigmentation, plays a major role in pathogenesis by reducing the black yeast's susceptibility to the host immune system [22]. In addition, melanin has an essential biomechanical function which enables the hyphae to penetrate host tissues [23]. Ecological flexibility and poly-extremophilia together, make of *E. dermatitidis* a fully successful microorganism and human pathogen.

As the fungus can cause infections even in healthy hosts, its presence in man-made environments generates alarm, besides raising questions about the infection route. As recently suggested, the black yeast's transition from natural habitat to humans might be achieved using natural spring water as a vector (Novak Babič et al., unpublished data). Moreover, spring water could act as an additional reservoir for this species. This hypothesis creates concern especially about the drinking water practice of central and north European countries, which is based on the routinely consumption on non-chlorinated water [24].

Despite the strain extremophilic character and pathogenicity are known, there is very little information regarding the molecular mechanisms which are at the base of its stress resistance.

Lately the molecular tractability of *E. dermatitidis* has increased the number of the transcriptomic [24,25] and genomic studies, especially since its sequenced genome became available (http://www.broadinstitute.org/annotation/genome/Black_Yeasts/MultiHome.html). However, our understanding of the system biology of this species is still limited mainly at the functional proteomic level. To this purpose, we describe here the first proteomic study of *E. dermatitidis*. Since the ability to cope with temperature stress has a crucial role in the fungus persistence in man-dominated habitat as well as for its virulence, our investigation had the aims to determine changes in the fungus proteome under sub- and supra optimal temperatures and to detect proteins and pathways that are involved in the response to temperature stress. The strain optimal temperature of growth at the laboratory conditions and the cell viability after temperature treatment were assessed prior to the experimental set up.

Different temperature conditions and exposure times were applied to evaluate their effects on the proteome. *E. dermatitidis* was thereby incubated at 37 °C and at 1 °C with the aim to simulate the cold natural habitat and putative reservoir (e.g. glaciers) from which the species — in meltwaters running off glaciers — might have access to the human environment. The incubation at 45 °C was instead performed in order to re-create temperatures well above that of humans, such as the body temperature of frugivorous animals (e.g. birds, bats), which are thought to have a natural habitat in association with *E. dermatitidis*. In addition, 45 °C can also be representative of the temperature of warm tap water as well as being in the range of temperatures recorded in steam bath and sauna facilities.

TEM observations were performed — with a special focus on melanin — in order to detect alterations of the cell structures due to temperature stress. 2D difference gel electrophoresis (2D-DIGE) and LC/MS–MS were used to characterize the temperature responsive proteins. Their abundance patterns, their functions and predicted interactions are discussed.

2. Materials and methods

2.1. Strains, cultivation and stress conditions

E. dermatitidis CBS 525.76 was purchased from the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands; Fig. 1). The strain was grown on 2% MEA (Malt Extract Agar) at 37 °C for seven weeks in order to obtain sufficient amounts of biomass for protein extraction. Consequently, it was exposed to 1 °C and 45 °C both for 1 h and for 1 week. Colonies grown at 37 °C were selected as control sample for the experiment. All tests were performed using four individuals — four petri dishes — as biological replicates, resulting in a total of 20 samples.

Temperature optima and colony viability after temperature treatment were tested prior to setting up the experimental conditions. Temperature tests were performed on 2% MEA plates at 1, 5, 10, 15, 20, 25, 28, 30, 35 and 37 °C. Higher temperatures were not tested since data supporting the growth of *E. dermatitidis* at 45 and 47 °C are already available in literature [6]. The diameter of the colonies was recorded each day for three weeks. The viability was evaluated after exposure to 1 and 45 °C for 12, 24, 48, 72 h and 1 week.

2.2. Transmission electron microscopy

A 1 mm^3 portion of *E. dermatitidis* colony from each experimental condition was fixed overnight in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 (v/v). The following steps were carried out according to Arcalis et al. [26] with a few modifications. After washing the sample with 0.1 M phosphate buffer, post-fixation was performed using 1% osmium tetroxide in phosphate buffer (0.1 M, pH 7.4, v/v) for 3 h on ice and in the dark. The sample was washed thereafter with phosphate buffer (0.1 M, pH 7.4), subsequently dehydrated through an acetone series [50, 70, 90, 96, 100% acetone in MilliQ water (Millipore), v/v] and then stepwise infiltrated (15, 30, 45, 60, 75, 90, 100% resin/acetone solution, w/v) in low viscosity epoxy resin (Agar Scientific) on ice. Polymerization was carried out at 60 °C for 48 h.

Ultrathin 70 nm sections were mounted on gold grids, stained in 2% (w/v) aqueous uranyl acetate followed by Reynold's lead citrate and inspected in both a Morgagni 268D transmission electron microscope (FEI) and in a Tecnai $G^2$20 (FEI) operated at 80 kV. Images were acquired using an 11 megapixel Morada CCD camera (Olympus-SIS) and an Eagle 4 k HS CCD Camera (FEI), respectively.

2.3. Preparation of protein extracts

Protein extraction was carried out according to a previously described protocol [27], however some modifications were applied in order to increase the protein yield [28] as follows: the biomass was disrupted using mortar and pestle under liquid nitrogen, then suspended in homogenization buffer (50 mM Tris–HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose, 2% SDS) with the addition of 0.1 M NaOH in a 1:1 ratio (biomass/NaOH, w/v) and further processed using a beating mill, to enhance the disintegration of the cell walls.

Tris-buffered phenol solution pH 8.0 (Sigma Aldrich) was added to the cell lysate and the phenolic phase was collected after centrifugation (3300 g for 20 min; Heraeus Megafuge 40R, Thermo Scientific). Proteins were precipitated overnight (−20 °C) by addition of 5 volumes of 0.1 M ammonium acetate in methanol (w/v). After centrifugation at 3300 g for 30 min, the precipitate was washed with ice-cold methanol (absolute) and then with ice-cold acetone (80%, v/v). The dried pellet was re-solubilized in 7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris HCl

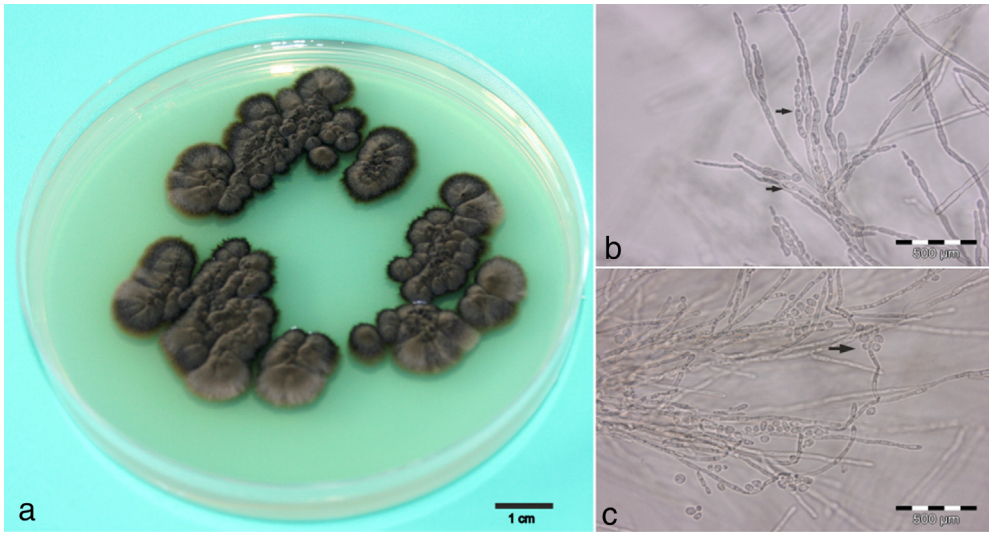


Fig. 1. *E. dermatitidis* CBS 525.76: colony morphology on 2% MEA (a); micrographies illustrating hyphae and conidia (b–c). Spherical and darkly pigmented nuclei are visible in (b), phialides, terminal cells from which the conidia are produced and conidia are shown in (c).

pH 8.5 by vigorous shaking for several hours at room temperature. Protein concentration was determined using the Bradford assay (BioRad). Equal amounts of the protein extracts were separated on a 5–10% SDS-PAGE gel and stained with silver nitrate in order to check for protein degradation.

2.4. Sample labeling and protein separation

Prior to 2D-DIGE analysis, protein extracts were labeled using Refraction-2D™ minimal dyes (NH DyeAGNOSTICS). An experimental plan was designed, according to which all biological replicates of control and temperature stressed samples were labeled with either G-Dye 200 or G-dye 300 (Table 1). A dye swap was also applied to minimize the effect of preferential labeling. To ensure normalization of spot intensities for protein abundance differences analysis, an internal standard (IS) was generated by pooling aliquots of equal amounts of all 20 samples and incorporated within each gel after labeling it with G-Dye 100. G-Dye 100, G-Dye 200 and G-Dye 300 labeled samples were combined to be separated on each gel. As a result 20 protein extracts and 10 internal standard were applied to 10 gels.

Table 1
DIGE experimental plan. All biological replicates of control and temperature treated samples were labeled with G-Dyes. Two samples and an internal standard (IS) were then combined to be separated on each gel.

DIGE experimental plan			
Gel No.	Fluorescent dyes		
	G-Dye^{100}	G-Dye^{200}	G-Dye^{300}
1	IS	A1	B1
2	IS	A2	D3
3	IS	E1	A3
4	IS	C1	A4
5	IS	D4	B2
6	IS	B3	E4
7	IS	B4	D1
8	IS	C2	E3
9	IS	D2	C3
10	IS	E2	C4

IS = internal standard.
A = control (37 °C).
B = exposure to 1 °C for 1 week.
C = exposure to 45 °C for 1 week.
D = exposure to 1 °C for 1 h.
E = exposure to 45 °C for 1 h.

Aliquots of 50 µg protein were separately labeled with 400 pmol minimal dye according to company's recommendations. Samples and IS were subsequently pooled together and incubated with 2× Lysis Buffer [7 M urea, 2 M thiourea, 2% CHAPS, 0.1% bromophenol blue, 130 mM DTT, 1% Servalyt™ 2–11 (Serva)] in a 1:1 ratio (protein extract/2× Lysis Buffer, v/v) on ice in the dark. Rehydration buffer containing 8 M urea, 2% CHAPS, 0.1% bromophenol blue, 13 mM DTT and 0.5% Servalyt™ 2–11 (Serva) was subsequently added to a final volume of 450 µl for loading on a non-linear IPG dry strip (24 cm, pH 3–10, GE Healthcare). The strips were rehydrated at room temperature for 16 h and consequently focused at 20 °C for 60.0 kVh, using a Protean IEF cell system (BioRad) and limiting the current to 50 µA per strip (Step 1: 500 V, 1 h; step 2: 1000 V, 1 h (gradient); step 3: 8000 V, 3 h (gradient); step 4: 8000 V, 45.0 kVh). Strip equilibration was carried out for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris HCl pH 8.4) with 2% DTT and subsequently for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris HCl pH 6.8) containing 0.1% bromophenol blue and 2.5% IAA. The volume of buffer used for each strip was 6 ml. In the second dimension proteins were resolved on 12% SDS-PAGE gel (20 × 26 × 0.15 cm) using the Ettan DALT six system (GE Healthcare) at 1 W/gel for 1 h, then at 15 W/gel, until the bromophenol blue line reached the bottom of the gels. Lower and upper running buffer were prepared by diluting 1:10 and 1:5 the 10× Running buffer (240 mM Tris, 1.92 M Glycine, 1% SDS), respectively. The chamber was cooled at 4 °C (Multi temp III, GE Healthcare).

Preparative gels — obtained in the same way as the analytical gels but using a higher protein amount (400 µg) — were stained with a mass spectrometry (MS) compatible silver staining as previously described [29]. Briefly, gels were fixed in 30% ethanol and 10% acetic acid for 1 h and then sensitized in a solution containing 0.5 M sodium acetate, 8 mM sodium thiosulfate pentahydrate and 30% of ethanol, at 4 °C overnight. Subsequently three washing steps with MilliQ water were performed, then the gels were incubated in a 0.2% silver nitrate solution for 30 min at 4 °C and further rinsed after the incubation. A 2.5% sodium carbonate and 0.01% formalin solution was applied for gel development until the desired intensity of staining was achieved. The staining process was stopped by incubating the gels in a 1% glycine solution, followed by a final washing step with MilliQ water.

2.5. Gel imaging and protein abundance analysis

The analytical gels were scanned in a Typhoon™ FLA 9500 (GE Healthcare) at a resolution of 100 µm using the 473 nm laser for

G-Dye 100, the 532 nm for G-Dye 200 and the 635 nm for G-Dye 300 and the BPB1 530 DF 20, BPG1 570 DF 20 and LPR (CH.2) R665 filters, respectively. Images were acquired using ImageQuant v8.1 (GE Healthcare) and analyzed by DeCyder™ 2D v7.0 (GE Healthcare) for spot count, gel matching and detection of changes in protein abundance. Protein spots were automatically detected and matched intra-gel using the batch processor module. The estimated number of spots was set to 10,000, spot slope and volume were set to 1.12 and 50,000 respectively. During the following stage of gel processing, spots intensities were normalized to the internal standard and inter-gel spot matching and quantification were performed using the BVA module (biological variation analysis). Matches were evaluated and improved by visual inspection. Experimental sets were then created in the EDA (extended data analysis) to compare all experimental conditions with each other and with the control. The percentage of spot maps where proteins had to be present was set to >75%. The results of differential abundances of spots were validated through statistical analysis. An independent Student's *t*-test and the one-way ANOVA were performed and the data were corrected applying the FDR (false discovery rate). Principal component analysis (PCA) and pattern analysis — i.e. hierarchical clustering (HC) — were further performed to search for outliers and for groups of co-varying spots. Protein spots with an average ratio increasing or decreasing more than 1.5-fold and with a p-value ≤ 0.05 were considered as significantly modulated and assigned to a pick list.

After formaldehyde free silver staining, preparative gels were compared with the analytical gels in order to match and localize the spots of interest. Protein spots were excised manually, using a 1.5 mm diameter one-touch spot picker (Gel Company) and subjected to digestion prior to MS analysis.

2.6. In-gel digestion

In gel tryptic digestion was carried out according to Shevchenko [30], with a few modifications. The spots of interest were de-stained with 100 mM sodium thiosulfate/30 mM potassium hexacyanoferrate (III) (1:1, v/v) at room temperature and subsequently rinsed with MilliQ water. The gel particles were then treated with acetonitrile and rehydrated by adding an equal volume of 100 mM ammonium bicarbonate buffer pH 8.5. After replacing the buffer with acetonitrile, the particles were incubated in a 100 mM ammonium bicarbonate pH 8.5/acetonitrile (1:1, v/v) solution and ultimately dried in a vacuum centrifuge (Unicryo MC2L-60, UniEquip). Gel pieces were incubated at 56 °C in a solution containing 10 mM DTT and 100 mM ammonium bicarbonate for protein reduction and thereafter in an alkylation solution (54 mM IAA, 100 mM ammonium bicarbonate) at room temperature and in the dark. A further incubation step with 100 mM ammonium bicarbonate and then with an equal amount of acetonitrile were performed prior to drying the spots in a vacuum centrifuge. Subsequently, a solution containing 95% 50 mM ammonium bicarbonate, 5% acetonitrile and trypsin (proteomics grade, Roche Diagnostics) at a final concentration of 10 ng/μl, were added. Digestion was carried out over-night at 37 °C, 350 rpm. Peptides were extracted with 50 mM ammonium bicarbonate, then acetonitrile and finally twice with 1% formic acid/acetonitrile solution (1:1, v/v). All extracts from one spot were combined and subsequently evaporated to complete dryness. Peptides were re-dissolved in 10 μl 0.1% formic acid solution and subjected to C18 Zip-Tip® purification (Merk Millipore). After elution with 0.1% formic acid/acetonitrile (1:1, v/v), the peptides were evaporated in a vacuum centrifuge and thereafter re-suspended in 10 μl 5% acetonitrile/0.05% formic acid solution.

2.7. nLC-ESI-MS/MS analysis

The tryptic peptides were subjected to nLC-ESI-MS/MS analysis performed on a nano-LC system (Ultimate 3000, Dionex) coupled with an HCT^{plus} (high capacity ion trap) mass spectrometer (Bruker Daltonics). The peptides were separated on an Acclaim PepMap 100 C18 reverse phase column (75 μm i.d. × 15 cm, 3 μm, 100 Å, Thermo Scientific). The mobile phase consisted of acetonitrile and MilliQ water, both containing 0.05% formic acid. A 90 min chromatography run was performed on each sample (0–10 min 5% acetonitrile; 10–60 min linear gradient from 5% to 50% acetonitrile; 60–61 min increase to 70% acetonitrile; 61–71 min 70% acetonitrile; 71–72 min decrease to 5% acetonitrile, 72–90 min column re-equilibration with 5% acetonitrile). Flow rate was set to 250 nl/min.

Mass Spectra were acquired in the positive-ion mode by using the enhanced scanning mode (m/z 300–1500) with a capillary temperature of 200 °C and dry gas equal to 0.5 l/min. The maximum accumulation time was 200 ms and the smart target, the averages and the rolling averaging were set to 100,000, 3 and 2, respectively. The parameter for fragmentation was set to one precursor ion using active exclusion (excluded after two spectra and released after 0.25 min). Time frame of repeated precursor ions was 25 s and the relative threshold was set to 5%.

2.8. Protein identification and bioinformatic analysis

The compound list, containing peak lists of all acquired MS/MS spectra were extracted using Data Analysis v3.2 (Bruker Daltonics) and submitted to the latest publically available version of MASCOT v2.5.1. (Matrix Science, www.matrixscience.com), through Biotools v3.2 (Bruker), for searches against the NCBI database (NCBInr 20140906; 48573147 sequences; 17378694133 residues), where the annotated genome from *E. dermatitidis* is available (accession number: AFPA01000000, The Broad Institute Genome Sequencing Platform). The following parameters were set prior to database search: one missed cleavage site by trypsin (porcine, Roche), MS tolerance of 0.5 Da, MS/MS tolerance of 0.3 Da, peptide charge of 1 +, 2 +, 3, carbamidomethylation of cysteine and oxidation of methionine as fixed and variable modification. Searches were performed using a taxonomy parameter set to Fungi (2952989 sequences).

The search results were evaluated on the base of the MASCOT score cut-off calculated for a significance threshold set to 0.05, number of matching peptides, sequence coverage, p*I* and molecular weight (Mw). Particular attention was dedicated to those proteins whose identification was based on one or two peptide sequences, by verifying the MS/MS spectra manually. BLASTP algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied for protein sequences identified as hypothetical or with uncharacterized function in order to search for homologues, selecting the non-redundant (nr) protein sequence database (scoring parameters: matrix BLOSUM62; Gap Costs Existence: 11 Extension:1; Compositional adjustments: conditional compositional score matrix adjustment). Knowledge about the single protein's function was gained by inputting each protein sequence into the UniProtKB database (http://www.uniprot.org/blast/) and detecting the respective Gene Ontology (GO) terms and annotations [31,32]. When terms were not assigned to a protein, the most closely related protein sequence from a different organism, whose GO terms were available, was used. In the case GO terms were not accessible even for homologous proteins, the protein sequence was submitted as query to InterProScan 5 (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) to scan for matches against the InterPro collection of protein signature databases using applications as PANTHER v9.0 (http://www.pantherdb.org) or SUPERFAMILY v1.75 (http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html). In the latter case, only terms with FDR <0.001 were selected.

GOstats [33] and KOBAS v2.0 (http://kobas.cbi.pku.edu.cn) — routinely used in transcriptomics with larger data volumes — were used to search for overrepresented cellular processes GO terms in the group of increased and decreased proteins, in order to elucidate their putative biological functions. GO terms with an uncorrected p-value < 0.05 were considered significantly enriched. The resulting lists of GO terms, i.e. one list per conditions' comparison, were summarized

with REVIGO (http://revigo.irb.hr) [34] by clustering semantically close GO terms and presented as pie chart with custom R scripts. The threshold for the categorization was set to 0.5.

KOBAS was additionally used to look for enriched pathways in the KEGG, BioCyc and Reactome [35–37] databases based on the set of up- and down-regulated genes. The genes were annotated with putative pathways by comparing them with genes with known annotation from *Saccaromyces cerevisiae* S288c. A pathway was considered significantly enriched when its uncorrected p-value was smaller than 0.05 [38]. If available, graphical representations of the overrepresented pathways were downloaded and annotated from the corresponding website.

Information about the sub-cellular localization of the differentially abundant proteins was gained by using the YLoc prediction system based on the YLoc + model for fungal proteins [39] (www.multiloc.org/YLoc).

3. Results

3.1. Growth temperatures and stress exposure

Tests for thermal preferences indicated the range from 25 to 37 °C as growth optimal temperatures for *E. dermatitidis* CBS 525.76 (Table 2). Accordingly, since thermo-tolerance has a crucial role in the fungus persistence in man-dominated habitat and in pathogenicity, 37 °C was chosen as temperature of growth prior to stress exposure. Fungal growth was still detected after temperature treatment at 1 °C — however at a slow rate — and at 45 °C for up to one week, thus ensuring the strain's viability at the chosen experimental conditions. The incubation temperatures and the respective time spans were selected to simulate different environmental conditions and to evaluate the effects of short- and long-term exposure on the proteome level.

3.2. Morphology of E. dermatitidis under different temperature conditions

TEM images of osmium tetroxide-fixed specimens of *E. dermatitidis* subjected to temperature treatment as well as the control sample are shown (Fig. 2).

A cross section of the control reveals cells with a big vacuole filled with abundant floccular material of high electron-density. Remarkably, the cell wall appears multilayered. Three layers can be distinguished: an inner and scarcely pigmented layer, a middle electron-opaque layer and an outer electron-dense layer, the latter most likely encrusted with melanin and surrounded by a fibrillar mucilage (Fig. 2a).

When comparing temperature stressed samples with the control, no dramatic alteration of the cell morphology can be observed. As for the organelles, big vacuoles occupying most of the cytoplasm can still be detected, as well as mitochondria located around nucleus (Fig. 2 b–f).

However, morphological changes at the cell surface level are observed at 1 °C, 1 week experimental condition, when the outermost cell wall layer appears to be highly melanized and floccular-like, being this last aspect particularly pronounced (Fig. 2b–c). Such floccular layer cannot be seen at 45 °C 1 week experimental condition, albeit melanization persists in the outer cell wall layer (Fig. 2d). The amount of floccular material appears thereby to be related to the strain prolonged exposure to low temperature.

As for the samples subjected to short term temperature treatment — i.e. exposure to 1 °C or 45 °C for an hour — only a slight increase in the pigmentation of the cell wall outer layer can be detected, by comparison with the control (Fig. 2e–f).

3.3. 2D-DIGE analysis and identification of differentially abundant proteins

2D-DIGE and nLC-ESI-MS/MS were performed to detect protein abundance changes caused by temperature stress. 2D-DIGE analysis of *E. dermatitidis* total protein extract was performed using four biological replicates for each of the four temperature treatments and for the control condition. The results of gel imaging on a total of 30 spot maps showed an average of 1700 protein spots being detected on each map.

Samples from all experimental conditions were compared with each other by creating experimental sets and protein spots exhibiting significant modulation ($p \leq 0.05$, Av.Ratio > 1.5 fold) were selected for identification via mass spectrometry. The majority of differentially abundant spots — i.e. 50 — could be detected when comparing 45 °C 1 week and 1 °C 1 week experimental conditions. The comparison of control and 1 °C 1 week condition yielded 47 significantly different protein spots. Samples exposed to 1 °C for 1 h also differed from samples subjected to 45 °C for 1 week but to a lesser extent, since only 15 protein spots changed in abundance. Among the 50 protein spots, 33 were detected as more abundant and 17 as less abundant in colonies exposed to 1 °C for 1 week. Of the 47 differentially abundant protein spots, 26 were increased and 21 decreased at 1 °C, 1 week condition; of the 15 spots, 13 showed increased abundance while 2 were less abundant at 1 °C, 1 week. Since some of the protein spots exhibited a change in abundance in more than one of the experimental sets, the total number of spots detected as significantly modulated was 75 (Fig. 3). From these 75 spots, 54 could be successfully analyzed and a total of 32 different proteins identified (Table 3). Table 3 also shows the alterations in spot abundance (as average ratios) for the three experimental sets where protein significant modulation was detected.

Out of the 54 identified protein, 5 spots most likely represent fragments of full-length proteins (e.g. spot N° 1507, 1582 1787, 943 and 947, Table 3) since only C- or N-terminal peptides were identified. Furthermore, a few spots with different p*I* but identical Mw are found to match the same particular protein (e.g. spot n° 1125, and 1896), this representing a quite common phenomenon in 2D electrophoresis, possibly involving posttranslational modifications. In some cases (e.g. spot N° 1582, 1799) from a single spot, peptides matching two or more different proteins were detected, thus not allowing unambiguous identifications.

The lack of sequence similarity in the database also represented an obstacle to protein successful identification. To elucidate the identity of the 5 observed hypothetical proteins, a search for homology was performed. In all cases the highest rate of sequence similarity is found with proteins belonging to the fungal species *Capronia coronata* and *C. epimyces,* both closely related to *E. dermatitidis* and forming *Exophiala*-like anamorphs in cultures. However, since the identity of the proteins from *Capronia* is also not known, next closely related proteins — whose identity is instead assigned — have been considered (Table 4). The major part of the matching proteins are stress response proteins, however in some cases — i.e. spots 1290 and 1197 — the BLAST results did not allow to unambiguously establish homology

Table 2
Thermal preferences of *E. dermatitidis* CBS 525.76.

Strain	Thermal preferences (°C)									
	1	5	10	15	20	25	28	30	35	37
E. dermatitidis CBS 525.76	0.17	0.33	2.33	5.50	13.33	14.33	16.50	16.33	13.83	16.67

Thermal preferences are reported as difference in size (mm) between inocula and colonies three weeks after the beginning of the experiment. The values represent the average of three different tests. All tests were performed on 2% MEA. CBS No.: strain number in the CBS-KNAV (The Netherlands) culture collection.

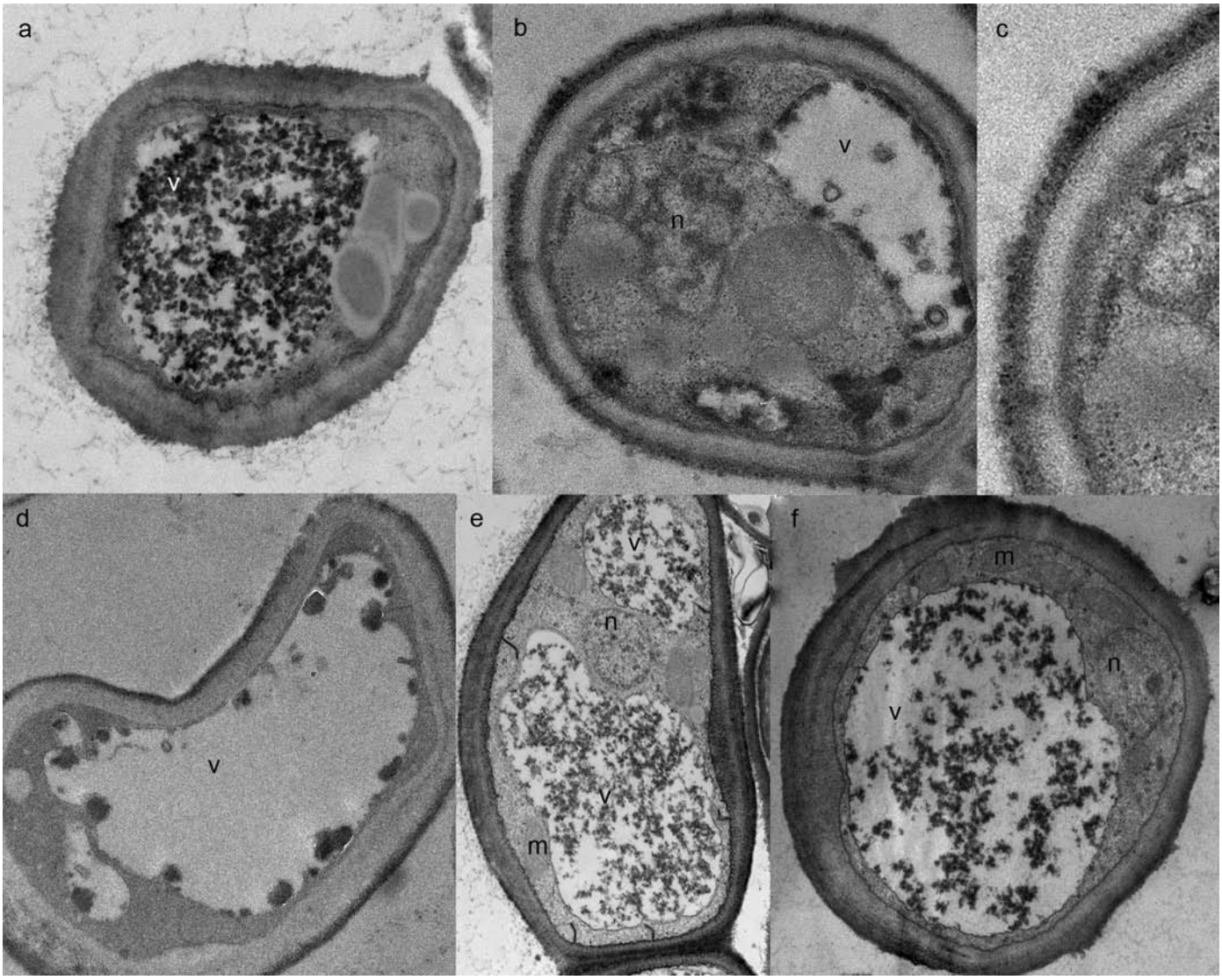


Fig. 2. Morphological analysis of *E. dermatitidis* CBS 525.76 under temperature treatments. a. Control condition (37 °C); b–d. 1 week exposure at 1 °C (b) or at 45 °C (d). c. Enlargement of the inset in b. e–f. 1 h exposure at 1 °C (e) or at 45 °C (f). Cells show a prominent vacuole (v) filled with floccular electron-dense material. The nucleus (n) and some mitochondria (m) are also visible. The cell wall consists of three layers, the outermost of which is pigmented due to melanization and exhibits a floccular-like consistency in relation to the prolonged exposure to low temperature (c). Bars = 0.5 μm.

between identified sequences and characterized proteins included in the NCBInr database.

Additionally, PCA analysis was performed on all matched spots across the gels to test the reproducibility of the four biological replicates and to verify the clustering of protein profiles according to presence or absence of similarity. As shown in Fig. 4a, all four 1 °C 1 week protein profiles cluster together, relative to the PC1 calculation. The same clustering pattern was observed for the control protein profiles. The PCA results demonstrated that, in all experimental sets, protein profiles of the four biological replicates from the same experimental condition group together while profiles from different experimental conditions are instead distinct. Component 1 explains 83% (3a), 90.6% (3b) and 93.4% (3c) of the variance, while component 2 explains 6.5%, 2.9% and 2.6%, respectively.

HC analysis — based on similarities of abundance patterns in the spot maps reveals clusters of co-varying spots graphically represented as dendrograms, for a standardized logarithmic abundance scale ranging from −1.0 to +1.0. As shown in Fig. 5, proteins with increased and with decreased abundance levels are visualized as large spots clusters in each of the experimental sets. Moreover, changes in the protein abundance are consistent within the four biological replicates of each experimental condition. In agreement with the PCA results, this demonstrates that the biological samples share a very similar spot map. Since the statistical analysis was performed only on proteins present in at least 75% of the spot maps, in a few cases data for a specific protein spot were not available. The absence of a protein spot in a spot map is shown as a gray box in the dendrogram.

3.4. Functional analysis of the identified proteins

In order to clarify the putative biological function of the identified proteins and their involvement in particular cellular pathways, protein functional analysis was carried out on the base of cellular process GO terms. This approach is generally used in transcriptomics, however, it could indicate the importance of the identified proteins for the cellular processes as well.

In the cases where the MS analyses of a single protein spot resulted in multiple protein identification, only the proteins characterized by the highest MASCOT score were considered for the analysis. If no term was available for a single protein, GO terms assigned to the most closely related protein sequence from a different organism, were used. No GO term could be assigned to roughly 5% of the identified proteins, namely

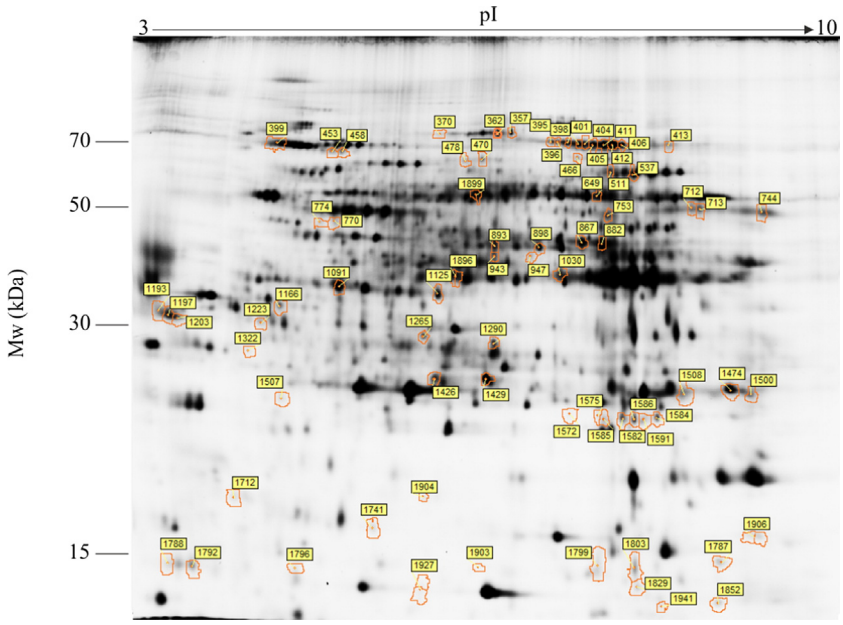


Fig. 3. 2D electrophoresis gel of total protein extracts of *E. dermatitidis* colonies under temperature treatment. All protein spots detected as differentially abundant in the three experimental sets are numbered on the gel.

to the hypothetical proteins gi|378733644 and gi|378726833, whose putative identity could be established through homology search.

Pie charts showing the over-represented GO terms for the proteins were obtained for each condition comparison using REVIGO. In each pie chart, semantically close terms are clustered into categories of cellular processes, being each category represented by a different color (Fig. 6, Table 5). Along with helping to elucidate the function of the differentially abundant proteins, this analysis provides information — as graphical representation — about the cellular processes, which are mostly affected by temperature treatment. The complete list of over-represented cellular process GO terms (uncorrected p-value <0.05) per condition comparison is shown in Table 1 in [40].

Knowledge about the proteins functional characterization was integrated with data about biological pathways. The identification of differentially abundant proteins indeed enabled us to discover biological pathways, which might accordingly be modulated in response to temperature exposure. KOBAS v2.0 was used to look for over-represented categories of pathways on the base of the sets of genes encoding for the increased and decreased proteins. A complete list of significantly enriched GO pathways (uncorrected p-value < 0.05) and links to the respective annotated graphical representations are shown in Table 2 in [40]. In each pathway the significantly regulated proteins are highlighted in blue and red according to decrease and increase in abundance, respectively. Green is used for the rest of the genes characterizing the pathway.

Both analyses were performed on the base of sets of identified proteins with changed abundance. Moreover, for each comparison of two experimental conditions, the two sets of proteins — with increased and with reduced abundance — were processed separately [41]. In a number of cases assessing the up- or down-regulation of a single protein was critical, especially when the protein actually resembled a fragment of the whole sequence based on the identification of only N- or C-terminal peptides. Despite that, the sets of data from each condition were directly submitted to proteins' functional characterization and pathway analysis, without prior rearranging them.

In a few cases the same cellular process and/or pathway are detected as both up- and down-regulated within the same experimental set. This concerns all fundamental cellular processes and pathways involving Hsp70-like proteins, acetyl-CoA synthetases, aldehyde dehydrogenases and glyceraldehyde 3-P dehydrogenase (indicated by their correspondent genes HMPREF1120_02626, HMPREF1120_06094, HMPREF1120_01109 and HMPREF1120_01270, respectively in Tables 1 and 2 in [40]).

In the case of Hsp70-like proteins (gene HMPREF1120_02626) three protein spots, probably corresponding to as many protein species and exhibiting different p*Is*, are found. Two of the spots show increased abundance at low temperature while the third one is decreased. Differences due to alternative splicing can be speculated, however it cannot be excluded the possibility that the three proteins are encoded by different genes, some of which have not been yet annotated. Post-translational modifications, phosphorylation or methylation, can be further hypothesized.

In the case of the acetyl-CoA synthetases, the only protein spot detected (i.e. 1507) presents increased abundance at low temperature. Aldehyde dehydrogenase is instead detected in 5 protein spots from different condition comparisons. While 2 spots resulted to have increased abundance (i.e. 943 and 947), three spots were detected as less abundant (i.e. 466, 649 and 1899). Nevertheless, all spots with increased abundance from both proteins show a lower Mw than expected. Since the masses do not match - although the protein identity match is high confidence — in this case a cleavage event can be hypothesized, which may be of evidence of protein degradation or truncation. Indeed, for spot 1507 only C-terminal peptides were identified in the mass spectra. Peptides spanning only the central part of the sequence were instead found in the mass spectra of protein spots 943 and 947, thus also indicating protein fragmentation. Since — as expected in such cases — a decrease in abundance concerning the proteins whose experimental Mw matches the expected Mw value was also observed, the decrease of the full-variant and the increase of the fragmented counterpart suggest the down-regulation of these proteins. Similarly, the glyceraldehyde 3-P dehydrogenase resembles a degradation product although an increase in its abundance is observed at low temperature. Hence, the respective proteins were considered as down-regulated.

In addition to protein functional characterization, the sub-cellular localization of all identified temperature responsive proteins was predicted. By using YLoc + interpretable server, the prediction was carried out on the base of each protein biological properties and performed into 10 different locations — also searching for multiple locations — taking

Table 3
Identified proteins with changed abundance following low temperature treatment for short and long term.

Spot No.[a]	NCBI accession number	ORF	Score [b]	Matched peptides [c]	% sequence coverage [d]	pI/Mw (kDa)[e]	Fold change [f]	Protein name
Experimental set: control v/s 1 °C, 1 week								
770	gi|378725903	HMPREF1120_00576	912	14	40	5.33/47.3	1.79	Enolase
774	gi|378725903	HMPREF1120_00576	183	3	10	5.33/47.3	1.61	Enolase
1166	gi|378725637	HMPREF1120_00315	266	7	27	4.96/30.7	3.1	14-3-3 family protein
1322	gi|378732712	HMPREF1120_07169	51	2	11	4.91/27.1	1.52	Proteosom component PUP2
1507[g]	gi|378731617	HMPREF1120_06094	380	7	15	5.84/74.1	2.17	Acetyl-coenzyme A synthetase
1572	gi|378734433	HMPREF1120_08836	54	1	9	9.06/25.7	1.97	Fe–Mn family superoxide dismutase
1582[g]	gi|378726610	HMPREF1120_01270	224	5	21	6.76/36.4	2.11	Glyceraldehyde-3-phosphate dehydrogenase
1585	gi|378734433	HMPREF1120_08836	207	4	23	9.06/25.7	1.65	Fe–Mn family superoxide dismutase
1586	gi|378734433	HMPREF1120_08836	184	3	23	9.06/25.7	1.58	Fe–Mn family superoxide dismutase
1591	gi|378730713	HMPREF1120_05220	186	6	31	9.56/29.8	1.66	Hypothetical protein HMPREF1120_05220
1787[g]	gi|19073387	HSP30	95	2	11	5.74/20.9	1.93	Hsp30
1799	gi|378729265	HMPREF1120_03849	197	4	34	6.75/14.7	1.98	Ubiquinol-cytochrome c reductase subunit 7
1803	gi|378728843	HMPREF1120_03444	96	3	27	7.74/17.1	2.09	Nucleoside diphosphate kinase
1829	gi|378731783	HMPREF1120_06254	316	6	81	7.66/14.0	1.57	Mitochondrial protein-transporting ATPase
1927	gi|378728179	HMPREF1120_02805	72	1	5	5.69/21.9	2.49	Minor allergen Alt a 7
357	gi|378731617	HMPREF1120_06094	716	15	23	5.84/74.1	−1.62	Acetyl-coenzyme A synthetase
362	gi|378731617	HMPREF1120_06094	814	16	27	5.84/74.1	−1.51	Acetyl-coenzyme A synthetase
398	gi|378732911	HMPREF1120_07361	645	15	22	6.14/75.0	−1.72	Transketolase
399	gi|378727999	HMPREF1120_02626	1092	22	34	5.1/70.6	−1.58	Hsp70-like protein
401	gi|378729152	HMPREF1120_03741	219	5	9	6.57/74.7	−1.63	Alcohol oxidase
404	gi|378729152	HMPREF1120_03741	193	4	7	6.57/74.7	−1.76	Alcohol oxidase
405	gi|378729152	HMPREF1120_03741	808	18	39	6.57/74.7	−1.99	Alcohol oxidase
406	gi|378729152	HMPREF1120_03741	422	11	23	6.57/74.7	−2.27	Alcohol oxidase
411	gi|378729152	HMPREF1120_03741	628	12	23	6.57/74.7	−2.13	Alcohol oxidase
412	gi|378729152	HMPREF1120_03741	954	22	48	6.57/74.7	−2.31	Alcohol oxidase
	gi|378726445	HMPREF1120_01109	495	9	25	6.29/54.1		Aldehyde dehydrogenase
470	gi|378731564	HMPREF1120_06041	501	12	27	5.77/62.0	−1.82	Phosphoenol pyruvate carboxykinase [ATP]
511	gi|378730878	HMPREF1120_05378	424	10	24	7.23/61.6	−1.87	Malate synthase, glyoxysomal
537	gi|378730878	HMPREF1120_05378	881	10	38	7.23/61.6	−1.68	Malate synthase, glyoxysomal
649	gi|378726445	HMPREF1120_01109	993	22	46	6.29/54.1	−1.60	Aldehyde dehydrogenase
713	gi|378728030	HMPREF1120_02657	208	5	11	9.2/50.2	−1.62	Elongation factor 1-alpha
744	gi|378728030	HMPREF1120_02657	316	7	16	9.2/50.2	−1.51	Elongation factor 1-alpha
867	gi|378730528	HMPREF1120_05043	663	14	43	6.29/40.6	−1.57	Formate dehydrogenase
882	gi|378730528	HMPREF1120_05043	683	14	47	6.29/40.6	−1.59	Formate dehydrogenase
898	gi|378730392	HMPREF1120_04915	501	11	41	6.23/45.6	−1.63	Beta-lactamase
	gi|378730838	HMPREF1120_05340	408	8	35	6.15/35.7		Malate dehydrogenase, NAD-dependent
1290	gi|378729668	HMPREF1120_04224	325	8	29	5.95/27.3	−1.76	Hypothetical protein HMPREF1120_04224
Experimental set: 45 °C 1 week v/s 1 °C, 1 week								
453	gi|378727999	HMPREF1120_02626	436	8	21	5.1/70.6	1.70	Hsp70-like protein
458	gi|378727999	HMPREF1120_02626	669	13	29	5.1/70.6	1.73	Hsp70-like protein
753	gi|378731870	HMPREF1120_06341	402	7	24	6.51/48.0	1.81	Hypoth. protein HMPREF1120_06341
770	gi|378725903	HMPREF1120_00576	912	14	40	5.33/47.3	1.54	Enolase
943[g]	gi|378726445	HMPREF1120_01109	484	9	23	6.29/54.1	1.51	Aldehyde dehydrogenase
947[g]	gi|378726445	HMPREF1120_01109	470	9	23	6.29/54.1	1.93	Aldehyde dehydrogenase
1166	gi|378725637	HMPREF1120_00315	266	7	27	4.96/30.7	3.05	14-3-3 family protein
1197	gi|378733644	HMPREF1120_08075	420	7	36	4.59/26.8	1.51	Hypoth. protein HMPREF1120_08075
1203	gi|378726833	HMPREF1120_01486	271	6	55	4.86/22.3	1.86	Hypoth. protein HMPREF1120_01486
1265	gi|378729910	HMPREF1120_04451	501	12	50	5.69/32.7	1.93	Haloalkanoic acid dehalogenase
1322	gi|378732712	HMPREF1120_07169	51	2	11	4.91/27.1	1.6	Proteosom component PUP2
1426	gi|378727822	HMPREF1120_02451	252	6	30	7.68/27.4	1.54	Oxidoreductase, short-chain dehydrogenase/reductase
1429	gi|378734310	HMPREF1120_08713	532	12	41	6.96/29.2	1.92	Tetrahydroxynaphthalene reductase
1474	gi|378730051	HMPREF1120_04591	345	10	32	8.59/29.9	1.65	Glutathione S-transferase
1500	gi|378726433	HMPREF1120_01097	330	7	40	8.76/26.5	1.52	7-Alpha-hydroxysteroid dehydrogenase
1507[g]	gi|378731617	HMPREF1120_06094	380	7	15	5.84/74.1	3.55	Acetyl-coenzyme A synthetase
1508	gi|378727822	HMPREF1120_02451	157	2	10	7.68/27.4	1.69	Oxidoreductase, short-chain dehydrogenase/reductase
1582[g]	gi|378726610	HMPREF1120_01270	224	5	21	6.76/36.4	3.88	Glyceraldehyde-3-phosphate dehydrogenase
1586	gi|378734433	HMPREF1120_08836	184	3	23	9.06/25.7	1.99	Fe–Mn family superoxide dismutase
1591	gi|378730713	HMPREF1120_05220	186	6	31	9.56/29.8	2.67	Hypothetical protein HMPREF1120_05220
1787[g]	gi|19073387	HSP30	95	2	11	5.74/20.9	3.16	Hsp-30
1799	gi|378729265	HMPREF1120_03849	197	4	34	6.75/14.7	2.43	Ubiquinol-cytochrome c reductase sub. 7
1803	gi|378728843	HMPREF1120_03444	96	3	27	7.74/17.1	3.82	Nucleoside diphosphate kinase
1927	gi|378728179	HMPREF1120_02805	72	1	5	5.69/21.9	4.05	Minor allergen Alt a 7
398	gi|378732911	HMPREF1120_07361	645	15	22	6.14/75.0	−1.59	Transketolase
399	gi|378727999	HMPREF1120_02626	1092	22	34	5.1/70.6	−1.58	Hsp70-like protein
401	gi|378729152	HMPREF1120_03741	219	5	9	6.57/74.7	−1.57	Alcohol oxidase
404	gi|378729152	HMPREF1120_03741	193	4	7	6.57/74.7	−1.73	Alcohol oxidase
405	gi|378729152	HMPREF1120_03741	808	18	39	6.57/74.7	−1.93	Alcohol oxidase
406	gi|378729152	HMPREF1120_03741	422	11	23	6.57/74.7	−2.22	Alcohol oxidase
411	gi|378729152	HMPREF1120_03741	628	12	23	6.57/74.7	−2.17	Alcohol oxidase
412	gi|378729152	HMPREF1120_03741	954	22	48	6.57/74.7	−2.35	Alcohol oxidase
470	gi|378731564	HMPREF1120_06041	501	12	27	5.77/62.0	−1.54	Phosphoenol pyruvate carboxykinase [ATP]
893	gi|378730392	HMPREF1120_04915	422	10	38	6.23/45.6	−2.04	Beta-lactamase
1030	gi|378730528	HMPREF1120_05043	299	8	26	6.29/40.6	−1.52	Formate dehydrogenase

Table 3 (*continued*)

Spot No.[a]	NCBI accession number	ORF	Score [b]	Matched peptides [c]	% sequence coverage [d]	pI/Mw (kDa)[e]	Fold change [f]	Protein name
Experimental set: 45 °C 1 week v/s 1 °C, 1 week								
1091	gi|378734037	HMPREF1120_08453	926	20	63	5.7/35.9	−1.54	Transaldolase
1899	gi|378726445	HMPREF1120_01109	383	9	21	6.29/54.1	−1.55	Aldehyde dehydrogenase
Experimental set: 45 °C 1 week v/s 1 °C, 1 h								
453	gi|378727999	HMPREF1120_02626	436	8	21	5.1/70.6	1.75	Hsp70-like protein
458	gi|378727999	HMPREF1120_02626	669	13	29	5.1/70.6	1.75	Hsp70-like protein
753	gi|378731870	HMPREF1120_06341	402	7	24	6.51/48.0	1.65	Hypoth. protein HMPREF1120_06341
1197	gi|378733644	HMPREF1120_08075	420	7	36	4.59/26.8	1.56	Hypoth. protein HMPREF1120_08075
1265	gi|378729910	HMPREF1120_04451	501	12	50	5.69/32.7	1.87	Haloalkanoic acid dehalogenase
1474	gi|378730051	HMPREF1120_04591	345	10	32	8.59/29.9	1.59	Glutathione S-transferase
1507[g]	gi|378731617	HMPREF1120_06094	380	7	15	5.84/74.1	2.93	Acetyl-coenzyme A synthetase
1582[g]	gi|378726610	HMPREF1120_01270	224	5	21	6.76/36.4	3.09	Glyceraldehyde-3-phosphate dehydrogenase
1586	gi|378734433	HMPREF1120_08836	184	3	23	9.06/25.7	1.72	Fe–Mn family superoxide dismutase
1591	gi|378730713	HMPREF1120_05220	186	6	31	9.56/29.8	2.4	Hypothetical protein HMPREF1120_05220
1803	gi|378728843	HMPREF1120_03444	96	3	27	7.74/17.1	3.67	Nucleoside diphosphate kinase
1896	gi|378730838	HMPREF1120_05340	821	17	53	6.15/35.7	−1.52	Malate dehydrogenase

[a] Protein spot number assigned by DeCyder and shown in Fig. 3.
[b] MASCOT protein score.
[c] Number of different peptides identified for each protein (multiple detection of the same peptide was not considered for the calculation).
[d] Percentage of protein sequence covered by matching peptides.
[e] Theoretical values.
[f] Fold change values from DeCyder v7.0 (positive values indicate an increase in protein abundance following exposure to 1 °C; negative values indicate a decrease in protein abundance after exposure to 1 °C).
[g] Protein valuated as a fragment of a longer sequence.

into account GO terms transferred from close homologous proteins. Protein localizations for each condition comparison are shown in Table 6. The complete list of all identified proteins and respective sub-cellular localization is presented in Table 3 in [40].

The results of protein functional characterization, of protein pathways analysis and of sub-cellular localization are presented and commented as follows. Since 27% of the differentially abundant protein spots could not be identified, additional protein functions and pathways are expected to play a role in *E. dermatitidis* response to temperature treatment. The same applies to the identified proteins that have been excluded from the functional analyses in order to avoid ambiguities in the prediction of up- and down-regulated cellular processes.

3.5. Functional characterization of proteins

According to the present data (Table 5), there are 4 important cellular processes to be affected by low temperature in the condition comparison control v/s 1 °C 1 week: acetate metabolism ($p = 1.1e-04$) and cell aging ($p = 0.006$), which show decreased levels at 1 °C and pyruvate metabolism and regulation of ligase activity, showing instead higher levels. Within the latter two categories, the most highly enriched sub-categories of cellular processes are: pyruvate metabolic process ($p = 4e-04$) and negative regulation of apoptotic process ($p = 0.02$), respectively.

Pentose phosphate shunt ($p = 9.95e-05$) and cellular aldehyde metabolism ($p = 0.001$) show decreased levels at low temperature when analyzing the 45 °C 1 week and 1 °C 1 week condition comparison. By contrast, acetate metabolism and cell aging are the main categories to exhibit higher levels at 1 °C 1 week, being the most highly enriched sub-categories of cellular processes oxidation-reduction process ($p = 3.19e-06$) and catabolic process ($p = 0.02$), respectively.

The main overrepresented GO categories in the set of proteins with decreased abundance at the condition 1 °C 1 h, by comparison with 45 °C 1 week, are tricarboxylic acid metabolism — representing tricarboxylic acid cycle ($p = 0.004$) the most highly enriched sub-categories of cellular processes within this category — and cell aging ($p = 0.01$). Pyruvate metabolism ($p = 0.003$) and negative regulation of intracellular transport ($p = 0.01$) are instead the main categories to be significantly increased at 1 °C 1 h.

Some of the GO terms clustering in the categories of up-regulated biological processes such as pyruvate metabolism (i.e. the terms displayed as XV a, c, d, e, f, k, q, r, s, u and w, in Table 5), acetate metabolism (I c, e, j, p, u, w, x, y, γ, ε, and θ) and cell aging (VI a, b and c) are associated to truncated or degraded forms of glyceraldehyde 3-P dehydrogenases, acetyl-CoA synthetases and aldehyde dehydrogenases. Accordingly, the evaluation of these biological processes as of actually up-regulated processes is critical.

Table 4
Homologues of the hypothetical proteins. BLASTP algorithm was applied in order to search for characterized homologue proteins within the NCBInr protein database. The matching proteins, whose identity was known — having the highest max score were selected.

Spot No.[a]	NCBI accession number	ORF	Name of the homologous protein/species	NCBI accession number of homologue	Max score	Identity (%)	Positives (%)	Gap (%)	Expected value
1591–1582	gi|378730713	HMPREF1120_05220	Nitroreductase-like protein/Macrophomina phaseolina MS6	gi|407921871	263	63	75	0	1e−84
1290	gi|378729668	HMPREF1120_04224	30 kDa heat shock protein/Fusarium oxysporum f. sp. cubense race 1	gi|477507425	73.6	28	48	5	2e−12
753	gi|378731870	HMPREF1120_06341	Peptidoglycan binding domain containing protein/Aspergillus kawachii IFO 4308	gi|358369269	282	42	53	16	1e−86
1197	gi|378733644	HMPREF1120_08075	Putative stress response protein/Colletotrichum gloeosporioides Nara gc5	gi|596668023	57	33	43	19	4e−06
1203	gi|378726833	HMPREF1120_01486	ABC transporter, permease protein/Thermus aquaticus	gi|489133849	38.5	45	53	10	0.73

[a] Protein spot number assigned by DeCyder and shown in Fig. 3.

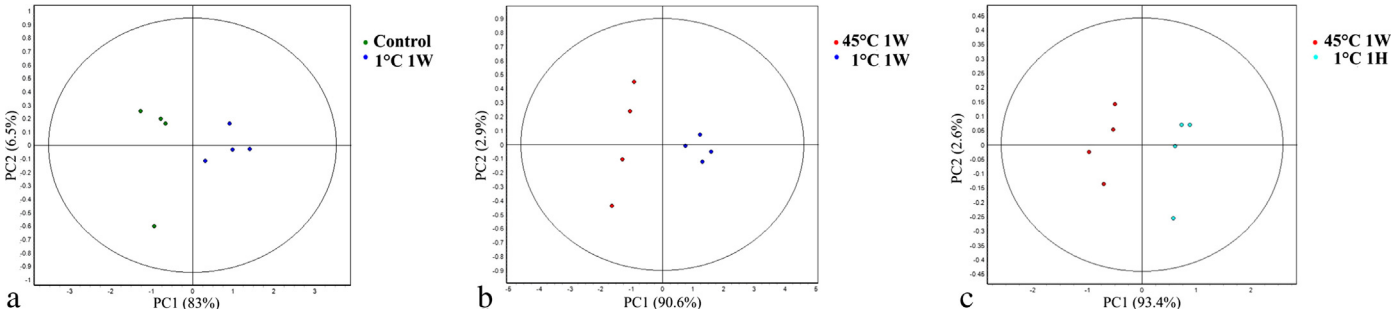


Fig. 4. PCA analysis showing the clustering of protein profiles in the three experimental sets. Each data point in the PCA represents a protein profile and the subset of associated proteins, whose ratios varied 1.5-fold or more ($p < 0.05$). Values of variance for component 1 and 2 are shown. DIGE protein profiles from the condition 1 °C 1 h are shown in light blue, protein profiles from 1 °C 1 week are shown in blue protein profiles from 45 °C 1 week are shown in red and protein profiles from the control are shown in green.

3.6. Identification of most affected pathways

The identification of temperature modulated proteins in *E. dermatitidis* allowed to discover biological pathways that are possibly also affected by the exposure to temperature treatment.

Based on the results of pathway prediction, pyruvate metabolism ($p = 8.55e-05$) shows the most significant down-regulation at low temperature, when comparing 1 °C 1 week experimental with the control sample. All proteins involved in the pyruvate metabolism and exhibiting reduced abundance are shown in Fig. 7. Direct links to similar graphical representations are available for the rest of the pathways (Table 2 in [40]). Four additional significantly down-regulated pathways can be observed: carbon metabolism ($p = 0.002$), glyoxylate and dicarboxylate metabolism ($p = 0.003$), ethanol degradation ($p = 0.004$) and cellular response to heat stress ($p = 0.005$). Among the most up-regulated pathways at 1 °C 1 week condition, glycolysis/gluconeogenesis ($p = 0.006$) and carbon metabolism ($p = 0.034$) are detected.

Carbon ($p = 0.013$) and pyruvate metabolism (0.038) are identified as down-regulated pathways at low temperature also when comparing 1 °C 1 week to 45 °C 1 week experimental conditions. However, the pentose phosphate ($p = 0.001$) is the most significantly decreased pathway. As for the up-regulated pathways instead, the beta-oxidation of very long chain fatty acids ($p = 0.0007$) are detected along with glycolysis/gluconeogenesis ($p = 0.004$), peroxisomal lipid metabolism ($p = 0.006$) and cellular response to stress ($p = 0.008$). In addition, the pathway cellular responses to stress also shows up-regulation ($p = 0.036$).

Aspartate degradation, TCA cycle and glyoxylate and dicarboxylate metabolism are detected as down-regulated at low temperature in *E. dermatitidis* when comparing 45 °C 1 week to 1 °C 1 h. However, since only one protein is regulated in each of the pathways, these data cannot be considered as significant. The up-regulated pathway at 1 °C 1 h are instead cellular response to stress ($p = 0.012$) and the glycolysis/gluconeogenesis ($p = 0.031$).

Since most of the proteins involved in glycolysis/gluconeogenesis — i.e. acetyl-CoA synthetases, glyceraldehyde-3-phosphate-dehydrogenases and aldehyde dehydrogenases — actually resemble truncated or degraded forms of the full-variant, it is likely that only enolases contribute to this pathway. Such issue applies to all condition comparisons where this pathway is detected.

3.7. Prediction of sub-cellular localization of the identified proteins

As shown in Table 6, most of the proteins decreased at 1 °C 1 week — as compared to the control sample — are predicted to be localized in the cytoplasm (52.2%), in the peroxisome (34.8%) and in the mitochondrion (8.7%). As for the localization of the increased proteins, cytoplasm (61.1%) and mitochondrion (22.2%) are the most represented cellular compartments. No protein is predicted to be located in the peroxisome, while a low percentage of proteins seem to be found at the nuclear and plasma membrane level. The remaining 5.5% should instead be represented by secreted proteins.

Similarly to what observed in the previously described condition comparison, most of the proteins decreased at 1 °C 1 week — as compared to 45 °C 1 week condition — are predicted to be cytoplasmic (40%) and peroxisomal (46.6%). About 60% of increased proteins are predicted to be located in the cytoplasm, followed by a 25.7% of mitochondria-associated proteins. The remaining 14.2% includes nuclear, secreted and membrane proteins.

All decreased proteins at 1 °C 1 h condition — as compared to 45 °C 1 week condition — are predicted to be cytoplasmic. Most of the increased proteins are predicted to be located in the cytoplasm, while the remaining 15.4% should be equally distributed between nucleus and mitochondrion.

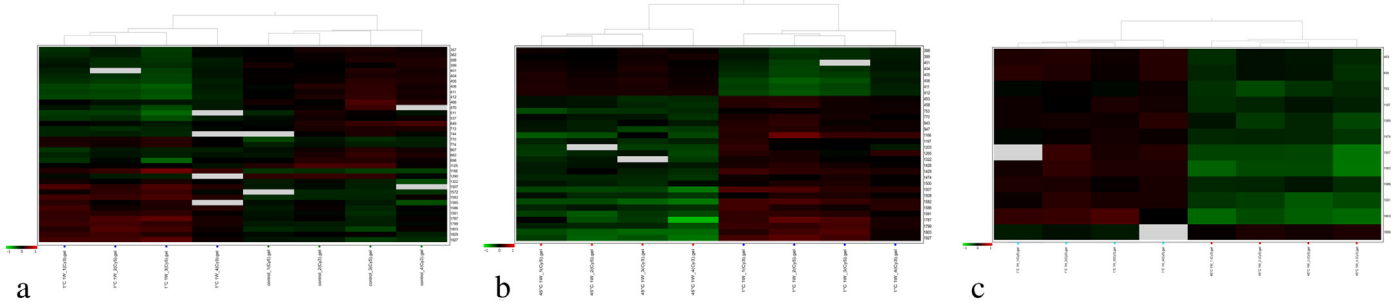
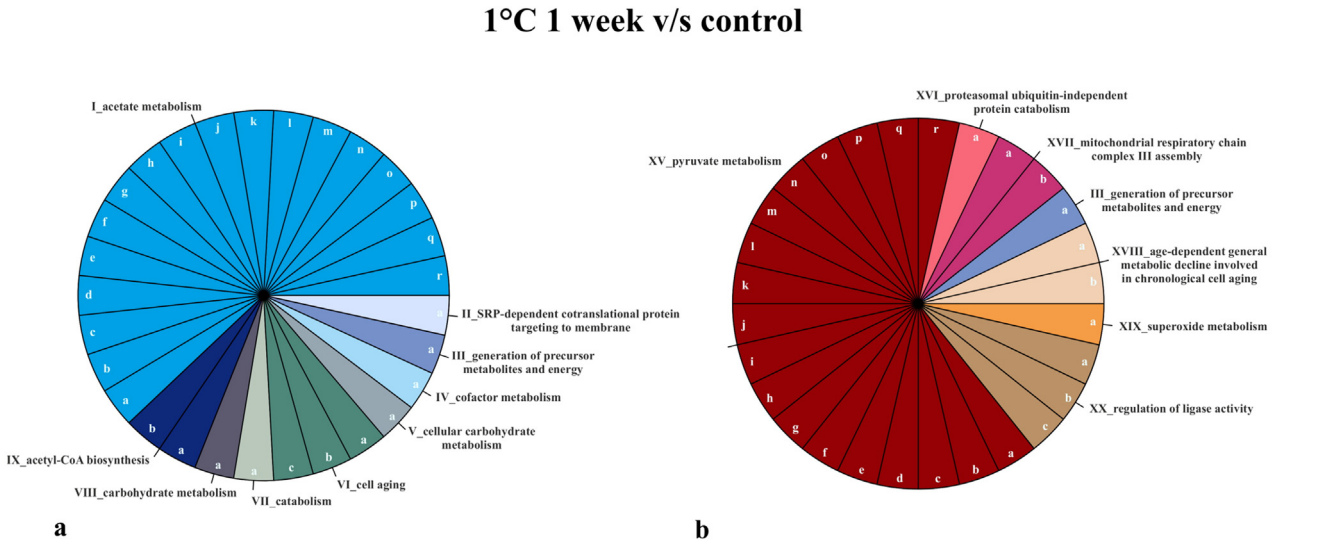
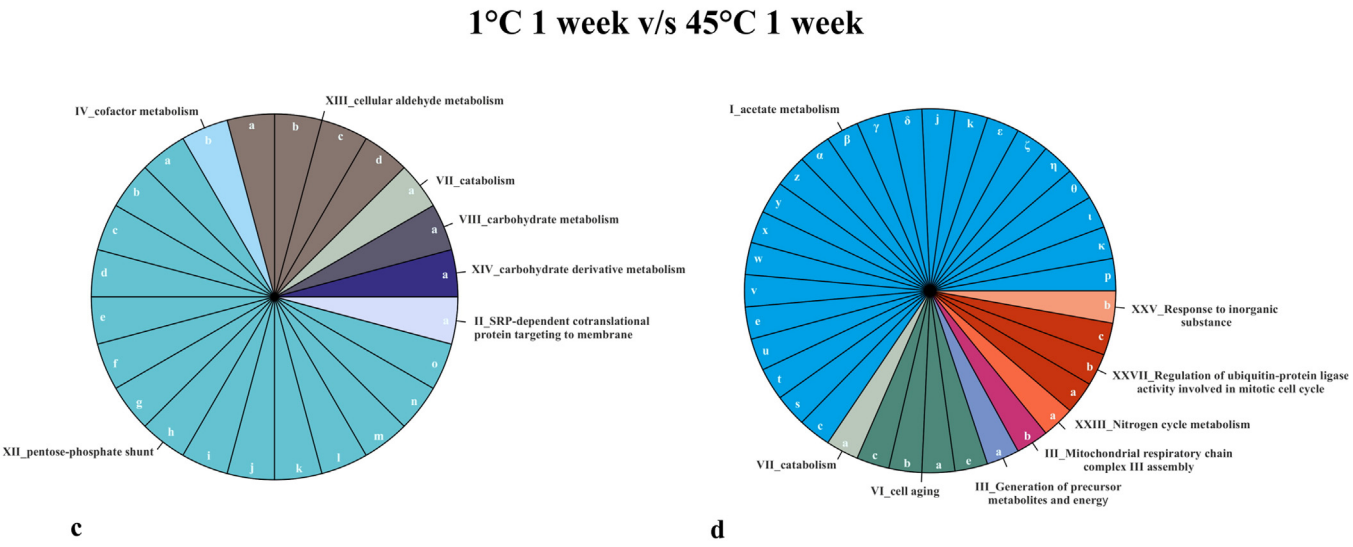


Fig. 5. Pattern analysis comparing the abundance of the identified protein between different experimental conditions. A dendrogram exhibiting clusters of co-varying spots is shown for each experimental set: (a) control v/s 1 °C 1 week; (b) 45 °C 1 week v/s 1 °C 1 week; (c) 45 °C 1 week v/s 1 °C 1 h. Values of standardized logarithmic abundance are displayed as a heat map. Red indicates increased abundance while green indicates decreased abundance (as calculated in comparison to the mean spot abundance). Gray boxes indicate that no data were available for the corresponding protein spot.





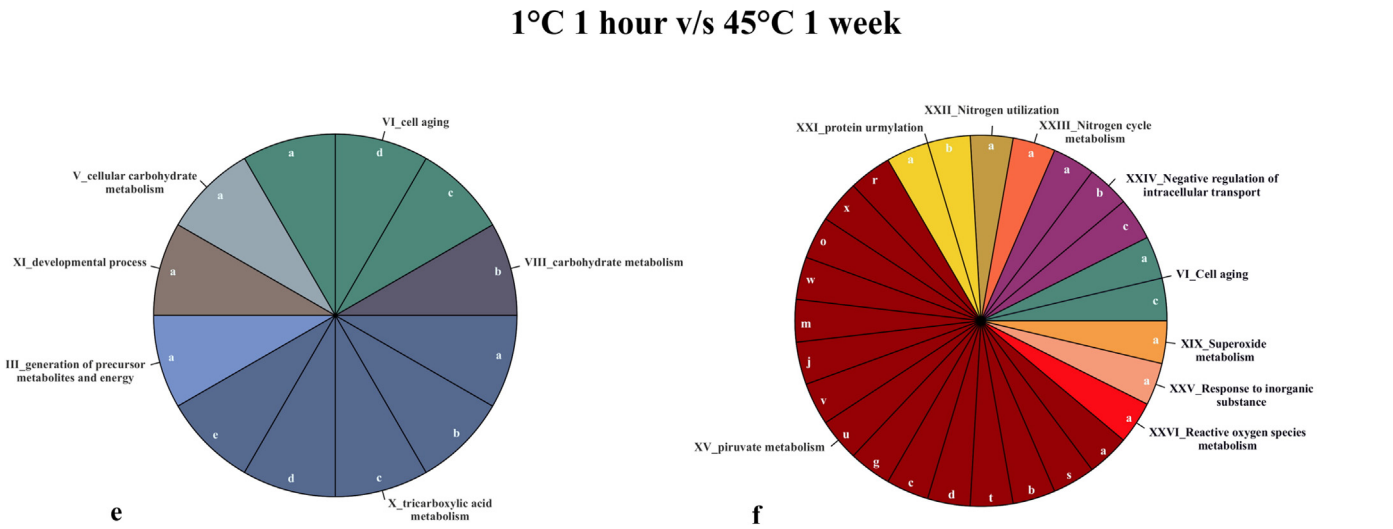


Fig. 6. GO terms based functional characterization of proteins identified from *E. dermatitidis*. In each pie chart, semantically close GO terms clustered into categories of cellular processes are displayed using different colors. Sub-categories of processes are also shown. a–c–e: down-regulated processes at 1 °C 1 week (a,c) and at 1 °C 1 h (e); b–d–f: up-regulated processes at 1 °C 1 week (b,d) and at 1 °C, 1 h (f).

4. Discussions

The present study represents the first proteomic contribution on *E. dermatitidis*, by the investigation of changes at the protein expression level at different temperature conditions and a comprehensive identification of the temperature responsive proteins. The results confirm the enormous ecological plasticity of this fungus, which can be interpreted as a major basis for understanding its transmission from the natural environment to the human habitats and the human host.

The pathogenic black yeast has recently received increasing attention, due to its frequent isolation from human-made environments such as sauna facilities and dishwashers. Its presence in such proximity to humans generates alarm especially since phaeoid (darkly pigmented) fungi collectively cause infections frequently not treatable with the available antifungal therapies and thereby lead to death [42,43]. For a number of reasons, *E. dermatitidis* can be considered as a good representative of most other phaeoid pathogens and can therefore be employed as model organism for studies about virulence of human pathogen species [44]. Its polymorphism and thus the ability to produce various morphological structures (i.e. mold-like stage, true and pseudo hyphae and sclerotic bodies in tissues) allows comparisons with different fungal species in terms of growth and developmental pathways (43, 44). Moreover, the incredible tolerance to a combination of stressors rarely observed in a single fungus, makes *E. dermatitidis* an excellent candidate for studies of the extremophile nature of fungi. As a result, the number of studies using the black yeast as model organisms are growing. However, knowledge regarding the molecular mechanisms at the base of the strains resistance to stress is still missing, especially at the functional proteomic level.

In this study four incubation conditions — two temperatures (1 and 45 °C) and two time spans (1 h and 1 week) — were selected to simulate the short- and long-term exposure to the temperatures which characterize the human-made and the natural habitats where the fungus has been isolated from (i.e. sauna facilities, tropical rain forest and glaciers). The strain temperature optimum (37 °C) was chosen as control condition. All experiments were carried out using four biological replicates. Morphological observation of the treated and control samples were performed to determine stress-induced alterations of the cell structures. A DIGE approach was applied in order to identify proteins whose abundance changed by temperature treatment. By doing that, particular reference was given to the protein functional characterization along with the prediction of protein pathways and subcellular localization. An additional purpose of the identification of the temperature reacting proteins was to evaluate the presence of a general stress response in the extremophile and pathogenic strain, also trying to assess whether stress affects its virulence.

4.1. Temperature preferences and morphological hints of temperature tolerance

Thermal preference tests performed on *E. dermatitidis* demonstrate that the fungus is capable to thrive at a wide range of temperatures, therefore it exhibits a certain ecological plasticity. The recorded optimal temperatures of growth lie indeed in the range of 25 to 37 °C, the latter representing the preferred temperature. Moreover, the black yeast shows good tolerance to temperature treatment up to one week, as confirmed by viability tests.

These data are consistent with the TEM observations, which reveal no dramatic alteration of the intracellular structures in *E. dermatitidis* under temperature treatments. However, an increase in melanization is detected at 1 °C and 45 °C compared to the optimal growth temperature (37 °C). In addition, the long-term exposure to low temperature (1 °C, 1 week) seems to have a greater impact on the cell morphology: under this condition, a thickening of the outermost cell wall layer is observed. The multilayered structure of the cell wall indeed persists and the exterior layer rather acquires a fibrillar-floccular consistency.

The accumulation of melanin in the fungus cell wall serves to multiple purposes. Melanin acts indeed as virulence factor in pathogenic species and has in addition a remarkable antioxidant activity, protecting the cells from both the host immune response and temperature stress [46]. ROS scavenging and tolerance to temperature stress are directly linked [47] and it is not surprising that a high proportion of melanin-producing fungi are isolated from environmentally stressed habitat, where the exposure to extreme temperatures is prolonged in time [48–50]. It follows that, in the present study, a higher abundance of melanin can undoubtedly be correlated to the necessity of the fungus to tolerate sub- and supra-optimal temperatures (i.e. 45 and 1 °C). The cell wall thickening occurred at the 1 °C, 1 week condition might serve a similar function: by acting as a barrier between the microorganism and the external environment it protects the cell from the possible damages caused by exposure to sub-optimal temperature or to freezing [51]. As for the floccular layer, a similar structure — enriched in glycosylated mannoproteins — has been previously described in pathogenic fungi as *Candida albicans* as a crucial element mediating the attachment to the host tissues during the initial steps of infection [52,53]. Unlike *C. albicans*, in *E. dermatitidis* such floccular layer is not detected in the present study at 37 °C or at higher temperature, therefore its correlation with the pathogenic activity can possibly be excluded. Further, it can be hypothesized that the fibrillar-floccular texture of the outermost layer of the wall is the result of the deposition of extracellular polysaccharides (EPS), which might also act as sheltering molecules and prevent cell freezing by slowing the accumulation of ice crystals at the cell wall. The attempt to increase the degree of freezing tolerance in response to low non-freezing temperatures was described in many species as part of the cold acclimation [54].

4.2. Temperature-related alterations in the cellular proteome

In *E. dermatitidis* and in other human pathogens growth at 37 °C is essential for pathogenesis, this temperature switch representing the signal for expression of virulence genes. As it is well known, high temperatures also contribute to protein denaturation especially in less adapted species and the synthesis of heat shock proteins (HSPs) indeed represents a general response to temperature stress [55].

The analysis of protein profiles from *E. dermatitidis* revealed changes in the protein abundance beyond the established cut-off value (p value < 0.05, average ratio > 1.5), only in response to the prolonged exposure to low temperature. The protein pattern does not undergo any remarkable increase or decrease in the number of protein spots as well as any statistically significant changes in protein abundance under the other temperature treatments. Even when lowering the average ratio to >1, protein modulation is not detected. This indicates that the short-term exposure to both high and low temperature does not trigger a rearrangement in the *E. dermatitidis* proteome, which is in complete contrast to what was hitherto found in other fungi and in other pathogenic species [56]. Previous studies conducted on human pathogens such as *C. albicans* and *Aspergillus fumigatus* actually detected re-arrangements of the protein pattern — including the over-expression of HSPs among other proteins — after a shift from 37 to supra-optima temperatures (i.e. 45 and 48 °C) [57,58]. Less is known about the reaction of fungal pathogens to low temperature. However, a correlation between cold and the over-expression of temperature responsive proteins — defined as cold-stress markers — has been previously described in the yeast *S. cerevisiae* [59].

The results of this study clearly show that the short-term exposure to sub- and supra-optimal temperatures (i.e. 45 °C and 1 °C) is not perceived as a stress by *E. dermatitidis*. Over-expression of chaperones, HSPs and cold shock proteins (CSPs) or on the contrary the down-regulation of the protein expression — representing the most important effects to temperature stress [27,60] — are not detected. Interestingly, this also applies to the long-term exposure of the fungus to high temperature (45 °C, 1 week), when the synthesis of temperature-

Table 5
All overrepresented GO terms, as clustered in categories of cellular processes.

N°	Main cellular process[a]	Letter	Sub-categories of cellular processes[b]
I	Acetate metabolism	a	Energy derivation by oxidation of organic compounds
		b	Primary alcohol metabolic process
		c	Organic acid metabolic process
		d	Cellular aldehyde metabolic process
		e	Oxidation–reduction process
		f	Misfolded or incompletely synthesized protein catabolic process
		g	Alcohol catabolic process
		h	Tricarboxylic acid cycle
		i	Tricarboxylic acid metabolic process
		j	Small molecule catabolic process
		k	Small molecule metabolic process
		l	Organic hydroxyl compound catabolic process
		m	Purine nucleobase metabolic process
		n	Nucleobase metabolic process
		o	Organic substance catabolic process
		p	Monocarboxylic acid metabolic process
		q	Ethanol metabolic process
		r	Acetate metabolic process
		s	CTP metabolism
		t	Guanosine-containing compound metabolic process
		u	Acetyl-CoA biosynthetic process
		v	Lipid oxidation
		w	Pyruvate metabolic process
		x	Glycolytic process
		y	gluconeogenesis
		z	Guanosine-containing compound biosynthetic process
		α	Mitochondrial electron transport, ubiquinol to cytochrome c
		β	Pyrimidine nucleoside triphosphate biosynthetic process
		γ	Carbohydrate biosynthetic process
		δ	Nucleoside diphosphate metabolic process
		ε	Single-organism catabolic process
		ζ	Nucleotide phosphorylation
		η	Pyrimidine ribonucleoside triphosphate metabolic process
		θ	Monocarboxylic acid biosynthetic process
		ι	Response to inorganic substance
		κ	Monocarboxylic acid catabolic process
II	SRP-dependent cotranslational protein targeting to membrane	a	SRP-dependent cotranslational protein targeting to membrane
III	Generation of precursor metabolites and energy	a	Generation of precursor metabolites and energy
IV	Cofactor metabolism	a	Cofactor metabolism
		b	Cofactor metabolic process
V	Cellular carbohydrate metabolism	a	Cellular carbohydrate metabolic process
VI	Cell aging	a	Aging
		b	Single-organism metabolic process
		c	Cell aging
		d	Single-organism developmental process
		e	Age-dependent response to oxidative stress involved in chronological cell aging
VII	Catabolism	a	Catabolic process
VIII	Carbohydrate metabolism	a	Carbohydrate metabolic process
		b	Carbohydrate metabolism
IX	Acetyl-CoA biosynthesis	a	Glucose 6-phosphate metabolic process
		b	Acetyl-CoA biosynthetic process
X	Tricarboxylic acid metabolism	a	Citrate metabolic process
		b	Energy derivation by oxidation of organic compounds
		c	Dicarboxylic acid metabolic process
		d	Tricarboxylic acid cycle
		e	Tricarboxylic acid metabolic process
XI	Developmental process	a	Developmental process
XII	Pentose-phosphate shunt	a	Pentose-phosphate shunt
		b	Organophosphate metabolic process
		c	Monocarboxylic acid metabolic process
		d	Ethanol metabolic process
		e	Organic substance acid biosynthetic process
		f	Monocarboxylic acid biosynthetic process
		g	Glucose 6-phosphate metabolic process
		h	Alcohol catabolic process
		i	Glyceraldehyde-3-phosphate metabolic process
		j	Misfolded or incompletely synthesized protein catabolic process
		k	Organic hydroxy compound catabolic process
		l	Acetate metabolic process
		m	Small molecule catabolic process
		n	Primary alcohol metabolic process
		o	Pyridine-containing compound metabolic process

(*continued on next page*)

Table 5 (*continued*)

N°	Main cellular process[a]	Letter	Sub-categories of cellular processes[b]
XIII	Cellular aldehyde metabolism	a	Small molecule metabolic process
		b	Single-organism metabolic process
		c	Nucleobase-containing small molecule metabolic process
		d	Cellular aldehyde metabolic process
XIV	Carbohydrate derivative metabolism	a	Carbohydrate derivative metabolic process
XV	Pyruvate metabolism	a	Acetate metabolic process
		b	CTP metabolic process
		c	Acetyl-CoA biosynthetic process
		d	Oxidation-reduction process
		e	Glycolytic process
		f	Gluconeogenesis
		g	Guanosine-containing compound biosynthetic process
		h	Mitochondrial electron transport, ubiquinol to cytochrome c
		i	Monosaccharide metabolic process
		j	Pyrimidine nucleoside triphosphate biosynthetic process
		k	Carbohydrate biosynthetic process
		l	Carbohydrate catabolic process
		m	Nucleoside diphosphate metabolic process
		n	Fungal-type cell wall polysaccharide metabolic process
		o	Nucleotide phosphorylation
		p	Purine ribonucleoside triphosphate metabolic process
		q	Monocarboxylic acid metabolic process
		r	Pyruvate metabolic process
		s	Organic acid metabolism
		t	Guanosine-containing compound metabolic process
		u	Oxoacid metabolic process
		v	Pyrimidine nucleoside triphosphate metabolic process
		w	Small molecule metabolic process
		x	Pyrimidine ribonucleoside triphosphate metabolic process
XVI	Proteasomal ubiquitin-independent protein catabolism	a	Proteasomal ubiquitin-independent protein catabolic process
XVII	Mitochondrial respiratory chain complex III assembly	a	Protein import into mitochondrial inner membrane
		b	Mitochondrial respiratory chain complex III assembly
XVIII	Age-dependent general metabolic decline involved in chronological cell aging	a	aging
		b	Age-dependent general metabolic decline involved in chronological cell aging
XIX	Superoxide metabolism	a	Superoxide metabolic process
XX	Regulation of ligase activity	a	Signal transduction involved in filamentous growth
		b	Negative regulation of apoptotic process
		c	Regulation of ligase activity
XXI	Protein urmylation	a	Misfolded or incompletely synthesized protein catabolic process
		b	Protein urmylation
XXII	Nitrogen utilization	a	Nitrogen utilization
XXIII	Nitrogen cycle metabolism	a	Nitrogen cycle metabolic process
XXIV	Negative regulation of intracellular transport	a	Regulation of establishment of protein localization
		b	Regulation of nitrogen utilization
		c	Negative regulation of intracellular transport
XXV	Response to inorganic substance	a	Response to inorganic substance
		b	Negative regulation of intracellular transport
XXVI	Reactive oxygen species metabolism	a	Reactive oxygen species metabolic process
XXVII	Regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	a	Regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle
		b	Acetate metabolic process
		c	Negative regulation of apoptotic process

[a] Main categories of cellular processes created by clustering semantically close GO terms.
[b] Overrepresented GO terms for cellular processes.

responsive and stabilizing proteins would occur — in most organisms — and serve as survival strategy at the base of the acclimation process. Acclimated organisms are indeed able to start a quick response if exposed to even more extreme temperatures [61]. Nevertheless, by promoting metabolic homeostasis despite environmental changes, acclimation requires energetic costs [62]. On this basis, we strongly believe that in the case of *E. dermatitidis* — where the costs may outweigh the benefits — a lack of response represents the best adaptation. This

Table 6
Predicted sub-cellular localizations of the proteins decreased (a) and increased (b) at 1 °C 1 week experimental condition, as compared with the control.

Strain	Experimental set	Number of protein spots (%)						
			CYT	NUC	MIT	PER	EXT	MEM
E. dermatitidis CBS 525.76	Control v/s 1 °C, 1 week	Increased	61.1	5.5	22.2	–	5.5	5.5
		Decreased	52.2	4.3	8.7	34.8	–	–
	45 °C, 1 week v/s 1 °C, 1 week	Increased	60	8.6	25.7	–	2.8	2.8
		Decreased	40	6.6	6.6	46.6	–	–
	45 °C, 1 week v/s 1 °C, 1 h	Increased	84.6	7.7	7.7	–	–	–
		Decreased	100	–	–	–	–	–

CYT = cytoplasm; NUC = nucleus; MIT = mitochondrion; PER = peroxisome; EXT = extracellular space; MEM = plasma membrane.

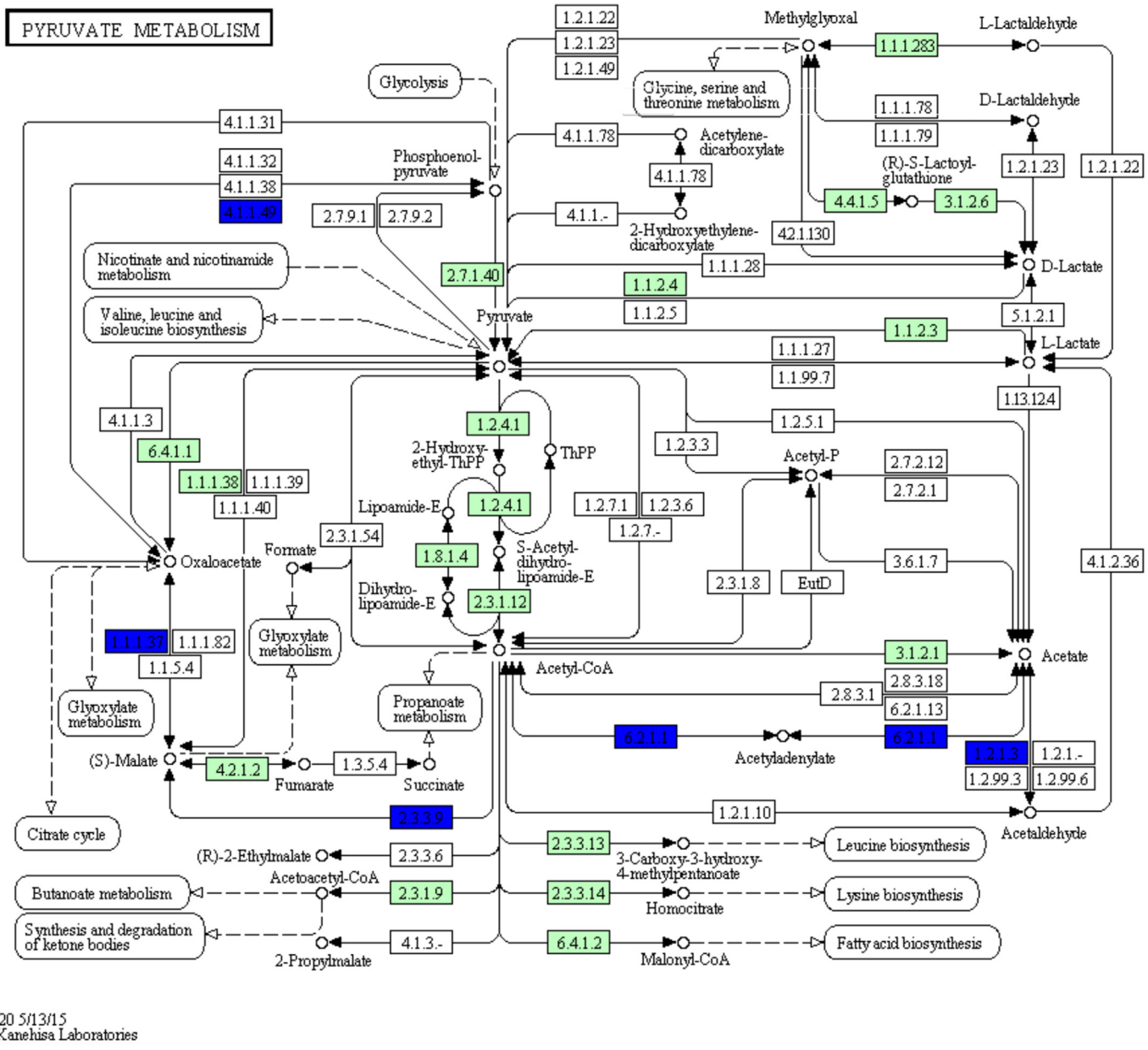


Fig. 7. Graphical representation of the piruvate metabolism, down-regulated pathway in *E. dermatitidis* at 1 °C 1 week, as compared with the control. All identified proteins decreased in abundance under temperature treatment are highlighted in blue. (4.1.1.49 = phosphoenol pyruvate carboxykinase; 1.1.1.37 = malate dehydrogenase, NAD-dependent; 2.3.3.9 = malate synthase, glyoxysomal; 6.2.1.1 = acetyl-CoA synthetase; 1.2.1.3 = aldehyde dehydrogenase).

idea finds support in the recourse of black fungi to energy saving mechanisms, which was already demonstrated to be crucial for the organism survival in both changing and constant environments [27,63,64]. Moreover, it can be speculated that *E. dermatitidis* temperature tolerance is based on the thermo-stability of the basic set of proteins which, by remaining functional under temperature shifts, allow the organism to save precious energy. This might additionally explain the absence of a shock response. A combination of housekeeping, stabilizing and putative virulence proteins might per se guarantee the organism's survival once it is exposed even to long lasting unfavorable temperature condition, without any novel protective proteins. However, other unknown cellular strategies may also have a crucial role in the tolerance and adaptation. Recently, the fusion of distinct mRNAs to produce the necessary proteins once in need has been proposed as a solution for thriving in the natural and in the human-made environment as well as in the human host [65].

4.3. Proteins involved in the response to low temperature

In *E. dermatitidis* the greatest impact on the cellular protein level is observed under the 1 °C 1 week incubation condition. In accordance with the findings of a recent study on the transcriptome of this fungus, the highest number of cellular processes is altered by the exposure to low temperature [65], thus indicating the strain's effort to cope with the cold.

The major part of proteins whose abundance changed significantly under low temperature are cytoplasmic and mitochondrial; peroxisomal proteins can only be found among the proteins decreased at low temperature. The performed proteome analysis enabled to highlight those biological processes and metabolic pathways mostly affected by low temperature, therefore providing first indicators — on the proteome level — for regulated pathways during temperature stress.

A large number of the temperature-modulated proteins is associated with the carbohydrate synthesis and metabolism. The results of both

protein functional characterization and pathways prediction show that especially proteins involved in the pyruvate, acetate and carbon metabolism exhibit lower levels at 1 °C. This is in agreement with what previously reported in yeasts following the incubation to low temperature [66] and suggests a reduced metabolism. Further components of basic energy metabolism such as malate synthase and malate dehydrogenase — which are responsible for the formation of malate from acetyl-CoA and of oxaloacetate from malate respectively in the glyoxylate shunt — show lower levels too at 1 °C. The glyoxylate shunt is an ancillary cycle to the TCA cycle and is essential for growth on two-carbon compounds, along with being required for fungal virulence [67]. It is not surprising that also the ethanol degradation, whose product acetyl-CoA can be metabolized by reacting with the intermediates of the glyoxylate cycle, results to be down-regulated. Moreover, as expected in response to the thermal downshift, proteins involved in the response to heat stress such as Hsp-70s, elongation factor 1-alpha and the hypothetical protein gi|378729668 identified as Hsp-30 through homology search, also show decreased levels [68]. Similarly, the down-regulation of cell aging at low temperature can be interpreted as direct consequence of the slowing of the metabolic rate, which is linked to a reduction of energy consumption and of ROS [69].

As already shown in the methylotrophic yeast *Pichia pastoris*, at low temperature and on non-methanolic growth substrates [66], also in *E. dermatitidis* a decrease in the abundance of alcohol oxidases is detected. Multiple spots with different electrophoretic mobility are actually identified as alcohol oxidase, thus suggesting the presence of isoforms [70]. Moreover, the spots show different ratios of abundance, the ones with most basic p*I* resulting to be more decreased. Although this study does not focus on the regulation of post-translational modifications of single proteins, the observation of concerted regulations of protein species — for e.g. the above mentioned alcohol oxidases isoforms — corroborates the finding that the pathways they are involved to, are also modulated. Depending on the species, the alcohol oxidases' function can be related to methanol metabolism, however a methylotrophic activity is not known for *E. dermatitidis*.

Interestingly, a few proteins involved in carbohydrate metabolism (i.e. glycolysis/gluconeogenesis, carbon and pyruvate metabolism) show a higher abundance at 1 °C. In accordance with the morphological changes in the cell wall observed at low temperature condition — i.e. thickening and floccular-like aspect — and with some of the enriched biological process GO terms (i.e. Table 5, XV k, l, n), the induction of these proteins might have the aim to enhance the synthesis of cell wall polysaccharides which have a physical protective function for the wall. However, the presence of truncated and/or degraded proteins interferes with the above mentioned categories of biological processes and pathways, thus their evaluation as of generally actually up-regulated processes is critical. The examination of the MS/MS spectra showed in fact that peptides with higher identification scores covered only parts of the total protein sequence. This phenomenon concerns other up-regulated biological processes and pathways as well. Protein fragments can be the product of both truncation and degradation, two phenomena that have different impact on the protein function. Unlike degradation, truncation can represent transcript splicing or functional cleavage [71]. Furthermore, it should be considered that the procedure for protein extraction can also have an impact. In the current proteomic study, down-regulation is suggested in all cases where the proteins with increased abundance resemble a degradation product and the full-length protein counterpart is found to be decreased. The Hsp30 (spot N° 1787) represents a good example: one protein spot identified as a fragment of Hsp30 with experimental Mw of approximately 10 kDa and p*I* around 9.0 shows higher abundance at 1 °C. A second spot identified as the *E. dermatitidis* hypothetical protein gi|378729668 and homologous of a 30 kDa Hsp of *Fusarium oxysporum,* exhibits instead a decreased level. This spot has experimental Mw and p*I* values of approximately 30 kDa and 5.9 respectively, which indeed match the theoretical values of the full-length Hsp30. Hsp30 is accordingly deemed down-regulated at low temperature. Similarly, the glycolysis/gluconeogenesis pathway and the methane metabolism are not to be considered as up-regulated.

An additional process to be up-regulated is the regulation of ligase activity, which based on the associated GO terms, is involved in the cell cycle, filamentous growth and the negative regulation of apoptotic process.

4.4. High and low temperatures comparison

Significant re-arrangements of the proteome are also detected between the low and the high temperature incubation conditions. Similarly to what observed when comparing the low temperature incubation condition with the control, the processes to be down-regulated are primarily related to carbohydrate metabolism. By comparison with 45 °C 1 week condition, at 1 °C 1 h the down-regulation is limited to the TCA cycle and the glyoxylate metabolism. One of the enzymes of the glyoxylate shunt — the malate dehydrogenase — that shows lower levels at 1 °C at all condition comparisons represents an interesting case of protein regulation. Two different protein spots (spot n°1896 and 1125) having diverse Mw and p*I* were identified as the enzyme, thus denoting the presence of post translational modified forms of the same protein being modulated at different growth conditions [72]. After the incubation to 1 °C for a week, pentose-phosphate shunt, carbon and pyruvate metabolism are down-regulated. Enzymes of the pentose phosphate pathway are actually known to have a heat shock-dependent regulation in yeasts, thus suggesting their role in the depletion of ROS and in balancing the organism redox state [58,73]. Cellular processes involving aldehydes, other components of basic energy metabolism which are responsible for interconversion of acetaldehyde and acetate [66], also show lower levels at 1 °C 1 week. Similarly, at 1 °C 1 h proteins involved in the aspartate degradation exhibit lower abundance. Since aspartate can be converted to intermediates of multiple pathways and can additionally act as precursor for the biosynthesis of many other compounds such as lysine and NAD^+, it actually represents a link between amino acid and carbohydrate metabolism [74].

Proteins involved in the beta-oxidation of fatty acids and peroxisomal lipid metabolism show instead higher levels at 1 °C 1 week. This may be explained by the fact that modifications in membrane lipid composition are a typical response to temperature changes in both uni- and multicellular organisms [75]. With the lowering of growth temperature also the fluidity of the cell membrane decreases, thus affecting membrane-associated cellular functions and ultimately compromising the organism survival [76]. Shortening the length of the membrane fatty acid chains represents a strategy to counteract the effect of cold stress by maintaining a constant degree of membrane fluidity [77]. No evidence of modulation of lipid-related pathways is observed at 45 °C, thus indicating that this temperature has no impact on the membrane status. By having a role in the breakdown of storage lipids, β-oxidation additionally represents an advantageous mechanism to produce energy by the use of existing molecules instead of investing energy in the synthesis. This aspect becomes more relevant under non-optimal conditions of growth.

The up-regulation of the ubiquitin-protein ligase activity at low temperature can actually denote the degradation of target proteins, which enables the organism to degrade unnecessary proteins and maximize the survival under different conditions, such as heat and oxidative stress [78]. Since poly- and mono ubiquitination — as one of the fundamental post-translational modifications for regulating protein function — indeed control many aspects of cellular function in yeasts and in all eukaryotes [79], it is not possible to exclude that other cellular processes, such as membrane transport and transcriptional regulation [80] are also involved.

The abundance of proteins related to the cell cycle control is increased after both short- and long-term exposure to 1 °C. Proteins like glutathione S-transferase and the 14-3-3 family proteins play a

regulatory role in all eukaryotic cells, having important cell signaling implications [81,82]. The up-regulation of 14-3-3 may also result in the alteration of target proteins' function as well as in protein sequestering. Spatial sequestration of misfolded and stress-denatured proteins aiming to protein degradation, may actually enhance cellular fitness during stress [83]. Moreover, according to the nature of the protein ligand, the 14-3-3 binding may lead to cell cycle arrest [84]. Both in prokaryotes and in eukaryotes, the slowdown of the cell cycle is known to be induced by cold stress [77], being triggered in yeasts by near-freezing temperatures [83].

Similarly, at both 1 °C 1 week and 1 °C 1 h condition as compared to 45 °C 1 week, proteins involved in carbohydrate metabolism (e.g. acetate and pyruvate metabolism) show higher abundance. The synthesis of cell wall polysaccharides can, in this case, be speculated. However, due to the fragmentation of the involved proteins, these processes can hardly be considered as actually up-regulated. The same applies to the up-regulated categories of cell aging.A small number of proteins involved in the cellular responses to stress — mostly superoxide dismutases, isoform of Hsp-70s and 14-3-3 family proteins are also recorded as up-regulated at 1 °C — at all condition comparisons. In combination with the down-regulation of the metabolism and the re-arrangements at the cell membrane level, this is a clear hint that low temperature is perceived by the fungus as non-optimal condition.

5. Conclusions

Being the present study the first contribution on the proteome of *E. dermatitidis* in relation to the effects of temperature, we aimed at the identification of key proteins for cellular processes regulated during temperature changes. The following conclusions can be drawn from our results: a) tests for thermal preferences and viability after heat and cold treatment confirm that *E. dermatitidis* is capable to thrive at a wide range of temperatures. Whereas 25–37 °C supports fast growth, the fungus is able to survive and to grow — albeit with slow growth rates — at temperatures far from the optimum (1 and 45 °C). This is in accordance with the morphological, ultrastructural observations as well as with the proteomic analysis. While the exposure to 1 °C is perceived by the fungus as non-optimal condition, thus leading to the down-regulation of the metabolism and to re-arrangements at the cell membrane level, 45 °C seems not to induce any stress response. Unlike other human fungal pathogens, no heat shock response is recorded at high temperature. On this basis, a fine-tuning regulation of the protein expression under temperature treatment — mostly involving housekeeping proteins such as the components of the major metabolic pathways — is suggested. Together with the thermo-stability of the basic set of proteins and the morpho-physiological adaptations, this is possibly what makes *E. dermatitidis* a fully successful microorganism and human pathogen.

b) In view of the fungus distribution, infection route and its temperature tolerance, a transition from glaciers — as a natural reservoir — to the water reservoirs (tap water system) and from there to the human environment is likely. Such transition is possibly accomplished through a series of morpho-physiological evolutionary adaptations that involve a basic set of stable proteins and, especially, a fine-tuning regulation of the protein expression. In combination with the down-regulation of the cellular metabolism, this set of properties would ensure survival under diverse temperature condition enabling access to multiple and even harsh habitat, bypassing problematic aspects such as the ones represented by the oligotrophic conditions in the water, and the switch from low to high temperature.

An in depth analysis of single proteins envisions implications not only in view of virulence but also for biotechnological applications especially regarding thermo-stable proteins and functions. Proteins involved in the glyoxylate shunt like formate and malate dehydrogenase, or alcohol oxidases and 14-3-3 family proteins, which participate in the basic cellular metabolism and whose levels are down-regulated by lowered temperature, represent good candidates for knock-out experiments. Further, the information gained about the sub-cellular localization of the identified proteins can be verified by immunocytochemistry by aiming at target proteins. Improving the understanding of the identified "key metabolic proteins" involved in tolerance fine-tuning can be implemented into biotechnological applications, regarding recombinant product formation. Additionally, the investigation of identified metabolic processes ultimately supports the development of antimycotic strategies.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in the version.

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RESEARCH ARTICLE

From Glacier to Sauna: RNA-Seq of the Human Pathogen Black Fungus *Exophiala dermatitidis* under Varying Temperature Conditions Exhibits Common and Novel Fungal Response

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Abstract

Exophiala dermatitidis (Wangiella dermatitidis) belongs to the group of the so-called black yeasts. Thanks in part to its thick and strongly melanized cell walls, *E. dermatitidis* is extremely tolerant to various kinds of stress, including extreme pH, temperature and desiccation. *E. dermatitidis* is also the agent responsible for various severe illnesses in humans, such as pneumonia and keratitis, and might lead to fatal brain infections. Due to its association with the human environment, its poly-extremophilic lifestyle and its pathogenicity in humans, *E. dermatitidis* has become an important model organism. In this study we present the functional analysis of the transcriptional response of the fungus at 1°C and 45°C, in comparison with that at 37°C, for two different exposition times, i.e. 1 hour and 1 week. At 1°C, *E. dermatitidis* uses a large repertoire of tools to acclimatize, such as lipid membrane fluidization, trehalose production or cytoskeleton rearrangement, which allows the fungus to remain metabolically active. At 45°C, the fungus drifts into a replicative state and increases the activity of the Golgi apparatus. As a novel finding, our study provides evidence that, apart from the protein coding genes, non-coding RNAs, circular RNAs as well as fusion-transcripts are differentially regulated and that the function of the fusion-transcripts can be related to the corresponding temperature condition. This work establishes that *E. dermatitidis* adapts to its environment by modulating coding and non-coding gene transcription levels and through the regulation of chimeric and circular RNAs.

Introduction

Black yeasts are a polyphyletic morphological group within the Ascomycetes that is characterized by melanized cells and yeast-like growth states (multilateral and polar budding cells) in

addition to hyphal growth. Some species, like *E. dermatitidis*, exhibit meristematic growth and form morula-like colonies in animal and human tissue or in natural stone [1,2]. All black yeasts and meristematic fungi share a number of universally present characters, such as strong melanization, thick and even multi-layered cell walls and exo-polysaccharide production, resulting in an extraordinary ability to tolerate chemical and physical stresses such as extreme pH, high and low temperature and desiccation [3–5]. Moreover, some of those fungi—including *E. dermatitidis*—show increased cell growth—division and cell size—when exposed to ionizing radiation [6,7].

The black fungi associated with humans are represented by the typical black yeasts belonging to the genera *Exophiala*, *Fonsecaea*, *Capronia*, *Phaeococcomyces* and *Cladophialophora*. Phylogenetically these genera are accumulated in the order of Chaetothyriales and the family of Herpotrichiellaceae. Within this family the genus *Exophiala* seems to be an evolutionary hot spot with a high diversification and emerging adaptation towards animals—e.g. *Exophiala salmonis*—towards the human host, in the case of *Exophiala dermatitidis* and—or to human environments such as bathrooms, sauna facilities or dishwashers [8,9]. *E. dermatitidis* is of special medical importance since it causes a variety of severe illnesses in humans: it is a causative agent of keratitis, of subcutaneous phaeohyphomycosis, and of chromoblastomycosis, and may cause pneumonia. Further, the fungus is neurotropic and causes fatal brain infections, and it plays an important role in patients with cystic fibrosis [8,10–13].

For many years the black yeasts and the microcolonial fungi were regarded as typical inhabitants of extreme environmental habitats such as the phylloplane, or rock in semi-arid and desert environments [5,14–16]. However, species of *Exophiala* show high prevalence in the human environment and seem to be rather frequent in sauna and steam bath facilities, in sink drains, and in drinking water [17–19]. Examinations of bathwater and sludge in drainpipes that are warmed daily to over 42°C have identified several species of this medically important genus [20]. Recently, mass growth of dark fungal biofilms on water taps and associated habitats was observed in various German drinking water distribution systems [21,22]. Here, *E. lecaniicorni* was found to be the major component in 10 out of 13 biofilms analyzed. Biofilm-forming *Exophiala mesophila* has been isolated from chlorine-dioxide-treated dental unit waterlines. *E. dermatitidis* and *E. phaeomuriformis* were reported to form stable communities in dishwashers, and extensive global studies showed that more than 50% of all rubber or silicone sealings in dishwashers are colonized with these fungi [23,24]. This finding generated considerable public attention and raised many questions concerning the routes of entry, the natural reservoir and the virulence for the human host of this fungus.

Interestingly, besides the human related habitats which are characterized by rather high temperatures, strains of *E. dermatitidis* have been isolated from glaciers—e.g. the Calderone glacier in the Apennines [25]—as well as in the Arctic and Antarctic environments [26]. Furthermore, Gunde-Cimerman (personal communication) is suggesting that natural spring water is a possible source of the fungi and is the vehicle by which it enters the human environment. This ability to adapt to the temperatures and nutrients found in environments as diverse as cold glaciers, hot saunas and dishwashers, as well as the more temperate human body, requires an important phenotypic plasticity, that might, in turn, explain the success of *E. dermatitidis* as a human pathogen.

E. dermatitidis, due to its association to the human environment and due to its phylogenetic relation to many environmental black fungi (which are known as poly-extremophilic or poly-extremotolerant) has become an important model organism for system biology studies, including proteomics and transcriptomics [9,27]. However, the knowledge of the system biology of this fungus is still limited. In order to better understand the capacity of this fungus to adapt, we

have reported the results of a transcriptomic study of gene expression responses under different temperature regimes and exposition times.

With this aim, *E. dermatitidis* was incubated at 1°C—thus simulating the cold environmental habitat—at 37°C—which is the optimal growth temperature for this strain—and at 45°C in order to simulate raised temperatures in the human environment—e.g. hot tap water.

Materials and Methods

Fungal strain and experimental conditions

Exophiala dermatitidis (CBS 525.76) was cultured in Malt extract agarose media (2% malt extract, 2% D-glucose, 0.1% bacto-peptone and 2% agar). For the RNA-seq experiment, the cells were grown in Petri dishes for 2 weeks at 37°C and then as follows: one week at 37°C (control, 37C), 1 week at 45°C (45C1W), 1 week at 37°C plus 1 hour at 1°C (1C1H) or at 45°C (45C1H), 1 week at 1°C (1C1W) and 1 week at 45°C (45C1W), for a total of four treatments.

RNA-seq

Total RNA was extracted with FastRNA PRO RED KIT (MP Biomedicals, Santa Ana, CA) according to the instructions of the manufacturer. The mRNA was isolated with the Dynabeads mRNA DIRECT Micro Kit (Ambion by Life Technologies, Carlsbad, CA) and the following transcriptome library preparation was performed with the Ion Total RNA-Seq Kit v2 (Life Technologies, Carlsbad, CA).

Total RNA, isolated mRNA and the final cDNA library were all qualitatively and quantitatively evaluated by mean of Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RNA-seq was realized by means of Ion Torrent technology coupled with the Ion Proton sequencer (Life Technologies, Carlsbad, CA). The average read length was 175 bp for all five samples. Total reads generated per sample varied between 57,611,573 and 99,965,344.

Gene Annotations

Protein annotation and functional annotation were retrieved from previous publications [4,5,27]. Interproscan5 with default parameters was used to improve the Gene Ontology annotations [28].

NcRNAs were annotated based on methods using sequence and structure homologies. Infernal [29] with default parameters was used to find ncRNAs in a general way. tRNAs were annotated with tRNAscan [30] with default parameters. RNAmmer was used to find rRNAs genes [31] while snoBoard [32] was used to search for snoRNAs. Finally BLAST [33] was used to look for putative miRNAs, rRNAs, sRNAs. RnasP and RnaseMP. Overlapping non-coding loci from different families were kept. In cases where more than one member of the same ncRNA family (snoRNA, miRNA, snRNA, tRNA) were assigned to the same locus, the member with the highest similarity to the search model/search sequence was kept.

Read Mapping and Analysis

Trimmed reads were downloaded from our local ion proton server. Segemehl [34] version 1.9 with the split-read detection option set, was used to map the reads on the recently published *E. dermatitidis* genome [27] while lack [34] was used to remap reads that were not mapped during the first mapping step. Lack utilizes *de novo* splice junction information from alignments reported by state-of-the-art split-read aligners. In contrast to other methods, lack is able to map reads across multiple splice junctions. Splice sites detection and classification was done with testrealign [35] with default settings. Reads that were split-mapped were assigned to one of the

following three categories: "normal," same strand, same chromosome and insert between 15 and 200 kb and matched fragments co-linear with the genomic DNA; "circular," same strand, same chromosome and junction distance less than 200 kb with fragment order inverted relative to genomic DNA; "(strand)switched," same chromosome, junction distance less than 200 kb and fragments located on opposite strands. Overlaps between mapped reads and annotation data were computed with the aid of BEDTools [36].

Fused transcripts were detected by looking at split reads connecting two different transcripts. In order to get high confidence fused transcripts we concentrated on split-reads whose ends were mapping less than 20nts from a canonical splice site, as suggested in [35]

. The presence of monoexonic circular transcripts, where a single intron or exon is circularized, was investigated by looking at split reads with the start and end located on the same exon/intron and with ends separated by less than 400nts. Furthermore, at least 8 reads should support the circular RNA and at least 50% of the spliced reads mapping at the splice has to support the circRNA.

Cuffdiff [37] was used to look for differential expression of genes, in a similar manner to the methods presented in [38]. The mapping output of segemehl was modified to fit the input requirements of cufflinks using a custom script. CummeRbund [39], a R module [40], was used to search for significant (at least 8 folds) differential expression between 37C and the other 4 temperature conditions (1C1H, 1C1W, 45C1H, 45C1W). AnnotationForge, GOstats [41], GSEABase [42] and KOBAS [43] were used to analyse GO and KEGG pathways enrichment of the up- and down-regulated genes.

Enriched GO terms and KEGG pathways with an uncorrected p-value < 0.05 were considered significant. REVIGO with default parameters was used to summarize the list of returned GO terms [44].

The mapping of the regulated genes on the pathways was performed with custom scripts using the KEGG database [45].

Results

To understand the molecular mechanisms behind the acclimatization of *E. dermatitidis* under a wide range of temperature adaptations, the fungus was exposed to 45°C and 1°C and the gene expression was analyzed through RNA-seq technology. In order to investigate the fungal response to short (stress) and long-term changes (acclimatization), *E. dermatitidis* was exposed for one hour and for one week, respectively, at both temperatures. The control condition is represented by the exposure to 37°C.

Tests for thermal preferences conducted prior to setting up the experimental conditions, indicated 37°C as the optimum temperature for *E. dermatitidis* and showed the presence of fungal growth at both 45°C—as also previously demonstrated [23]—and at 1°C. In addition, the fungal colonies proved to be viable after prolonged treatment at all the selected temperatures (data not shown, Tesei et al. in preparation).

The transcriptome analysis was performed comparing pairs of experimental conditions, providing a list of over-represented Gene Ontology and KEGG terms of the differentially expressed genes. In order to deepen our knowledge of the dependence of cell regulation upon temperature changes, the annotation of *E. dermatitidis* was extended with ncRNA annotation. Furthermore, circular RNAs and trans-spliced genes were also studied.

Overview of RNA sequencing

In order to get an overview of the alteration of *E. dermatitidis* transcriptome upon temperature changes, the number of strongly (8 fold) up- and down-regulated coding and non-coding

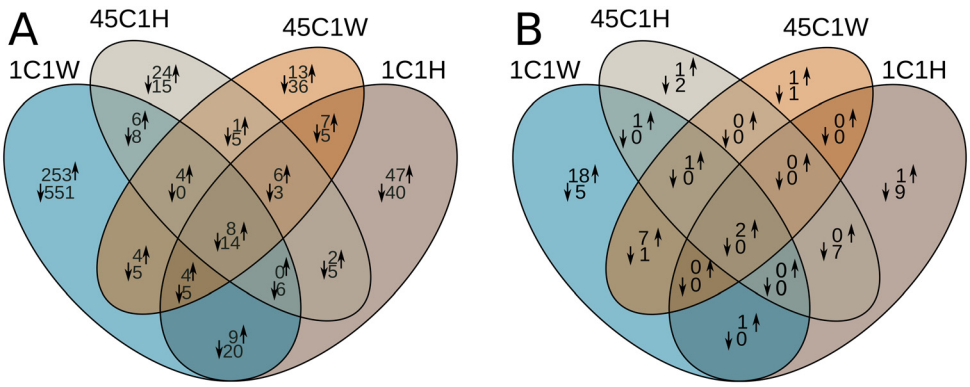

Fig 1. Representation of up- and down-regulated genes for cold and hot conditions, compared to 37°C. Upwards and downwards arrows represent up- and down-regulated genes, respectively. Protein coding genes (A) show the highest number of differentially expressed genes following the treatment at 1°C for 1 week (1C1W), with a total of 609 and 288 down- and up-regulated genes, respectively. NcRNA genes (B) show the highest number of differentially expressed genes at the same experimental condition (1°C for 1 week, 1C1W), with a total of 30 and 6 up- and downregulated genes, respectively. For both coding and non-coding genes, the smallest number of differentially expressed genes is found when the fungus have been exposed at 45°C for 1 week (45C1W).

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genes were derived by comparing their transcription level at 37C and 1C1W, 1C1H, 45C1H and 45C1W. While we are confident that our mRNA isolation protocol successfully selected polyA-transcripts, we also found reads coming from ncRNAs loci. This is in line with previously- reported polyadenilated ncRNAs found in various prokaryotes and eukaryotes [46]. For the protein-coding genes, the largest number of differentially expressed genes is found for 1C1W, where 609 genes were down-regulated and 208 genes were up-regulated. The smallest number of differentially expressed genes is found for 45C1W, where only 72 and 45 genes were down- and upregulated, respectively (Fig 1A). Pie charts with up- and downregulated GO terms were produced for each temperature exposure in comparison with the control. The most informative are represented in Fig 2 and the others are listed in the supporting information.

Similar to the protein-coding genes, the highest number of differentially expressed ncRNAs is found for 1C1W, where in contrast to proteins, more genes were up-regulated (30) than down-regulated (6). As for the coding genes, the smallest number of strongly regulated genes is found for 45C1W (Fig 1B).

Exposure to 1°C compared to 37°C

After a short-term exposure to 1°C, the major changes in the metabolic profile of. *E. dermatitidis* are visible in the lipid metabolism, and in particular the glycero- and glycerophospho-lipids (Fig 2A and S1 Table). Post-chaperonin tubulin folding pathway and cellular development are the next up-regulated processes in this dataset (Fig 2A and S1 Table). This is in line with results showing that cold shock response induces genes related to cytoskeleton rearrangement [47,48]. Genes related to the cell cycle and DNA-checkpoints are also up-regulated (S1 and S10 Tables).

In contrast, processes related to the nuclear envelope, the protein export from the nucleus, the protein-DNA complex and the DNA conformation change are down-regulated (S2 Table). Moreover, *E. dermatitidis* reacts to 1°C temperature by slowing down mitosis and the consequent cellular division, as well as the transcription machinery.

After exposure to 1°C for 1 week the metabolism of glycerol 3-phosphate (G3P) was strongly upregulated (Fig 2B and S3 Table). Other overrepresented BP terms of upregulated genes are related to hexose, carbohydrate and carbohydrate derivative metabolism (S3 Table).

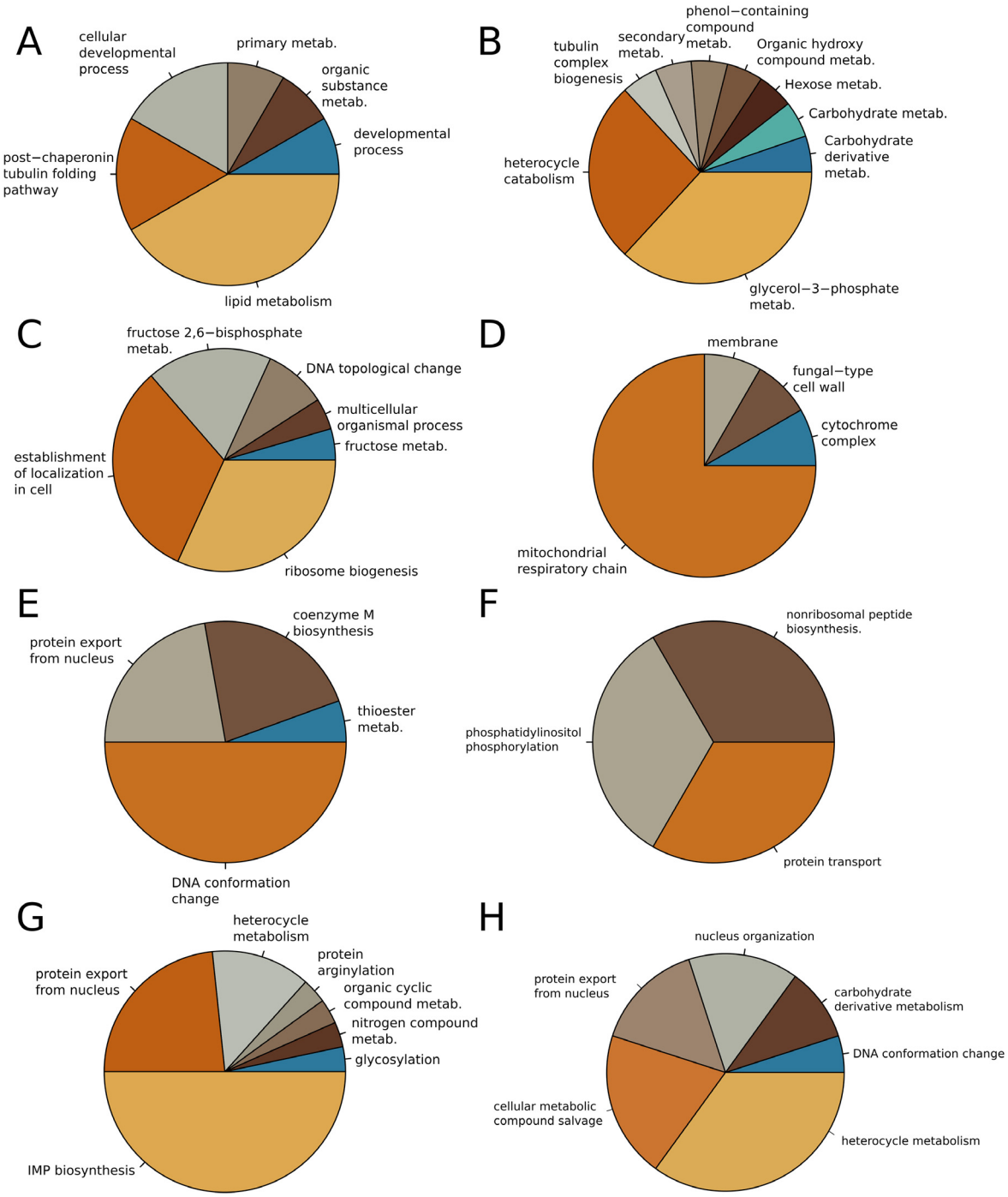

Fig 2. Pie chart of the summarized Gene Ontology (GO) terms for different conditions. (A) Over-represented BP-related GO terms for the genes upregulated at 1°C for 1 hour. (B) Over-represented GO terms related to biological processes (BP) for the genes up-regulated at 1°C for 1 week. (C) Over-represented BP-related GO terms for the genes down-regulated at 1°C for 1 week. (D) Over-represented CC-related GO terms for the genes up-regulated at 1°C for 1 week. (E) Over-represented BP-related GO terms for the genes down-regulated at 45°C for 1hour. (F) Over-represented BP-related GO terms for the genes up-regulated at 45°C for 1 week. (G) Over-represented BP-related GO terms for the genes down-regulated at 45°C for 1 week. (H) Over-represented BP-related GO terms for the genes up-regulated at both 45C1H and 45C1W.

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Similarly to the 1C1H stress condition, *E. dermatitidis* down-regulates the ribosome biogenesis and rRNA processing at 1C1W (Fig 2C), indicating that, in general, cold stress represents a stimulus for slowing down the transcription machinery. We also see a concomitant decrease of histone acetylation ($p = 0.035$), known to be correlated with decreased levels of gene expression [49]. The nuclear transport and nuclear organization are highly represented among the down-regulated genes, confirming that the fungus had slowed down nuclear activity. Exocytosis, membrane docking and vesicle mediated transport are also down-regulated processes. Finally, on the cellular component level, there is an enrichment of terms related to the cellular and mitochondrial respiratory chain for the up-regulated genes (Fig 2D), while for the down-regulated genes, the nuclear envelope is over-represented (S11 Table).

We examined further the genes that were similarly regulated during the short and long exposure to 1°C compared to 37°C. At 1°C, the genes that are commonly up-regulated are related to the lipid metabolism, post-chaperonin tubulin folding pathway and cellular developmental process. The common down-regulated genes are instead related to protein export from nucleus and purine nucleoside salvage.

Exposure to 45°C compared to 37°C

After 1 hour exposure to 45°C, the up-regulated genes are all related to DNA metabolism. In particular, as shown in S5 Table, we could observe an over-representation of biological processes related to deoxyribose phosphate metabolism, alanyl-tRNA aminoacylation and DNA replication initiation, strongly indicating that the fungus is actively synthesizing DNA. This is further supported by the finding that the MCM (mini chromosome maintenance) complex, the core of eukaryotic replicative helicase, is also up-regulated (S14 Table). Among the down-regulated processes (Fig 2E and S6 Table) DNA conformational change was found, as well as DNA packing and mitotic chromosome condensation, indicating that short exposure to 45°C shifted the fungus into an active replication stage.

The main biological processes activated after a 1 week exposure to 45°C are related to phosphatidylinositol (PI) phosphorylation, protein transport and nonribosomal peptide biosynthesis (Fig 2F and S7 Table). Phosphatidylinositol phosphorylation leads to phosphatidylinositol 4-phosphate (PI-4-P) and the latter is known to be prevalent in Golgi apparatus membranes, where it is responsible for recruiting proteins that need to be carried to the cell membrane [50]. The large number of biological processes that are down-regulated at 45C1W, like the nuclear envelope organization or protein export from the nucleus, (Fig 2G and S8 Table for the complete list) seems to indicate a cell in a kind of quiescent state compared both to the exposure to its optimal temperature and to the stress exposure at 45°C.

No gene was found to be significantly down-regulated at both 45C1W and 45C1H compared to 37°C. On the other hand, 30 GO terms were found to be significantly over-represented in the group of genes that are strongly up-regulated at both 45C1H and 45C1W (Fig 2H). Those terms are related to DNA conformation change, protein export from the nucleus, cellular metabolic compound salvage, carbohydrate derivative metabolism; heterocyclic metabolism and nucleus organization.

Aside from comparing two temperature conditions, we also looked at genes with similar expression patterns along the temperature profile with cummeRbund [51]. We concentrated on the group of genes with high expression at low temperature and low expression at 45°C (S3 Fig). Interestingly the genes in this cluster were enriched in GO terms related to trehalose, a potent cryoprotectant whose concentration was shown to increase with decreasing temperature [47,52]. HMPREF1120_00310, one of the member of the cluster, is involved in the desaturation of membrane fatty acid, a process that fluidizes the cell membrane at low temperature [53,54].

Pathway analysis

Similar to the GO enrichment terms, KEGG pathway enrichment was performed for all four conditions. In contrast to the GO terms analysis, significant enrichment of KEGG terms was only found for genes down-regulated at 1C1W. Under this condition the fatty acid degradation pathway, as well as the tryptophan metabolism, contain significantly more down-regulated genes than expected, with p-values of $3.3x10^{-6}$ and $1.6x10^{-7}$, respectively. While fatty acids are of utmost importance for the regulation of the cell membrane fluidity, it was only recently shown that tryptophan plays a role in fungi under cold conditions [55].

Pathways related to cell wall biosynthesis, cell wall integrity, melanin biogenesis and trehalose metabolism, were analyzed for genes that were highly (more than eight-folds) up- or down-regulated.

The highest number of pathways containing genes strongly regulated was seen again at 1C1W. For example, under that condition, the three melanin pathways contain mainly down-regulated genes (S17 Table) indicating that melanin production is probably diminished under cold condition.

From the 6 pathways related to cell wall stress response, only 1,3-α-glucan synthesis and processing and 1,3-β-glucan synthesis and processing showed important regulation of their genes upon temperature shift. More precisely Alpha-amylase (HMPREF1120_03460), the only significantly regulated gene in the 1,3-α-glucan synthesis and processing pathway, was up-regulated at 1C1W, in line with results previously found in wheat grain [49].

From the 4 differentially expressed genes in the 1,3-β-glucan synthesis pathway, Glucan 1,3-beta-glucosidase, extracellular cell wall glucanase Crf1 and Glycosyl transferase are down-regulated at 1C1W, while endo-1,3(4)-beta-glucanase is up-regulated at 1C1W.

Trehalose is very well known for its involvement in abiotic stress resistance in plants and fungi and its synthesis has been related to salt, drought, heat and cold stress in many organisms [47,52,56]. Since the induction of trehalose synthesizing enzymes is a typical response to near freezing temperatures in yeasts [47,52], we pointed out which genes of the trehalose metabolism were regulated in the case of long-term exposure to 1°C. Trehalose is synthesized through the processing of α-D-glucose-1P. The pathway starts with α-D-glucose-1P which is converted by UTP-glucose-1-phosphate transferase, an enzyme that is expressed four times more at 1C1W than at 37C, to UDP-glucose. UDP-glucose is then processed by UDP-glucose 6-dehydrogenase or glycogen synthase, two enzymes that do not lead to trehalose, or by trehalose 6-phosphate synthase, leading to trehalose-6P and subsequently to trehalose. At 1C1W the 6-dehydrogenase and glycogen synthase enzymes are down-regulated by a factor of at least 4, increasing the amount of UDP-glucose to be processed by trehalose 6-phosphate synthase, which is itself up-regulated by a factor of at least 4, into trehalose-6P and subsequently into trehalose (S1 Fig).

Fused transcripts

A totally novel finding in *E. dermatitidis* is the presence of fused transcripts, i.e. annotated transcripts connected by split-reads. They are found under all conditions, with the highest and lowest number of fused transcripts found at 1C1W (873) and 37C (82), respectively (S19 Table). The number of split-reads and the corresponding connected genes were dependent on the experimental conditions. At 1C1W, the fused transcript with the highest number of split-reads (130) is made out of MC family mitochondrial carrier protein (HMPREF1120_06233) and L-fuculose-phosphate aldolase (HMPREF1120_06484) (Fig 3A). Aldolase was previously shown to be up-regulated under cold stress in *Arabidopsis thaliana* [57].

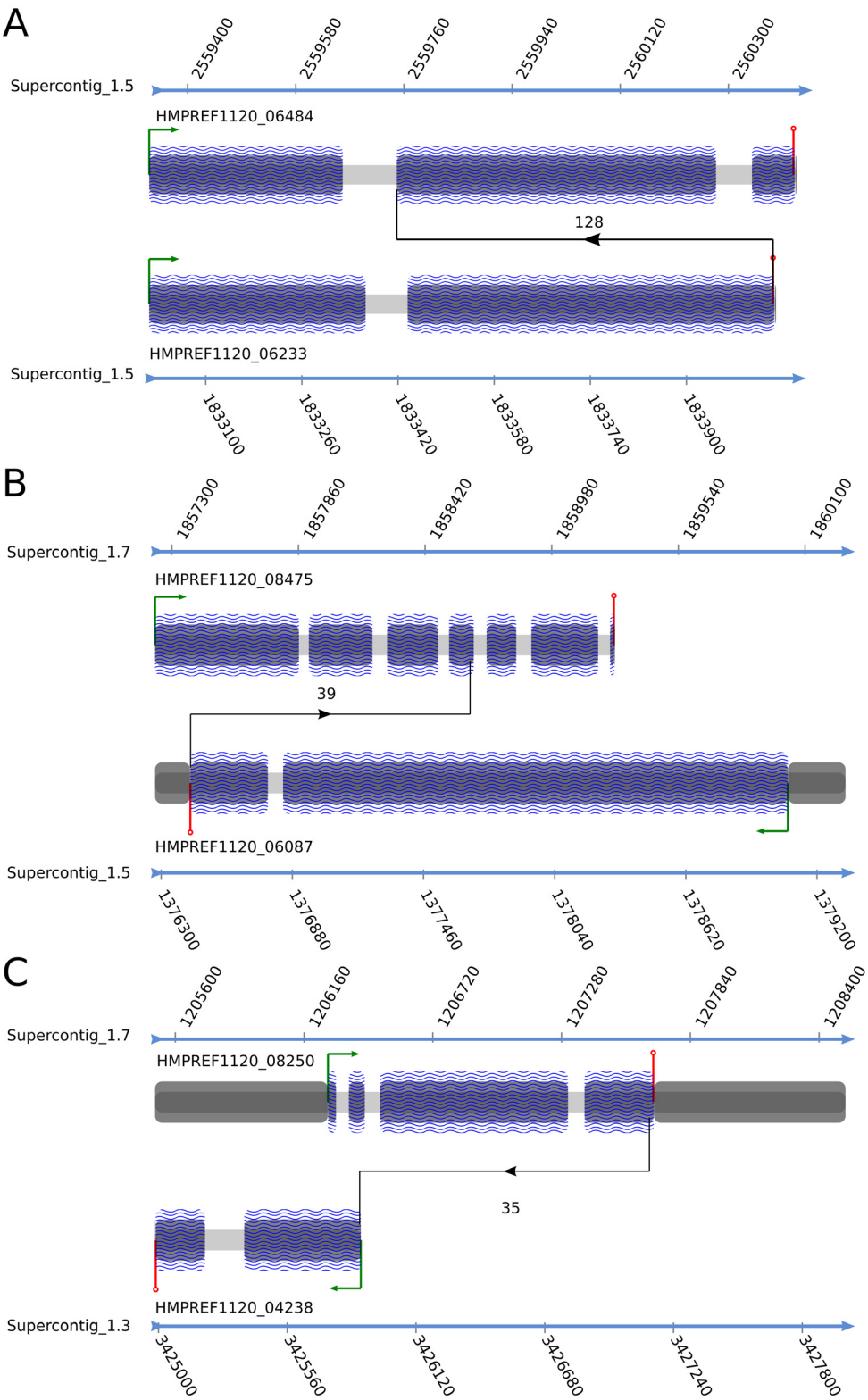

Fig 3. Graphical representation of the chimeric RNAs with the highest number of split reads for different temperatures. Fusion transcripts present at the following experimental conditions: 1°C for 1 week (A), 37°C (B) and 45°C for 1 week (C). The thick blue arrow represents the Supercontig with the corresponding scale. The start codon is represented by the green arrow. The stop codon is shown as a red

vertical line. The splice connecting both genes is shown in black and the number of supporting split reads is located between both fused genes. The blue regions represent the Coding DNA Sequence (CDS), the dark grey regions represent the Untranslated Regions (UTRs), while the light gray regions represent introns. The splicing direction is given by the black arrow head.

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At 1C1H, the fusion transcript with the highest number of trans-splicing events (128) is composed of an amidase and translation initiation factor eIF-4F. At 37C, HMPREF1120_08475, an ankyrin-containing protein, is connected to V-type proton ATPase subunit A (HMPREF1120_06233), two proteins involved in the energy metabolism (Fig 3B). Interestingly, in the bat *Myotis brandtii*, V-type proton ATPase subunit B is reported to contain 3 ankyrin domains [58].

At 45C1H, the second coding exon of Glucose-6-phosphate isomerase (HMPREF1120_08503) is connected to the first coding exon of HMPREF1120_03038 through 36 split reads, an homolog to Adhesin protein Mad1. Finally at 45C1W, the whole coding region of HSP40 (HMPREF1120_08250), a molecular chaperone involved in the heat shock response, is ligated through 35 reads to the whole transcript of HMPREF1120_04238, a homolog of an uncharacterized Glycosylphosphatidylinositol (GPI)-anchored protein found in various fungi (Fig 3C). This fusion transcript, with its heat shock domain, fits nicely with the condition and with the up-regulated phosphatidylinositol phosphorylation process at 45C1W.

NcRNAs annotation

A total of 221 ncRNAs were annotated in the genome. All major ncRNA families, with the exception of miRNAs, were found (S20 Table). Similar to the protein coding genes, ncRNAs are differentially expressed. The highest and lowest number of regulated genes are found at 1C1W (30 up- / 6 down-regulated) and 45C1W (11 up- / 2 down-regulated) (Fig 1B), similar to what was found for the protein coding genes. However, in contrast to mRNAs, at 1C1W more ncRNAs are positively regulated than negatively. Eight ncRNAs, all belonging to the class of snoRNA, are located in introns of protein coding genes. Previous reports on intronic snoRNAs [59] showed that the regulation pattern between the intronic snoRNAs and their host genes might differ, something that is also seen in *Exophiala dermatitidis* (S2 Fig).

All tRNAs, with the exception of trp-tRNAs, were found. 26 tRNAs contain an intron of varying length. Both tRNA-halves and introns of these spliced-tRNAs showed temperature-dependent expression modulation (Fig 4A).

Circular RNA

Circular RNAs (circRNAs) were investigated by examining reads that contained apparent splice junctions connecting the end (start) of a split read fragment to the start (end) of a downstream (upstream) fragment. The number of such split-reads was strongly influenced by the experimental conditions (S18 Table). The highest number of circular split-reads were found at 1C1H (35877) and the smallest number at 1C1W (4215). Circular RNAs were divided into short circular RNAs, e.g. transcripts covered by circular split reads whose ends were not separated by more than 200 nts and long circular RNAs, where the circular split reads connected distinct exons/introns.

Like the other classes of RNAs, circRNAs show a temperature dependent expression. This is clearly seen for the circRNA located inside HMPREF1120_01344. Its expression is at its peak at 1C1W, while under other conditions, the expression level is strongly down-regulated (Fig 4B). Two cases of circular RNAs overlapping ncRNAs were found. At 1C1H and 45C1H, the intron of the spliced His-tRNA located at Supercontig_1.5 1803599 1803694, is reported to

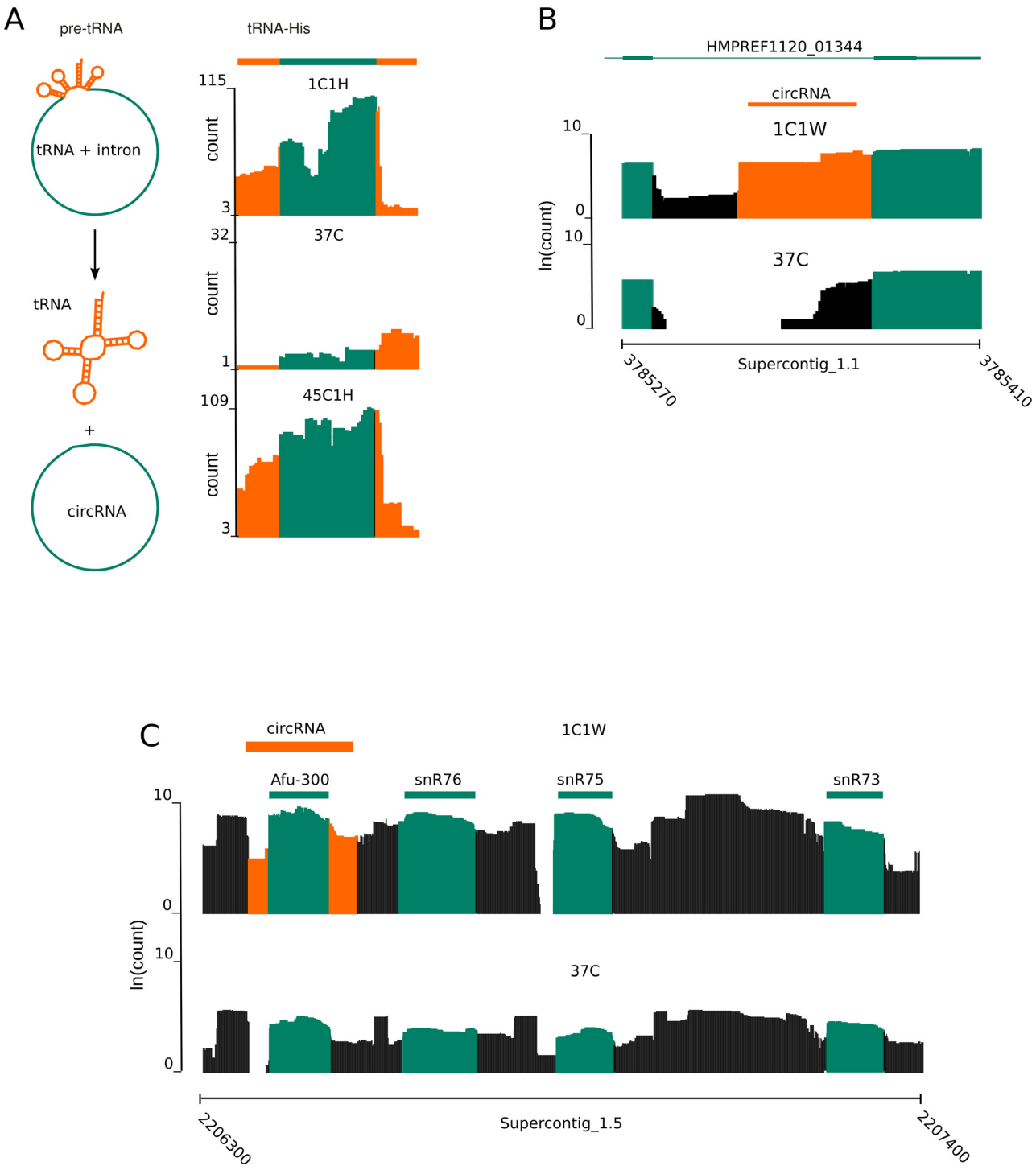

Fig 4. Circular RNAs are differentially expressed. (A) Circular RNA found inside the intron of a tRNA. The two tRNAhalves are shown in orange, while the circular RNA is shown in green. The circularization of the tRNA-His intron happens only after the short-term treatment (1 hour) at 1°C and 45°C. Both halves of the tRNA-His show unconstrained temperature-dependent regulation. *(B)* Log10Count of the reads mapping in the region around the circRNA (orange) located in the intron of HMPREF1120_01344 (green). The expression level of the circRNA is higher upon exposure at 1°C for 1 week than at the control condition (37°C). (C) Expression profile log10(count) at 1°C for 1 week and 37°C of a circRNA overlapping with an homolog of Afu_300 snoRNA (green). For completeness, the snoRNAs belonging to the same snoRNAs cluster are also shown.

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be circularized (21 split reads at 1C1H, 19 split reads at 45C1H) (Fig 4A). Circularized tRNA introns were previously reported in *Haloferax volcanii* [60] and various other archea [61].

The other example is found for 1C1W where the snoRNA homolog to C/D-snoRNA Afu_300 is reported to be located inside a circularized region of 165 nucleotides (Fig 4C). Circularized snoRNAs were previously reported in *Nanoarchaeum equitans [62]* as well as *Pyrococcus furiosus* [61]. The region located around Afu_300 is interesting as it contains a cluster of 4 snoRNAs (snR73, snR76, snR78, Afu_300) that is found in other fungi [63] (Fig 4C).

The circular RNA located in the his-tRNA and the most highly expressed circular RNA at 1C1W (115 sustaining split reads) were tested with RT-PCR (reverse transcriptase PCR) and both were confirmed (see S4 Fig).

Discussion

In this study we focused on the transcriptome of *E. dermatitidis* and its variations under three temperature conditions corresponding to its known environments, i.e. cold water (1°C), human body (37°C) and the warm sites found in different human habitats (45°C).

At 1°C, the cell is mainly focused on becoming adapted to the cold and regulates different processes. The lipid metabolism is the pathway that is most strongly regulated. The modification of the lipidome is a typical response to temperature stresses in both uni- and multicellular organisms [47]. This can be explained by the fact that, at low temperatures, the cell membrane becomes rigid, compromising membrane-associated cellular functions, hindering membrane-bound enzymes, slowing down diffusion rates and inducing cluster formation of integral membrane proteins [64]. However, cells have a large repertoire of processes to increase the membrane fluidity: e.g. the modification of the ratio of polyunsaturated to saturated fatty acids as well as the integration of more sterols into the membrane phosphate bi-layer ensuring membrane integrity and stability [47,53,65]. In our data we found that HMPREF1120_00310, a gene whose yeast homolog is involved in the desaturation of fatty acid, is up-regulated at low temperature and that ergosterol biosynthesis pathway is enriched in genes that are up-regulated at low temperature (p-value 0.02).

Glycerol 3-phosphate metabolism (G3P) is the main active process when the fungus is exposed long-term to 1°C. It has already been established that G3P is mainly involved in the anabolism of triacylglycerols, being the backbone for the biosynthesis of all phospholipids, and it can enter the glycolytic pathway after being oxidized to dihydroxyacetone phosphate. Nevertheless, it is also a major donor of electrons in the mitochondrial respiratory chain. According to our set of genes up-regulated at 1°C, it seems that the latter could be its role under long-term cold exposure (Fig 2D). Thus, the fungus is metabolically active at 1°C.

The heterocyclic catabolism is, after the G3P metabolism, the second most represented process at 1W1C. It leads to the proline catabolic processes and to glutamate biosynthesis (S3 Table). The important role of glutamate in cold stress resistance has previously been reported in different eukaryotes [66–68]. Moreover, Takagi *et al.* have demonstrated how, in yeasts, proline and charged amino acid such as arginine and glutamate exhibit cryoprotective activities almost comparable to that of threalose and glycerol [56]. In summary, with the support of the data related to trehalose pathway, we can conclude that the cryoprotection in this fungus is achieved by the increase of both proline and intracellular trehalose.

Genes related to cell cycle checkpoints are up-regulated after short-term exposure to 1°C, presenting a cell in an alert state. The cell cycle slowdown is a typical response to cold stress, since it has been observed both in prokaryotes [65] and eukaryotes [47,66]. In yeasts, in particular, it is described as a characteristic response to near-freezing temperature (<10°C), although *S. cerevisiae* still actively grows when exposed to temperatures between 10 and 18°C [47].

Finally, genes up-regulated over both long and short exposures to 1°C are enriched in GO terms related to the post-chaperonin tubulin folding pathway as well as the tubulin complex biogenesis processes. Interestingly, it was previously reported in *S. cerevisiae* that the cytoplasmic chaperonin CCT is a cold shock protein and that its main cellular substrates are actine and tubulin [69].

The number of processes modified by the exposure of *E. dermatitidis* at 1°C is much larger than those found at 45°C. For example, no alteration of the lipid-related pathways could be observed at 45°C, indicating that this temperature does not represent a stress condition for the membrane. Furthermore DNA packing and mitotic chromosome condensation are down-regulated during the short 45°C exposure, indicating that the fungus is in a fully replicative state. Taken together the replicative state and the missing modification of membranes indicates that 45°C—albeit being beyond the optimal growth temperature—does not yet induce any cellular stress.

Finally, increased activity of the Golgi apparatus was observed following long-term exposure to 45°C. The importance of the Golgi apparatus at 45C1W is underlined by the enrichment in genes related to the COPII vesicle coat, a type of coat protein that transports other proteins from the endoplasmic reticulum to the Golgi apparatus [70].

Beyond the mere mRNAs, ncRNAs, fusion transcripts as well as circRNAs exhibit temperature-dependent regulation. Moreover, intronic snoRNAs and circRNAs show a regulation pattern different from that of the host-gene. Environmental changes can modulate the expression of house-keeping ncRNAs, like snoRNAs, snRNAs or Rnase [59,71]. With the exception of a few examples [59], the role of these basal RNAs in case of stress conditions is still poorly understood. Even if the function of circRNAs in fungi is still unknown [72,73], their proven resistance to denaturation and to enzymatic degradation [74] might play a role in fungal stress resistance as well as virulence.

Conclusion

Exophiala dermatitidis is an important model organism in view of fungal ecology but also in view of new and emerging mycosis threatening human health. As mentioned in the introduction, the fungus has been isolated from glaciers, from cold and hot tap water, from dishwashers and sauna facilities, and the clinical occurrence of the fungus as an agent of cutaneous, subcutaneous and systemic mycosis is increasing. Hitherto, effective therapies and drugs have not been developed, a consequence of the lack of knowledge of the ecology of the fungus and its virulence factors. In this study the acclimatization of *E. dermatitidis* to temperatures ranging from 1°C to 45°C was studied because temperature plays an important role in the fungus pathogenicity. Our molecular observations confirm that *E. dermatitidis* can survive at a wide range of temperatures. While it shows some stress responses when exposed to 1°C—like DNA and cell cycle checkpoints and modification of membranes—45°C does not seem to induce any stress response in the cell. However, the fungus—after having acclimatized to 1°C—is metabolically active even at this temperature.

The wide ecological amplitude and the ability to be active at low and high temperatures, which might in part be due to the capacity of the fungus to fuse distinct mRNAs to create the necessary proteins on-the-fly, and is probably responsible for the success of *Exophiala dermatitidis* in thriving from its natural habitat into the warm human environment and as a pathogen into the human host.

Our study is part of an emerging research trend that applies the use of Next Generation Sequencing tools to the study of many relevant pathogenic fungi [75–77] with the aim of deepening the knowledge of the gene expressions that underlie their pathogenicity. Together with the

work published in [27] and by Robertson *et al.* [6] that also focus on the transcriptome of *E. dermatitidis*, our work is pioneering for this fungus.

Getting a deeper understanding of the cellular mechanisms of the fungus, both on the protein and RNA levels, is a first step towards the understanding of *E. dermatitidis* pathogenicity and will pave the way for the development of novel drug targets for effective therapies against the emerging mycosis caused by this fungus.

Supporting Information

S1 Fig. Starch and sucrose metabolic metabolic pathway. Each rectangle correspond to an enzyme. White rectangles correspond to enzymes not found in S. cerevisiae. Green rectangles correspond to enzymes present in S. cerevisiae. Red coloured rectangles are downregulated genes at 1C1W with respect to 37C. Blue coloured rectangles are upregulated genes at 1C1W compared to 37C. ⇡ and ⇣ represent a up- and downregulation smaller than a factor two. ↑ and ↓ represent a 2 to 4 fold up- and downregulation, respectively. ⇑and ⇓represent a 4 to 8 fold up- and downregulation, respectively. ⤊ and ⤋represent a more than 8 fold up- and downregulation, respectively.
(TIFF)

S2 Fig. Expression profile of an intronic snoRNA homolog to Afu_191. The snoRNA corresponds to the green line located in the host gene HMPREF1120_04106 (yellow line). The log10 (count) are reported for each temperature. Upon exposure at 1°C for 1 week, the snoRNA is at its highest expression, while the host gene is lowly expressed, clearly indicating that the snoRNA and mRNA expressions are not always related.
(TIFF)

S3 Fig. Expression profile of the 10 closest genes with respect to the medoid of cluster 1.
(TIFF)

S4 Fig. Agarose gel of RT-PCR of tested circular RNAs. cRNA1.4 is the most highly expressed circRNA at 1C1W with 115 sustaining reads. The expected size of the amplicon is 80bp. cRNA1.5a/b are located inside the his-tRNA intron (See Fig 4a). Two different primer pairs were used for cRNA1.5a and cRNA1.5b, leading to an amplicon size of 71bp and 70bp, respectively. The second amplicon at around 700bp is probably due to an unspecific binding of the primers couple on transcript HMPREF1120_08504T0 as reported by RNAplex.
(EPS)

S1 Table. List of over-represented GO terms in the Biological Process category for the genes up-regulated at 1°C for 1 hour.
(DOC)

S2 Table. List of over-represented GO terms in the Biological Process category for the genes down-regulated at 1°C for 1 hour.
(DOCX)

S3 Table. List of over-represented GO terms in the Biological Process category for the genes up-regulated at 1°C for 1 week.
(DOCX)

S4 Table. List of over-represented GO terms in the Biological Process category for the genes down-regulated at 1°C for 1 week.
(DOCX)

S5 Table. List of over-represented GO terms in the Biological Process category for the genes up-regulated at 45°C for 1 hour.
(DOCX)

S6 Table. List of over-represented GO terms in the Biological Process category for the genes down-regulated at 45°C for 1 hour.
(DOCX)

S7 Table. List of over-represented GO terms in the Biological Process category for the genes up-regulated at 45°C for 1 week.
(DOCX)

S8 Table. List of over-represented GO terms in the Biological Process category for the genes down-regulated at 45°C for 1 week.
(DOCX)

S9 Table. List of over-represented GO terms in the Cellular Components category for the genes down-regulated at 1°C 1 hour.
(DOCX)

S10 Table. List of over-represented GO terms in the Cellular Components category for the genes up-regulated at 1°C for 1 hour.
(DOCX)

S11 Table. List of over-represented GO terms in the Cellular Components category for the genes down-regulated at 1°C for 1 week.
(DOCX)

S12 Table. List of over-represented GO terms in the Cellular Components category for the genes up-regulated at 1°C for 1 week.
(DOCX)

S13 Table. List of over-represented GO terms in the Cellular Components category for the genes down-regulated at 45°C for 1hour.
(DOCX)

S14 Table. List of over-represented GO terms in the Cellular Components category for the genes up-regulated at 45°C for 1 hour.
(DOCX)

S15 Table. List of over-represented GO terms in the Cellular Components category for the genes down-regulated at 45°C for 1 week.
(DOCX)

S16 Table. List of over-represented GO terms in the Cellular Components category for the genes up-regulated at 45°C for 1 week.
(DOCX)

S17 Table. Melanin biosynthesis pathway genes regulated by at least a factor 8 compared to the control at 37°C.
(DOCX)

S18 Table. Number of circular split reads returned by the segemehl:testrealign approach.
Splits with the passed flags were retained. * The number of splitreads is at least 10. The distance between the mapping position of the read end and read start is less than 200 nts. **Reads end and Reads start are not on the same exon, intron, the number of splitreads is at least 10 and

reads end and start are falling close (less than 20nts) to an intron/exon boundary.
(DOCX)

S19 Table. Number of linear splits returned by the segemehl:testrealign approach. Only splits with the passed flags were retained. **The number of split-reads is at least 10 and reads start and end are falling close (less than 20nts) to an intron/exon boundary.
(DOCX)

S20 Table. Number of annotated ncRNAs genes for different ncRNA families.
(DOCX)

S21 Table. Raw number of reads and mapped number of reads for the 5 experimental conditions.
(DOCX)

Author Contributions

Conceived and designed the experiments: DT KS. Performed the experiments: BB. Analyzed the data: HT BB. Contributed reagents/materials/analysis tools: HT. Wrote the paper: KS HT BB DT.

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Natural Science

A Review beyond the borders: Proteomics of microclonial black fungi and black yeasts

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ABSTRACT

Black microcolonial fungi and black yeasts are inhabitants of extreme environments like vulcanic, desert and polar regions, where they are exposed to enhanced temperature alterations and desiccation. They have developed, therefore, extraordinary biologic characteristics which are mainly based on the expression of proteins, however, these are rarely studied and known. The review article presented here is focused on the obstacles and solutions for the proteomic analyses of this very particular fungal species.

Keywords: Sample Preparation; Protein Solubilities; Extremophiles; Fungi

1. INTRODUCTION

Black micro-colonial fungi (MCF) and black yeasts are the most successful inhabitants of bare rock on the surfaces and inside in desert-like regions [1] as well as ubiquitous dwellers of rock in other climatic zones [2-5]. Generally they exhibit high tolerance to stress and climate changes [6]. The cell composition of the MCFs consists majorly of lipids (e.g. mono-, di- and triacylglycerols, phosphotidilcholine, phosphatidylethanolamines, sterols, sterol ethers, phosphatidilrthanolamines, and free fatty acids) and pigments (e.g. melanins, carotenoids in colorless and brown-red formations) and a very complex and dense cell wall made of chitin, melanin and polysaccharides [7]. The cell wall is also the main obstacle in protein extraction from fungal biomass. Biologically, however, all the components are involved in machinery, which enable the MCFs to be the most resistant eukaryotic organism on the Earth in an extremely stressful climate like deserts, volcanos or polar environments as well as in radioactive areas [8,9]. Experiments showed that theses terrestrial organisms are even capable of surviving the severe and hostile conditions in outer space over extended periods of time [10]. Some of these features might also be responsible for the human pathogenicity of some black yeasts like e.g. *Exophiala dermatitidis.*

The extreme stress tolerance of MCFs, however, is a virgin field of research. Except knowledge about their phenotypes, very slow growth rate and a complex structural composition, there are still very rare proteomic approaches generated about the system biology of black MCF [11,12]. The major challenge is to understand the expression, function/regulation of the entire set of genes/proteins encoded by fungal genomes. Up to now full genomic sequences of only two strains of black fungi—*Exophiala dermatitidis* and *Coniosporium apollinis*—were done and are available in international databases (http://www.broad.mit.edu). The genomic data about MCFs are relatively rare because DNA extraction and purification for Next Generation Sequencing approaches has several obstacles. As mentioned above this is mainly the rigid cell wall and the high melanin content. Sequencing of further strains, however, is in progress. As compared to functional genomics approaches by transcriptome analysis (e.g. by microarrays), while a proteomics approach is more direct, transcriptomics investigates the all over change of gene expression at mRNA level. Proteomics leads to more reliable conclusions, since proteins are directly related to biological functions and phenotypes [13].

The lack of proteomic and genomic data for MCFs indeed indicates major analytical and methodological challenges and therefore there are only a few reports on the proteomic stress response of MCFs to different environmental factors [11,12]. The main bottleneck, however,

is the sample preparation prior to analysis which is going far beyond the routine efforts.

2. SAMPLE PREPARATION

2.1. Sample Homogenisation

The fungus cell disruption is performed using different approaches by mechanical or enzymatic methodologies [14]. Since black fungi possess extraordinary rigid cell walls, the effective cell disruption is a crucial step for proteomic studies. The MCFs must be cultivated on a solid media or rigid background like cellulose sheets and the collection of the sample is, therefore, laborious and time consuming (**Figure 1**).

Mechanical cell disruption using glass beads in beating mills is, therefore, a preferred approach [15-19]. The mill can be also thermostatised at 4˚C avoiding increased temperatures by heat generation and protein lysis due to intracellular proteases or denaturation. The milling can be also performed using mortar and pestle with liquid nitrogen [20]. Freeze drying can be also used as a disruptor before the milling procedure.

In this frame, the mechanical cell disruption can be combined with different homogenization buffers to enhance the disintegration of cell walls and inhibition of proteases activity [14]. Different concentrations of urea (7 - 9 M) in combination with 2 M thiourea are used as a strong chaotropic to re-solubilize the proteins in filamentous fungi [21]. Note that 7 M urea is a saturated solution and any concentrations above this value enter the oversaturation level, which means that the buffer is only in liquid form at room temperature or higher. The ignoring of the saturation limit will end up in crystallization and drying of IPG-strips during the in-gel rehydration. Thiourea can also interfere with different protein determination procedures later on. Tris or phosphate buffers at 10 - 30 mM were used to keep the pH constant and/or to be compatible with DIGE-labeling [22]. Further, the addition of NaOH at 0.1 - 1 M can enhance the protein solubility and destabilize the cell wall tremendously [23]. As a non-ionic detergent CHAPS is used at the concentrations of 1% - 4%. It is recommendable to try different concentrations of CHAPS for the most desirable protein yield, which is to be optimized case to case [14]. DTT and ß-mercaptoethal were used as reducing agents in the homogenization buffer [24,25]. They reduce the disulfide bonds and enhance the protein solubility. Even ampholytes at a concentration of 0.5% to 0.8% are used for increased protein solubility, however it must be taken into consider for the focusing procedure [26]. Proteases inhibitor cocktails or PMSF are added to the homogenization buffer to deactivate the strong fungal proteases and lysis of the proteins [10]. Addition of proteases inhibitors can potentially disturb the iso-electric focusing and can be skipped carefully if urea and/or thiourea are available in the homogenization buffer or the procedure is performed at 4˚C.

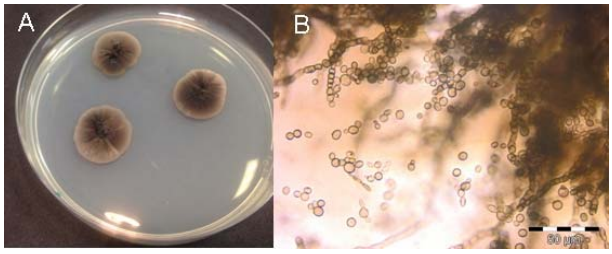

Figure 1. Colonies of *Exophiala dermatitidis* cultivated on solid media. Pictures taken by A) stereo microscope and B) optical microscope.

Enzymatic and chemical cell wall lysis was also used with less success for fungi samples [14]. Taken into consideration that black MCF is characterized by a more robust cell wall than filamentous fungi types, the solubilization of their proteins remain the main challenge for proteomic analyses. Therefore the selection of an appropriate method can enhance the initial protein amounts must be optimized for each species.

2.2. Protein Enrichment

An optimal proteomic analyses needs 1 - 5 µg/µL of soluble proteins. The most popular strategy for protein enrichment remains precipitation using organic solvents like TCA or phenol. Several approaches are described and optimized by different researchers; however there are still no general protocols available to extract proteins from any given sample [27]. The combination of TCA and acetone is reported as effective and easy for precipitation of fungal proteins [28,29]. Other strategies like 100 mM ammonium acetate in methanol were described also for fungal tissues [21]. However, TCA precipitation can disturb the iso-electric focusing even after multiple washing cycles using 80% acetone. The re-solubilization of protein pellet is significantly reduced after acidic precipitation, which affects the final protein yield. To avoid this problem the pellet must be dried very fast (1 - 2 minutes) before re-solubilization [14]. The main obstacles in protein extraction from black fungi are the high complexity of fungi matrices containing melanins, pigments, polysaccharides, chitin and lipids. Further, the fungal protein solubility is rather low and tends towards spontaneous precipitation. Both factors caused very low protein yield and high content of sample impurities which contain mainly melanins and various dark colored pigments interfering with protein determination and separation.

This might be also the reason for the lack of even one single proteomic study with black MCF or even with

black yeasts [11]. The troubles caused by impurities could be overcome using different precipitation strategies for 2DE analysis [12]. However, the extracted amounts of the proteins were limited and could be first visualized by silver staining.

In order to increase the yield of the protein during extraction and precipitation different strategies and additives were needed (**Table 1**). A TCA/Aceton/phenol-based precipitation protocol helped to separate the colored impurities from the protein extract before main protein extraction and precipitation. The protein pellet must then be re-solubilized by shaking with urea/thiourea for several hours. However, the yield of protein remained nearly the same, even by increased amounts of initial biomass. Since black MCFs are inhabitants of dry environments they comprise a protein repertory which is biologically active in water-free structures. This very unique characteristic implicates high abundance of less water-soluble proteins, which are susceptible to irreversible precipitation. It seems that the soluble proteins were at their solubility limits, so that their amount and concentrations could not be increased without the risk of a re-precipitation.

To increase the protein yield the addition of 2% SDS can be used during the homogenization before precipitation. As a second strategy NaOH was added to homogenization buffer during the milling of biomass. Both strategies could enhance the protein solubility signifi-

Table 1. Different sample preparation approaches to extract proteins from *Exophilia dermatitis*.

Species	Biomass (mg)	Grinding methods	Homogenization buffer	Solubilisation buffer	Average protein concentration (µg/µl)
Exophiala *dermatitidis*	500	Lyophilization	Washing steps with 10% TCA/Acetone, 0.1 M ammonium acetate in 80% MeOH an 80% acetone.	4 M Urea, 2% CHAPS	~ 0.60
Exophiala *dermatitidis*	500 - 1000	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	50 mM Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose	7 M Urea, 2 M Thiourea, 4% CHAPS	~1.5 - 2.0
Exophiala *dermatitidis*	1000 - 1500	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	50 mM Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose	4 M Urea, 2% CHAPS	~ 0.80
Exophiala *dermatitidis*	700	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	50 mM Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose, 1 mM TCEP, 2% SDS	7 M Urea, 2 M Thiourea, 1% CHAPS, 1 mM EDTA	~2.00
Exophiala *dermatitidis*	2700	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	50 mM Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1%PVPP, 30% Sucrose, 1 mM TCEP, 2% SDS	300 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS	0.80 - 1.70
Exophiala *dermatitidis*	2700	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	50 mM Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose, 1 mM TCEP, 2% SDS	300 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS	0.50 - 0.80
Exophiala *dermatitidis*	4700	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	50 mM Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1%PVPP, 30% Sucrose, 1 mM TCEP, 2% SDS	300 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS	1.10 - 1.20
Exophiala *dermatitidis*	700	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	0.5 M Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose, 2% SDS, 4% CHAPS	300 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS	~0.30
Exophiala *dermatitidis*	700	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	0.5 M Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose, 2% SDS	300 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS	~0.40
Exophiala *dermatitidis*	700	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	0.5 M Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose, 4% SDS	300 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS	~0.50 - 0.70
Exophiala *dermatitidis*	700	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	50 mM Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose, 2% SDS + 0.1 - 1 M NaOH	300 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS	2.5 - 3.0
Exophiala *dermatitidis*	700	Mortar grinding under liquid nitrogen followed cell disruption using a glass bead beating system	50 mM Tris-Buffer pH8.5, 5 mM EDTA, 100 mM KCl, 1% PVPP, 30% Sucrose, 4% SDS + 0.1 - 1 M NaOH	300 mM Tris, 7 M Urea, 2 M Thiourea, 4% CHAPS	1.60 - 2.80

cantly. It seems that NaOH can disintegrate the cell membranes more effectively than other additives; however, the time of incubation must keep as short as possible avoiding protein degradation. Both additives will be separated from the proteins by the phenolic extraction, so that they cannot disturb the process of iso-electric focusing or interfere with LC-MS.

3. PROTEIN SEPARATION

3.1. Gel-Based and Gel-Free Separation Techniques

Three main technologies are applied to resolve the fungal proteome. The first and the most powerful is the two-dimensional electrophoresis, which seems to be also the method of choice for the separation and visualisation of proteins from black MCF and black yeasts. The methodology shows its power to separate a high number of proteins and visualize the alteration of protein patterns [11,12,30, **Figure 2**]. One-dimensional electrophoresis has been used in combination with mass spectrometry and despite its simplicity for the separation of the fungal proteome, leading to the discovery of novel proteins like malate dehydrogenase and peptydil prolyl cis-trtans isomerase [31,32] However, SDS-PAGE remains a qualitative methodology and the separation of the proteins in the bands is limited, resulting in a heterogenous band containig different proteins of identical molecular weight.

The gel-free LC-MS-based methodologies are still rarely used. Isobaric tag for relative and absolute quatitation (iTRAQ) in combination with MALDI-TOF/TOF MS was reported for the analysis of plasmamembrane ancored proteins in filamentous fungi, [33,34]. The application of gel-free techniques seems to enhance the efficient sepaparation and sensitivity of protein identification. However, gel-free protein separation approaches are still not conducted and implemented for black microclonial fungi. It seems that differential gel electrophoresis (DIGE) and LC-Based approaches iTRAQ are also bottlenecked by the sample preparation and protein yields. The main challenge herein remains the soluble protein contents in an appropriate buffer, which is compatible with the separation technique. Although, all the methodologies shows high potentials, their successful application is, therefore, still very limited.

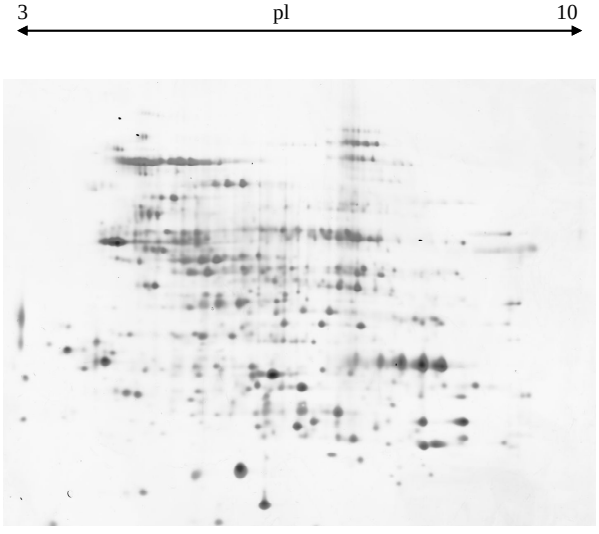

Figure 2. 2DE gel of an Exophiala *dermatitis* grown at optimal temperature of 37˚C and extracted with homogenisation buffer containing 2% SDS and 0,2M NaOH. The proteins are focussed using Immobiline gel strips in pI range of 3 - 10 obtained from GE-Healthcare. The second dimension is performed in 10% polyacrylamide gel and stained by mass spectrometry compatible silver staining.

3.2. Data Analysis and Bioinformatics

Proteomic analysis is exceptional for its capability of data generation. Even a simple 2D-gel provides information for at least some hundreds of proteins. Therefore, data analysis and implementation of bioinformatics gain a central role for the interpretation of obtained data.

The data generated in the field of black microclonial fungi and black yeasts are still rare. The lack of genomic sequence data makes the protein identification to an odyssee of homology searches in several available proteomic data bases like UniProt (http://www.uniprot.org), for plant pathogenic fungi Munich Information Center of Protein Sequence MIPS (http://www.helmholtz-muenchen.de/en/ibis), the Sanger Institute Fungal Sequencing (http://sanger.ac.uk/Projects/Fungi/), Central Aspergillus Database Repository (CADRE, http://www.cadre-genomes.org.uk/) and FungalGenome (http://fungalgenomes.org/).

The more universal databases, however, gain on importance for the homology searches. These are to find at World-2DPAGE Repository from ExPASy (http://world-2dpage.expasy.org/repository/), Proteomic IDEntifications database (PRIDE, www.ebi.ac.uk/pride/) and MASCOT (http://www.matrixscience.com). Despite of large depositories, huge amounts of data and publications, the available informations in the databases are infected with erroneous results, low-confidence protein identification and insufficient statistical evaluation [35]. HUPO Proteomic standard Initiative tries to bypass the disharmonies in experimental design and data generation by the quidance modules [36].

In the example of black microclonial fungi and the black yeasts, however, we must still wait for the completion of genomic sequence data by the novel technology of the next generation sequencing and annotations, which allow functional exploration of the protein expression of these unknown biological entities.

4. CONCLUSIONS

The technological developments and construction of huge proteomics databases allow the exploration of system biologic processes and protein composition in various fungal species. However, the proteomics of black microcolonial fungi are still in the beginning due to limitations caused by the nature of species and protein solubility. Conventional approaches to extract the protein failed to enrich the protein even if the amounts of biomass was increased 2- to 3-fold. The ambitious and challenging issue could be bypassed by rigorous intervenetions like over average basic millieu and the use of SDS. The separation technologies are now capable of analysing the samples and extracts are compatible to different micro-technologies.

Updates in genomic seqencing methodologies and techniques empower biology to obtain full genomic sequence in less than two months. The main challenge will be then the functional annotation of the gene sequences and to extract the available data in the databases from erroneous information and weak data.

At the time being, the road is paved and open for conclusive experiments at physiologic as well as stress-tolerance biology. The future will show how MCFs cope with extreme environments and are able to maintain their survival and growth.

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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

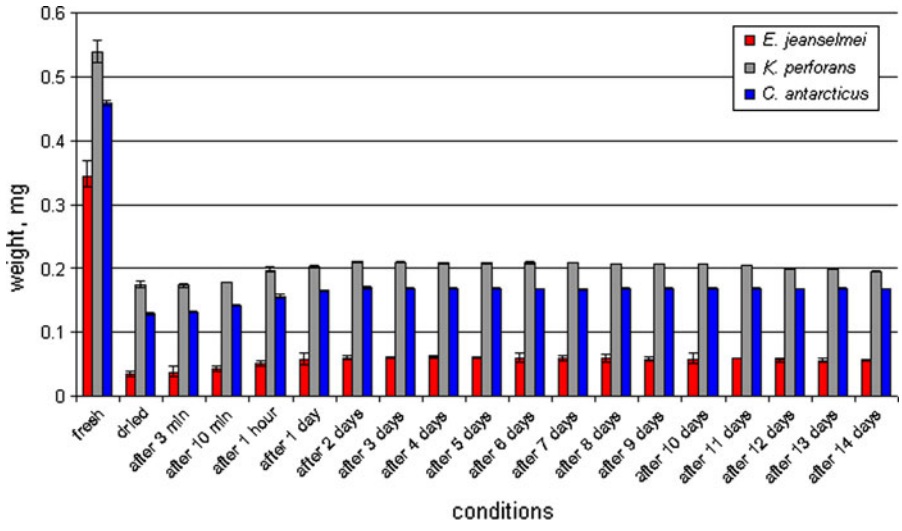

Fig. 1 Rehydration of MCF and *black* yeast. Fresh biomass was subjected to the desiccation until the constant weight was reached (on 6th day), and then dried biomass was rehydrated for 14 days. The weight was estimated for all the samples at different conditions: starting from 3 min of rehydration till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

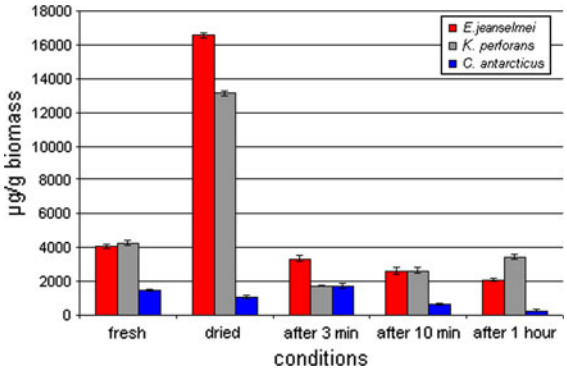

Fig. 2 Absolute protein concentration of MCF and *black* yeast. Protein amount of the biomass was measured before and after desiccation, and also at different conditions: after 3 min, 10 min and 1 h of rehydration

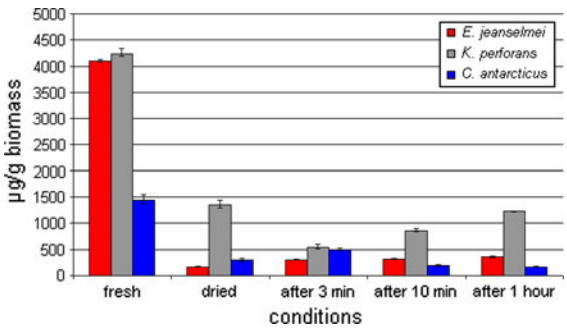

Fig. 3 Relative protein concentration of MCF and *black* yeast. Protein content was re-calculated to the hydrated biomass according to the water loss of the samples

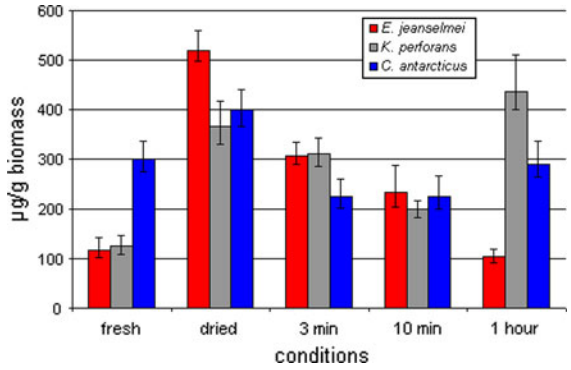

Fig. 4 Absolute RNA concentration of MCF and *black* yeast. RNA concentration of the biomass was measured before and after desiccation and also at different conditions: after 3 min, 10 min and 1 h of rehydration

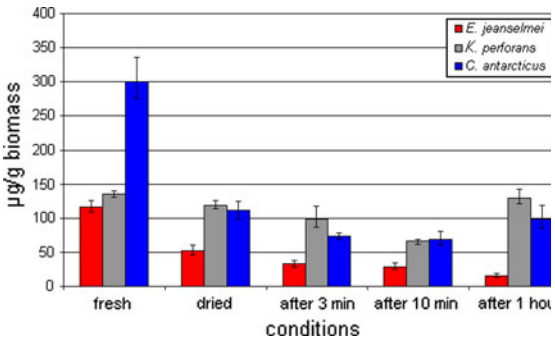

Fig. 5 Relative RNA concentration of MCF and black yeast. RNA concentration was re-calculated to the hydrated biomass according to the water loss of the samples

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,

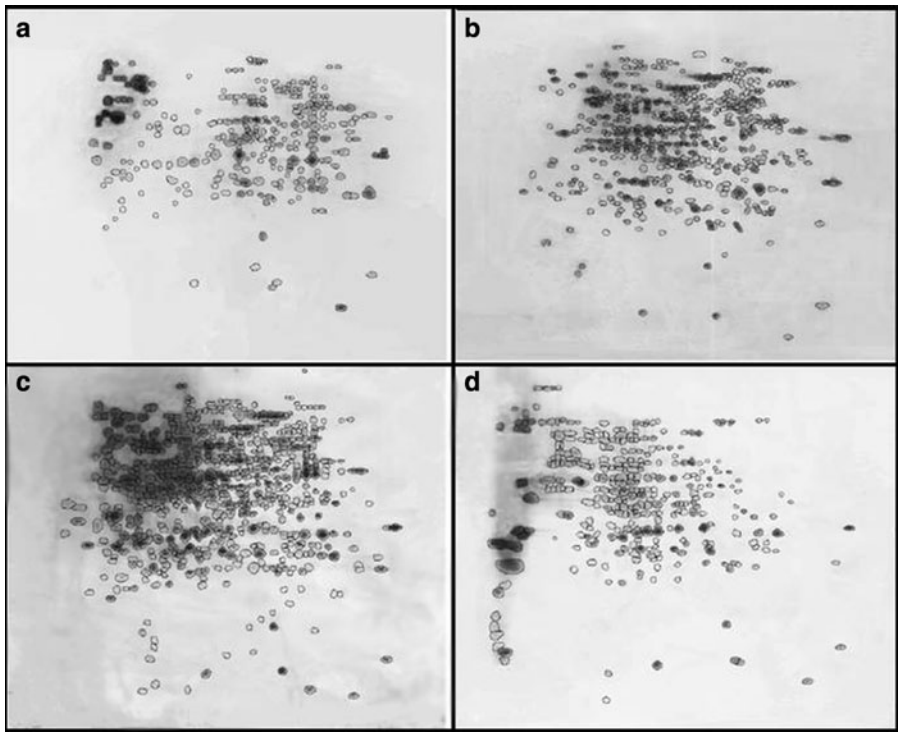

Fig. 6 Comparison of 2D gel protein expression profiles of *K. perforans*: **a** fresh biomass, **b** after desiccation for 6 days, **c** after rehydration for 3 min and **d** after rehydration for 1 h. For each gel, 20 µg of protein was applied. IEF separation was performed using 13-cm strips pH 3-10NL

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

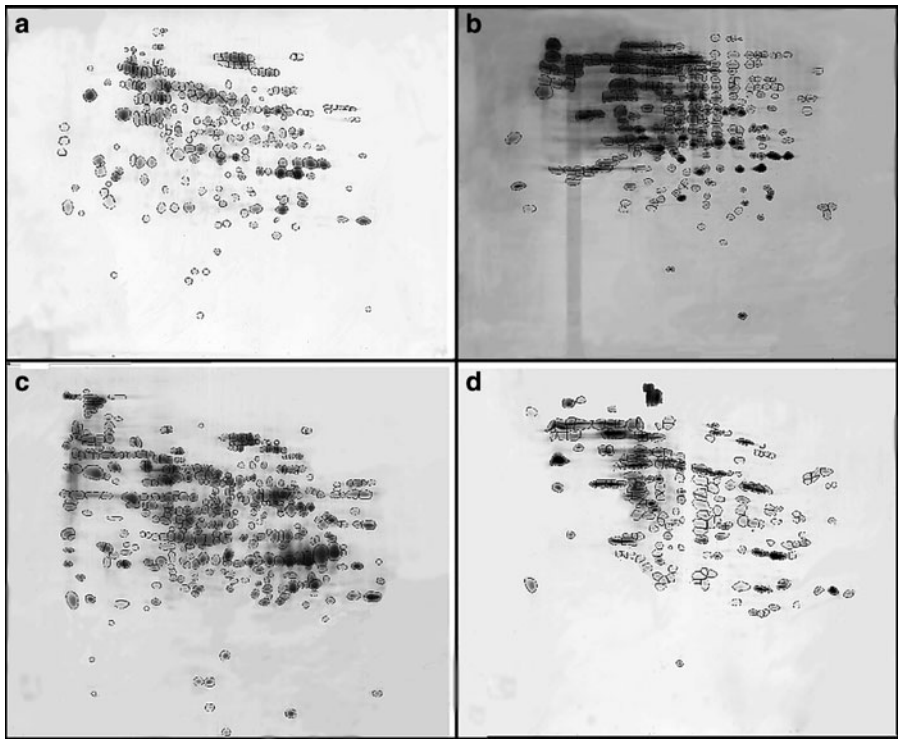

Fig. 7 Comparison of 2D gel protein expression profiles of *E. jeanselmei*: **a** fresh biomass, **b** after desiccation for 6 days, **c** after rehydration for 3 min and **d** after rehydration for 1 h. For each gel, 20 µg of protein was applied. IEF separation was performed using 13-cm strips pH 3-10NL

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

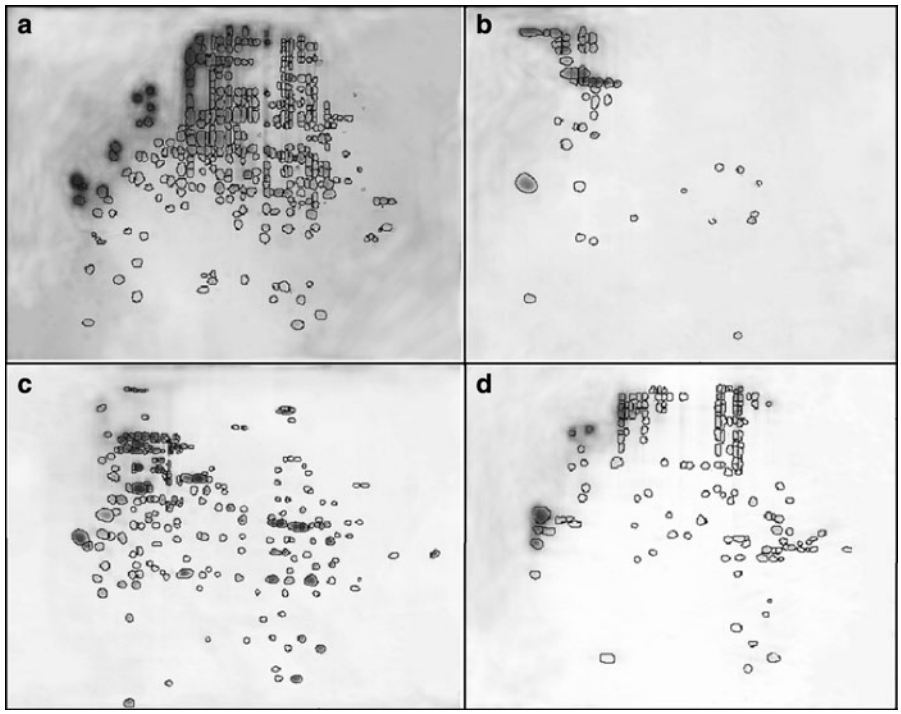

Fig. 8 Comparison of 2D gel protein expression profiles of *C. antarcticus*: **a** fresh biomass, **b** after desiccation for 6 days, **c** after rehydration for 3 min and **d** after rehydration for 1 h rehydration for 1 h. For each gel, 20 µg of protein was applied. IEF separation was performed using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

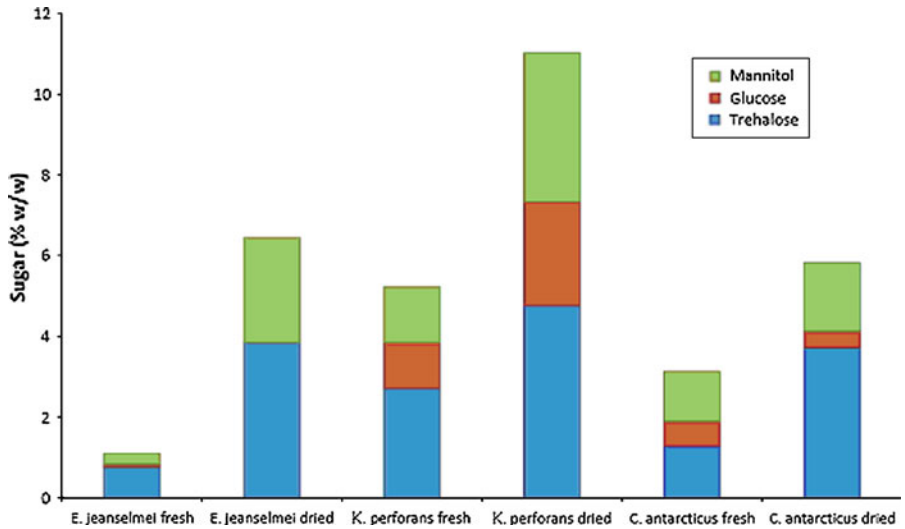

Fig. 9 Sugars (% w/w) present in fresh (fully hydrated) biomass and dried biomass (after desiccation for 6 days). The amount of mannitol, glucose and trehalose estimated for each sample

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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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

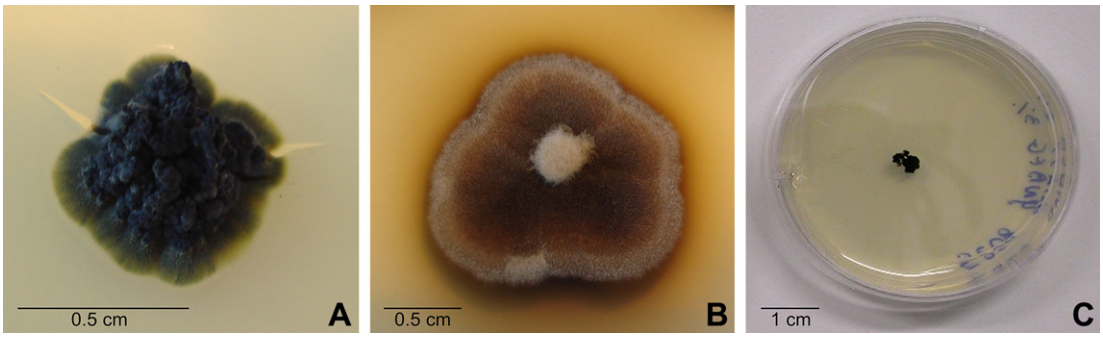

Fig 1 – Colony morphology of the analysed black fungi grown on MEA: (A) *Coniosporium perforans* MA 1299; (B) *Exophiala jeanselmei* MA 2853 (photos Tesei D); and (C) *Friedmanniomyces endolithicus* CCFEE 5208 (photo Selbmann L).

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

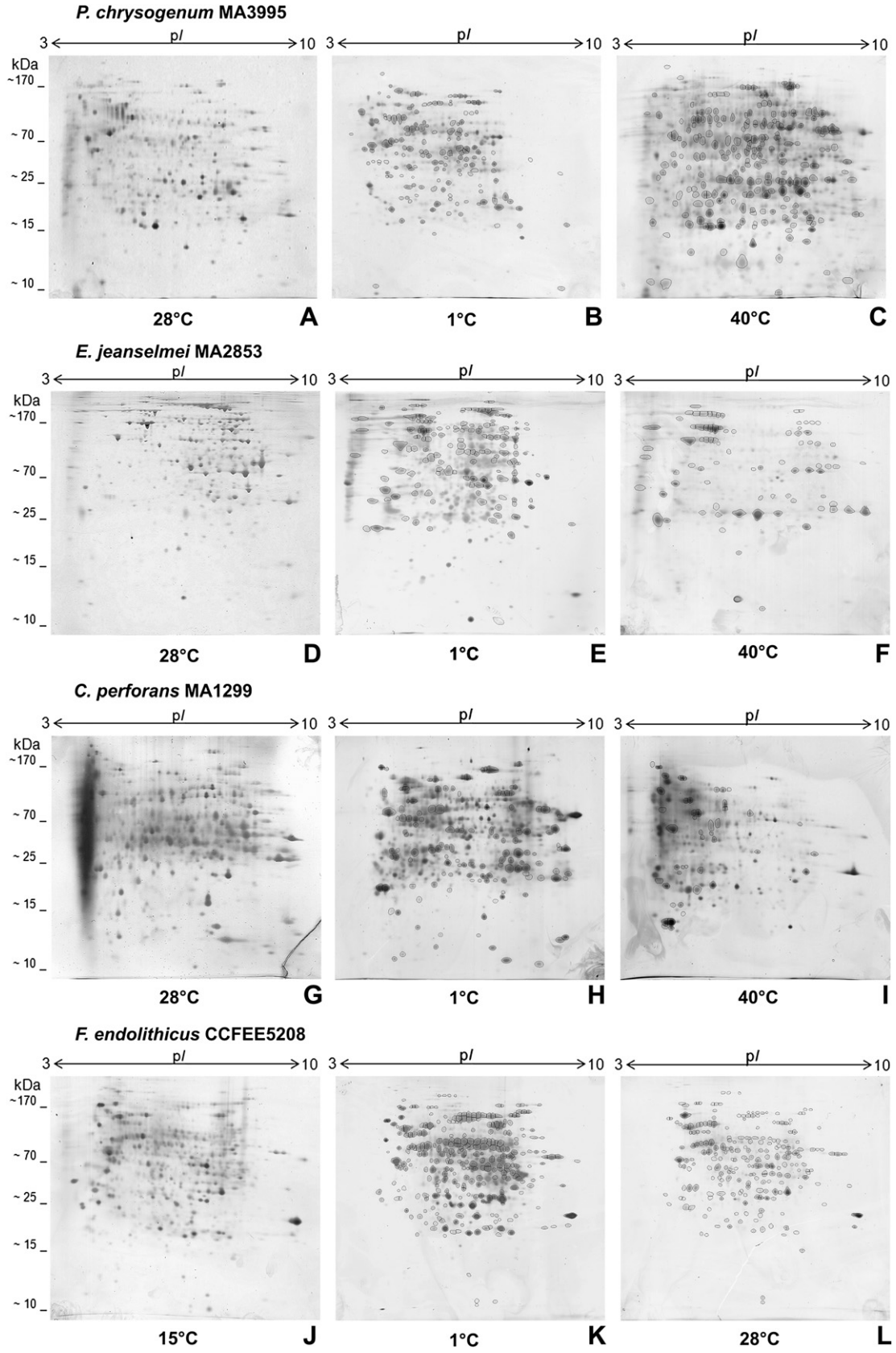

Fig 2 – 2D gel protein patterns obtained after exposure to different temperatures. *P. chrysogenum* MA 3995 (A–C), *E. jeanselmei* MA 2853 (D–F), *Coniosporium perforans* MA 1299 (G–I), and *F. endolithicus* CCFEE 5208 (J–L). Pairs of protein spots detected by overlapping are highlighted in black.

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 hyphomyctes 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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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

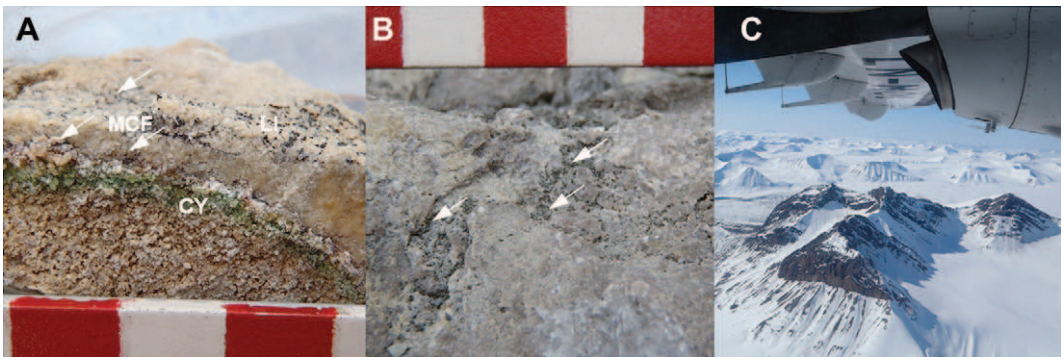

Fig 1 – (A) Epi- and endolithic rock community in the Arctic environment with black fungi (MCF) and lichen (LI) on the top and in the first mm of the rock, and cyanobacteria (CY) within the rock (bar 1 cm). (B) Rock surface in Svalbard (Arctic) with fungal colonies (MCF) located in depressions and fissures. (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).

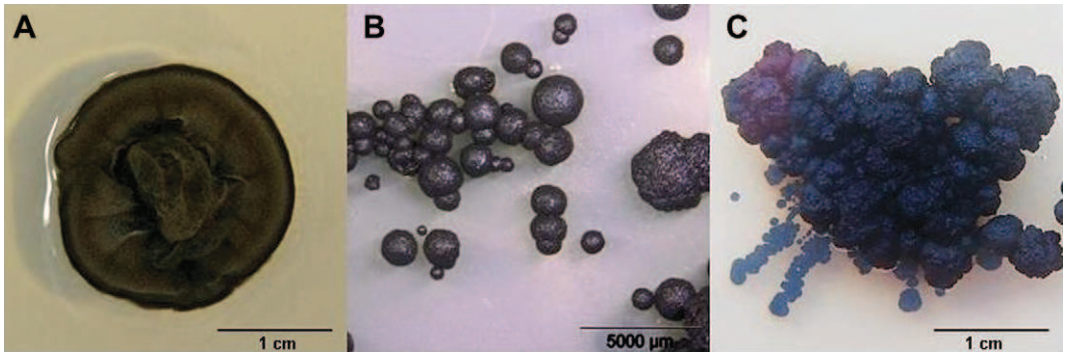

Fig 2 – Typical colony morphologies of MCF grown on agar plates: (A) *Coniosporium perforans*, (B) *Friedmanniomyces endolithicus*, (C) *Cryomyces minteri*.

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

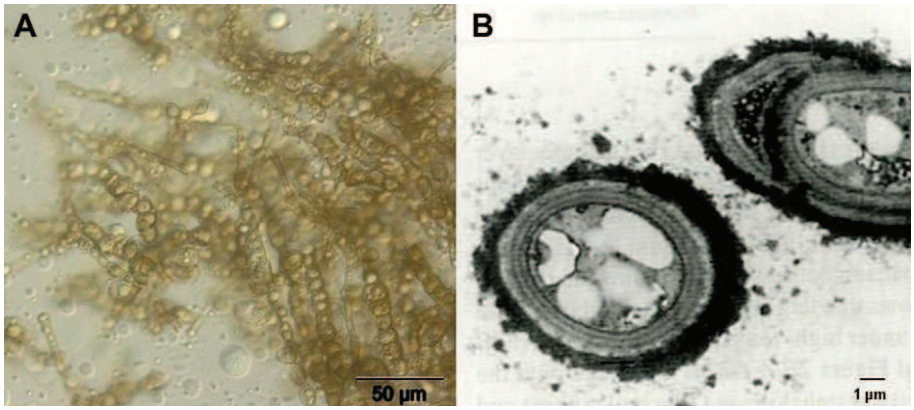

Fig 3 – (A) Meristematic growth and (B) TEM photograph of microcolonial fungi showing the multi-layered cell wall (photos: Sterflinger).

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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