Department of Biotechnology

VIBT EQ Extremophile Center

Supervisor: Assoc. Prof. Dr. Katja Sterflinger

RNA SEQUENCING OF THE HUMAN PATHOGEN *EXOPHIALA DERMATITIDIS*

Doctoral Thesis
for obtaining a doctorate degree at the
University of Natural Resources and Life Sciences

Submitted by
DI Caroline Poyntner Bakk. techn.

Acknowledgement

I would like to express my sincere gratitude to Prof. Katja Sterflinger, for giving me the opportunity to work in her group for the last years, for providing the infrastructure, knowledge and helpful ideas for this thesis. She is always able to frame results in a broader biological picture and explain the unexpected things black yeasts are doing. I am outmost thankful that she spread her enthusiasm for the fungal kingdom and that she could always restore motivation. Additionally, she stayed a thoughtful, empathic human-being caring also for non-scientific topics.

Further I would like to thank Dr. Hakim Tafer for his bioinformatic work, for giving sense and structure to the endless floods of data, for his patience answering questions and for the group effort producing two papers. I also want to thank Dr. Barbara Blasi, for introducing me to the RNA lab-work, sequencing, hungry RNases, Bioanalyzer TV and for the enjoyable conference time together. I am also thankful for the discussions with Dr. Guadalupe Piñar, for showing me the metagenomics world and for being able to feed my microbial ecology related interests but also for bringing some happy spirit into the lab. I would like to thank the whole black yeast group, Dr. Donatella Tesei for black yeasts discussions, Dr. Ksenija Lopandic for the curious questions, Christian Voitl for always assisting with every problem, and Stephanie Stohl for being in her happy, helpful mood.
I would also like to thank collaborating scientists: Prof. Mira Mirastschijski, Dr. Elsa Arcalis, Regina Weinmüllner, Elena Stelzer, Dr. Luci Terlecki and Prof. Grillari - for their help, interest and skin expertise.

I would like to acknowledge the EQ-BOKU VIBT GMBH and the Austrian Science Fund (FWF) project (P 25119-B16) for funding this research.

Additionally, I would like to thank all the people in the DG for making the PhD life easier, especially Silvia, Christina, Miriam, Linda and of course the motivated, funny Sarah.

I am deeply grateful for the support I always got from my family. Without their help I would not have been able to pursue these years of studying – Danke Irene, Alfred, Lukas und Birgit. The PhD years would have been a lot different without Gabriel, who shared the science enthusiasm even on Sundays and brought me back on track in frustrating times. Thanks for enduring all the doubts, the corrections, the distractions and for being honest, supportive and kind.

Last but not least I would like to thank a few people outside of Boku and maybe even outside of Austria who supported me with their friendship, open ears for complaints or non-scientific adventures: Eva, Vicky, Dani, Dodo, Lukas, Sabine, Phil, Souhila, Antonio, Andrea, Chris, Clara,…..

In fungal pathogens, some virulence factors are shown openly.

But others are hidden. Therefore looking on a molecular level is needed.

adapted from: J. Czichos (1987).

Abstract

Exophiala dermatitidis is a pathogenic, polyextremophilic fungus belonging to the group of black yeasts. The fungus colonizes extreme niches in households such as e.g. sauna facilities and washing machines. In the environment, the strain was isolated from extreme habitats e.g. salterns and glaciers. The strain tolerates wide ranges of pH values, temperatures, UV radiation and desiccation. Moreover, it is an opportunistic pathogen for humans and animals and can lead to subcutaneous, cutaneous and systemic infections. These mycoses affect immunocompromised as well as immunocompetent patients. The colonization of human lungs with *E. dermatitidis* is especially common in cystic fibrosis patients. Although reports of *E. dermatitidis* infections increase, infection mechanisms and host invasion are not understood in detail. This thesis aims to study these mechanisms especially in the first phase of contact between the pathogen and the host. This phase is hypothesized to be crucial for host invasion.

To this aim two studies were conducted. In the first study, an artificial ex-vivo skin model infection was set up to monitor the first phase of *E. dermatitidis* infection. Virulence factors and important mechanisms during this first infection phase were determined. RNA sequencing was applied to study changes on the transcriptome level. Additionally, the infection was monitored microscopically and findings indicated that melanin played a key role in the infection mechanism. The second study is based on the knock-out of the PKS1 gene, which is responsible for the production of DHN melanin. The CRISPR/Cas9 technology was used for the mutation. A comparative study between the wildtype and the mutant showed that this type of melanin is crucial for skin invasion. Additionally, the role of the three different melanin types and a possible Trojan-horse like mechanism for keratinocyte invasion are described. Overall, a number of mechanisms and virulence factors during the first phase of infection were identified during the studies. The findings are of importance to get a deeper insight into the infection mechanisms of *E. dermatitidis.*

Keywords: *black yeasts, Exophiala dermatitidis, melanin, total RNA-Sequencing*

Kurzfassung

Exophiala dermatitidis ist ein pathogener, polyextremohiler Pilz, der zur Gruppe der schwarzen Hefen gezählt wird. Der Pilzstamm besiedelt extreme Nischen in Haushalten, wie zum Beispiel Waschmaschinen oder Saunabäder, sowie natürliche extreme Habitate, wie zum Beispiel Salinen. *E. dermatitdis* kann einem weiten Bereich an pH-Werten, Temperaturen, UV-Strahlung und Austrocknung standhalten. Zusätzlich ist der Pilz ein opportunistischer Krankheitserreger. *E. dermatitidis* löst subkutane, kutane und systemische Infektionen aus, welche sowohl immunkompetente als auch immunsupprimierte Patienten betreffen können. Sehr häufig wird *E. dermatitidis* aus Lungen von Patienten, die an Mukoviszidose erkrankt sind, isoliert. Obwohl die Anzahl an Erkrankungen in Verbindung mit *E.dermatitidis* zunimmt, ist sehr wenig über dessen Virulenzmechanismen und Wirtsbefall bekannt. Das Ziel der vorliegenden Arbeit ist, die der Virulenz zugrunde liegenden Mechanismen zu untersuchen und grundlegende Faktoren während der ersten Kontaktphase des Erregers mit dem Wirt festzustellen. Diese erste Phase gilt als entscheidend für den erfolgreichen Wirtsbefall.

Daher wurden zwei Studien durchgeführt: (1) In der ersten Studie wurde ein Modell zur künstlichen Hautinfektion etabliert, welches möglich macht, die erste Phase des Pilz-Haut Kontaktes zu beobachten. Die RNA Sequenzierung wurde als Methode gewählt, um die Änderungen auf der Transkriptomebene zu untersuchen. Zusätzlichen wurden mikroskopische Techniken angewendet. Die Ergebnisse der ersten Studie deuten darauf hin, dass Melanin eine wichtige Rolle in der Hautinfektion spielt. Die in der ersten Studie erfassten Virulenzfaktoren und Mechanismen waren die Basis für die (2) zweite Studie: Diese basierte auf dem Knock-Out des PKS1 genes, welches zur DHN Melanin Produktion nötig ist. Die Mutation wurde mit Hilfe der CRISPR/Cas9 Technologie durchgeführt. Die anschließende vergleichende Studie zwischen Wildtyp und Mutant konnte zeigen, dass DHN Melanin entscheidend für den erfolgreichen Hautbefall ist. Die unterschiedlichen Rollen der drei Melanintypen und ein mit einem trojanischen Pferd vergleichbarer Invasionsmechanismus wurden beschrieben.

Zusammenfassend wurde eine Reihe an Virulenz Mechanismen und Faktoren erkannt, die während der ersten Phase des Kontaktes von Wirt und Erreger entscheidend sind. Diese Ergebnisse tragen dazu bei, den Infektionsmechanismus von *E. dermatitidis* besser zu verstehen.

Schlüsselwörter: *Schwarze Hefen, Exophiala dermatitidis, Melanin, total RNA-Sequenzierung*

Table of Contents

1 Introduction

Fungi are defined as eukaryotic, heterotrophic, absorptive organisms that develop a rather diffuse, branched, tubular body and reproduce by means of spores (Kendrick, 2000). To date, approximately 100 000 species are described (Tedersoo et al., 2014) and it is estimated that 5.1 million species exist (Hawksworth, 1991). The fungal kingdom is highly diverse on a taxonomic, ecological and morphological level. Fungi are known for their mutualistic symbiosis such as lichens, for parasitism and for hyperparasitism (Webster and Weber, 2007). Moreover, fungi are saprotrophs having an important role in the ecosystem due to their ability to degrade organic material. The fungal degradation mechanism is based on extracellular digestion with the use of enzymes followed by absorption (Webster and Weber, 2007). Therefore, fungal strains are used as enzyme producers in biotechnology. Another function applied in biotechnology is their ability to produce antibiotics. The first fungal derived antibiotic, Penicillin, was described by Alexander Fleming in 1928 (Susan Aldrich, 1999) and it is still used today. Rainforests are suggested to harbor the largest number of potential drug-producing organisms although the most successful drug producing fungi were isolated from temperate climates (Blackwell, 2011). Surprisingly, a high number of fungal strains was reported in Alaskan boreal forest soil (Taylor et al., 2010). A large number of hitherto unexplored environments were thought to be sterile, such as deserts, stones, deep sea sediments or toxic environments. However, fungal strains were isolated also from these habitats (Nagano and Nagahama, 2012; Poyntner et al., 2018b; Selbmann et al., 2014b; Sterflinger et al., 2012). In order to survive in extreme habitats, fungal strains have developed a high degree of environmental stress resistance. Thereby, some fungi are specialized on cold or osmotic niches while others are able to thrive in a wide variety of different environmental conditions (Blackwell, 2011).

Many fungal strains have harmful effects to humans as they are pathogens for animals, including mammals, and for plants of agricultural importance. Additionally, they cause food spoilage, colonize interiors and lead to allergic reactions triggered by spores and hyphal fragments. Hyphomycetes and yeasts, e.g. *Aspergillus fumigatus* or *Candida alibcans,* are common infection agents for humans.

1.1 Black yeasts - colonizers of extreme environments

Fungal strains of the black yeast group have a very high degree of environmental stress resistance. The group is heterogeneous from a phylogenetic and taxonomic point of view but shares - as the name suggests - a black pigment in the outer cell wall: melanin (Sterflinger, 2005). Three different morphologies can be found within the group, mostly dependent on the growth conditions: the microcolonial growth form, the hyphal growth form and the yeast-like budding growth form.

In environmental samples, black yeasts were overseen for a long time because their detection was hampered by fast growing fungi that are usually dominating the agar plates used for isolation. Moreover, the dimorphic growth on the one hand and the lack of morphological differentiation on the other hand caused difficulties for morphological identification (Selbmann et al., 2014a). However, the development in molecular methods including Sanger sequencing and next generation sequencing allowed the distinction of different species on the DNA level. Application of these methods revealed an unexpected large species diversity and a world-wide environmental distribution. Most of the black yeasts belong to the order of *Chaetothyriales* and are known for their ability to survive in hostile environments such as glaciers (Branda et al., 2010), hot- (Pulschen et al., 2015) or salty environments (Gunde-Cimerman et al., 2000). Some strains were isolated from pollutant rich environments and appear to be able to assimilate alkylbenzene hydrocarbons. Therefore, they are possible candidates for degradation of toxic xenobiotics (Blasi et al., 2016; Prenafeta-Boldú et al., 2004, 2006). Black yeasts are resistant to a broad pH range (pH 1 to 12.5), (Döğen et al., 2013b; Hölker et al., 2004), desiccation, temperature, ozone, UV, salt concentrations and radioactivity. For example, the black yeast *Hortaea acidophila* was isolated from lignite, at a pH of 1 (Hölker et al., 2004). Air dried mycelia of black yeasts can withstand 120°C for at least 0.5 hours (Sterflinger, 1998).

Some strains of the black yeast group are able to live endolithic in all climate regions of the Earth and even in Mars-like conditions (Onofri et al., 2012; Zakharova et al., 2013, 2015), the latter making them appropriate model organisms for exobiology studies. One of the most resistant fungi is the endolithic black yeast strain

Friedmanniomyces, which is able to survive at temperatures lower than zero in the Antarctic ice-free area of the Victoria Land, one of the coldest and driest habitats on Earth.

The ability of black fungi to survive and be metabolically active in extreme conditions is extraordinary but also poses problems from the anthropogenic point of view. For example, in cultural heritage, endolithic black fungi cause a special deterioration phenomenon, so-called biopitting on antique marble (Sterflinger, 2005) and form dark biogenic crusts on whole rock surfaces. Other black yeasts have the ability to cause severe diseases in humans and animals. Interestingly the ability to colonize and penetrate rock is supposed to be connected to their ability to penetrate human skin and tissue and thus to cause subcutaneous and cutaneous infections. Additionally, the ability to use alkylbenzene hydrocarbons as energy source might be in relation to the neurotropism of black yeasts, as neurotransmitters have similar molecular shapes (Prenafeta-Boldú et al., 2006). Thus, there seem to exist a relation between environmentally derived morphological and physiological traits and a shift to animals as novel habitats.

In recent years, an emerging adaptation of black yeasts towards human environments has been observed. Many extreme niches in our households became common habitats of black yeasts as for example dishwashers, sauna facilities or sink drains (Döğen et al., 2013b; Gümral et al., 2015; Matos et al., 2002; Novak Babič et al., 2016; Zalar et al., 2011; Zupančič et al., 2016). The reasons for the increase of black yeast colonization in our households and their increasing impact on human health is not fully understood. Most probably, the fungi are distributed via the fresh water systems to our households, resulting in colonization of water related machines and equipment. The usage of programs at low tempartures such as 30°C in washingmachines and dishwashers are contributing to the fungal survial, colonization and growth in these niches.

One of the key agents to survive under extreme conditions is melanin, a negatively charged, hydrophobic macromolecule composed of oxidative polymerized phenolic or indolic monomers and often complexed with proteins and carbohydrates (Butler and Day, 1998a; Casadevall et al., 2000). Melanin is essential for the riceblast causing

fungus *Mangnaportae oryzae.* It enables the fungus to generate a turgor pressure to enter the host cell and act as a barrier against the efflux of solute from the appressorium (Chumley, 1990; Wilson and Talbot, 2009). Although such turgor related mechanisms have not been reported in black yeasts, the black pigment itself serves as protection agent against environmental stress. Melanin is able to scavenger free radicals, it helps to survive phagocytic cell burst (Schnitzler et al., 1999) and binds to hydrolytic enzymes which are able to attack the plasma membrane (Revankar and Sutton, 2010). Thus, in black yeasts, melanin appears to be an essential factor for pathogenicity and survival in environmental stress conditions. Another group of pigments present in black yeasts are carotenoids (Figure 1). Carotenoids are assumed to shelter the fungus against harmful irradiation such as UV (Schnitzler et al., 1999). In the black yeast strain *Knufia petricola* A95, five different carotenoids were found and the content varied among different conditions such as desiccation and rehydration (Gorbushina et al., 2008).

Another stress survival factor is the ability to build extracellular polymeric substances (EPS) (Selbman et al., 2005) which are essential for the formation of biofilms. In the black yeast *Exophiala dermatitidis* the thermotolerance is directly connected to EPS production (Yurlova and de Hoog, 2002) and might therefore be important for pathogenicity and stress tolerance. Capsule building is a known mechanism to survive stressful environments in microbes. In the black yeast *Exophiala spinifera,* the production of capsules was observed. Moreover its genome contains orthologous of known capsule building genes (Song et al., 2017; Yurlova and de Hoog, 2002).

The ability of black yeasts to switch between hyphal and yeast-like growth increases their environmental plasticity and adaption to environmental stressful and quickly changing environments such as changes in temperature, water availability or pH.

Figure 1 *Exophiala. dermatitidis* wildtype and mutant on MEA plates. The wildtype strain is completely black due to melanin (first row). The *PKS1* mutant is able to produce any melanin and therefore is white when incubated without light source (second row left). Grown in daylight, the *PKS1* mutant turns pink due to carotenoid production (Second row right).

1.2 Pathogenic black yeasts

In animals black yeasts can be found as symbionts, e.g. in ants (Mayer et al., 2018; Vasse et al., 2017), but also as pathogens as e.g. in salmon (*Exophiala salmonis,* Carmichael, 1967) or trout (*Exophiala psychrophila,* Pedersen and Langvad, 1989). In mammals, especially in humans, a number of black yeasts is able to cause systemic infections. For example *Cladophialophora bantiana*, due to its ability to pass the blood-brain barrier, is the most common cause of cerebral phaeohyphomycosis (Revankar et al., 2004). Next to *C. bantiana*, many cases of infections are caused by the black yeasts *E. dermatitidis* and *Rhinocladiella mackenziei* (Li and de Hoog, 2009). In contrast to most life threating fungal infections, primary phaeohyphomycosis mostly affects immunocompetent patients and adults in their second or third decade of life (Jung and Kim, 2014) with a mortality rate of 73% (Revankar et al., 2004). Moreover, the disease “chromoblastomycosis” caused by black yeasts was added to the World Health Organization (WHO) list of neglected tropical diseases in 2016. Forty-eight species within the order of *Chaetotyriales* are described as clinically relevant (Song et al., 2017).

Prenafeta-Boldú et al. (2006) suggested that there is a connection between the ability to metabolize alkylbenzene hydrocarbons and the ability of black yeasts to cause serious human disease with a tendency towards neurotropism. *Cladophialophora immunda* for instance is capable of assimilating toluene and is found in the same

phylogenetic clade as the lethal, neurotropic pathogen *C. bantiana* (Blasi et al., 2016, 2017). *Exophiala mesophila* harbors similar features. The tendency to assimilate akylbenzene hydrocarbons can generally be found throughout the whole *Herpotrichiellaceae* family. A recent study on the genome of *R. mackenziei* (Moreno et al., 2018a) reported a high number of conserved protein coding genes linked to the degradation of alkylbenzenes. Homologs of these genes are shared among recognized neurotropic and alkylbenzene assimilating black yeasts. Additionally, pathways overlap between the nutrient acquisition pathways in environmental stress conditions and energy producing pathways in the host. For example phenylalanine, a precursor of tyrosine, can either lead into the citrate cycle or to the production of melanin (Moreno et al., 2018a; Poyntner et al., 2018a).

Black yeasts can be both - mere environmental strains or environmental and pathogenic - and were grouped in the following way by Seyedmousavi et al. (2014):

- Environmental strains not known from vertebrate disorders or not causing any symptoms.
- Strains being able to thrive in the environment but also being able to cause superficial, cutaneous and subcutaneous infections in humans and animals.
- Highly virulent strains causing systemic infections that can only be isolated from infections in humans or a living animal as bait.

The clinical picture of chromoblastomycosis can be caused by traumatic infection from the environment. It leads to an initial cutaneous lesion at the inoculation site and progressive and chronic involvement of cutaneous and subcutaneous tissue (Queiroz-Telles et al., 2017). The fungal infection causes hyperproliferation of host tissue and the fungal pathogen is mostly present in the muriform cell form. Phaehyphomycosis is the second typical clinical picture caused by black yeasts and usually leads to tissue necrosis instead of proliferation of cells. Most patients reported are immunocompromised. The fungal cells present in the patients are in yeast-like budding states and in hyphal states.

1.3 The *Exophiala* clade

The genus *Exophiala* is an evolutionary hotspot in the group of black yeasts with many genotypically divergent species (de Hoog et al., 2003; Sterflinger, 2005). So far, 30 different species have been described (Woo et al., 2013), common representatives among these are *E. spinifera, E. xenobiotica, E. aquamarina* and *E. dermatitidis*. Nineteen of the thirty species have been described or suggested as causative agents for human infections and colonization (Zeng et al., 2007). Additionally, various *Exophiala* strains were isolated from toxic environments such as *E. sideris,* isolated from a heavily polluted mine (de Hoog et al., 2011b), or *E. dermatitidis,* isolated from creosote containing railway sleepers (Gümral et al., 2014). *Exophiala* strains are very similar from a morphological point of view, defined by annellidic conidiogenesis with production of slimy heads of conidia (de Hoog et al., 2011a).The order *Chaetotyriales* comprises numerous opportunistic pathogenic *Exophiala* strains such as *E. dermatitidis, E. spinifera, E. oligosperma* or *E. xenobiotica* (Figure 2).

One of the most studied strains is *E. dermatitidis*, formerly known as *Wangiella dermatitidis,* which is supposed to originate from the rainforest (Sudhadham et al., 2008). *E. dermatitidis* tolerates wide ranges of pH values, temperature and salt concentrations (Zalar et al., 2011). Additionally, ionizing radiation induces enhanced growth of the fungal strain (Dadachova et al., 2007; Robertson et al., 2012). These tolerances are rarely observed in one single fungus (Chen et al., 2014). Due to these environmental stress tolerances and the ability to cause infections in humans and animals (Sudhadham et al., 2008) the strain serves as a good model organism within the black yeasts. In stressful conditions the fungus is mostly prevalent in its yeast-like form but being pleomorphic, it is also able to build hyphal forms (Figure 3). Some strains change to a granular, meristematic forms at 42°C (de Hoog et al., 1994). *Exophiala* has a known teleomorph form, *Capronia*, and together with *E. spinifera* it is one of the only two *Exophiala* species which can produce EPS around its yeast-like cells (Song et al., 2017).

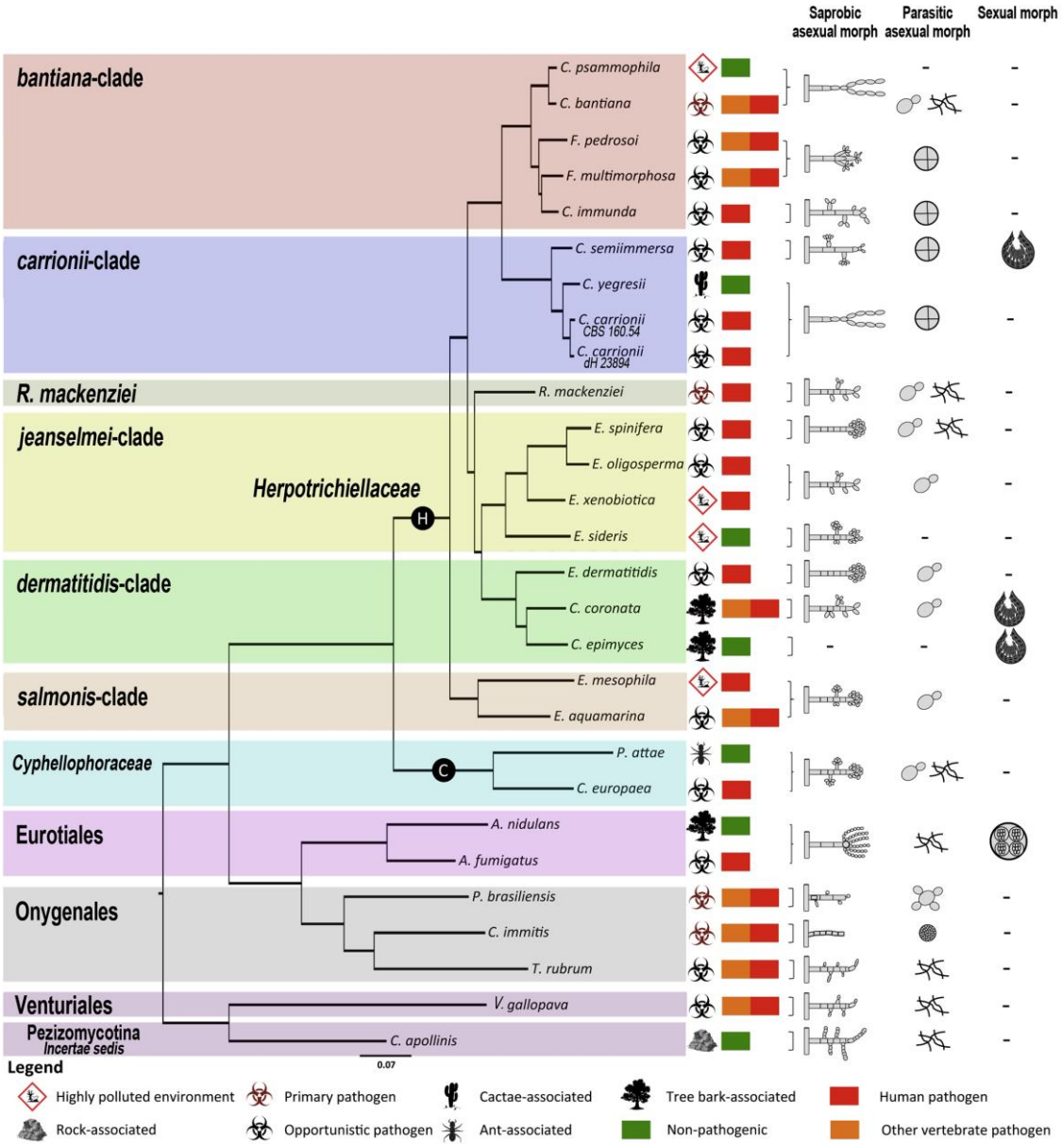

Figure 2 Phylogenetic tree of the most known black yeasts *Chaetothyriales* and related. The main families, *Cyphellophoraceae* family (C) and *Herpotrichiellaceae* (H) are marked and the isolation sources, anamorphs, teleomorphs form are shown (adapted from Teixeira et al., 2017).

E. dermatitidis is the most common black yeast in clinical routine (Sudhadham et al., 2010) and is regarded as an emerging pathogen. The strain causes cutaneous and subcutaneous phaeohyphomycosis (e.g. keratitis, otitis externa) and systemic infections (Hoog et al., 2000; Matsumoto et al., 1993). In the collected data of Song et al., 34 of 39 cases of systemic infections caused by *E. dermatitidis* concerned immunocompetent patients (Song et al., 2017). In recent years, reports describing *E. dermatitidis* colonization in lungs of cystic fibrosis patients increased (Lebecque et

al., 2010; Pihet et al., 2009). This raises the question where and in which way patients encounter the black yeast. One hypothesis is the exposure to black yeasts in households. As mentioned above, *E. dermatitidis* was isolated from different locations in houses such as dishwashers, sink drains or sauna facilities (Döğen et al., 2013b; Gümral et al., 2015; Novak Babič et al., 2016; Zalar et al., 2011; Zupančič et al., 2016). Once entering the human body, lungs of cystic fibrosis patients provide a good habitat for the fungus because they contain high salt concentrations. This again corresponds with the extremophilic nature of the fungus. Besides of cystic fibrosis other reported risk factors are a compromised immune system due to other underlying diseases or a mutation of the CARD9 gene. This gene is a risk factor due to its necessity in the adaptive immune response (Wang et al., 2014).

There are three different genotypes (A, B and C) known in *E. dermatitis*, genotypes A and B being reported more frequently than C. Genotypes A and B differ genetically in only three nucleotides in the ITS1 region. Genotype A is more frequently encountered in patients while genotype B dominates in tropical fruit-eating animal feces. This suggests that human-made extreme environments may stimulate evolution and generate pathogenic genotypes which otherwise would not have evolved (Sudhadham et al., 2008). Genotype C is reported to have a greater biofilm forming ability in comparison to genotype A and B (Sav et al., 2016).

Molecular mechanisms underlying the stress resistance of *E. dermatitidis* have been intensively studied in the recent years: studying the protein patterns at different temperatures (1°C, 37°C and 45°C), Tesei et al. (2015) report that the fungus is fine tuning its stress response in different temperatures on the proteome level. The study by Tesei et al. concludes that the major portion of proteins in *E. dermatitidis* is thermotolerant, showing a response at 1°C but no heat-shock response at 45°. A similar pattern is reported on a transcriptome level - a stress response at cold temperature and little response at high temperature (Blasi et al., 2015). This supports the theory that the fungus might be distributed form glaciers to melting waters, to water pipes and finally into our households, as it can easily adapt to changing conditions during this journey.

Figure 3 Hyphal growth and yeast-like budding. *Exophiala dermatitidis* can grow in a hyphal state and yeast-like budding state (marked in picture). Cultures with both morphologies at the same time or only one morphology are possible.

1.4 Black yeasts in the NGS era

Next generation sequencing (NGS) technology is a useful tool to understand the differences in environmental and pathogenic strains, their distribution from their niches to the host but also their virulence factors. Known virulence markers in *E. dermatitidis* are ureases, catalases and DNases. They can be important for evasion of the host immune system. Additionally, proteinases are found which are restricted to environmental isolates. The virulence profile of *E. dermatitidis* does not show significant differences compared to the strict saprobe strains as *E. crusticola* (Sav et al., 2016). Another virulence factor is the possibility to switch between hyphal and yeast-like growth. This is a known pathogenic trait also in other pathogenic fungi as *Candida albicans* (Mayer et al., 2013), *Histoplasma capsulatum* and *Penicillium marneffei* (Boyce and Andrianopoulos, 2015). The most discussed virulence factor of *E. dermatitidis* is melanin. The pigment has an impact during infection of mice in comparison to non-melanized *E. dermatitidis* mutants (Dixon et al., 1987; Feng et al., 2001). Melanin has an impact on the immune system recognition of pathogens. More precisely, melanized fungal cells are resistant against the phagolysosomal oxidative burst of human neutrophils (Schnitzler et al., 1999) and antifungal drugs (Paolo et al., 2006).

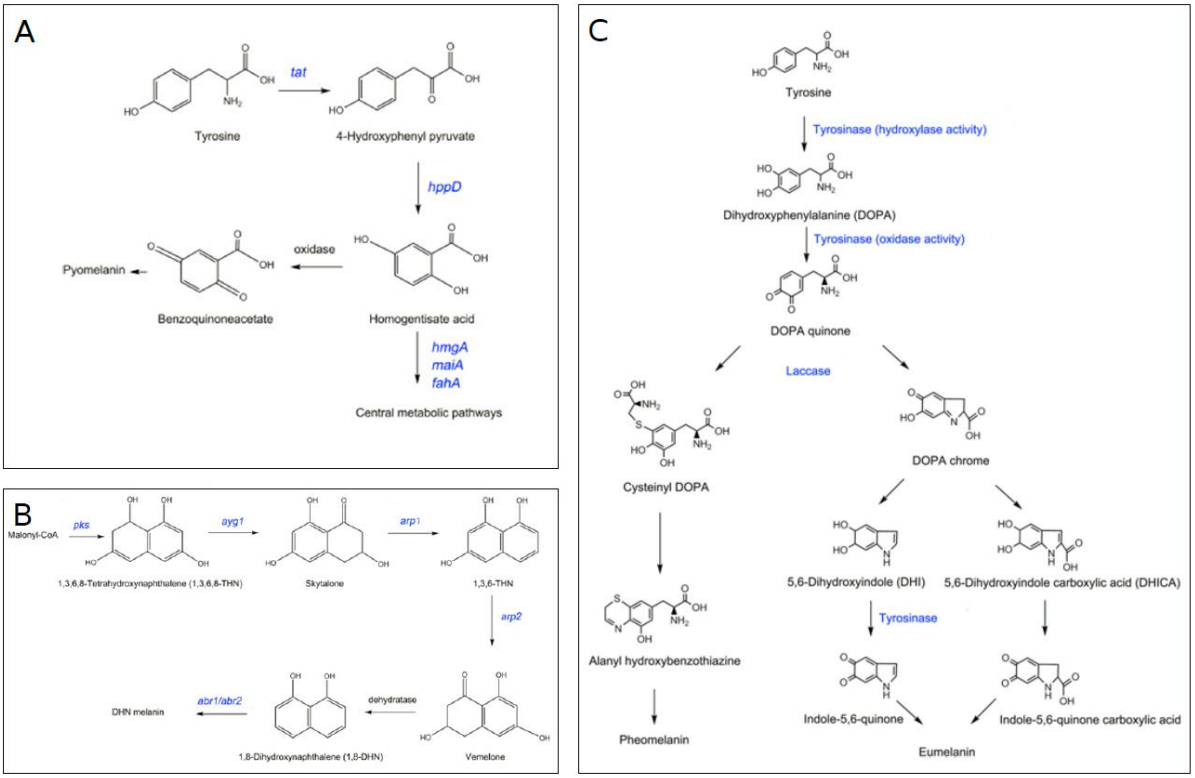

Figure 4 Melanin pathways. (A) L-tyrosine pathway resulting in pyomelanin production or in the alternative pathway into the central metabolic pathway. (B) DHN melanin pathway resulting in DHN melanin. (C) L-Dopa melanin pathway resulting in eumelanin or pheomelanin production (adapted from Li et al., 2016).

Chen et al. (2014) published the whole genome of *E. dermatitidis* and described genes for three different melanin pathways, the L-Dihydroxyphenylalanine (L-Dopa), the 1-8-Dihydroxynaphthalene (DHN) and the L-tyrosine pathway. The genome of *E. dermatitidis* was the first whole genome published within the group of black yeast. The transcriptome study of Poyntner et al. 2016 (see chapter 3) describes the molecular mechanisms of *E. dermatitidis* that give the fungus an enormous physiological and ecological versatility although having a rather small genome of 26.4 Mbp. These mechanisms include multifunctional sites which can be transcribed at the same time or at different time points. Transcriptome studies help to reveal unknown functions such a long non-coding RNAs, circular RNAs or fusion transcripts. These three categories of RNA were also found in the black yeast *E. dermatitidis* (Blasi et al., 2015).

To date, 27 whole genomes of black yeasts are published (Moreno et al., 2018b). The whole genome sequencing data allow to compare the different black yeasts e.g. genome sizes, specific genes present and their function.

The technology of transcriptome sequencing enables to determine the identity and abundance of RNA sequences in a sample. This data can be linked to pathways and mechanisms used by the cell during the specified conditions. Additionally, novel protein coding transcripts and RNA species, for instance non-coding RNA species can be found. These RNA species play an important role for regulatory mechanisms. They evolved evolutionary in parallel to DNA and proteins (Mercer et al., 2009). Two types of non-coding RNAs exist: long non-coding RNAs, which seem to have a regulatory function and are longer than 200bp (Mercer et al., 2009), and small RNAs. Most known small RNAs are: small interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi-associated RNAs (piRNAs) (Großhans and Filipowicz, 2008) (Table 1). MiRNAs play important roles in fungi as for example in the plant pathogen *Fusarium oxysporum* during infection of *Arabidopsis thaliana.* Another interesting mechanism are fungal small RNAs which hijack host RNA interference (RNAi) pathways (Weiberg et al., 2013). In *E. dermatitidis,* small nucleolar RNAs (snoRNAs) were found upregulated during artificial infection (see chapter 3) and in the black yeast *C. immunda* during growth on toluene (Blasi et al., 2017).

In addition to the NGS technologies, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing method is a newly developed tool which can be applied in scientific research. This method is based on the bacterial and archaeal adaptive immune system mechanism using CRISPR RNAs to defend themselves against viruses and plasmids (Jinek et al., 2012). The same mechanism can be used in vitro to insert site specific DNA strandbreaks, using single guided RNAs and the Cas9 protein. This is one of the most promising techniques and it has been awarded the Science break through award in 2015 (Science News Staff, 2015). It is described as one of the most direct, cheap and easy techniques for gene editing. Nevertheless a lot of ethical concerns are raised, especially concerning the application in gene therapy and precision (Plaza Reyes and Lanner, 2017). Crispr/Cas9 is a useful tool also in research on black yeasts. Site specific knock-outs of known virulence and stress resistance factors can be created.

Overall, –omics technologies can be useful tools for answering open questions in the black yeast group concerning virulence factors and molecular mechanisms. Especially in combination with host interactions, interesting functions are revealed which may help in the development of antifungal drugs.

Table 1 Types of non-coding RNAs with known functions. Adapted from (Esteller, 2011).

Name	Size	Location	Functions
Short ncRNAs			
miRNAs	19–24 bp	Encoded at widespread locations	Targeting of mRNAs and many others
piRNAs	26–31bp	Clusters, intragenic	Transposon repression, DNA methylation
tiRNAs	17–18bp	Downstream of TSSs	Regulation of transcription?
Mid-size ncRNAs			
snoRNAs	60–300 bp	Intronic	rRNA modifications
PASRs	22–200 bp	5′ regions of protein-coding genes	Unknown
TSSa-RNAs	20–90 bp	−250 and +50 bp of TSSs	Maintenance of transcription?
PROMPTs	<200 bp	−205 bp and −5 kb of TSSs	Activation of transcription?
Long ncRNAs			
lincRNAs	>200 bp	Widespread loci	Examples include scaffold DNA–chromatin complexes
T-UCRs	>200 bp	Widespread loci	Regulation of miRNA and mRNA levels?
Other lncRNAs	>200 bp	Widespread loci	Examples include X-chromosome inactivation, telomere regulation, imprinting

2 Scope and aims

This thesis aims to contribute to the overall understanding of virulence factors in the black yeast *E. dermatitis*. The strain was chosen as model organism within the black yeasts, being an opportunistic, emerging pathogen for humans and animals. The strain is colonizing extreme niches in households. Therefore, humans are constantly exposed to spores and cells of this fungus. Compared to the wide distribution in households, the number of reported cases of human infection with *E. dermatitidis* is still surprisingly low. Astonishingly, the reported infections are mainly affecting immunocompetent patients which is in contrast to common fungal pathogens affecting mostly immmunodeficient patients. In general, infection mechanisms of *E. dermatitidis* affecting the human host, such as cell invasion, neurotropism and interactions with the immune system, are poorly understood. Thus, the understanding of *E. dermatitidis* virulence mechanisms and interaction with the human host is of high medical and scientific importance.

Based on previous research it is hypothesized, that the first contact between the fungal pathogen *E. dermatitidis* and the human host is a key factor for successful host invasion. Furthermore, it is hypothesized that during this first contact, specific mechanisms and changes in the fungal cell are crucial for the infection which might be connected to their environmental stress resistance. Specifically, (i) the understanding of the mechanisms that are performed on a transcriptome basis during infection, (ii) the identification of novel RNA species and of RNA species which might play a similar role in pathogenicity of other fungal pathogens (Weiberg et al., 2013) and (iii) the influence of putative virulence factors through genomic knock-outs especially.

In order to verify this hypothesis and to examine the first phase of the pathogen-host interaction, experiments were set up mimicking a cutaneous infection. To this aim, e*x-vivo* skin models from human patients undergoing abdominoplasty or brachioplasty were chosen and artificially infected with *E. dermatitis*. The system used had the advantage of being rather simple, being close to the real infection and including both - host and pathogen - response. Beforehand three layered human skin

models were used for the experiments, however this had the disadvantage of delivering insufficient fungal biomass due to the small size of the skin models (data not shown). Changes of the transcriptome during the mimicked infection were analyzed in addition to the establishment of the *ex-vivo* skin models. To this aim, new bioinformatics pipelines had to be established and suitable databases for data interpretation had to be chosen. Further microscopic staining methods had to be adapted to monitor the skin model colonization and invasion.

In the first study (see chapter 3) putative virulence factors were detected and melanin was found to be an important factor for skin infection. Therefore, in the subsequent study (see chapter 4) melanin was knocked out using Crispr/Cas9 for the first time in black yeasts to further investigate the role of melanin for skin infection. New skin infection experiments were performed to investigate differences in virulence between the wildtype and the mutant of *E. dermatitidis.*

3 The Transcriptome of *E. dermatitidis* during Ex-vivo Skin Model Infection

Caroline Poyntner[1], Barbara Blasi[1], Elsa Arcalis[2], Ursula Mirastschijski[3], Katja Sterflinger[1], and Hakim Tafer[1]

[1]Department of Biotechnology, VIBT EQ Extremophile Center, University of Natural Resources and Life Sciences, Vienna, Austria

[2]Department for Applied Genetics and Cell Biology, Molecular Plant Physiology and Crop Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

[3]Klinikum Bremen-Mitte, Department of Plastic, Reconstructive and Aesthetic Surgery, Faculty of Biology and Chemistry, Center for Biomolecular Interactions Bremen, University Bremen, Bremen, Germany

Results published in:
Frontiers Cellular and Infection Microbiology – Fungal Pathogenesis 2016; 6: 136.
doi: 10.3389/fcimb.2016.00136

3.1 Abstract

The black yeast *E. dermatitidis* is a widespread polyextremophile and human pathogen that is found in extreme natural habitats and man-made environments such as dishwashers. It can cause various diseases ranging from phaeohyphomycosis and systemic infections, with fatality rates reaching 40%. While the number of cases in immunocompromised patients are increasing, knowledge of the infections, virulence factors and host response is still scarce. In this study, for the first time, an artificial infection of an *ex-vivo* skin model with *E. dermatitidis* was monitored microscopically and transcriptomically. Results show that *E. dermatitidis* is able to actively grow and penetrate the skin. The analysis of the genomic and RNA-sequencing data delivers a rich and complex transcriptome where circular RNAs, fusion transcripts, long non-coding RNAs and antisense transcripts are found. Changes in transcription strongly affect pathways related to nutrients acquisition, energy metabolism, cell wall, morphological switch, and known virulence factors. The L-Tyrosine melanin pathway is specifically upregulated during infection. Moreover the production of secondary metabolites, especially alkaloids, is increased. Our study is the first that gives an insight into the complexity of the transcriptome of *E. dermatitidis* during artificial skin infections and reveals new virulence factors.

3.1 Introduction

Black yeasts are a special group of Ascomycetes characterized by melanized cells and the ability to form hyphal and yeast-like budding states. They are known for their capacity to survive in extreme habitats, which range from hot and cold deserts, bare rock surfaces, glaciers (Isola et al., 2015; Sterflinger et al., 2012) contaminated soils and rivers. They also thrive in man-made extreme environments like dishwashers or sauna facilities (Gümral et al., 2015; Matos et al., 2002). In both immunocompetent and immunocompromised humans, black yeasts can colonize the skin and bones as well as the lymphatic and nervous systems (Seyedmousavi et al., 2014). A serious and life-threatening problem can be fungal wound infections in burn patients with large total charred body surface areas or compromised immune response (Schaal et

al., 2015). The clinical picture caused by black yeasts includes mere superficial blackening of the skin, for example Tinea nigra caused by *Hortea werneckii*, necrotic lesions of the skin and formation of cysts, granuloma and tumors, eumycetoma and chromoblastomycosis triggered by species of *C., Fonsecea* and *E.*, to deep systemic infection of the lymphoid system and passage of the blood brain barrier with fatal lesions in the human brain, e.g., provoked by species of *C.* and *E.*. Black yeasts are involved in a broad range of diseases and detailed knowledge of the ways of infection, the factors leading to virulence and outbreak of the diverse clinical pictures are still elusive.

Since 1990, an increasing number of phaeohyphomycosis, i.e., infections caused by dematiaceous or pigmented filamentous fungi that contain melanin in their cell wall, has been reported in immunocompromised patients (Revankar et al., 2002) and the list is expected to grow. This is not only explained by the availability of better diagnostic tools (Seyedmousavi et al., 2014) but particularly by the outstanding evolutionary diversification and adaptation of black yeast to their mammalian hosts. Especially the genus *E.* can be regarded as an evolutionary hotspot with a high diversification and emerging adaptation toward many environments (de Hoog et al., 2003). *Exophiala dermatitidis* in particular is a polyextremophilic, poikilohydric and poikilotrophic fungus tolerating low and high temperatures, wateravailability and pH values. As a consequence the fungus is commonly found in a wide range of natural and anthropogenic environments. In fact steam bath facilities, saunas and dishwashers are man-made extreme environments where *E. dermatitidis* is thriving (Gostinčar et al., 2011; Matos et al., 2002; Zalar et al., 2011).

Infection with *E. dermatitidis* may affect cutaneous and subcutaneous regions, leading among other to otitis externa, keratitis and onychomycosis (Hoog and Guarro, 1995; Matsumoto et al., 1993). Colonization might also happen in the lung of cystic fibrosis patients, which represent 2–8% of the susceptible patient population (Chotirmall and McElvaney, 2014; Horré et al., 2004), or the intestinal tract (de Hoog et al., 2005). *Exophiala dermatitidis* is also the etiologic agent of life threatening systemic infections that are predominantly found in patients with diabetes mellitus, rheumatic arthritis, lymphocytic leukemia or of Asian descent (Sudhadham et al., 2008). While the incidence of infection with *E. dermatitidis* is low, its mortality rate for

systemic infections of 40% is high (Chen et al., 2016). In case of neurotrophic infection the fatality rate has been reported to be over 80% (Patel et al., 2013).

The principles of pathogenesis, the host response as well as the difference in incidence in humans of different etiology and predisposition are poorly understood. The mechanisms sustaining the polyextremophily of *E. dermatitidis* are both involved in the pathogenicity and the antifungal resistance of this black yeast (Seyedmousavi et al., 2014). Melanin, which is not essential for growth and development, plays a crucial role in virulence and pathogenicity, allows the fungus to escape phagocytosis and protects him against free radicals (Paolo et al., 2006; Revankar and Sutton, 2010) Thermotolerance, cellular plasticity and the ability to assimilate aromatic hydrocarbons are features that emerged in order to adapt to harsh environments and that are used by *E. dermatitidis* to successfully infect and invade its host (Abramczyk et al., 2009; Seyedmousavi et al., 2014; Ye and Szaniszlo, 2000).

E. dermatitidis has become the most studied species within the group of black yeasts due to its human pathogenicity, its polyextremophily, its association to the human environment as well as its close relation to many other black fungi. While studies looking at the transcriptome or the proteome have been released recently, their focus was limited to fungal response under pH and temperature stress (Blasi et al., 2015; Chen et al., 2014; Tesei et al., 2015). This is in contrast to other pathogenic fungi where transcriptomes studies of the infection are available both for the host and the pathogen (Enguita et al., 2016).

In this work we present for the first time results of an artificial traumatic infection of human skin models by *E. dermatitidis*. *Ex-vivo* human skin explants were wounded and inoculated with *E. dermatitidis* and incubated at 37°C to simulate human body temperature for 1 week. At the end of the experiment, attachment and fungal growth on the skin grafts were confirmed microscopically and biomass was harvested for RNA sequencing. These data, together with multiple genomes alignments, were used to improve the current *E. dermatitidis* annotation (Chen et al., 2014) by identifying new UTRs, adding splicing variants and reporting new coding and (long) non-coding elements. Transcriptome data from *E. dermatitidis* grown on a nylon membrane in cell culture medium were used to find new coding and non-coding transcript, improve the *E. dermatitidis* genome annotation and identify differentially expressed coding

and non-coding genes. We observe differential regulation of known virulence factors as well as newly annotated coding and non-coding genes. These findings will help to better understand which mechanisms are employed by *E. dermatitidis* to infect skin.

3.2 Materials and Methods

Fungal strain, skin grafts, and culture conditions

Skin for the *ex-vivo* skin wound model was obtained from two healthy patients (60 and 38 years) undergoing breast reduction from the Department of Plastic, Reconstructive and Aesthetic Surgery, Klinikum Bremen-Mitte, Germany. Skin was either a full thickness graft or a partial thickness graft. Skin stripes were cut into 5 × 5 cm pieces and on the surface cuts were done with a scalpel to simulate wounds. On top of each wound *E. dermatitidis* (CBS 525.76) was inoculated with a sterile inoculation loop taken from a 7 days old culture grown on malt extract agar (2% malt extract, 2% D-glucose, 0.1% bacto-peptone and 2% agar). The skin was cultured as described by Mirastschijski et al. (2002): Culture media composed of DMEM (4500 mg/L Glucose, D5671, Sigma Aldrich, St. Louis, MO), Penicillin—Streptomycin (100 U Penicillin per mL, 100 µg Streptomycin, P4333 Sigma Aldrich, St. Louis, MO) and 10% v/v heat-inactivated bovine serum (Gibco, Life Technologies Carlsbad, CA) was carefully added to the skin grafts. A surface-liquid interface was built, allowing the skin to float in media but keeping the surface unsubmerged to prevent the inoculum from being washed away. The cultures were kept at 37°C for 7 days and medium was exchanged every second day. For every part and patient a control without fungal inoculum was kept. As negative control, *E. dermatitidis* was inoculated on a prewetted Nylon membrane (Whatman 0.45 µm) for 2 days at 37°C. Culture medium, as described, was added and kept for 1 week at 37°C. *Exophiala dermatitidis* cells growing on the skin models were collected by scrapping them from the skin surface.

Ethics statement

The use of human skin for this study was approved by the ethics committee of the Medical Council of Bremen (No 336-2012). Written consent was obtained from all donors prior to the operation.

Microscopy

Light microscopy After fixation in formalin, pieces of tissue were embedded in paraffin and sections were cut with a microtome. Sections were stained on SuperFrost microscope slides using common Haematoxylin and Eosin (HE) staining. Pictures were taken with a light microscope (Olympus BX51) of the HE stained samples and the skin pieces.

Transmissions electron microscopy (TEM) Preparation of the samples were done according to Arcalis (2004) with small modifications. Samples were cut into 1 mm3 pieces and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4, v/v) for 2 h at room temperature. Samples were washed several times with phosphate buffer (0.1 M) and then kept in buffer over night at 4°C. The samples were post-fixed with osmium tetraoxide (1%) and potassium ferricyanide (0.8%) for 3 h at room temperature. Washing steps were performed with phosphate buffer (0.1 M). On ice, acetone series were used for dehydration. Infiltration with epoxy resin (Agar low viscosity resin kit, Agar Scientific Ltd., Essex, UK) was done in several steps for 3 h on ice. The sample was kept at 4°C in pure resin overnight. Polymerization was done in pure resin in inclusion molds at 60°C for 24 h. From the blocks, ultrathin section were cut and mounted on copper grids. Staining was done with 1% (w/v) aqueous uranyl acetate. The ready prepared sample grids were inspected with a FEI Tecnai G2 operating at 160 kV.

RNA extraction and sequencing

The FastRNA Pro RED KIT (MP Biomedicals, Santa Ana, CA) was used to extract total RNA out of three independent biological replicates. Fungal biomass was scratched from the skin surface to perform RNA extraction. Following the Life

Technologies manual, of 1–8 µg total RNA, mRNA were poly(A)-enriched with the Dynabeads mRNA DIRECT Micro Kit (Ambion by Life Technologies, Carlsbad, CA) and the libraries were created using the Ion Total RNA-Seq kit v2 (Life Technologies, Carlsbad, CA). In all the steps quality and quantity of the RNA and in later steps cDNA was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qbit Fluorometer 2.0 (Life Technologies, Carlsbad, CA). Targeted length of the library was selected to 290 bp with Pippin Prep instrument (Sage Science, Beverly, 7 MA). Sequencing was performed using Ion Proton Technology and the HiQ sequencing kit.

Bioinformatics

Reads mapping All reads were mapped with STAR 2.4.1d (Dobin et al., 2013) with -alignIntronMin 15 -alignIntronMax 2000 -outFilterIntronMotifs RemoveNoncanonicalUnannotated -chimSegmentMin 12 -chimJunctionOverhangMin 12 -alignSJDBoverhangMin 10 against the concatenated human and *E. dermatitidis* genomes. This allowed us to assess how many reads were preferentially mapping to human. Unmapped reads were mapped against a concatenated database of rRNAs by using mapping parameters allowing a higher sensitivity –outFilterScoreMinOverLread 0 –outFilterMatchNminOverLread 0 –outFilterMatchNmin 20 The reads mapping against *E. dermatitidis* were then used to find differentially expressed genes, detect chimeric RNAs and to improve the current *E. dermatitidis* annotation. Count of mapped reads on annotation elements was done with featureCounts v1.4.6p2 (Liao et al., 2014). The identification of differentially expressed genes was done with the R (R Core Team, 2016) module edgeR (Robinson et al., 2010), while the functional enrichment of the significantly regulated genes was done with GoStat and Kobas, with BlastP (*E*-value < 1·10−08) (Falcon and Gentleman, 2007). Revigo (Supek et al., 2011) was used to summarize the lists of overrepresented Gene Ontology terms. Sample to sample distance was assessed with R. Fusion transcripts were detected with the STAR-Fusion pipeline: https://github.com/STAR-Fusion/STAR-Fusion while circular RNAs (circRNAs) were detected by looking directly at the read mapping patterns. In order to be reported, circular RNAs had to be supported by at least 5 split reads in two replicates of the

same experimental condition. Fusion transcripts were reported if they appeared in at least three replicates of the same condition and with at least ten sustaining reads in each replicate.

Annotation The annotation of ncRNAs was taken from Blasi et al. (2015) while the protein coding annotation was obtained from Chen et al. (2014). Genes annotation were enriched by assembling the reads into transcripts. To this end the sequenced reads were assembled with Trinity 2.0.6 and Cufflinks 2.2.1 (Haas et al., 2013; Trapnell et al., 2012). The assembled transcripts were then combined with PASA (Haas et al., 2003). These transcripts were used to update (splice variants, UTR) and enrich the protein-coding gene annotation. Transdecoder (Haas et al., 2003) was applied to the PASA-assembled transcripts to find new putative protein-coding genes. PASA-Transcripts that were not considered coding by Transdecoder and by CPAT (Wang et al., 2013) (p-value < 0.01), that did not show significant (p-value > 0.001) sequence homology against SWISSPROT when searched with Blast (Altschul et al., 1997) and that did not contain Pfam-domain (Finn et al., 2014) (p-value > 0.001) when searched with HMMER (Rawlings et al., 2014) were classified as non-coding. The transcripts that could not be unequivocally classified as either coding or non-coding were classified as transcript with unknown functions. Samtools (Li et al., 2009) were used to process the mapped reads. Annotation overlap were studied with the Bedtools (Quinlan and Hall, 2010). Interproscan (Jones et al., 2014) as well as the CaZy-, Merops- and TCDB-databases (Cantarel et al., 2009; Rawlings et al., 2014; Saier et al., 2006) were used to functionally annotated protein coding genes. Conserved coding and non-coding elements were detected with RNAcode (Washietl et al., 2011) (p-value < 0.01) and RNAz (Washietl et al., 2005b) (probability P > 0.9), respectively. To this aim a multiple genomes alignment of *E. dermatitidis, C. immunda, Fonsacaea pedrosi, Hortea werneckii* and *Candida albicans* was generated with the multiz pipeline (Blanchette et al., 2004).The pipelines for the RNAseq mapping, differential expression, chimeric RNA annotation and gene functional annotation were implemented with snakemake (Köster and Rahmann, 2012).

3.3 Results

RNA sequencing of E. dermatitidis

The transcriptional landscape of *E. dermatitidis* during infection was studied by growing *E. dermatitidis* on human *ex-vivo* skin models as well as on a prewetted Nylon membrane for 1 week (see Materials and Methods). The skin infection and control experiments were sequenced in triplicates on the Ion Proton platform, yielding a total of 286 million reads with an average length of 170 nts. The reads were mapped against the concatenated human and *E. dermatitidis* genomes, in order to separate the reads based on their human or fungal origin. From the 286 million reads, between 32% and 78%, depending on the sequencing run, mapped to the fungal genome, i.e., 154.5 million reads could be mapped to the *E. dermatitidis*. 173,133 reads were mapped to the human genome (Supplementary Table 1 for more details). The reads mapping against the human genome were discarded from further analyses. The unassigned reads were remapped to a set of saccharomycetes and human rRNAs with STAR parameters increasing the mapping sensitivity (see Materials and Methods). Between 83% and 98.76% of the unmapped reads were assigned to rRNAs, indicating that the poly (A) enrichment protocol did not completely discard rRNAs. Despite the rRNA contamination, sample expression similarity assessed with principal component analysis fits well with the expectation from the experimental design (Figure 5).

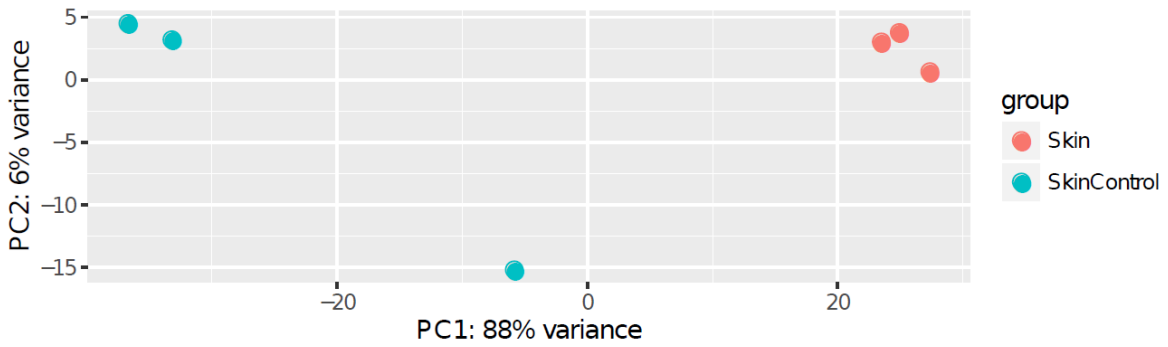

Figure 5. Similarity assessment of the RNA sequencing runs with principal component analysis. The clustering pattern fits well with the expectation from the experimental design.

Infection

The inoculated skin regions were found to be populated with *E. dermatitidis* four days post infection. After 7 days the fungus had completely covered the skin surface (Figure 6 A-C). Further the fungus successfully invaded the skin as can be seen from the HE stained microscopic specimen shown in Figure 6D. *E. dermatitidis* also started to colonize the undersurface of the skin (Figure 6H).

The successful skin entry and growth of fungal cells were also validated by TEM pictures (Figure 6 E-G) where the incorporation of fungal cells into skin fibers can clearly be seen. The black electron dense bodies scattered in the fungal cells are evidence of melanosomes comparable to findings reported in the black yeast *Fonsacaea pedrosi* (Franzen et al., 1999, 2008). Another electron dense matter can be seen as melanin layer surrounding the cell wall. Morphological forms, especially melanosome-like electron dense spots, are heterogeneously scattered between individual cells (Figure 6 E-G) which might be due to differences in contact between skin and individual fungal cells.

The observed increased melanin production is in line with the strong upregulation of Tyrosine aminotransferase (16-fold) and Hydroxyphenylpyruvate dioxygenase (4-fold), two enzymes involved in the production of melanin through the L-Tyrosine degradation pathway (Figure 6D).

Genome re-annotation

Conserved elements The *E. dermatitidis, C. immunda, Fonsacaea pedrosi, Hortea werneckii* and *Candida albicans* genomes were aligned with multiz (Blanchette et al., 2004). RNAz (Washietl et al., 2005a) and RNAcode (Washietl et al., 2011) were run on it in order to detect non-coding and coding elements, respectively. A total of 11168 conserved regions covering 58% of the genome were returned by the alignment pipeline. RNAz detected a total of 895 conserved non-coding loci with a *P*-score>0.9. 683 hits overlapped 681 protein coding genes among (348 UTRs, 711 CDS). 182 elements overlapped with non-coding transcripts, 30 with annotated non-coding RNAs and 89 hits were intergenic.

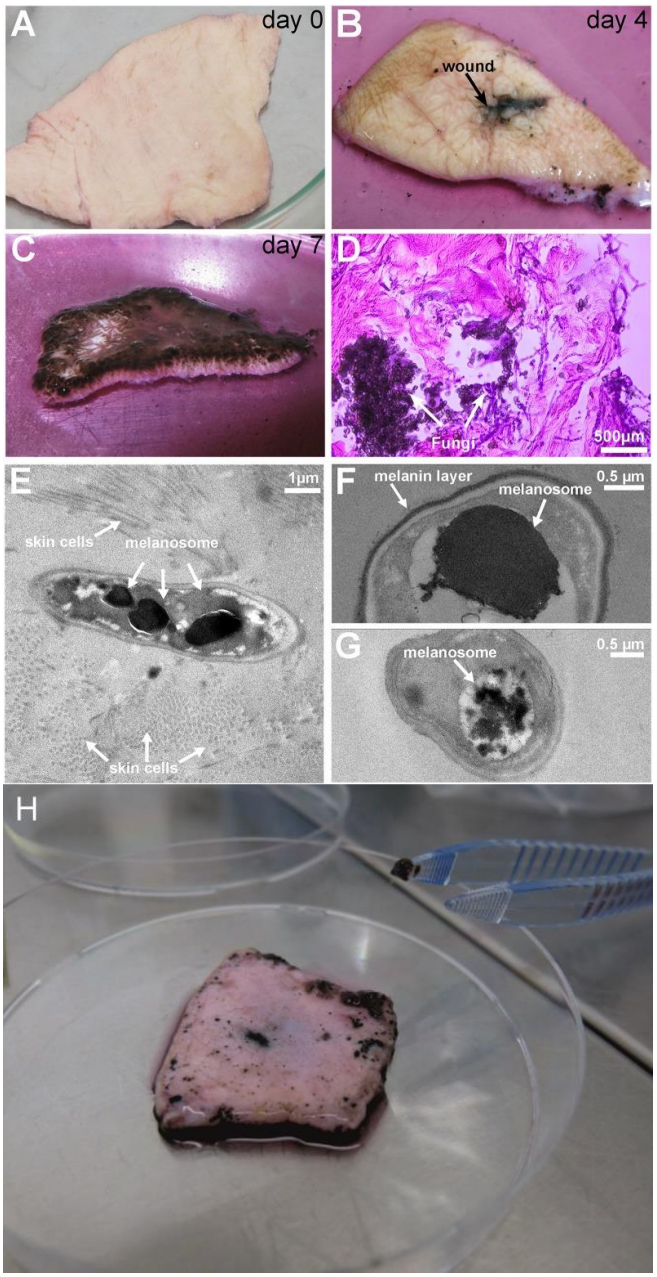

Figure 6 Pictures of the colonization and invasion of *ex-vivo* skin samples by *E. dermatitidis* at 37° and TEM pictures after 1 week. (A) At day 0, *ex-vivo* skin samples were cut in approximately 5 × 5 cm pieces and inoculated with *E. dermatitidis*. (B) At day 4 growth in and around the wound (black spot in the middle) could already be observed. (C) At the end of the experiment (day 7), the upper skin surface was completely covered by the fungus. (D) HE staining of the infected skin. Skin is stained in pink and the fungus is stained in deep purple. After 7 days *E. dermatitidis* had penetrated deep inside the skin. (E) Melanosomes inside a fungal cell surrounded by skin cells. (F) Vacuole filled with flocculated melanin and melanin layer surrounding the cell. (G) Flocculated melanin in a vacuole. (H) After 1 week, *E. dermatitidis* grew through the wound and was visible on the undersurface of the skin model (Black spot in the middle).

As RNAz hits have no strand information a single hit might overlap to transcript located on both strands. RNAcode predicted 24,613 conserved coding elements with a *p*-value < 10−3. Among them 23,200 (94.2%) mapped to annotated proteins, 312 mapped to non-coding transcripts and 150 mapped to the list of transcripts that could not be strictly classified as coding or non-coding.

Three unstructured but conserved snoRNAs, i.e., snR72, snR74, and snR4 were found by specifically looking at conserved intronic regions in *E. dermatitidis* and subsequently by searching for homologous sequences in fungi with RNAlien (Eggenhofer et al., 2016) (Figure 8 A,B).

RNAseq-based re-annotation The current transcript annotation of *E. dermatitidis* was updated by using RNA-Seq data from the skin and skin-control experiments (See Methods and Figure 7 for more details).Out of the 9577 protein coding transcripts annotated in *E. dermatitidis*, 3391 were not modified by PASA while 4168 new splice variants were detected (see Supplementary Table 1). In 18 cases two neighboring transcripts were merged into one (see Supplementary Table 1). Further a total of 2284 coding transcripts, mapping to 1241 loci and without overlap with known CDS, were found. Among them 555, mapping to 348 loci, had no UTR overlap with the official genome annotation (see Supplementary Table 1).Given the genome size of *E. dermatitidis* the total number of 10907 protein-coding loci is in-line with that of *E. mesophila* (29Mb,9121loci), *E. sideris* (29Mb,10114 loci) and *E. spinifera* (32MB, 12049 loci). 684 gene loci overlapped with genomic regions contained in the multiple genomes alignment. The 2284 predicted protein-coding transcripts were blasted against the nr-database. 578 proteins from 131 loci had a hit with a E-value < 0.001. 92 out of the 131 loci overlapped with regions of the multiple genomes alignment. 387 proteins from 133 loci could be functionally annotated (see Methods).Further we looked at the presence of new non-coding RNAs by fetching all PASA assembled transcripts that did not overlap with protein-coding loci, that did not contain pfam-domain, that were not reported as coding by CPAT (Wang et al., 2013) (Wang et al., 2013) (coding p-value >0.001) and that did not show sequence homology to SwissProt (Blastp E-value >0.001).

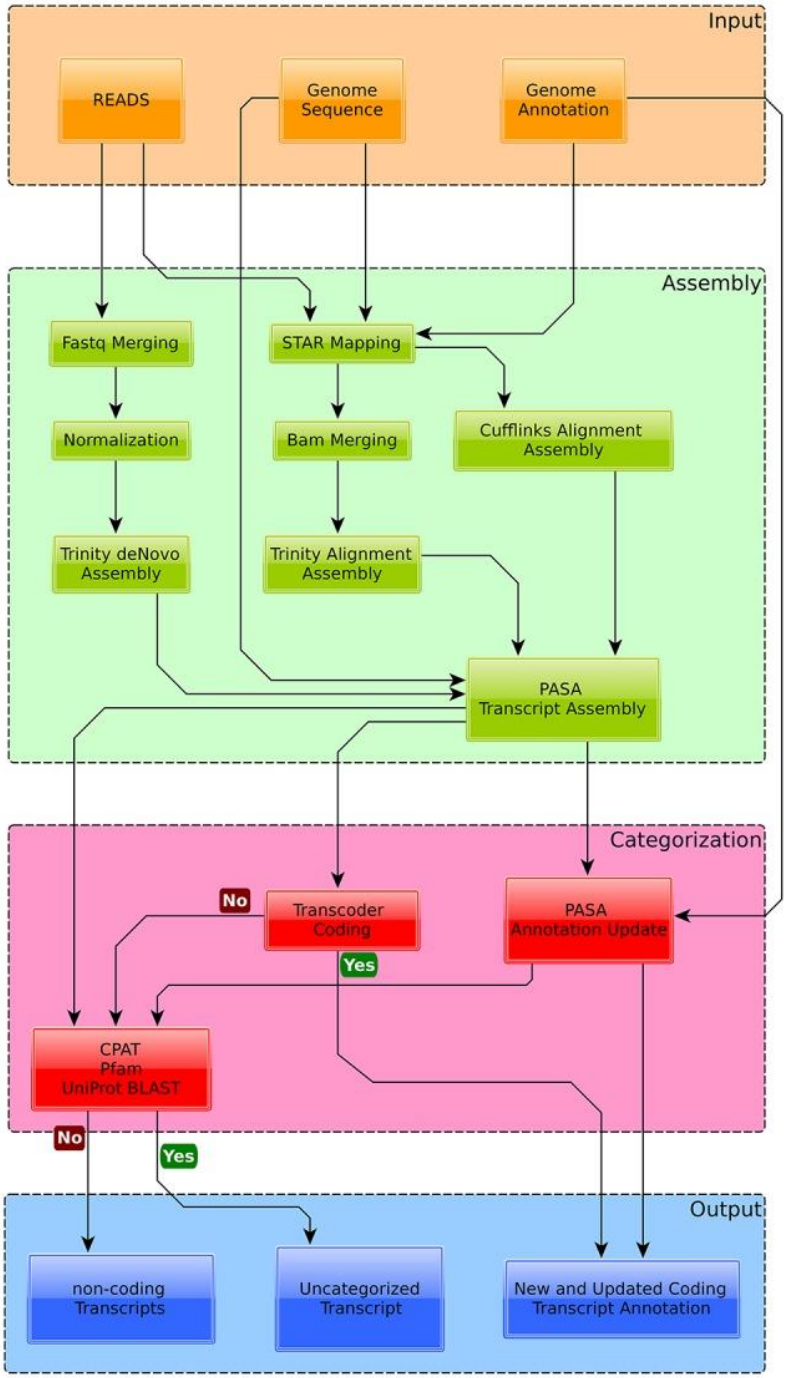

Figure 7 Overview of the annotation pipeline used to augment the current genome annotation of *E. dermatitidis*. Transcripts were assembled with Trinity and Cufflinks and mapped back to the genome with PASA. Transcripts overlapping with *E. dermatitidis* coding regions were used to find new splice variants and (re-)annotate UTRs. Transcripts that were considered coding by Transdecoder were classified as new proteins. The other transcripts were tested for their coding potential with CPAT, Pfam and by blasting them against Uniprot in order to differentiate between truly non-coding transcripts and non-coding transcripts with coding potential.

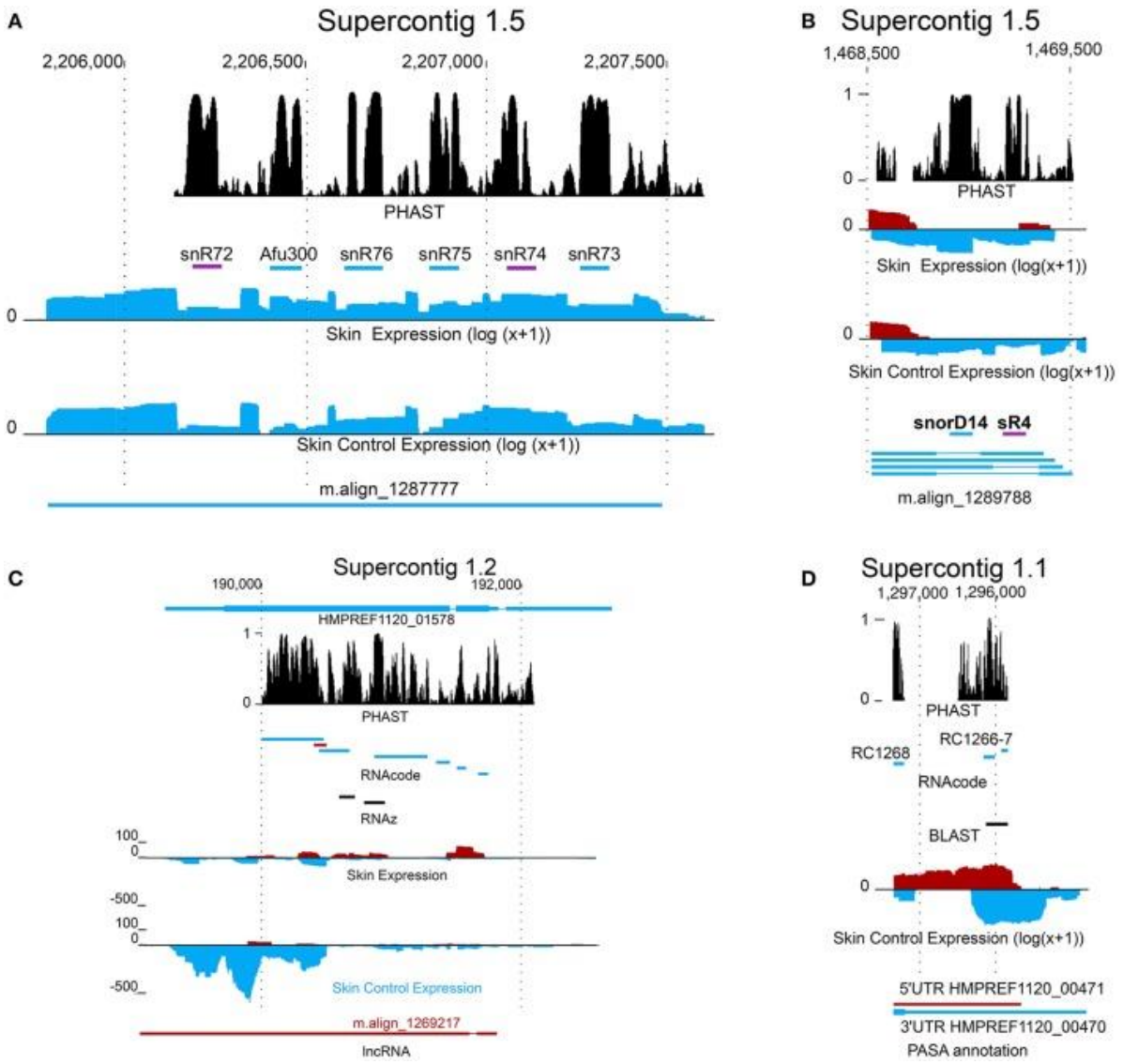

Figure 8 New coding and non-coding genes in *E. dermatitidis*. (A) m.align_1287777 is a spliced non-coding transcript containing intronic snoRNAs. snR72 and snR74, both shown as were annotated in this study**.** (B) The transcripts encoded in region Supercontig_1.5 1468500-1469500 contains two intronic snoRNAs, snorD14 and sR4. snorD14 is upregulated when *E. dermatitidis* grows on the *ex-vivo* skin samples. (C) lncRNA m.align_1269217 and HMPREF1120_01578 are antisense and exhibit opposite regulation pattern. They are located in a conserved region where the secondary structure and the coding potentials are constrained**.** (D) A homolog of *Candida albicans* metallothionein, shown as black bar, was found in a conserved region overlapping with the 3UTR of HMPREF1120_00470. The coding potential of the region encoding the metallothionein is further sustained by the RNAcode predictions RC1266 and RC1267.

A total of 7778 non-coding transcripts mapping to 5017 gene loci were found. Four snoRNAs, homologous to snR4, snorD14, snosnR55, and snosnR61 are located in introns of long non-coding transcripts (Figure 8B). Each snoRNA is flanked by canonical splice sequences (GT/AG).

The transcripts that were neither classified as coding by transdecoder nor rejected as non-coding by CPAT, Pfam or Blast (see Methods) were classified as transcript of ambiguous type (TAT). A total of 6630 TATs from 5800 loci were found. Among these 5800 loci, 3133 overlapped with RNAcode predictions. While no information on the TAT function is available, one TAT, align_m.1287777 is hosting a snoRNA cluster containing snR72, snR74, snR75, snR76, snR77, Afu300 (Figure 8A). SnoRNA clusters have been detected in a multitude of different eukaryotic organisms, including *Saccharomyces cerevisiae*, which may suggest an ancient origin of this gene arrangement (Liang et al., 2002). Similar to the snR4-snorD14 cluster, each snoRNA is flanked by canonical splice sites indicating that they are actively spliced out of the TAT transcript.

Circular and chimeric RNA The occurrence of circular RNAs and fusion transcripts in the transcriptome data was investigated. CircRNAs were searched 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. Five circularized transcripts were found in at least two replicates of the skin with at least 6 supporting read and 4 were found in the skin control.

The presence of fused transcripts in *E. dermatitidis*, i.e., distant transcripts connected by split reads were recently reported in Blasi et al. (Blasi et al., 2015). In this study, 126 fusion transcripts supported by at least 10 reads in all replicates of a given condition were found. Sixty two of the fusion transcripts were specific to the control condition, 43 were found exclusively in the skin experiment and 21 were found in both conditions.

Transcript expression

Functional enrichment of the 100 most highly expressed coding genes in the skin and control samples was analyzed. In the control samples, genes with domains related to Band 7 proteins were enriched (HMPREF1120_07454, HMPREF1120_01950, HMPREF1120_06159) (fdr = 0.001) and Histone-related proteins (HMPREF1120_06310, HMPREF1120_01816, HMPREF1120_06252). The 100 most highly expressed proteins in the skin experiment were related to Heat shock protein 70 (Hsp70) (HMPREF1120_02626, HMPREF1120_07756, HMPREF1120_08142,fdr = 0.002; 3/5 genes), phosphorylcholine metabolism (HMPREF1120_09233, HMPREF1120_04356 fdr = 0.004, 2/2) and translation elongation (HMPREF1120_08281, HMPREF1120_00844, fdr = 0.004, 2/2). Hsp70 is known for having function in protection and repair of cells after stress. In addition, in *Candida albicans* two members of the Hsp70 family are expressed on the cell surface and function as receptors for antimicrobial peptides (López-Ribot et al., 1996; Sun et al., 2010a). In the pathogenic fungus *Paracoccidioides brasiliensis* Hsp70 was also found to be induced in the mycelial to yeast transition (da Silva et al., 1999). Phosphorylcholine (PC) locates on the cell surface of various pathogenic prokaryotes and eukaryotes. While PC is targeted by the host immune system, PC can also modulate the response of the immune system, allowing the pathogen to remain undetected. Through modifications of the PC, the pathogenic microbes and bacteria can hide from or modulate the immune response (Clark et al., 2012; Clark and Weiser, 2013).

Differential expression

ncRNAs It was checked if ncRNAs as annotated in Blasi et al.(Blasi et al., 2015) were differentially expressed. While no non-coding RNA was significantly downregulated in the skin experiment, 4 ncRNAs (SRP, Afu_300, snorD14,U6) were upregulated with a fdr < 0.05 (see Supplementary Table 1). snorD14 (U14/snR128), which is upregulated 10 times (corrected fdr = 0.026) in the skin experiment vs. control, is a C/D Box snoRNA that is found in plants, fungi and animals. Genetic depletion and mutation of snorD14 in yeast showed that it is required for growth and

that loss of snorD14 disrupts pre-rRNA processing (Samarsky et al., 1996). Interestingly snorD14 was shown to be contained in the extracellular vesicles (EV) of *Saccharomyces cerevisiae* and of human fungal pathogens (*Cryptococcus neoformans, Paracoccidioides brasiliensis* and *Candida albicans*)(Peres da Silva et al., 2015). Similarly we could identify this snoRNA in the extracellular vesicles of *E. dermatitidis* and *C. immunda* growing at 37°C (manuscript in preparation).

Like snorD14, SRP-RNA (7SL) was also found in EV of *E. dermatitidis* and was strongly upregulated in the skin experiment. With the exception of Srp74 that was significantly upregulated (fdr = 0.006), the other SRP components annotated in *E. dermatitidis* were not differentially expressed.

Sixty four putative lncRNAs were significantly (fdr ≤ 0.05) upregulated (see Supplementary Table 1). We looked at the set of significantly regulated proteins overlapping with the group of significantly regulated lncRNAs. We found that 7 protein-coding loci were significantly upregulated (HMPREF1120_06710, HMPREF1120_00114, HMPREF1120_01575, HMPREF1120_04063, HMPREF1120_ 01423, HMPREF1120_01437, HMPREF1120_01107), and 4 were significantly downregulated (HMPREF1120_01578, HMPREF1120_06121, HMPREF1120_00784, HMPREF1120_04399) (Figure 8C).

The region covered by HMPREF1120_01578 is interesting as it contains signals for coding and non-coding elements. There is a long non-coding RNA antisense to the coding locus. The genomic region is well conserved as can be seen from the PHAST score in Figure 8C. RNAcode tells us that both strands have significant coding potential, i.e., that the antisense lncRNA might also have at least one conserved ORF. Finally we find RNAz hits that overlap with one of the RNAcode predictions, the non-coding RNA and the coding locus. These annotations indicate that the protein and/or lncRNA loci encode transcripts that might be multi-functional, working either as a coding elements and/or as ncRNAs.

Fourteen putative TAT were significantly upregulated and 5 were downregulated. Two upregulated TAT overlapped with two upregulated protein coding genes.

Functional enrichment of regulated coding genes Over 2000 protein coding genes were differentially expressed (DEG) with an fdr < 0.05 and $|\log_2 FC| > 1$ between the skin and control experiments (see Supplementary Table 1). Even at a more stringent threshold on the fold change ($|log_2 FC| > 2$), 1016 DEG were found (490 upregulated and 526 downregulated) with an fdr < 0.05 between the skin and control experiments (see Supplementary Table 1).

Upregulated genes The largest enriched functional category in the set of upregulated genes was DNA replication (fdr = $3.3 \cdot 10^{-4}$). Together with the categories DNA metabolic process (fdr = $5.0 \cdot 10^{-3}$) and rRNA metabolic process (fdr = $7.0 \cdot 10^{-3}$), it indicates that *E. dermatitidis* seems to be in a replicative state when growing on skin. The enrichment analysis of protein domains in the upregulated genes is in line with the GO analysis. P-loop containing nucleotide triphosphate hydrolase (NTPase) (G3DSA:3.40.50.300, fdr = $4.9 \cdot 10^{-15}$, 101 genes; IPR027417, fdr = $1.67 \cdot 10^{-14}$, 104 genes; SSF52540, fdr = $4.56 \cdot 10^{-14}$; 104 genes) is the most significantly enriched term. In *Toxoplasma gondii* NTPase are essential for the parasite proliferation (Nakaar et al., 1999). Helicase C (Pfam00271, fdr = $1.25 \cdot 10^{-8}$, 32 genes), helicase_CTER (PS51194, fdr = $5.66 \cdot 10^{-8}$, 32 genes), DEAD-like helicase superfamily (SM00487, fdr = $5.57 \cdot 10^{-6}$, 19 genes), Helicc (SM0049, fdr = $5.57 \cdot 10^{-6}$, 19 genes), helicase ATP binding (PS51192, fdr = $4.67 \cdot 10^{-8}$, 32 genes), helicase superfamily 1/2 (IPR014001, fdr = $7.97 \cdot 10^{-9}$, 32 genes) and helicase C terminal (IPR001650, fdr = $7.97 \cdot 10^{-9}$, 32 genes) are enriched and correspond to the overrepresented GO terms related to DNA replication. RNA helicases and especially DEAD box RNA helicases are important for RNA synthesis and also function as pre-mRNA processing and in ribosome biogenesis (Franzen et al., 2008). Mini chromosome maintenance complex (PS50051, fdr = 0.0157, 5 genes) is important in the initiation and elongation phases in DNA replication. AAA+ ATPase domain (IPR003593, fdr = 0.0002, 29 genes) is important for membrane fusion, proteolysis and DNA replication (Ogura and Wilkinson, 2001). SNF2-related, N-terminal domain (IPR000330, fdr = 0.0090, 11 genes) is related to transcription regulation, DNA repair, DNA recombination and chromating unwinding.In Merops two families of proteases are overrepresented, C26 (fdr = 0.0010, 13 genes) and S16 (fdr = 0.0043, 6 genes). C26 is a gamma-glutamyl hydrolase which is either secreted or located in

lysosome while S16 Lon protease is a known bacterial and fungal virulence factor (Breidenstein et al., 2012; Cui et al., 2015; Takaya et al., 2003) In TCDB the Peroxisomal Protein Importer (PPI) Family (3A20, fdr = 0.016, 8 genes), the ATP-binding Cassette (ABC) Superfamily (3A1, fdr = 0.0038, 15 genes) and the nuclear mRNA Exported (mRNA-E) Family (3A18, fdr = 0.0038, 17 genes) were enriched in the set of upregulated proteins. An overview of the most enriched protein domains and GO terms can be found in Figure 9A-B.

Downregulated genes Oxidoreductase activity and carbohydrate transport were the most significantly enriched term in the set of downregulated genes. 39 protein domain annotations were enriched (see Supplementary Table 1). NAD(P)-binding domain (G3DSA:3.40.50.720, fdr = 0.0043, 24 genes; G3DSA:3.90.180, fdr = 0.0422, 7 genes; IPR016040, fdr = 0.0019, 61 genes, IPR011032, fdr = 0.0042, 18 genes; PTHR11695, fdr = 0.0502, 7 genes; SSF51735, fdr = $5.2191 \cdot 10^{-5}$, 62 genes) is contained in many different dehydrogenases and is related to the utilization of nitrogen source (Gough et al., 2001) in yeast. IPR005828 and IPR005829 are domains involved in the binding and transport of various carbohydrates and particularly sugars (IPR005829, fdr = 0.082, 14 genes; PF00083, fdr = 0.086, 16 genes; PS00217, fdr = 0.0015, 13 genes; TIGR00879, fdr = 0.0046, 12 genes).

KEGG pathways related to metabolism of xenobiotics by cytochrome P450 (ko00980, fdr = 0.0317, 3 genes), Glycosphingolipid biosynthesis (ko00603, fdr = 0.0317, 2 genes) and N-glycan biosynthesis (ko00513, fdr = 0.0317, 2 genes) are overrepresented. An overview of the enriched protein domains can be found in Figure 9C.

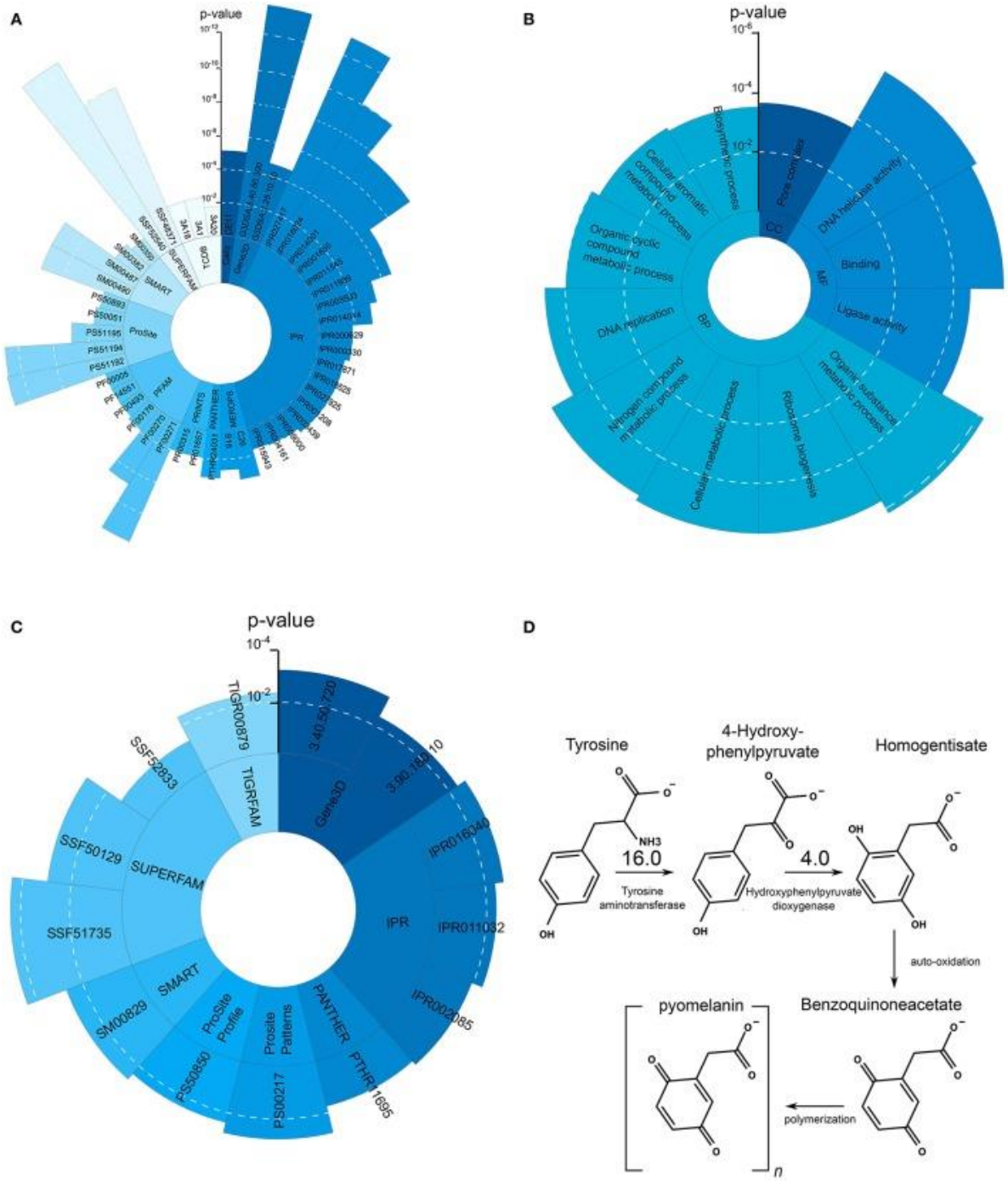

Figure 9 Significantly overrepresented protein domains and GO terms as well as L-Tyrosine melanin metabolism. (A) Overrepresented protein domains in the set of upregulated genes during growth on skin. Each blue shade represent another enriched domain category. (B) Overrepresented GO terms in the set of upregulated genes during growth on skin. (C) Overrepresented GO terms in the set of downregulated genes during growth on skin. (D) L-Tyrosine degradation pathway leading to the production of pyomelanin. Compared to the control condition Tat and HppD are upregulated 16 and 4 times, respectively.

Nutrient acquistion

Carbon In yeast, Snf1 kinase is activated under gluconeogenic conditions leading to the activation of gluconeogenesis and glyoxylate cycle. Snf1 in *E. dermatitidis* is strongly upregulated (fdr = 0.003; 5.46) (see Supplementary Table 1, Figure 10). Since most of the transcriptional regulators triggering gluconeogenesis in *Saccharomyces cerevisiae* are also available in *Candida albicans* and *E. dermatitidis*, similar regulatory mechanisms might exist in these fungi (Fleck et al., 2011; Turcotte et al., 2010). In fact, in the skin environment both the gluconeogenesis pathway and the glyoxylate cycle of *E. dermatitidis* is regulated in a way similar to what is seen in *Candida albicans* upon phagocytosis (Lorenz et al., 2004) (see Supplementary Table 1, Figure 10). The acetyl-CoA needed by the glyoxylate cycle is probably obtained from the β-oxidation pathway as all of its enzymes are significantly upregulated in the skin condition in *E. dermatitidis* (see Supplementary Table 1). This is similar to what is seen in *Candida albicans* (Fleck et al., 2011).

Nitrogen Homologs of *Candida albicans* aspartyl proteases, which are associated with the dissociation of amino acids from the host (Fleck et al., 2011), were found in *E. dermatitidis* but none of them were upregulated.

In *Onygena corvina* a mix of three protease families, S8, M28, and M3 was shown to be sufficient to break down keratin (Huang et al., 2015). Nine S8 proteases were found in *E. dermatitidis*. Only HMPREF1120_07613 and HMPREF1120_06376 were significantly regulated in skin w.r.t. control by a factor of 2.56 and 0.22, respectively. Six genes are members of the M28 protease family but only HMPREF1120_00135 is significantly regulated (fdr = 0.0027; 0.015). Two M03 proteases, HMPREF1120_07166 and HMPREF1120_06271, were found in *E. dermatitidis* but only the former was significantly regulated (fdr = 0.0077; 3.18).

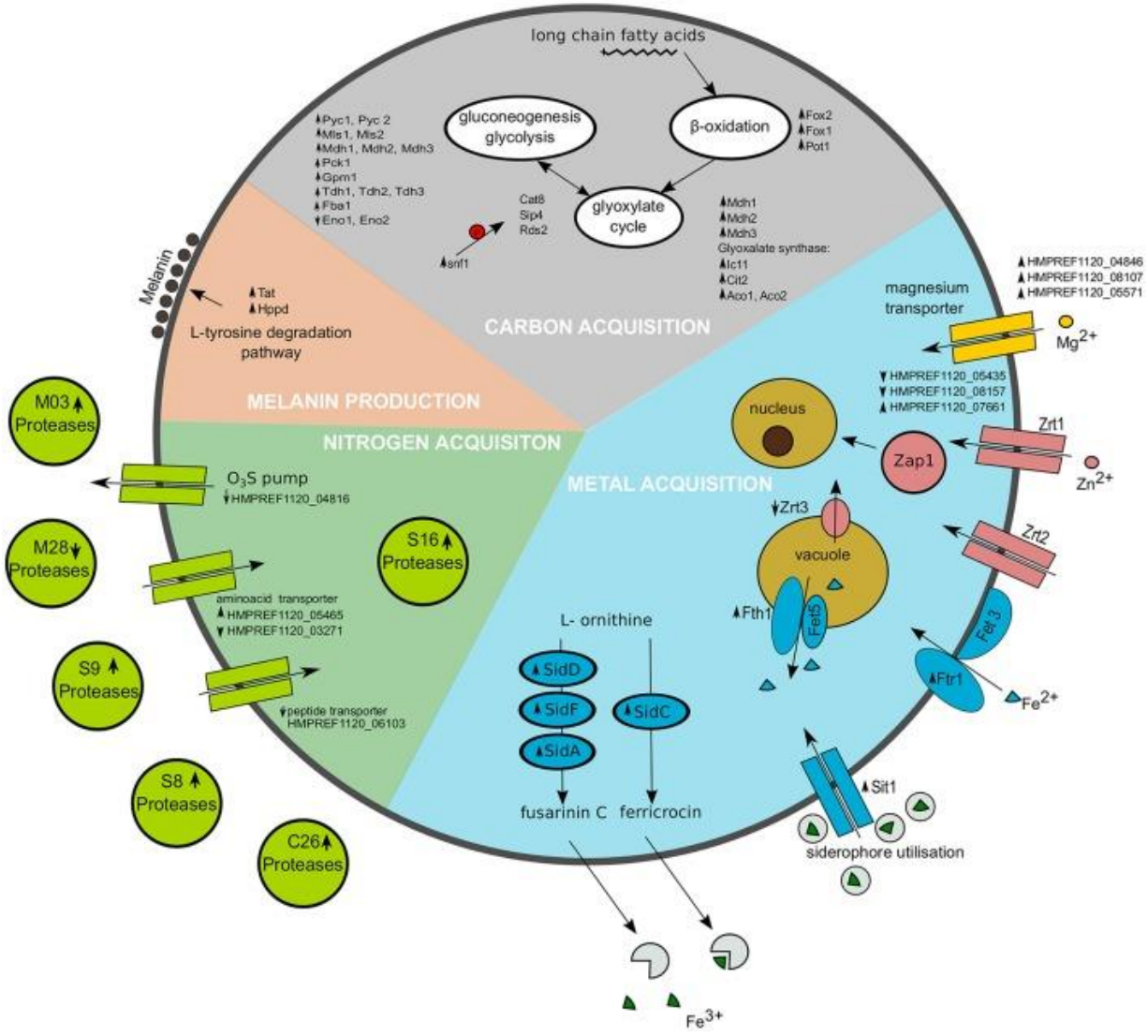

Figure 10 Representation of selected cellular processes significantly regulated in *E. dermatitidis* when growing on skin. Metal acquisition is shown in the blue region, Nitrogen acquisition in the green region and carbon acquisition in the gray region. Melanin production is found in the salmon pink region. Upregulated genes are marked with upward pointing arrows, while downregulated genes are marked with downward pointing arrows. The red P in the carbon acquisition part indicates phosphorylation.

HMPREF1120_07218, a prolyl oligopeptidase, is the second most significantly upregulated protease (fdr = 0.0029; 4.41), and was shown to be a virulence factor in the bacterium *Porphyromonas gingivalis* (Nelson et al., 2003) and might be connected to virulence in the dermatophytes *Trychomyces rubrum* and *Trychophyton mentagrophytes* (Kaufman et al., 2005).

In *E. dermatitidis* 12 proteins are classified as amino acid transporters. HMPREF1120_05465 is upregulated 4.86 times during skin infection, HMPREF1120_03271 is downregulated 3.81 times in skin, while the other genes are not significantly regulated. Seven oligopeptide transporters are found in *E. dermatitidis* among which three are related to *Saccharomyces cerevisiae* OPT (oligopeptide transporter) family. HMPREF1120_06103 is the only significantly regulated peptide transporter (fdr = 0.03; 0.31).

Metal In *E. dermatitidis* during skin infection, three out of 13 annotated magnesium transporter, i.e., HMPREF1120_04846, HMPREF1120_08107 and HMPREF1120_05571, are significantly upregulated by a factor 6.53, 2.87, and 3.05, respectively.

During skin infection, the *E. dermatitidis* Zap1 homolog (HMPREF1120_08244; Blast *E*-value $4 \cdot 10^{-25}$) is significantly upregulated (fdr = 0.01; 2.56). *Exophiala dermatitidis* contains 7 zinc transporters homolog to Zrt1 and Zrt2. Two of them are downregulated (HMPREF1120_05435, HMPREF1120_08157) while one is upregulated (HMPREF1120_07661) when the fungus is growing on skin. Zrc1 and Cot1, two proteins involved in zinc internalization (Conklin et al., 1992; Zhao and Eide, 1997)are not significantly regulated, while Zrt3, a protein associated with zinc mobilization (MacDiarmid et al., 2000), is downregulated (fdr = 0.044; 0.43).

In *E. dermatitidis*, no copper transporter and no metallothionein, are annotated in the genome. Still the region located in Supercontig_1 between 1295848 and 1296128 was found to be significantly (tBlastn *E*-value < 10^{-19}) similar to Cdr2 (Figure 8), a metallothionein (Ding et al., 2014). This is a conserved region with significant RNAcode signals overlapping with the 3'UTR region of HMPREF1120_00470. Other genes involved in Cu uptake, like Crt2 (HMPREF1120_07198), Crt3 (HMPREF1120_00028) Fre (HMPREF1120_00949), Ccc2 (HMPREF1120_05014), Atx1 (HMPREF1120_03801) and Sur7 (HMPREF1120_00735) (Ding et al., 2014) were found in the genome, but were not significantly regulated in the skin experiment.

Genes related to the reductive Iron uptake pathway, like Fres (HMPREF1120_ 00949), Ccc1 (Li et al., 2001) (HMPREF1120_04139, HMPREF1120_03992), or the gene pairs Fth1/Fet5 and Ftr1/Fet3 (HMPREF1120_01589, HMPREF1120_ 01590; HMPREF1120_02753, HMPREF1120_02754; HMPREF1120_04509, HMPREF1120_04510) were found in *E. dermatitidis*. Still only HMPREF1120_ 04509 (putative Ftr1 or Fth1) is significantly regulated in the skin experiment (fdr = 0.025; 6.76).

While we could not find homologs to *Candida albicans* heme- or ferritin-transporters, 8 genes homolog to Sit1, a siderophore protein responsible for iron uptake in *Candida albicans, Saccharomyces cerevisiae* and *Cryptococcus neoformans*, were found in *E. dermatitidis* (Ding et al., 2014). Three of them (HMPREF1120_07838, HMPREF1120_02555, HMPREF1120_01434) were significantly regulated (fdr = 0.006, 0.011, 0.031; 14.26, 7.07, 4.473) in skin w.r.t. control. Further the enzymes responsible for the synthesis of the siderophores fusarinin C and ferricrocin, i.e., SidD, SidF, SidA and SidC (HMPREF1120_01440, HMPREF1120_01438, HMPREF1120_07635, HMPREF1120_07636) (Chen et al., 2014) were upregulated when *E. dermatitidis* grew on skin (fdr = 0.012, 0.010, 0.0334, 0.0028; 8.74, 4.55, 2.45, 7.31).

Virulence related genes

We checked for differential expression of known virulence factors of *E. dermatitidis, Blastomyces dermatitidis, Candida albicans, Aspergillus fumigatus* and *Cryptococcus neoformans* found in the PHI database (Winnenburg et al., 2008). Three genes related to virulence were found to be upregulated: 60S ribosome biogenesis protein Brx1 (PHI:2546, Q4WKJ9, HMPREF1120_02997, fdr = 0.0061, logFC = 2.18), ATP dependent RNA helicase mak5 (PHI:2549, Q4WMS3, HMPREF1120_06261, fdr = 0.0063, logFC = 2.46) and nuclear export protein Noc3 (PHI: 2551, Q4WZG4, HMPREF1120_05871, fdr = 0.0069, logFC = 2.39).

Dimorphism *E. dermatitidis*, like *Candida albicans* (Albrecht et al., 2006; Schaller et al., 1999), is a polymorphic fungus that can adopt yeast-like form or the hyphal form. Both morphologies were found in various cases of Phaehyphomycosis caused by *E. dermatitidis* (Myoken et al., 2003; Park et al., 2011; Woollons et al., 1996). In the HE stained specimen it can be observed that the majority of the fungal cells are in yeast form (Figure 6D). We searched for homologs of eight dimorphism related genes reported in Mayer et al. (Mayer et al., 2013) (see Table 1). Hgc1, a gene necessary for the formation of hyphae, showed a significant regulation (fdr = 0.012, logFC = 3.13). Further the expression of HMPREF1120_05541, a gene shown to be essential for the yeast-hyphal switch (Ye and Szaniszlo, 2000) and homolog to *Candida albicans* Efg1, is increased by a factor 7 during skin infection, indicating that a part of the fungal population growing on skin is switching to the hyphal form.

Table 2 List of fungal virulence factors found in *E. dermatitidis*. Genes with at least one homolog in *E. dermatitidis* are reported. Each entry contains the function, the original organism, the gene name, the corresponding homolog in *E. dermatitidis*, the homology type (B, Blast E-value; PD, protein domain SUPERFAMILY E-value), the regulation factor, in case it is significant, and/or the expression level of the gene if it belongs to the top 100 expressed genes in the skin experiment and a reference.

Function	Species	Gene	Homolog	Type of homology	DEG (fdr;fold) Rank	References
Shape	*Candida albicans*	Hgc1	HMPREF1120_04169	B:$4\cdot10^{-12}$,PD:$6\cdot10^{-35}$	0.012; 3.13	(Mayer et al., 2013)
	E. dermatitidis	Efg1 StuA	HMPREF1120_05541	B:$4\cdot10^{-168}$	0.0037; 7.01	(Ye and Szaniszlo, 2000)
Adherence	*Aspergillus fumigatus*	AfCalAp	HMPREF1120_05161	B:$1\cdot10^{-23}$,PD:$1.5\cdot10^{-4}$		(Upadhyay et al., 2009)
			HMPREF1120_03984	B:$7\cdot10^{-13}$,PD:$5.1\cdot10^{-9}$		
			HMPREF1120_05769	PD:$2.22\cdot10^{-48}$		
	Candida albicans	Ecm33	HMPREF1120_03851	B:$6\cdot10^{-49}$		(Martinez-Lopez et al., 2006)
	Candida albicans	Phr1	HMPREF1120_03477	B:$7\cdot10^{-177}$		(Calderon et al., 2010)
			HMPREF1120_07283	B:$1\cdot10^{-85}$	85	
			HMPREF1120_01763	B:$4\cdot10^{-84}$		
			HMPREF1120_01682	B:$2\cdot10^{-66}$		
	Microsporum	Sub3	HMPREF1120_08439	B:$2\cdot10^{-75}$		(Baldo et al.,

	canis					2010)
Protease	*Candida albicans*	Sap1-10	HMPREF1120_00212	PD:$8{\cdot}10^{-93}$ B:$8{\cdot}10^{-19}$		(Mayer et al., 2013)
			HMPREF1120_00818	PD:$3{\cdot}10^{-77}$ B:$3{\cdot}10^{-16}$		
			HMPREF1120_01981	PD:$5{\cdot}10^{-6}$		
			HMPREF1120_03062	PD:$8{\cdot}10^{-19}$		
			HMPREF1120_03766	PD:$2{\cdot}10^{-81}$ B:$3{\cdot}10^{-26}$		
			HMPREF1120_05067	PD:$3{\cdot}10^{-22}$		
			HMPREF1120_05119	PD:$9{\cdot}10^{-90}$ B:$7{\cdot}10^{-61}$		
			HMPREF1120_06343	PD:$3{\cdot}10^{-115}$ B:$4{\cdot}10^{-33}$		
			HMPREF1120_06360	PD:$7{\cdot}10^{-77}$ B:$9{\cdot}10^{-46}$		
			HMPREF1120_06819	PD:$9{\cdot}10^{-74}$ B:$2{\cdot}10^{-14}$		
			HMPREF1120_07351	PD:$6{\cdot}10^{-36}$		
			HMPREF1120_08062	PD:$7{\cdot}10^{-76}$ B:$1{\cdot}10^{-46}$		
			asmbl_11606|m.22873	PD:$8{\cdot}10^{-25}$		
	Trychomyces rubrum	Ssu1	HMPREF1120_04816	B:$5{\cdot}10^{-115}$	0.046; 0.40	(Lechenne et al., 2007)
Invasion	*Cryptococcus neoformans*	Plb1	HMPREF1120_00190	B:$6{\cdot}10^{-100}$		(Noverr et al., 2003)
			HMPREF1120_06300	B:$1{\cdot}10^{-61}$		
	Candida albicans	Ssa1 (Hsp70)	HMPREF1120_01564	B:0	0.0043;6.19	(Sun et al., 2010a)
			HMPREF1120_02459	B:$4{\cdot}10^{-30}$	0.025;3.24	
			HMPREF1120_02626	B:0	7	
			HMPREF1120_04200	B:$1{\cdot}10^{-61}$	0.003;14.02	
			HMPREF1120_07756	B:0	0.004;30.06/28	
			HMPREF1120_08142	B:0	0.016;4.02/81	
			HMPREF1120_09114	B:$3{\cdot}10^{-80}$		
	E. dermatitidis	StuA	See above			

Biofilm	*Aspergillus fumigatus*	Laea	HMPREF1120_06677	B:1·10^{-72}		(Fanning and Mitchell, 2012)
			HMPREF1120_03377	B:1·10^{-57}		
			HMPREF1120_07722	B:2·10^{-57}		
			HMPREF1120_08797	B:3·10^{-55}		
			HMPREF1120_02912	B:2·10^{-51}		
			HMPREF1120_08429	B:3·10^{-49}		
			HMPREF1120_01429	B:3·10^{-43}		
			HMPREF1120_08930	B:1·10^{-40}		
			HMPREF1120_05890	B:2·10^{-37}		
			HMPREF1120_05291	B:5·10^{-32}		
			HMPREF1120_02485	B:8·10^{-24}		
	Candida albicans	Efg1	HMPREF1120_05541	B:6·10^{-57}	9.31·10^{-5}; 7.01	(Dieterich et al., 2002)
	Candida albicans	Bgl2	HMPREF1120_04141	B:7·10^{-51}		(Taff et al., 2012)
	Candida albicans	Phr1	See above			
	Candida albicans	Exg1	HMPREF1120_04506	B:1·10^{-109}		(Taff et al., 2012)
			HMPREF1120_06180	B:2·10^{-62}		

Signaling pathways HMPREF1120_02538 (MAP kinase) and HMPREF1120_04310 (Map kinase kinase) are two members of the HOG signaling pathway and are significantly regulated (fdr 0.0031, 0.015; 5.45, 3.23) in the skin experiment. In pathogenic fungi the HOG pathway is a major controller of cellular responses to diverse external stimuli (Lenardon et al., 2010). In the Ca^{2+}/calcineurin signaling pathway, the voltage-gated high-affinity calcium channel is upregulated (HMPREF1120_08350) during growth on skin (fdr 0.0086, 5.426). Results from Chen et al.(Chen et al., 2014) show that the gene is upregulated upon acidic pH stress, which is in line with the skin having a surface pH of around 5. Interestingly two calcium transporting ATP-ase, i.e., HMPREF1120_07859 and HMPREF1120_00316, which are responsible for the transport of Ca^{2+} outside the cell, are upregulated during fungal growth on skin.

Adherence We searched for the 45 adhesins from six human pathogenic fungi reported by de Groot et al. (de Groot et al., 2013). Four of them, CalA from *Aspergillus fumigatus* and Ecm33, Car10, Phr1 from *Candida albicans*, were found in *E. dermatitidis* (Table 2). While none of those genes were differentially expressed, Phr1, which promotes binding to laminin and murine lung cells (Upadhyay et al., 2009) ranked among the 100 most expressed genes in the skin experiment.

Among all genes involved in biofilm formation in *Candida albicans*, only Phr1 showed a significant regulation (Table 2). It was also ranked among the 100 most expressed genes in the skin experiment.

Invasion We looked for genes responsible for endocytosis or active penetration. In *E. dermatitidis* 7 homologs of Ssa1, a gene involved in endocytosis in *Candida albicans* (Phan et al., 2007; Sun et al., 2010b) and 3 homologs of Plb1, a gene that gives rise to endocytosis in *Cryptococcus neoformans* (Noverr et al., 2003), were found in the genome. While Plb1-homologs were neither highly expressed nor differentially regulated, 5 Ssa1 homologs were significantly upregulated and three of them belonged to the set of 100 most highly expressed genes (Table 2).

Secondary metabolites SMASH (Weber et al., 2015) and SMURF (Khaldi et al., 2010) were used to find secondary metabolite biosynthesis gene clusters. SMASH returned a total of 4 NRPS, 1 type I PKS, 1 type 3 PKS, 3 terpene and 3 clusters of unknown type. SMURF returned 13 dimethylallyl tryptophan synthases (DMATS), 4 NRPS, 3 NRPS-Like, 3 PKS and 1 PKS-like clusters. A complete list can be found in Table 3. As reported by (Chen et al., 2014), *E. dermatitidis* has fewer PKS and NRPS than *Aspergillus oryzae, Aspergillus fumigatus* or *Aspergillus nidulans* (Yu and Keller, 2005). It exhibits however 13 DMATS while the three *Aspergilli* cited above exhibit only 2,7 and 2 DMATS, respectively (Inglis et al., 2013). DMATS are catalyzing the first biosynthesis step of the ergot alkaloid (Tudzynski et al., 1999), a potent mycotoxin.

Table 3 List of backbone genes in *E. dermatitidis* involved in the production of secondary metabolites. *Exophiala dermatitidis* genome contains 13 DMATS, 8 NRPS/NRPS-like, 3 TERPENE and 8 PKS/PKS-like backbone genes. 4 DMATS, 2 terpene and 3 NRPS/NRPS-like backbone-enzymes were upregulated during skin invasion.

Gene	Type	Comment (fold, fdr)	Gene	Type	Comment (fold, fdr)
DMATS	*HMPREF1120_00352*	*(4.11, 0.009)*	***NRPS***	*HMPREF1120_01440*	
	HMPREF1120_01260	*(2.86, 0.026)*		*HMPREF1120_02993*	*Acetylaranotin, toxin*
	HMPREF1120_01968			*HMPREF1120_04809*	
	HMPREF1120_02200			*HMPREF1120_06043*	*(7.511, 0.0045)*
	HMPREF1120_06543			*HMPREF1120_07636*	*(2.46, 0.033)*
	HMPREF1120_07933		***NRPS-like***	*HMPREF1120_00598*	*(13.55, 0.005)*
	HMPREF1120_08032			*HMPREF1120_03318*	
	HMPREF1120_08132			*HMPREF1120_07093*	
	HMPREF1120_09268		***PKS***	*HMPREF1120_03173*	
	HMPREF1120_09269			*HMPREF1120_06568*	
	HMPREF1120_08670	*(3.55, 0.011)*		*HMPREF1120_06570*	*t1PKS*
	HMPREF1120_09038	*(3.20, 0.008)*		*HMPREF1120_07394*	*t3PKS*
	HMPREF1120_09090		***PKS-Like***	*HMPREF1120_08091*	
TERPENE	*HMPREF1120_02863*				
	HMPREF1120_03149	*Terpene Cyclase (3.46, 0.012)*			
	HMPREF1120_09198	*(3.11, 0.008)*			

Other pathways Various cell wall genes are significantly (fdr < 0.05) regulated in the skin experiment. Chs1 (HMPREF1120_07981, 9.49), Phosphoacetylglucosamine mutase (HMPREF1120_02062, 4.38), Skt15 (activator of chitin synthase 3, HMPREF1120_06335, 10.32 ; HMPREF1120_05528, 2.33), Chia (Class III chitinase, HMPREF1120_03399, 14.03), Chitin deacetylase (HMPREF1120_08023, 5.11), Crh1 (transglycosidase, HMPREF1120_00627, 6.99), Mlg1 (mixed linked glucanases) (HMPREF1120_09051, 8.64), CelA (cellulose synthase, HMPREF1120_ 04699, 5.68) are upregulated. On the other hand N-acetyl-beta-glucosaminidase (HMPREF1120_06285, 0.23; HMPREF1120_06035, 0.27), HMPREF1120_01790 (UDP-N-acetylglucosamine 6-dehydrogenase, 0.26) and HMPREF1120_08078 (1,3-β-transglucosylases) are downregulated.In *E. dermatitidis* under skin condition, members of the HOG and Ca^{2+}/Calcineurin pathways are strongly regulated, which might explain the significant regulation of cell wall and chitin-related genes, known also in *Candida albicans* (Lenardon et al., 2010). Three light sensing genes were upregulated upon growth on skin, i.e., HMPREF1120_06318 (Wc-1, 3.72), HMPREF1120_00072 (VelB, 3.75) and HMPREF1120_07867 (Phy-1, 3.98). In contrast the carotenoid oxygenase (HMPREF1120_02864, 0.22) is downregulated.

3.4 Discussion

In this study an experimental setup for fungal growth on *ex-vivo* skin explants was established. To our knowledge this is the first time that *E. dermatitidis* infection of *ex-vivo* skin models was successfully performed under experimental conditions in the lab. Based on this infection system, the transcriptome of *E. dermatitidis* during its first contact with *ex-vivo* skin tissue was reproducibly sequenced. With the help of these transcriptome data, genes playing a role during the first contact of the fungus with the *ex-vivo* skin models were clearly identified. Further, state of the art bioinformatics pipelines for genomic annotation, functional annotation and differential expression analysis pipelines were designed for this study, allowing us to reproducibly investigate the sequencing data and genome data.

Based on this analysis, we could substantially increase the number of annotated protein-coding genes and found new homologs to ncRNAs, i.e., snR4, snR72,

snR74. For the first time long non-coding transcripts were reported in the group of black yeasts. While it is not clear what the function of these transcripts could be, it is known that lncRNAs, as a cis-regulatory element, are able to control the transcription of the loci in their neighborhood (see Quinn and Chang, 2015 for a review). Chacko et al. (Lin et al., 2015) showed that a long non-coding RNA, RZE1, regulates the yeast-to-hyphal transition in *Cryptococcus neoformans*. Further in Kaposi sarcoma-associated herpes virus it was shown that the lncRNA β2.7 has a role in preventing stress response and apoptosis in the host cell (Amaral et al., 2013).

In line with the studies of Blasi et al. (Blasi et al., 2015), circular RNAs and fusion transcripts were detected, two kind of transcripts that were previously connected to infections in archaea and virus (Lau et al., 2014). We could also show that circRNAs and fusion-transcripts were also differentially regulated and seem therefore important for infection in *E. dermatitidis*. The finding that various ncRNAs that are exported outside the cell in vesicles are also upregulated during skin infection, might indicate that they might be useful during the host infection, as was previously reported for *Botrytis cinerea* (Weiberg et al., 2013).

E. dermatitidis grows successfully on and into the skin. This was supported both by the microscopic pictures (Figure 6) and the functional enrichment analysis. The most significantly enriched gene functions are related to active replication and transcription such as MCM complex, which is the eukaryotic replicative helicase (Bochman and Schwacha, 2009), ribosome biogenesis, AAA+ ATPases (Ogura and Wilkinson, 2001), helicases and SF2-related N-terminal domain, which is involved in all aspects of RNA and DNA metabolism (Fairman-Williams et al., 2010). The genome *E. dermatitidis* contains four adhesins, CalA, Ecm33, Car10, and Phr1 that were shown to successfully promote adhesin in either *Candida albicans* or *Aspergillus fumigatus* (de Groot et al., 2013) While none of them are significantly regulated, Phr1 is the 85th most highly expressed gene in the *E. dermatitidis* transcriptome. In *Candida albicans* Phr1 is involved in adherence to laminin, murine lung cells (Upadhyay et al., 2009), abiotic surfaces and human epithelial cells (Calderon et al., 2010).

Seven out of eight homologs of *Candida albicans* Hsp70 (Ssa1), are either highly upregulated or highly expressed during fungal growth on skin. Hsp70 was shown to

be involved in cell proliferation, cell death and morphological switch toward the yeast-form in *Paracoccidioides brasiliensis* (da Silva et al., 1999). Besides its role in morphology, Hsp70 was shown to be responsible for cell invasion in *Candida albicans* (Phan et al., 2007; Sun et al., 2010b). While the yeast is prevalent in *E. dermatitidis* during growth on the skin model (Figure 6D), there are evidence from the upregulation of Hgc1 and Efg1, two genes that were previously connected to the hyphal growth (Dieterich et al., 2002; Mayer et al., 2013), that some cells in the experiment might be switching to the hyphal form. This discrepancy could be explained by the fact that *E. dermatitidis* growing on and in the skin might adopt different morphologies. Due to the lack of biomass both populations were sequenced together.

The skin environment further triggers the upregulation of various secondary metabolites clusters. *Exophiala dermatitidis* exhibits a high number of DMATS compared to other filamentous fungi and 4 DMATS, 2 Terpene and 3 NRPS/NRPS-like clusters are upregulated while none of the backbone genes involved in the production of secondary-metabolites are downregulated. One of the upregulated NRPS cluster is a putative antibiotic precursor (HMPREF1120_06043) which might help the fungus to overcome the skin microbiota barrier. DMATS upregulation, which might be linked to an increased production of ergot alkaloid, might be used to attack skin cells. The cytotoxicity of ergot alkaloids was previously shown in human renal cell lines (Haarmann et al., 2005).

Beyond the known fungal virulence factors, *E. dermatitidis* seems to employ mechanisms to infect and invade its host that were not previously reported. Apparently only pyomelanin, i.e., the melanin originating from the L-Tyrosine pathway, is upregulated during infection. In *Aspergillus fumigatus*, the production of pyomelanin was shown to be upregulated during infection but dispensable for virulence (Heinekamp et al., 2013). In the fungal pathogen *Sporothrix brasiliensis* pyomelanin was shown to protect this fungal pathogen against antifungal drugs (Almeida-Paes et al., 2016). Further melanin and genes involved in melanin production were shown to be crucial for iron uptake (Ding et al., 2014), in *Cryptococcus neoformans*. Similarly, in the bacterial pathogen *Legionella pneumophila* pyomelanin is involved in iron uptake (Zheng et al., 2013). We can

hypothesize that the upregulation of pyomelanin in *E. dermatitidis* during the skin model infection is probably connected to the upregulation of iron acquisition, similar to what is observed in *Cryptococcus neoformans*. The other melanin-production pathways were not regulated during the growth on the *ex-vivo* skin model. Inversely the L-Tyrosine pathway was not upregulated during salt, UV and ozone (24 ppm) stress (manuscript in preparation).

During the infection of the skin model, *E. dermatitidis* experiences a complete reprogramming of its carbon metabolism. Snf1 (HMPREF1120_02538), which responds to declining level of glucose and is the central player in the glucose repression pathway in *Saccharomyces cerevisiae* (Conrad et al., 2014; Usaite et al., 2009), undergo a five-fold upregulation (fdr = 0.003) when *E. dermatitidis* is growing on skin. Snf1 was shown to be crucial for the growth on any non-fermentable carbon source (Young et al., 2003). Even though glucose from the cell culture media is available during the artificial infection, it seems that *E. dermatitidis* is at least in part switching to the gluconeogenesis pathway. It was previously shown that *Candida albicans* was also showing a switch to gluconeogenesis when internalized in macrophages (Lorenz et al., 2004). It can be seen through the upregulation of the Fox1,2 and Pot1 genes that the production of acetyl-CoA, which is needed for generation of malate from glyoxylate through the malate synthase (fc = 21, fdr = 0.003) in the glyoxylate cycle, is increased. Mdh1-3, which are needed in glyoxylate cycle for the production of oxaloacetate, are upregulated, and therefore lead to phosphoenolpyruvate, the substrate of the gluconeogenesis pathway. The upregulation of nearly all genes involved in the gluconeogenesis pathway indicates that phosphoenolpyruvate is actively converted into glucose (see Supplementary Table 1, Figure 10).

While the carbon acquisition metabolism was significantly regulated during the skin experiment, we could not clearly derive which genes were responsible for the nitrogen acquisition mechanism. Two protease families, i.e., classified as C26 and S16 in merops, were significantly enriched in the set of the upregulated genes. The S16 Lon protease family is a known bacterial and fungal virulence factor (Breidenstein et al., 2012; Cui et al., 2015; Takaya et al., 2003). It is unclear if keratin is actively degraded by *E. dermatitidis*. Two members of the M03 and one member of

the S8 merops class, were upregulated upon infection. Huang et al. (Huang et al., 2015) showed that these types of proteases in combination with M28, which in our case was downregulated when *E. dermatitidis* grew on skin, are able to degrade keratin. Ssu1, a sulfite efflux pump which plays a crucial role in the catabolism of keratin in dermatophytes (Grumbt et al., 2013), is downregulated in *E. dermatitidis* during infection. HMPREF1120_05465, an aminoacid transporter protein is upregulated, while HMPREF1120_03271, another aminoacid transportes is downregulated. HMPREF1120_06103, a peptide transporter, is strongly downregulated.

In order to sustain its accelerated growth on the skin the fungus is actively acquiring metals. Based on gene homology with genes reported in *Saccharomyces cerevisiae, Cryptococcus neoformans, Candida albicans* and *Aspergillus fumigatus* (Ding et al., 2014) we could determine that *E. dermatitidis* is uptaking iron via the reductive iron uptake and the use of siderophores. In the reductive iron uptake pathway, Ftr1 and Fth1 are significantly upregulated indicating that this pathway might be used. *Exophiala dermatitidis* upregulates SidD, SidF, SidA and SidC, which are the enzymes responsible for siderophore synthesis (Chen et al., 2014). Further Sit1, a siderophore transporter, is upregulated (Heymann et al., 2000).

Other metal transporters upregulated are the magnesium transporters, namely HMPREF1120_04846, HMPREF1120_08107 and HMPREF1120_05571. This is in line with results of (Giles and Czuprynski, 2004), where it was shown that in *Blastomyces dermatitidis*, a dermatophyte, a lack of magnesium even for short period of time is detrimental to its proliferation. Transporters of zinc and related genes were found to be downregulated or not regulated in the skin environment which leads to the conclusion that zinc might not be crucial for the first week of infection.

Overall the improved annotation of coding and non-coding genes as well as the thorough quest for known virulence factors and mechanism from other fungal pathogens allows us to get a better insight into the virulence mechanism and adaptation of *E. dermatitidis* during host invasion. Due to the restrained knowledge on this fungus, further studies are necessary in order to better apprehend the mechanisms in play during host infection by *E. dermatitidis*. Based on this study, the

first knock-out experiments using the Crispr/Cas9-System in *E. dermatitidis*, are being conducted on various identified virulence factors in our lab.

3.5 Conflict of Interest Statement

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

3.6 Acknowledgments

The computational results presented were achieved, in part, through the use of the Vienna Scientific Cluster (VSC). We would like to thank Regina Weinmüllner of the group Grillari DBT BOKU, who helped with the HE-stainings.

3.7 Supplementary Material – Raw data

The raw data can be found online in the supplementary table: https://www.frontiersin.org/article/10.3389/fcimb.2016.00136

4 Transcriptome Study of an *E. dermatitidis* *PKS1* Mutant on an *Ex-vivo* Skin Model: is Melanin Important for Infection?

Caroline Poyntner[1], Ursula Mirastschijski[2,3], Katja Sterflinger[1], Hakim Tafer[1]

[1]Department of Biotechnology, VIBT EQ Extremophile Center, University of Natural Resources and Life Sciences, Vienna, Austria

[2]Wound Repair Unit, Center for Biomolecular Interactions Bremen, University of Bremen, Bremen, Germany

[3]Division of Plastic and Aesthetic Surgery, Rotkreuzklinikum München, Munich, Germany

Results published in:
Frontiers Microbiology - Fungi and Their Interactions. 2018; 9: 1457.
doi: 10.3389/fmicb.2018.01457

4.1 Abstract

The black yeast *E. dermatitidis* is a polyextremophilic human pathogen, especially known for growing in man-made extreme environments. Reported diseases caused by this fungus range from benign cutaneous to systemic infections with 40% fatality rate. While the number of cases steadily increases in both immunocompromised and immunocompetent people, detailed knowledge about infection mechanisms, virulence factors and host response are scarce. To understand the impact of the putative virulence factor melanin on the infection, we generated a polyketide synthase (*PKS1*) mutant using CRISPR/Cas9 resulting in a melanin deficient strain. The mutant and the wild-type fungus were inoculated onto skin explants using an *ex-vivo* skin organ culture model to simulate *in-vivo* cutaneous infection.

The difference between the mutant and wild-type transcriptional landscapes, as assessed by whole RNA-sequencing, were small and were observed in pathways related to the copper homeostasis, cell wall genes and proteases. Seven days after inoculation the wild-type fungus completely colonized the stratum corneum, invaded the skin and infected keratinocytes while the mutant had only partially covered the skin and showed no invasiveness. Our results suggest that melanin dramatically improves the invasiveness and virulence of *E. dermatitidis* during the first days of the skin infection.

4.2 Introduction

The Ascomycete *E. dermatitidis* belongs to the group of black yeasts, a heterogeneous taxonomic and phylogenetic group that shares melanized cell walls and yeast-like budding as common features (Sterflinger, 2005). Black yeasts are known for their exceptional abilities to survive extreme environments e.g. salterns (Gunde-Cimerman et al., 2000) and glaciers (Branda et al., 2010) but also man-made extreme environments e.g. sauna facilities (Matos et al., 2002) and dishwashers (Döğen et al., 2013b). In humans, *E. dermatitidis* colonization is often reported in cystic fibrosis patients with a prevalence rate varying from 4.8 to 15.7% in Germany

and Belgium (Pihet et al., 2009). Cutaneous and subcutaneous abscesses (Zeng et al., 2007), septic arthritis, endocarditis, catheter-associated fungemia (Byrne and Reboli, 2017) but also systemic infections have been reported (Hiruma et al., 1993). A huge difference is observed in etiology and severity (Sudhadham et al., 2008). Theories to explain these differences exist (Matos et al., 2002; Poyntner et al., 2016) but detailed studies about the infection mechanisms are scarce. One of the proposed virulence factors is melanin (Taborda et al., 2008), a negatively charged, hydrophobic macromolecule composed of oxidative polymerized phenolic or indolic monomers and often complexed with proteins and carbohydrates (Butler and Day, 1998b; Casadevall et al., 2000). It was shown that melanin protects *Cryptococcus neoformans* against oxidants produced by the host and that its production increases with high pH, illumination with visible light and zinc addition (Wang et al., 1995) and might therefore help the fungus to survive in extreme environments. Melanin confers an increased resistance against the phagolysosomal oxidative burst of human neutrophils (Schnitzler et al., 1999) and antifungal drugs (Paolo et al., 2006). A melanin deficient mutant showed a substantial decrease in virulence and case fatality rate in albino mice but retained its neurotropic potential (Dixon et al., 1987). In this study we sought to assess the impact of melanin on the first phase of fungal skin infection. To this aim we used the CRISPR/Cas9 technology in the black yeast *E. dermatitidis* to knockout the *PKS1* gene, leading to the disruption of the DHN melanin production. The fungal growth of both strains on *ex-vivo* skin models was monitored microscopically and macroscopically while differences in gene regulations in the mutant and the wild-type were assessed by total RNA sequencing seven days after inoculation.

The wild-type caused epidermolysis, ballooned keratinocytes, dermal infiltration and covered the whole skin model, while the mutant partially colonized the epidermis but did not grow invasively. On the transcriptome level only minor changes were observed. Our results indicate that melanin produced by the DHN pathway is important for the invasion. We hypothesize that the fungus might use the ability of human keratinocytes to absorb exogenous melanin in order to introduce fungal melanin and other virulence factors like proteases, ureases and DNases into the keratinocytes like a like a “trojan horse”, which ultimately leads to dermal disintegration.

4.3 Materials and Methods

CRISPR/Cas9 editing

As described before by Feng et al. (Feng et al., 2001) a mutation of the *PKS1* gene leads into a melanin deficient strain. Based on this study we used sgRNA scorer 2 (Chari et al., 2017) to find single guided RNA (sgRNA) targets against the gene HMPREF1120_03173 on both strands. We selected the five highest scoring targets mapping 5' half of the gene (Supplementary material Table 1). The corresponding sgRNAs and Cas9 protein were provided by the BCF Protein Technologies Facility, CRISPR Lab, Vienna Biocenter Core Facility (www.vbcf.at).

E. dermatitidis (CBS 525.76) was cultivated in YPD broth (2% peptone, 1% bacto yeast extract, and 2% dextrose) for 24 hours at room temperature. Cells were counted in a hemocytometer and 10^6 cells were centrifuged (600 g, 5 minutes) and chilled on ice for 30 minutes. The pellet was washed twice with cold 10% glycerol and resuspended in 10% glycerol (200 µl). SgRNA(12 µg), 1x cleavage buffer (10x: 200 mM Hepes, 1.4 M KCl, 5 mM DTT, 100 mM $MgCl_2$ and 1 mM EDTA) was concentrated to 5 µl and mixed with Cas9 protein (5 µg, WT Cas9 from *Streptococcus pyogenes* with two nuclear localization signals). The mixture was incubated at 37°C for 10 minutes. Electroporation was carried out in a Gene Pulser Xcell (Bio-Rad, Hercules, CA) at 2,5 kV field strength, 300 Ω resistance and 25 mF capacitance. Electroporated cells were incubated at 25°C for 3 hours, inoculated on YPD media and incubated at 37°C. For each transfection untreated fungal cells were used.

Cultures with tricyclazole, L-Dopa, L-tyrosine

Malt extract agar (2% malt extract, 2% D-glucose, 0.1% bacto-peptone and 2% agar) plates with 50 mg/L tricylazole (LGC Standards, UK, Li et al., 2016) were inoculated with the wild-type and the mutant strains and incubated at 37°C for 10 days. The tricyclazole served as inhibitor of the pentaketide melanin biosynthesis (DHN pathway) (Franzen et al., 2006). Using a minimal medium (15.0 mM glucose, 10.0

mM $MgSO_4$, 29.4 mM KH_2PO_4, 13.0 mM glycine, and 3.0 µM thiamin) the mutant and wild-type were also inoculated in liquid cultures with 1 mM L-Dopa (L-Dopa, Merck, Germany) or 1 mM L-tyrosine (Merck, Germany, Paolo et al., 2006). The cultures were kept in the dark to withstand autopolymerisation of L-Dopa at 37°C for 10 days.

Whole genome sequencing of the mutant

DNA extraction and whole genome sequencing was performed as previously described (Tesei et al., 2017) using the PGM Sequencing platform (Life Technologies, Carlsbad, CA). Sheared genomic DNA (Bioruptor™ CD-200 TS Sonication System, Diagenode, Belgium) were used for the library builder (AB Library Builder™ System, Life Technologies, Carlsbad, CA). The resulting library was quantified (Ion Library TaqMan Quantitation Kit, Life Technologies, Carlsbad, CA) and loaded on the Chef instrument (Life Technologies, Carlsbad, CA) followed by single-end sequencing. Samtools/Bcftools (Li et al., 2009) and Vcftools (Danecek et al., 2011) were used to identify putative CRISPR/Cas9 mutated sites and SNPeff was used to assess their impacts (Cingolani et al., 2012).

Skin culture experiments

Skin culture experiments were conducted as previously described (Poyntner et al., 2016). Skin for the *ex-vivo* skin wound model was obtained from a patient undergoing brachioplasty at Department of Plastic, Reconstructive and Aesthetic Surgery, Klinikum Bremen-Mitte, Germany. The skin was defatted and full-thickness skin explants were cut into pieces with a size of 5 × 5 cm. With a scalpel blade, incisional wounds were inflicted on the epidermal surface. Wild-type (14 skin explants, CBS 525.76) or mutant (13 skin explants) were inoculated with a sterile inoculation loop taken from a 7 days old culture grown on 2% malt extract agar. The skin was cultured as described by Mirastschijski et al. (Mirastschijski et al., 2002), with the explants placed at the liquid-air-interface for cultivation of the fungi on top of the skin mimicking pathological conditions. The cultures were kept at 37°C for seven days and medium was exchanged every second day. A skin control without fungal inoculum was cultured in parallel. For a negative control, wild-type or mutant from a

seven days old MEA culture were inoculated on a prewetted Nylon membrane (0.45 µm, Whatman, Maidstone, Kent, UK) and incubated with culture medium under the same conditions as the skin model. Biomass was collected for three replicates followed by RNA extraction using the FastRNA Pro RED KIT (MP Biomedicals, Santa Ana, California).

RNA Sequencing and reads mapping

From the total RNA extracted, mRNA was selected using the Dynabeads mRNA DIRECT Micro Kit (Ambion by Life Technologies, Carlsbad, CA) and the library was constructed using Ion Total RNA-Seq kit v2 (Life Technologies, Carlsbad, CA). Quality and quantity were measured using an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA) and a Qubit 2.0 (Life Technologies, Carlsbad, CA). Targeted library length of 290 bp was selected with a PippinPrep (Sage Science, Beverly, MA). Sequencing was performed using the Ion Torrent Proton and the HiQ sequencing kit (Life Technologies, Carlsbad, CA). From the sequenced raw data, reads were mapped as described previously (Poyntner et al., 2016). We removed reads originating from human sequences by mapping the total set of reads against the concatenated human (GRCh37) and *E. dermatitidis* genomes with STAR 2.4.1d (Dobin et al., 2013). Reads that mapped against *E. dermatitidis* were used to assess the differentially expressed genes. Reads from the mutant were mapped against the wild-type genome and annotation. Counting of mapped reads on annotation elements was done with featureCounts v1.4.6p2 (Liao et al., 2014) and identification of differentially expressed genes as well as assessment of sample to sample distance was done with R (R Core Team, 2016) and the edgeR package (Robinson et al., 2010). The functional enrichment of the significantly regulated genes was done with GoStat (Falcon and Gentleman, 2007). Revigo (Supek et al., 2011) was used to summarize the lists of overrepresented Gene Ontology terms. Gene families annotation was obtainend from Poyntner et al. 2016 (Poyntner et al., 2016) and Chen et al 2014 (Chen et al., 2014).

Ethics statement

The skin models were approved by the ethics committee of Bremen (No 336-2012) and all donors signed a written consent prior operation.

Microscopical analyses

Skin samples were fixed in formalin, embedded in paraffin and processed as previously described (Mirastschijski et al., 2002) followed by Haematoxylin and Eosin (HE) staining. Microscopic images were taken using an Olympus BX51 microscope.

4.4 Results

CRISPR/Cas9 genome editing

The mutant strain was obtained by using the CRISPR/Cas9 system directed against the gene *PKS1* (HMPREF1120_03173), since its disruption was previously shown to yield melanin deficient *E. dermatitidis* strains (Feng et al., 2001). We first transfected *E. dermatitidis* with five sgRNAs (2.4 µg each) in one experiment. 20% of the treated cells turned white, while the other fungal cells did not exhibit any change in color. The white fungi however were unstable and changed to light brown after few days. We repeated the experiment sequentially i.e. by using one sgRNA at a time but at a fivefold amount (12 µg, see Material and Methods). Only the sgRNA targeting region 5 (Supplementary material Table 1) successfully produced a stable melanin deficient strain by effectively knocking out *PKS1* through non-homologous end joining (NHEJ).

CRISPR/Cas9 mediated a four nucleotides deletion leading to a frame-shift and a stop codon seven amino acids downstream of the mutation site (Figure 11). The deletion site which is located close to a non-canonical PAM sequence (Zhang et al., 2015), does not correspond to any of the regions originally targeted (Figure 1). Still the deletion was seen both by PCR and by mapping the reads from the whole genome sequencing of the mutant strain to the wild-type strain. The mutation is supported by 33 reads out of the 35 overlapping with the locus (IMF 94.3%).

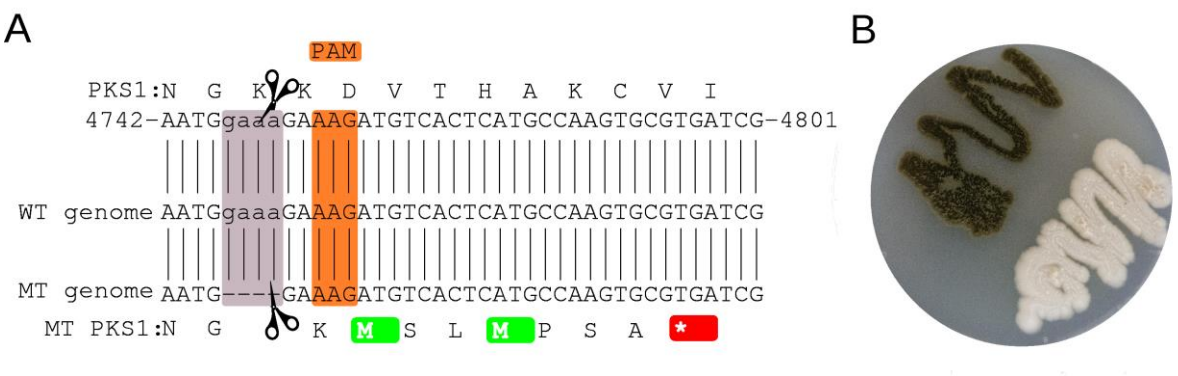

Figure 11 Mutated site in the CRISPR/Cas9-targeted gene. The local PKS1 amino acid and nucleotide sequence in the WT is shown at the top (A). The sequence of the reference genome is shown in the middle, while the assembled sequence of the mutant is shown at the bottom of the figure. The orange and mauve rectangles highlight the PAM and deleted sequences, respectively. The mutation leads to a stop codon 7 amino acids downstream of the deleted region (red). Culture of the mutant and wild-type strains (B).

Infection development and microscopic observations

The wild-type was able to grow on the full-thickness skin explants in our *ex-vivo* skin wound model. After 7 days, the whole skin surface was covered by fungal biomass (Figure 12 A-B) similar to previous results (Poyntner et al., 2016). In contrast, the mutant strain partially covered the skin and adhered around the cut (Figure 12C-D). By HE histology we found that the skin explants were morphologically intact and vital when cultured without any fungal inoculum under the same conditions of the fungal experiments (Figure 12 E and J). Skin inoculated with the wild-type showed signs of disintegration with the epidermis separated from the dermis, keratinocytes being pyknotic and increasing epidermal degradation. The fungus changed the color of the stratum corneum (Figure 12F and K) and was able to invade and infect the dermis (Figure 12G and L). Wild-type cells were bigger in size compared to the mutant. After seven days the mutant was colonizing the epidermis with growth on top of the stratum corneum. No dermal (Figure 12I and N) invasion but attachment to medium exposed surfaces was found (Figure 12H and M). No color change of the stratum corneum was visible and only few keratinocytes were pyknotic.

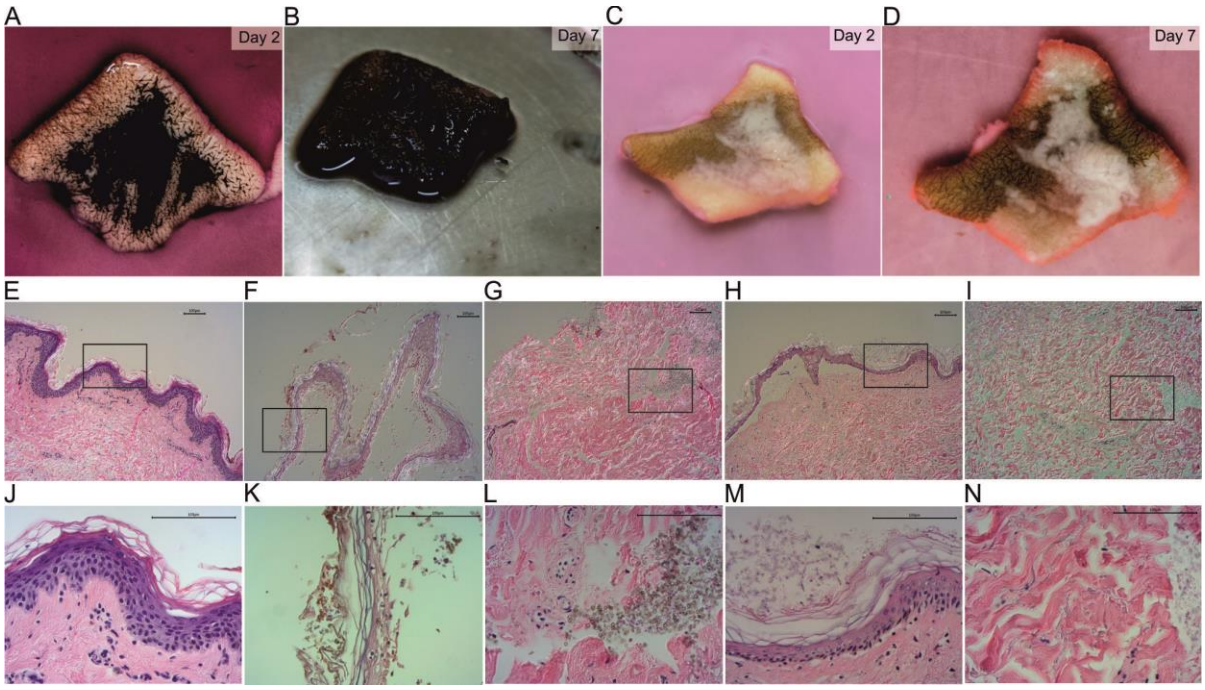

Figure 12 HE stained sections of inoculated *ex vivo* skin explants. *Ex vivo* skin explants after 2 days (A,C) and 7 days (B,D) inoculated with the wild-type (A,B) and the mutant (C,D) at 37°C. The brown color in (C) and (D) derives from surgical ink used for incisional markings in plastic-reconstructive surgery and does not originate from the mutant fungus. HE stained sections of *ex vivo* skin explants without fungal inoculum (E,J), the wild-type (F,G,K,L) and the mutant (H,I,M,N). Epidermal samples of the wild-type (F,K) and the mutant (H,M) and dermal tissue of wild-type (G,L) and mutant (I,N) are shown. Areas marked with a black box (E–I) are shown in higher magnification in the lane below (J–N). Scale bars: 100 µm.

Whole genome sequencing and RNA sequencing

The genome of the mutant strain was sequenced (see Material and Methods) resulting in 4247797 reads and 1229354886 sequenced bases. The reads were assembled with Newbler 2.9 into a genome containing 263 contigs, 26351726 bases with a coverage of 46 and a N50 of 198974. This Whole Genome project has been deposited at DDBJ/ENA/Gen Bank under the accession QEYA00000000. The version described in this paper is version QEYA01000000. The WGS reads were uploaded to NCBI (SRR7081810).

The skin infection and control experiments for the wild-type and mutant strains were sequenced in triplicates on the Ion Proton Platform. Between 45% and 75% of the

reads were mapping against *E. dermatitidis,* and between 0.9%and 7.3% of them had a human origin. The numbers of sequenced and mapped reads for each experiments are shown in the Supplementary material Table 1. Principal component analysis of the read counts for each replicate in each experiment shows that the control experiments are well separated from the skin experiments, and that the mutant strain and wild-type strains have a similar transcriptional landscape (Figure 13).

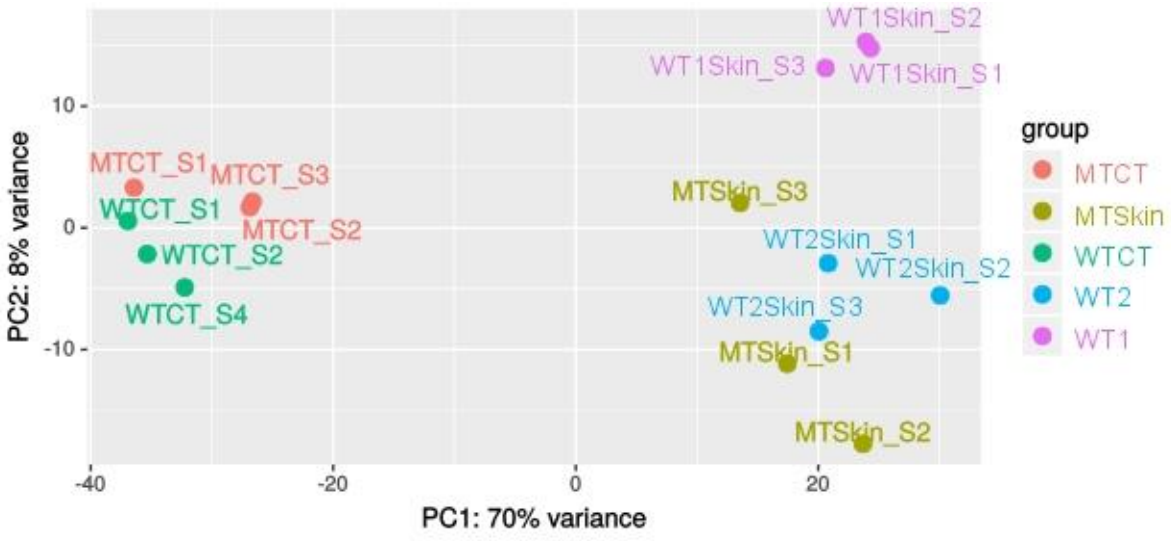

Figure 13 PCA based on the count vector obtained from feature Counts for the 15 RNA sequencing runs. MT stands for mutant, WT for wild-type, CT is the control experiment and Skin is the skin experiment. Skin dataset with the Old suffix are from the Poyntner et al., (2016) paper on skin infection.

Comparison of enriched functional terms in the set of regulated genes in wild-type and mutant

We looked at the differentially expressed genes between the mutant, the wild-type and the wild-type from our previous experiment (Poyntner et al., 2016). For clarity, we name the sequencing data from the mutant experiment as MT, from the current experiment as WT2 and from the previous experiment as WT1. Two genes were significantly differentially expressed between the WT2 and MT in the control

experiment. HMPREF1120_02141 (Gentisate 1, 2 - dioxygenase) and HMPREF1120_02142 (Salicylate hydroxylase). No other genes were significantly regulated between WT and MT in the control and skin experiment.

Sixty enriched functional terms are shared by the set of upregulated genes in the skin vs the control for WT1 (Figure 14, WT1U), the set of upregulated genes in WT2 (WT2U) and the set of upregulated genes in mutant (MTU). Among those terms, 55 gene ontologies were clustered into three categories (DNA-dependent DNA replication, monocarboxylic acid metabolism and cellular component biogenesis, Supplementary material Table 1) by Revigo (Supek et al., 2011) indicating an increase in DNA replication and active growth during the skin experiment compared to control. The enrichment of DEAD-Box RNA helicase family and nuclear mRNA exporter (The Transporter Classification Database (TCDB) 3.A.18) indicates an increase in transcriptional activity.

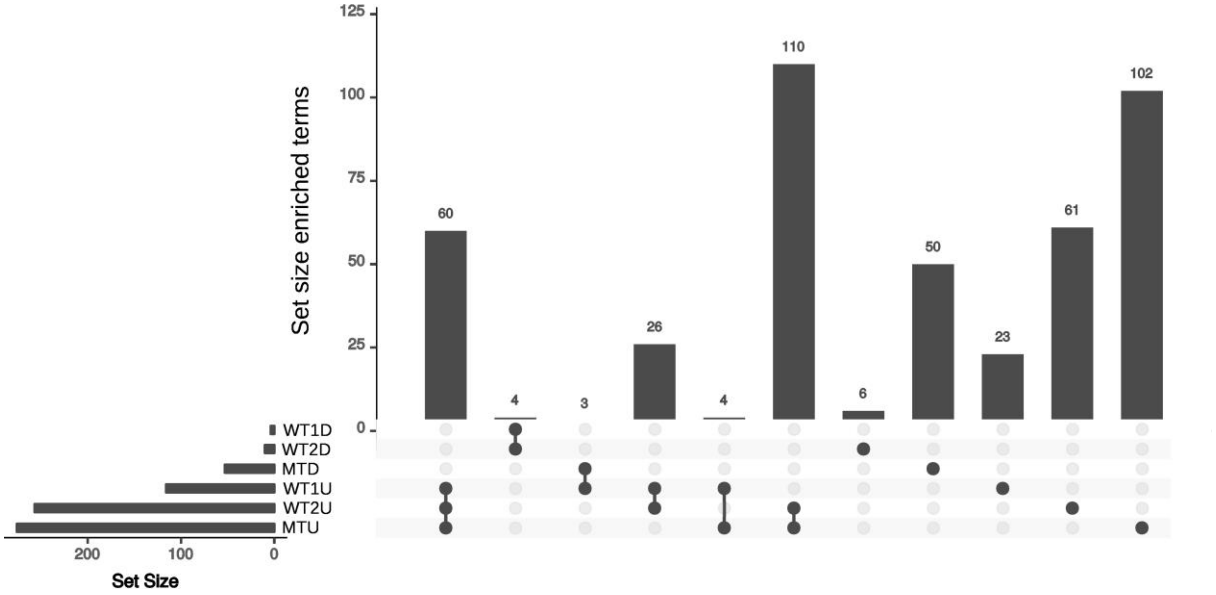

Figure 14 Upset plot for the sets of enriched annotation for the skin experiments. Set of enriched annotations found in the WT1, WT2, and MT. U and D represent the set of enriched annotation for the up- and down-regulated genes, respectively.

Fifteen gene ontology (GO) terms specific to WT2U and WT1U (Supplementary material Table 1) are clustered into DNA replication, MCM complex, helicase activity and carbohydrate derivative binding. Accordingly, the enriched protein domains are related to the MCM complex, translation, transcription and large substrate binding. Finally, genes annotated with the Lon Protease S16 domain are enriched in the set of upregulated genes in the WT experiments but not in the MT. Lon proteases were reported to contribute to pathogenicity in fungi (Li et al., 2015) and are known bacterial virulence factors involved in biofilm formation, motility and macrophage survival (Breidenstein et al., 2012; Cui et al., 2015; Takaya et al., 2003).The ensemble of enriched genes in MTU (Supplementary material Table 1) contains 102 terms, distributed into 91 GO terms, 7 Interpro (IPR) terms and 4 TCDB terms. The TCDB entries are related to the trafficking between the cytoplasm and the mitochondria, while the IPR terms are related to tryptophan synthase, energy homeostasis, translation, secretory proteins and riboflavin synthase. On the GO level, terms related to respiration, translation and transcription indicate that the MT is metabolically active on the *ex-vivo* skin samples. The MTD is enriched in terms related to transport (MFS transporter, TCDB *2A1*) and transcription (Supplementary material Table 1). The group of GO terms were enriched in regulation of biological processes, organic cyclic compound biosynthesis, transmembrane transporter activity and zinc ion binding, indicating that the mutant cells are decreasing their metabolism and transport compared to the control and the WT experiments. This is in line with the slower growth of the MT on the *ex-vivo* skin models compared to the WT. For WT2D and WT1D four terms were enriched: oxidoreductase activity, NAD (P)-binding domain, GroES-like superfamily and alcoholdehydrogenase superfamily (Supplementary material Table 1).

Quantitative analysis

The regulation of genes involved in melanin production, nutrient uptake, metal acquisition, secondary metabolite synthesis and gluconeogenesis was analyzed.

Melanin Three melanin pathways are present in *E. dermatitidis* (Chen et al., 2014) and during skin infection only the L-tyrosine degradation pathway had consistently upregulated genes (Poyntner et al., 2016). This is also seen in the present work in both the WT and MT strains (Table 1), where tyrosine aminotransferase and 4-hydroxyphenylpyruvate dioxygenase are upregulated, indicating an increased conversion of L-tyrosine to homogentisate acid, which upon auto-oxidation and polymerization is converted to pyomelanin. In parallel *FahA* (HMPREF1120_03825) and *HmgA* (HMPREF1120_03827), two genes that degrade homogentisate acid to fumarate and acetoacetate, are upregulated (Table 1 and Supplementary material Table 1) in WT and MT by at least a factor six. The L-Dopa melanin pathway (Eisenman et al., 2007) is downregulated during growth on skin as was previously reported. Finally, the *Abr1* (Multicopper ferroxidase) component of the DHN melanin pathway is downregulated in WT and MT while *Abr2* (Laccase) is upregulated in all three experiments (Table 1).

We further condcuted an inhibition test of DHN melanin production with tricyclazole on the wild-type and mutant strain. It induced an increase in melanin production but no depigmentation. Further melanin was excreted by the fungus into the medium. Interestingly, the mutant was able to take up melanin from the medium (Figure 15). The mutant did not exhibit any changes in presence of tricyclazole. L-Dopa melanin production was successfully induced in the mutant by using liquid minimal medium containing L-Dopa. Finally the use of L-tyrosine in liquid minimal media did not induce melanin production in the mutant (Figure 15).

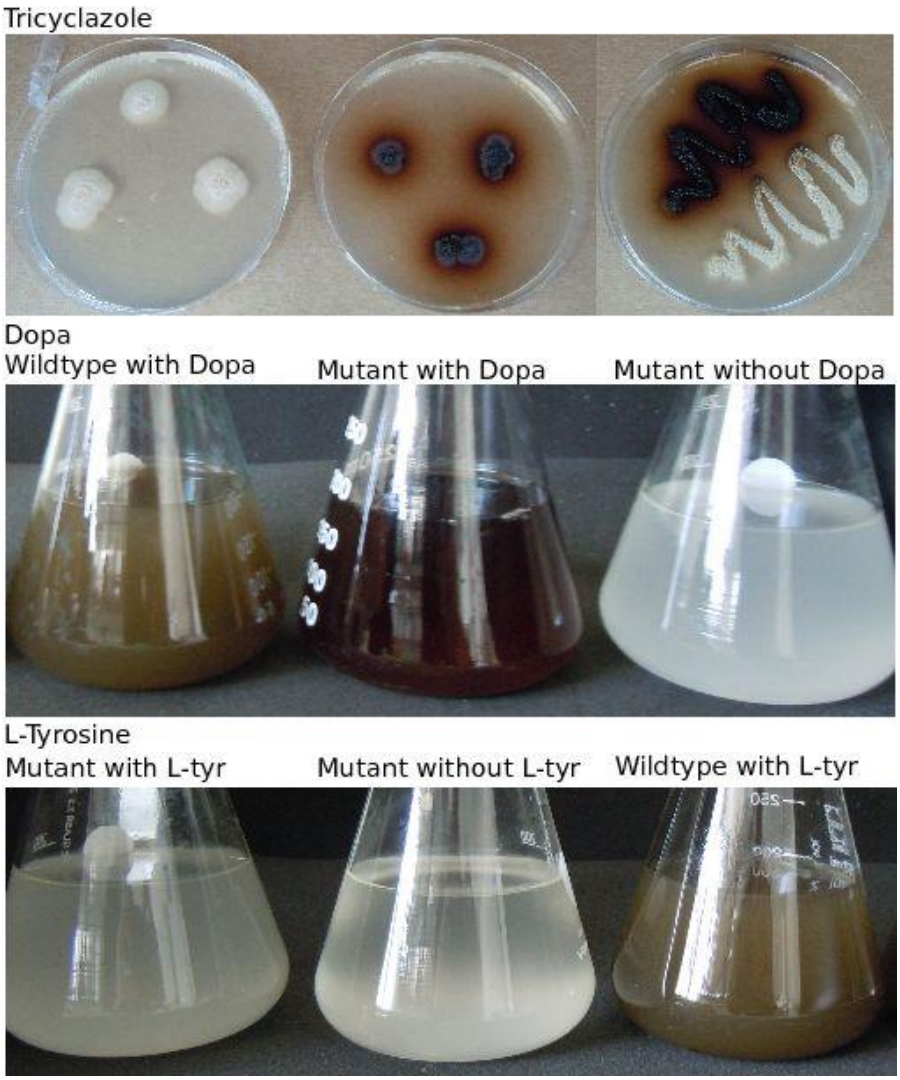

Figure 15 Malt extract agar plates with either tricyclazole (50 mg/L, first row) inoculated for 10 days at 37°C with the mutant (left plate), wild-type (middle plate) or mutant and wild-type together. Liquid minimal medium with L-Dopa (1 mM, second row) or L-tyrosine (1 mM, third row) inoculated with the wild-type or mutant did show color change for the mutant with L-Dopa but not with L-tyrosine.

Carbon uptake During growth on skin, WT *E. dermatitidis* activates the gluconeogenesis, glyoxylate and beta-oxidation pathways (Poyntner et al., 2016). In this work, WT2 and MT showed a similar trend. In the beta-oxidation pathway *POT1* (3-oxoacyl CoA thiolase), which cleaves 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA, is enriched in the WT1U, WT2U and MTU. Acetyl-CoA is converted to oxaloacetate in the glyoxylate synthase pathway, where the key enzymes *Icl1* (isocitrate lyase) and *Cit2* (citrate synthase) are enriched in WT1U, WT2U and MTU,

while *Aco1* (aconitase) is exclusively enriched in WT1U and WT2U. Oxaloacetate is then processed by the gluconeogenesis pathway to yield glucose. In *E. dermatitidis*, this happens with the help of *Mae1* and *Pyc1* which are upregulated in the wild-type and mutants during growth on skin. The next step involves *PCK* (phosphoenolpyruvate carboxykinase) which is upregulated in WT1 and MT. Further genes regulated along the gluconeogenesis pathway in WT1, WT2 and MT are phosphoglycerate mutase (*GPM3*) and fructose-biphosphate aldolase class II (*FBA2*), while glyceraldehyde-3-phosphate dehydrogenase (*TDH*) and fructose-biphosphate are upregulated only in the MT (Table 1).

Metal acquisition Iron uptake in *E. dermatitidis* happens over the siderophore pathway and through reduction of Fe^{2+} to Fe^{3+} (Poyntner et al., 2016). The enzymes responsible for the synthesis of siderophores *SidD*, *SidF*, *SidA* and *SidC* (Chen et al., 2014) and their transporter *Sit1,* are upregulated in all three skin experiments. Similarly, two genes related to the reductive uptake pathway, *Ftr1* and *Fet3,* are upregulated in WT1, WT2 and MT (Table 2).

In contrast, genes related to copper transport from the environment into the cell, like *Crt* (Ding et al., 2014; Puig et al., 2002) and *Atx1,* are specifically upregulated in the mutant (Table 2). In *Cryptococcus neoformans Atx1*, a copper chaperone, is not only associated with copper transport but also with melanin production and iron uptake (Walton et al., 2005).

Environment Sensing Among the genes responsible for the photoreception, only carotenoid oxygenase was downregulated in all three experiments, while the other genes were not consistently regulated (Supplementary material Table 1). Among the genes involved in the MAPK pathway, *Ypld1*, which mediates the multistep phosphotransfer reaction is upregulated in the MT leading to the downregulation of *Ssk1* (Suppressor of Sensor Kinase HMPREF1120_04973). A homologue to *Tco2* (Sensory transduction histidine kinase, HMPREF1120_5233) is solely upregulated in the MT.

Cell Wall Regulation Based on the annotation of the cell wall genes in *E. dermatitidis* (Chen et al., 2014) our results show that five out of nine chitin synthase genes and four out of eight chitin degradation genes are significantly regulated in the MT (Table 1), indicating that this strain is reorganizing more thoroughly its cell wall upon growth on skin than the WT. Among those genes one chitin synthase, *Chs1*, and one chitin degradation gene, *ChiA*, are also regulated in WT1 and WT2. The chitin degradation gene *NagA* is significantly downregulated in WT experiments but not in the MT (Table 1).In the set of beta-glucan synthesis and processing genes, two genes are upregulated in all three skin experiments (Table 1), while four genes are downregulated only in the MT (*SunB*, *Kre6*, *CelA*, HMPREF1120_05230) and 2 genes are upregulated in the MT. Finally, HMPREF1120_03513, a gene belonging to the family of endo-mannanase, with a putative role in glycosylphosphatidylinositol-bound cell-wall protein incorporation (Chen et al., 2014), is downregulated specifically in the MT (Table 1). The production of ergosterol, the main component of fungal cell walls (Weete et al., 2010), increases in all three skin experiments. *ERG1*, *ERG24*, *ERG5*, *ERG6* and *MVD1* are all significantly upregulated in the WT and MT, while *ERG3* is upregulated only in the WT strains (Table 1).

Virulence related genes Homologues of genes related to virulence mechanisms including adherence, signaling pathways, invasion and dimorphism reported in other pathogenic fungi were examined. Among the eight homologous genes related to dimorphism reported in Mayer et al. (Mayer et al., 2013) (Supplementary material), only one member of the protease *Sap1-10* genes set is significantly downregulated in the MT (HMPREF1120_05067).

Ssa1 is a member of the HSP70 family proteins reported to be expressed on the cell surface. It functions as a receptors for antimicrobial peptides in *Candida albicans* (Sun et al., 2010a) and has an important role in endocytosis in epithelial cells. *Exophiala dermatitidis* has seven known homologues of *Ssa1*. Four are significantly upregulated in the three experiments and one gene is upregulated only in WT1 and WT2 (Supplementary material).

We further looked at the expression patterns of extracellular proteases. HMPREF1120_01991, a *S41* peptidase, is upregulated in both WT skin experiments but not in MT. The *S41* protease family is hypothesized to be involved in the basal fungal metabolism and to have occasionally developed specific characteristics connected to virulence (Muszewska et al., 2017). MT downregulated specifically a *S08* (HMPREF1120_08439, Cerevisin), *S09* (HMPREF1120_01940) and *S10* (HMPREF1120_5855) peptidases when growing skin. A DNase, (HMPREF1120_05403) is regulated both in the MT and the WT during skin infection. DNase were previously linked to virulence in *E. dermatitidis*, *Cryptococcus neoformans* and *gattii* (Sánchez and Colom, 2010; Sav et al., 2016).

Secondary metabolites Among the genes involved in secondary metabolites synthesis (Poyntner et al., 2016), a homologue of dimethylallyl tryptophan synthase (HMPREF1120_01968, Table 1), a gene involved in the synthesis of ergot alkaloid, was upregulated in the MT experiment. Linear gramicidin synthetase subunit C (HMPREF1120_00598), a non-ribosomal peptide synthetase involved (NRPS), was upregulated in all skin experiments.

4.5 Discussion

In this work the CRISPR/Cas9 gene knockout method has been successfully used in *E. dermatitidis*. The targeted gene, *PKS1*, which is required for the melanin production of the DHN melanin pathway (Feng et al., 2001), was disrupted successfully. The MT and WT strains were inoculated and cultured on vital human skin using identical *ex-vivo* culture conditions and were monitored microscopically and transcriptomically. While both strains grew at a similar rate on the nylon membrane control, the WT proliferated significantly faster than the MT on the *ex-vivo* skin models. Macroscopic findings were confirmed by HE microscopy and in the HE-stained sections it was clearly visible that the WT was able to infect and disintegrate the skin and invade into deeper parts of the dermis, while the MT was only colonizing the surface of the epidermis. The skin model inoculated with the MT maintained its

vital structure compared to the WT, where separation of epidermis and dermis as well as pyknotic keratinocytes were visible. This indicates that the absence of melanin reduced the ability of the MT to colonize the skin. The changes in transcriptional landscapes between WT and MT in the control and in the skin experiments were minimal. We looked therefore at differences in the enriched categories of the differentially expressed genes between the skin experiment and control of the WT or MT for each strain separately (WT1, WT2, and MT).

From the transcriptome point of view, the main differences between the mutant and wild-type were seen in the regulation of copper transporters. It is known that melanin increases copper biosorption (Gadd and de Rome, 1988; Glass et al., 2014), probably increasing the copper concentration in vicinity of the WT membrane. Therefore, the MT might upregulate the copper transporters to compensate for the reduced copper concentration in the proximity of the fungal cell. Additionally, copper is a crucial component of melanin synthesizing enzymes like laccase and tyrosinase (Li et al., 2016). The nitrogen acquisition pathway was also impacted by the disruption of *PKS1*. In MT, four peptidases are downregulated in the *ex-vivo* skin model experiment, among which two are metallopeptidases (*M28*) that can be activated by divalent cations including copper. Further the lack of melanin did impact the regulation of cell-wall components like chitin and b-glucan as well as the ergosterol biosynthesis indicating a reorganization of the cell wall in the MT. The enrichment in MCM complex is only found in the WT strains, supporting the reduced growth seen macroscopically and microscopically in the MT experiment compared to the WT experiments.

In all three experiments we find negative regulation of the genes involved in the synthesis of DHN and L-Dopa melanin. The DHN pathway seems to be crucial for the invasion of the skin model as the mutation of the *PKS1* gene of the DHN pathway leads to completely different microscopic and macroscopic pictures. L-Dopa added to minimal liquid media induced the production of melanin in MT, in line with similar experiments (Paolo et al., 2006).

The L-Tyrosine pathway was upregulated even in the melanin deficient mutant but did not lead to a blackening of the mutant on skin. The addition of L-tyrosine in liquid media did not lead to pyomelanin production in the mutant either. The reason might be that the phenolic homogentisate acid, which is a precursor of the pyomelanin in this pathway, is also degraded to fumarate and acetoacetate (Keller et al., 2011). One of the genes responsible for the degradation (*fahA*) is upregulated 45 times in the mutant (Table 1). This indicates that the degradation products of L-tyrosine are primarily used in the Krebs cycle and not for pyomelanin production. The neurotropism of *E. dermatitidis* may be associated with its ability to use L-tyrosine as an energy source. *Exophiala dermatitidis* and other black yeast species were isolated from mono- and poly-aromatic compounds rich places such as creosote covered railway sleepers (Döğen et al., 2013a) or toxic hydrocarbons (Blasi et al., 2016; Prenafeta-Boldú et al., 2006; Zhao et al., 2010) and seem to be able to grow on aromatic substances. A link between the capabilities of fungal strains to degrade contaminants and being neurotropic agents for warm-blooded vertebrates is hypothesized (Prenafeta-Boldú et al., 2006).

The difference in skin invasion between the WT and MT are probably due to the disruption of DHN melanin production. Lack of skin invasiveness of an albino fungal strain compared to the pigmented one, has previously been reported in an experimental rat model with sporotrichosis (Barros et al., 2011). Similarly, DHN melanin extracted from the black yeast *Aureobasidium pullulans,* was shown to significantly inhibit the proliferation of cultivated human keratinocytes (Blinova et al., 2003). Melanin may play various roles in host intrusion. It is involved in the formation of the appressorium, a dome-shaped cell that applies physical force to rupture and invade host plant cells (Howard and Ferrari, 1989). While appressoria are not reported to be formed by zoopathogenic fungi, it was shown that melanised *E. dermatitidis* hyphae grew faster through thick agar than their albino counterparts (Brush and Money, 1999). Recently, it was shown that DHN melanin synthesized by the banana pathogen *Mycosphaerella fijiensis* can photogenerate singlet molecular oxygen, which may be involved in the damaging of the plant (Beltrán-García et al., 2014).

In our study the category of Lon Protease S16, which among other contributes to pathogenicity of bacteria and fungi (Breidenstein et al., 2012; Cui et al., 2015; Li et al., 2015; Takaya et al., 2003), was exclusively enriched in the set of upregulated genes in the WT experiments. Further a putatively excreted serine protease (HMPREF1120_01901, *S41*) is upregulated only in the WT during growth on skin and three excreted serine proteases were exclusively downregulated in the mutant during skin growth (HMPREF1120_01940 (*S09*), HMPREF1120_05855 (*S10*), HMPREF1120_08349 (*S08*, Cerevisin)). In humans, physiological melanin uptake by endocytosis, a process to protect the nucleus and DNA from influences of UV irradiation (Ando et al., 2012), occurs after activation of the protease-activated receptor-2 (*PAR*-2) (Seiberg et al., 2000a). The *PAR-2* receptor is activated by a serine protease (Seiberg et al., 2000a), while serine protease inhibition is accompanied with reduced melanin transfer and skin lightning (Seiberg et al., 2000b). Proteases from the skin pathogens *Serratia marcescens* and *Propionibacterium acnes* were shown to activate *PAR-2* (Koziel and Potempa, 2013), as did various serine proteases in a murine model (Andoh et al., 2012). *PAR-2* activation by trypsin, *SLIGRL* or *SLIGKV* increases the ability of keratinocytes to ingest fluorescently labeled microspheres or *E. coli* K-12 bioparticles (Sharlow et al., 2000). Interestingly, two recent studies showed that melanin absorbed by keratinocytes is located in non-degradative compartments (Correia et al., 2018; Hurbain et al., 2018).

Given the facts that human keratinocytes absorb exogeneous melanin (Huang et al., 2017) and that lung epithelial cells uptake of *PKSP* mutant conidia from *Aspergillus fumigatus* was much lower than of its melanised counterpart (Amin et al., 2014), it can be hypothesized that the fungus uses the melanin uptake system to either invade the keratinocytes or channels virulence factors into it in a “trojan horse” manner. Hence, lack of melanin along with the serine protease crucial for endocytosis may have accounted for the lack of invasion and virulence seen with the MT fungus. The use of endocytosis to promote infection in a “trojan horse” manner has been proposed for fungi, bacteria, virus and even bacteriophage (Nguyen et al., 2017; Peluso et al., 1985; Santiago-Tirado and Doering, 2017). More specifically, the yeast *Candida albicans* secretes aspartic proteases that are internalized to endosomes and lysosomes in epithelial cells and subsequently trigger apoptosis (Wu et al., 2013).

Overall the albino and the wild-type strains exhibit similar transcriptional landscapes but show profound differences in cutaneous pathogenicity with regard to invasiveness and skin desintegration in their microscopic appearance. The lack of melanin impacts mainly the fungus' cell wall structures, the copper homeostasis, protein degradation and ultimately the speed of growth and invasiveness of the mutant in the *ex-vivo* skin culture model, indicating that melanin, while not being strictly necessary during the first phase of the skin colonization, is crucial for invading the skin. This finding is in line with experiments showing that albino strains of *E. dermatitidis* is associated with reduced case fatality rates in mice (Dixon et al., 1987; Feng et al., 2001). Further the changes in the copper homeostasis indicate that this metal plays an important role in the first phase of infection.

In conclusion, the usage of CRISPR/Cas9 and RNA sequencing allowed us to knock-out the *PKS1* gene, disrupt melanin production and assess the impact on the virulence of the fungus. We could show that melanin plays a key role with regard to the fungus' virulence by facilitating skin invasion and disintegration.

In contrast to our *ex-vivo* skin model, fungi involved in human infections face host defense mechanisms, among others the phagocytosis by macrophages and oxidative burst (Lorenz et al., 2004). In order to better understand the relevance of melanin in oxidative stress resistance, we plan to grow the wild-type and the mutant under oxidative conditions and compare their transcriptional response and growth rate. To understand if human cells can distinguish between fungal and human melanin, we further plan to investigate the uptake mechanism of fungal melanin into the human skin cells and the impact of melanin on the first human immune response.

4.6 Conflict of Interest

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

4.7 Acknowledgement

We would like to thank Regina Weinmüllner from the Grillari Group, Department of Biotechnology, Boku for providing help with HE stainig. The computational results presented were achieved, in part, through the use of the Vienna Scientific Cluster (VSC).

4.8 Supplementary Material – Raw data

Supplementary table can be found in the Appendix

The raw data can be found online in the supplementary table: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01457/full#supplementary-material

5 Conclusions and Outlook

Fungal pathogens are an increasing threat for human and animal well-being. Pandemic incidents are expected by emerging and re-emerging pathogens in relation to climate change and increasing populations especially in urban areas (Morens et al., 2004). Moreover, emerging fungal pathogens are often neglected in clinical research (Rodrigues and Albuquerque, 2018). However, novel mycosis e.g. Candidiasis caused by the multidrug resistant fungal pathogen *Candida auris* (Lamoth and Kontoyiannis, 2018) demonstrate the importance of research on fungal pathogens. One important emerging fungal pathogen is *Exophiala dermatitidis*, a member of the group of black yeasts. Black yeasts are causative agents of chromoblastomycosis, which was recently added to the list of neglected disease of the WHO (WHO | Mycetoma, chromoblastomycosis and other deep mycoses, 2018). This shows the increased awareness for the pathogenic potential of black yeasts and their harmful effect on human and animal well-being. *Exophiala dermatitidis* is the most frequently reported black yeast in clinics (Sudhadham et al., 2010). The opportunistic pathogen causes systemic infections in immunocompetent and immunocompromised patients and thrives in man-made environments. Although the fungus is widely distributed in our households, its infection mechanisms and successful host invasion is not understood. For this reason, getting a better understanding of the virulence mechanisms of *Exophiala dermatitidis* is of utmost importance.

It was hypothesized that the first phase of infection is the key factor for successful host invasion. To test this hypothesis, two main studies were conducted within this thesis. The studies focused on the mechanisms underlying the cutaneous infection on the transcriptome level to understand the first phase of infection and thereof the virulence mechanisms of *E. dermatitidis*:

(1) An *ex-vivo* skin model infection was established for the first time within the black yeast group. The fungus was able to grow and invade human skin and the genes playing a role during the first contact with the skin were identified. The fungus was in an active replication state which was supported by upregulated terms such as the

MCM-maintenance complex, ribosome biogenesis or helicases. Several potential fungal virulence mechanisms on a cellular level were identified. For example Hsp70 was upregulated during the artificial skin infection, which is important on one hand for proliferation and on the other hand for the switch between yeast-like and hyphal growth.

Special non-coding RNAs were found, for example clusters of snoRNAS which were also differentially regulated during skin infection. These snoRNAs were upregulated during skin infection but also in another black yeast, *C. immunda*, during toluene degradation (Blasi et al., 2017) and in extracellular vesicles of *E. dermatitidis* (Blasi et al., manuscript in preparation). It can be hypothesized that these snoRNAs are exported from the fungal cell to attack the host tissue and play a similar role as in fungal plant pathogens where miRNAs are hijacking the host RNAi pathways (Weiberg et al., 2013). To better understand this mechanism, the geomicrobiology group at the University of Natural Resources and Life Sciences, Vienna, is currently looking at the effect of the snoRNA14 in host cell cultures.

We identified Long non-coding RNAs were identified which are known in other fungal pathogens for yeast to hyphal transition (RZE1) and also for prevention of stress response and apoptosis (β2.7) in the host. It is known that, in pathogenic fungi such as *Candida*, the switch between yeast and hyphal growth is an important virulence mechanism (Mayer et al., 2013). Therefore, the morphology may also be an important virulence factor of *E. dermatitidis*. Current results indicate the presence of more cells in a yeast-like state, but some hyphal structures are visible in the microscopic pictures during artificial skin infection. This is in line with the clinical picture description of phaeohyphomycosis were both forms are present at the same time. The results further indicate that the fungus might attack the host through ergot alkaloids, which were shown to be cytotoxic in humans (Haarmann et al., 2005) and which might be linked to the upregulation of DMATS.

During the artificial infection, the fungus was reprogramming the carbon acquisition by switching to the glyoxalate cycle and gluconeogensis pathway. In *Candida albicans,* this was seen during internalization in macrophages and might therefore be

a reaction of the *E. dermatitidis* during invasion. The upregulation of PHR1 was also shown to be important for adhesion in other pathogens.

Melanin is a known virulence factor which is reported to be important in black yeasts not only for withstanding extreme environments but also for pathogenicity as shown in albino mutant experiments in mice (Dixon et al., 1989). On a genome level, all three pathways (DHN, L-Dopa and L-tyrosine pathway) are present in the fungus as reported by Chen et al. (Chen et al., 2014). During the first contact with the skin the genes of the L-tyrosine pathway are upregulated but neither the L-Dopa nor the DHN melanin pathways are upregulated, indicating that this pathway is important for skin cell invasion.

(2) The second main study was performed in order to understand the role of melanin during the skin invasion. A mutation in the *PKS1* gene resulting in a melanin deficient mutant was performed. The CRISPR/Cas9 method was used for the first time in black yeasts to retrieve a DHN melanin deficient mutant. The fungus was inoculated with different substrates to analyze the different melanin pathways. Using L-Dopa as substrate in the culture the mutant was able to produce melanin and the culture turned black. In contrast, the mutant with L-tyrosine as substrate did not show any visible effect. This leads to the conclusion that, although the genes of the L-tyrosine pathway are upregulated during skin invasion, the fungus is not producing any pyomelanin but rather switches on an alternative pathway leading to the citrate cycle. This is in line with published studies reporting strains of the black yeast group which are able to degrade aromatic hydrocarbons. (Blasi et al., 2016, 2017; Moreno et al., 2018a; Prenafeta-Boldú et al., 2006). These results lead to the conclusion that *E. dermatitidis* can use L-tyrosine as an energy source and this might be linked to the fungal neurotropism because molecules in the human brain are similar in the structure of L-tyrosine.

In the comparative *ex-vivo* skin infection using the mutant and the wildtype *of E. dermatitidis,* no major differences on the transcriptome level were observed. Nevertheless, on a microscopic level, the skin samples inoculated with the mutant showed rather vital cells while the wildtype lead to pyknotic cells and separation of the dermis and epidermis. Additionally, the mutant only partially colonized the skin

whereas the wildtype covered the whole surface and was able to blacken the epidermis. This confirms the hypothesis that melanin has a crucial role for skin invasion of *E. dermatitidis.*

One hypothesis explaining the role of melanin in skin invasion is related to the upregulation of the Lon protease in the wildtype seen in both studies. In humans, melanin is transferred into the cell through a protease induced Par2 receptor (Hurbain et al., 2018; Seiberg et al., 2000a; Sharlow et al., 2000). Therefore, the fungal pathogen might use the human melanin uptake system using the Lon protease to induce Par2 and smuggle melanin and other virulence factors into the skin cell in the manner of a Trojan horse.

In all experiments trace elements played important roles for the wildtype magnesium, iron and zinc transporters were upregulated, whereas in the mutant experiments copper transporters were upregulated. Differences in copper uptake can be linked to melanin occurrence because copper is biosorbed to melanin. Accordingly, the mutant lacking melanin has a need to compensate for the lack of copper and upregulates these transporters. Further genes of the pyomelanin production were seen to be necessary for iron uptake in *Crypotcoccus neoformans* (Ding et al., 2014). Therefore, the upregulation of the genes of the L-tyrosine melanin pathway can be linked with the upregulation of iron siderophores.

In conclusion, the two studies successfully demonstrated important virulence factors and mechanisms during the first phase of skin infection with *E. dermatitidis.* The artificial skin model infection revealed itself as a good model system to study fungal cutaneous infection. Moreover, the role of the three melanin types during infection was described. DHN melanin was shown to be crucial for invasion of the skin while the upregulated L-tyrosine pathway leads to energy production. The usage of aromatic molecules for energy production is interesting, as it is in line with the hypothesis that these tools are used for the neurotropic infection by fungal pathogens. Additionally, the infection mode of melanin as Trojan horse might be a fundamental pathogenic mechanism in black yeasts but more experiments are necessary to confirm this hypothesis. Currently ongoing work indicates that extracellular vesicles and snoRNAs are putative virulence mechanisms and could be

connected to the Trojan horse hypothesis. Additionally, we are looking at the involvement of the different melanin pathways during environmental stress conditions in a climate chamber using UV, ozone and salt which correlate to oxidative stress conditions in the host (Tafer et al., in preparation). This will help to understand strategies of black yeasts to thrive in ecological extreme niches. Further shared mechanisms between environmental stress tolerance and pathogenic mechanisms not only of *E. dermatitidis* but also in other black yeasts can be studied. These results are expected to contribute to a better understanding of these mechanisms which is necessary for applications in medicine and biotechnology.

6 References

Abramczyk, D., Park, C., and Szaniszlo, P. J. (2009). Cytolocalization of the class V chitin synthase in the yeast, hyphal and sclerotic morphotypes of Wangiella (Exophiala) dermatitidis. *Fungal Genet. Biol.* 46, 28–41. doi:10.1016/j.fgb.2008.10.004.

Albrecht, A., Felk, A., Pichova, I., Naglik, J. R., Schaller, M., de Groot, P., et al. (2006). Glycosylphosphatidylinositol-anchored Proteases of *Candida albicans* Target Proteins Necessary for Both Cellular Processes and Host-Pathogen Interactions. *J. Biol. Chem.* 281, 688–694. doi:10.1074/jbc.M509297200.

Almeida-Paes, R., Figueiredo-Carvalho, M. H. G., Brito-Santos, F., Almeida-Silva, F., Oliveira, M. M. E., and Zancopé-Oliveira, R. M. (2016). Melanins Protect Sporothrix brasiliensis and Sporothrix schenckii from the Antifungal Effects of Terbinafine. *PLoS One* 11, e0152796. doi:10.1371/journal.pone.0152796.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–402. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9254694 [Accessed August 1, 2016].

Amaral, P. P., Dinger, M. E., and Mattick, J. S. (2013). Non-coding RNAs in homeostasis, disease and stress responses: an evolutionary perspective. *Brief. Funct. Genomics* 12, 254–278. doi:10.1093/bfgp/elt016.

Amin, S., Thywissen, A., Heinekamp, T., Saluz, H. P., and Brakhage, A. A. (2014). Melanin dependent survival of Apergillus fumigatus conidia in lung epithelial cells. *Int. J. Med. Microbiol.* 304, 626–36. doi:10.1016/j.ijmm.2014.04.009.

Ando, H., Niki, Y., Ito, M., Akiyama, K., Matsui, M. S., Yarosh, D. B., et al. (2012). Melanosomes Are Transferred from Melanocytes to Keratinocytes through the Processes of Packaging, Release, Uptake, and Dispersion. *J. Invest. Dermatol.* 132, 1222–1229. doi:10.1038/jid.2011.413.

Andoh, T., Takayama, Y., Yamakoshi, T., Lee, J.-B., Sano, A., Shimizu, T., et al. (2012). Involvement of Serine Protease and Proteinase-Activated Receptor 2 in Dermatophyte-Associated Itch in Mice. *J. Pharmacol. Exp. Ther.* 343, 91–96. doi:10.1124/jpet.112.195222.

Baldo, A., Mathy, A., Tabart, J., Camponova, P., Vermout, S., Massart, L., et al. (2010). Secreted subtilisin Sub3 from *Microsporum canis* is required for adherence to but not for invasion of the epidermis. *Br. J. Dermatol.* 162, 990–997. doi:10.1111/j.1365-2133.2009.09608.x.

Barros, M. B. de L., Paes, R. de A., and Schubach, A. O. (2011). Sporothrix schenckii and Sporotrichosis. *Clin. Microbiol. Rev.* 24, 633. doi:10.1128/CMR.00007-11.

Beltrán-García, M. J., Prado, F. M., Oliveira, M. S., Ortiz-Mendoza, D., Scalfo, A. C., Pessoa,

A., et al. (2014). Singlet molecular oxygen generation by light-activated DHN-melanin of the fungal pathogen Mycosphaerella fijiensis in black Sigatoka disease of bananas. *PLoS One* 9, e91616. doi:10.1371/journal.pone.0091616.

Blackwell, M. (2011). The Fungi: 1, 2, 3 ... 5.1 million species? *Am. J. Bot.* 98, 426–438. doi:10.3732/ajb.1000298.

Blanchette, M., Kent, W. J., Riemer, C., Elnitski, L., Smit, A. F. A., Roskin, K. M., et al. (2004). Aligning multiple genomic sequences with the threaded blockset aligner. *Genome Res.* 14, 708–15. doi:10.1101/gr.1933104.

Blasi, B., Poyntner, C., Rudavsky, T., Prenafeta-Boldú, F. X., Hoog, S. De, Tafer, H., et al. (2016). Pathogenic Yet Environmentally Friendly? Black Fungal Candidates for Bioremediation of Pollutants. *Geomicrobiol. J.* 33, 308–317. doi:10.1080/01490451.2015.1052118.

Blasi, B., Tafer, H., Kustor, C., Poyntner, C., Lopandic, K., and Sterflinger, K. (2017). Genomic and transcriptomic analysis of the toluene degrading black yeast Cladophialophora immunda. *Sci. Rep.* 7, 11436. doi:10.1038/s41598-017-11807-8.

Blasi, B., Tafer, H., Tesei, D., and Sterflinger, K. (2015). From Glacier to Sauna: RNA-Seq of the Human Pathogen Black Fungus Exophiala dermatitidis under Varying Temperature Conditions Exhibits Common and Novel Fungal Response. *PLoS One* 10, e0127103. doi:10.1371/journal.pone.0127103.

Blinova, M. I., Yudintseva, N. M., Kalmykova, N. V, Kuzminykh, E. V, Yurlova, N. A., Ovchinnikova, O. A., et al. (2003). Effect of melanins from black yeast fungi on proliferation and differentiation of cultivated human keratinocytes and fibroblasts. *Cell Biol. Int.* 27, 135–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12662970 [Accessed May 29, 2018].

Bochman, M. L., and Schwacha, A. (2009). The Mcm Complex: Unwinding the Mechanism of a Replicative Helicase. *Microbiol. Mol. Biol. Rev.* 73, 652–683. doi:10.1128/MMBR.00019-09.

Boyce, K. J., and Andrianopoulos, A. (2015). Fungal dimorphism: the switch from hyphae to yeast is a specialized morphogenetic adaptation allowing colonization of a host. *FEMS Microbiol. Rev.* 39, 797–811. doi:10.1093/femsre/fuv035.

Branda, E., Turchetti, B., Diolaiuti, G., Pecci, M., Smiraglia, C., and Buzzini, P. (2010). Yeast and yeast-like diversity in the southernmost glacier of Europe (Calderone Glacier, Apennines, Italy). *FEMS Microbiol. Ecol.* 72, 354–69. doi:10.1111/j.1574-6941.2010.00864.x.

Breidenstein, E. B. M., Janot, L., Strehmel, J., Fernandez, L., Taylor, P. K., Kukavica-Ibrulj, I., et al. (2012). The Lon Protease Is Essential for Full Virulence in Pseudomonas aeruginosa. *PLoS One* 7, e49123. doi:10.1371/journal.pone.0049123.

Brush, L., and Money, N. P. (1999). Invasive hyphal growth in Wangiella dermatitidis is induced by stab inoculation and shows dependence upon melanin biosynthesis. *Fungal*

Genet. Biol. 28, 190–200. doi:10.1006/fgbi.1999.1176.

Butler, M. J., and Day, A. W. (1998a). Fungal melanins: a review. *Can. J. Microbiol.* 44, 1115–1136. doi:10.1139/w98-119.

Butler, M. J., and Day, A. W. (1998b). Fungal melanins: A review. 44, 1115–1136. Available at: https://www.scopus.com/record/display.uri?eid=2-s2.0-0032447022&origin=resultslist&sort=cp-f&src=s&st1=melanin+fungi&nlo=&nlr=&nls=&sid=69a3c67ad0f8b8de8781ffac8044c026&sot=b&sdt=b&sl=28&s=TITLE-ABS-KEY%28melanin+fungi%29&relpos=4&citeCnt=368&searchTerm= [Accessed September 25, 2017].

Byrne, D. D., and Reboli, A. C. (2017). Rare Yeast Infections: Risk Factors, Clinical Manifestations, Treatment, and Special Considerations. *Curr. Clin. Microbiol. Reports* 4. doi:10.1007/s40588-017-0073-7.

Calderon, J., Zavrel, M., Ragni, E., Fonzi, W. A., Rupp, S., and Popolo, L. (2010). PHR1, a pH-regulated gene of Candida albicans encoding a glucan-remodelling enzyme, is required for adhesion and invasion. *Microbiology* 156, 2484–2494. doi:10.1099/mic.0.038000-0.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res.* 37, D233-8. doi:10.1093/nar/gkn663.

Carmichael, J. W. (1967). Cerebral mycetoma of trout due to a *Phialophora* -like fungus. *Med. Mycol.* 5, 120–123. doi:10.1080/00362176785190211.

Casadevall, A., Rosas, A. L., and Nosanchuk, J. D. (2000). Melanin and virulence in Cryptococcus neoformans. *Curr. Opin. Microbiol.* 3, 354–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10972493 [Accessed March 6, 2018].

Chari, R., Yeo, N. C., Chavez, A., and Church, G. M. (2017). sgRNA Scorer 2.0: A Species-Independent Model To Predict CRISPR/Cas9 Activity. *ACS Synth. Biol.* 6, 902–904. doi:10.1021/acssynbio.6b00343.

Chen, M., Zhang, J., Dong, Z., and Wang, F. (2016). Cutaneous phaeohyphomycosis caused by Exophiala dermatitidis: A case report and literature review. *Indian J. Dermatol. Venereol. Leprol.* 82, 173–7. doi:10.4103/0378-6323.171013.

Chen, Z., Martinez, D. A., Gujja, S., Sykes, S. M., Zeng, Q., Szaniszlo, P. J., et al. (2014). Comparative genomic and transcriptomic analysis of Wangiella dermatitidis, a major cause of phaeohyphomycosis and a model black yeast human pathogen. *G3 (Bethesda).* 4, 561–78. doi:10.1534/g3.113.009241.

Chotirmall, S. H., and McElvaney, N. G. (2014). Fungi in the cystic fibrosis lung: Bystanders or pathogens? *Int. J. Biochem. Cell Biol.* 52C, 161–173. doi:10.1016/j.biocel.2014.03.001.

Chumley, F. G. (1990). Genetic Analysis of Melanin-Deficient, Nonpathogenic Mutants of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 3, 135. doi:10.1094/MPMI-3-135.

Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., et al. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff. *Fly (Austin).* 6, 80–92. doi:10.4161/fly.19695.

Clark, S. E., Snow, J., Li, J., Zola, T. A., and Weiser, J. N. (2012). Phosphorylcholine allows for evasion of bactericidal antibody by Haemophilus influenzae. *PLoS Pathog.* 8, e1002521. doi:10.1371/journal.ppat.1002521.

Clark, S. E., and Weiser, J. N. (2013). Microbial modulation of host immunity with the small molecule phosphorylcholine. *Infect. Immun.* 81, 392–401. doi:10.1128/IAI.01168-12.

Conklin, D. S., McMaster, J. A., Culbertson, M. R., and Kung, C. (1992). COT1, a gene involved in cobalt accumulation in Saccharomyces cerevisiae. *Mol. Cell. Biol.* 12, 3678–88. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1508175 [Accessed July 27, 2018].

Conrad, M., Schothorst, J., Kankipati, H. N., Van Zeebroeck, G., Rubio-Texeira, M., and Thevelein, J. M. (2014). Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 38, 254–299. doi:10.1111/1574-6976.12065.

Correia, M. S., Moreiras, H., Pereira, F. J. C., Neto, M. V., Festas, T. C., Tarafder, A. K., et al. (2018). Melanin Transferred to Keratinocytes Resides in Nondegradative Endocytic Compartments. *J. Invest. Dermatol.* 138, 637–646. doi:10.1016/j.jid.2017.09.042.

Cui, X., Wei, Y., Wang, Y.-H., Li, J., Wong, F.-L., Zheng, Y.-J., et al. (2015). Proteins interacting with mitochondrial ATP-dependent Lon protease (MAP1) in Magnaporthe oryzae are involved in rice blast disease. *Mol. Plant Pathol.* 16, 847–59. doi:10.1111/mpp.12242.

Czichos, J. (1987). *What's so funny about Microbiology?* Madison, Wisconsin: Science Tech Publishers.

da Silva, S. P., Borges-Walmsley, M. I., Pereira, I. S., Soares, C. M. de A., Walmsley, A. R., and Felipe, M. S. S. (1999). Differential expression of an hsp70 gene during transition from the mycelial to the infective yeast form of the human pathogenic fungus Paracoccidioides brasiliensis. *Mol. Microbiol.* 31, 1039–1050. doi:10.1046/j.1365-2958.1999.01236.x.

Dadachova, E., Bryan, R. A., Huang, X., Moadel, T., Schweitzer, A. D., Aisen, P., et al. (2007). Ionizing radiation changes the electronic properties of melanin and enhances the growth of melanized fungi. *PLoS One* 2, e457. doi:10.1371/journal.pone.0000457.

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., et al. (2011). The variant call format and VCFtools. *Bioinformatics* 27, 2156–8. doi:10.1093/bioinformatics/btr330.

de Groot, P. W. J., Bader, O., de Boer, A. D., Weig, M., and Chauhan, N. (2013). Adhesins in Human Fungal Pathogens: Glue with Plenty of Stick. *Eukaryot. Cell* 12, 470–481. doi:10.1128/EC.00364-12.

de Hoog, G. S., Matos, T., Sudhadham, M., Luijsterburg, K. F., and Haase, G. (2005). Intestinal prevalence of the neurotropic black yeast Exophiala (Wangiella) dermatitidis in healthy and impaired individuals. *Mycoses* 48, 142–5. doi:10.1111/j.1439-0507.2004.01083.x.

de Hoog, G. S., Takeo, K., Yoshida, S., Göttlich, E., Nishimura, K., and Miyaji, M. (1994). Pleoanamorphic life cycle of Exophiala (Wangiella) dermatitidis. *Antonie Van Leeuwenhoek* 65, 143–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7979319 [Accessed June 14, 2018].

de Hoog, G. S., Vicente, V. A., Najafzadeh, M. J., Harrak, M. J., Badali, H., and Seyedmousavi, S. (2011a). Waterborne Exophiala species causing disease in cold-blooded animals. *Persoonia* 27, 46–72. doi:10.3767/003158511X614258.

de Hoog, G. S., Vicente, V., Caligiorne, R. B., Kantarcioglu, S., Tintelnot, K., Gerrits van den Ende, A. H. G., et al. (2003). Species diversity and polymorphism in the Exophiala spinifera clade containing opportunistic black yeast-like fungi. *J. Clin. Microbiol.* 41, 4767–78. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=254316&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

de Hoog, S., Grube, M., Gunde-Cimerman, N., Seyedmousavi, S., Badali, H., Chlebicki, A., et al. (2011b). Exophiala sideris, a novel black yeast isolated from environments polluted with toxic alkyl benzenes and arsenic. *Fungal Biol.* 115, 1030–7. doi:10.1016/j.funbio.2011.06.004.

Dieterich, C., Schandar, M., Noll, M., Johannes, F.-J., Brunner, H., Graeve, T., et al. (2002). In vitro reconstructed human epithelia reveal contributions of Candida albicans EFG1 and CPH1 to adhesion and invasion. *Microbiology* 148, 497–506. Available at: http://mic.sgmjournals.org/content/148/2/497.long [Accessed July 7, 2014].

Ding, C., Festa, R. A., Sun, T.-S., and Wang, Z.-Y. (2014). Iron and copper as virulence modulators in human fungal pathogens. *Mol. Microbiol.* 93, 10–23. doi:10.1111/mmi.12653.

Dixon, D. M., Polak, A., and Conner, G. W. (1989). Mel $^{-}$ mutants of *Wangiella dermatitidis* in mice: evaluation of multiple mouse and fungal strains. *Med. Mycol.* 27, 335–341. doi:10.1080/02681218980000451.

Dixon, D. M., Polak, A., and Szaniszlo, P. J. (1987). Pathogenicity and virulence of wild-type and melanin-deficient Wangiella dermatitidis. *J. Med. Vet. Mycol.* 25, 97–106. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3598824 [Accessed March 6, 2018].

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi:10.1093/bioinformatics/bts635.

Döğen, A., Kaplan, E., Ilkit, M., and de Hoog, G. S. (2013a). Massive Contamination of Exophiala dermatitidis and E. phaeomuriformis in Railway Stations in Subtropical Turkey. *Mycopathologia* 175, 381–386. doi:10.1007/s11046-012-9594-z.

Döğen, A., Kaplan, E., Oksüz, Z., Serin, M. S., Ilkit, M., and de Hoog, G. S. (2013b). Dishwashers are a major source of human opportunistic yeast-like fungi in indoor environments in Mersin, Turkey. *Med. Mycol.* 51, 493–8. doi:10.3109/13693786.2012.738313.

Eggenhofer, F., Hofacker, I. L., and Höner zu Siederdissen, C. (2016). RNAlien – Unsupervised RNA family model construction. *Nucleic Acids Res.* 44, 8433–8441. doi:10.1093/nar/gkw558.

Eisenman, H. C., Mues, M., Weber, S. E., Frases, S., Chaskes, S., Gerfen, G., et al. (2007). Cryptococcus neoformans laccase catalyses melanin synthesis from both D- and L-DOPA. *Microbiology* 153, 3954–62. doi:10.1099/mic.0.2007/011049-0.

Enguita, F., Costa, M., Fusco-Almeida, A., Mendes-Giannini, M., and Leitão, A. (2016). Transcriptomic Crosstalk between Fungal Invasive Pathogens and Their Host Cells: Opportunities and Challenges for Next-Generation Sequencing Methods. *J. Fungi* 2, 7. doi:10.3390/jof2010007.

Esteller, M. (2011). Non-coding RNAs in human disease. *Nat. Rev. Genet.* 12, 861–874. doi:10.1038/nrg3074.

Fairman-Williams, M. E., Guenther, U.-P., and Jankowsky, E. (2010). SF1 and SF2 helicases: family matters. *Curr. Opin. Struct. Biol.* 20, 313–324. doi:10.1016/j.sbi.2010.03.011.

Falcon, S., and Gentleman, R. (2007). Using GOstats to test gene lists for GO term association. *Bioinformatics* 23, 257–8. doi:10.1093/bioinformatics/btl567.

Fanning, S., and Mitchell, A. P. (2012). Fungal Biofilms. *PLoS Pathog.* 8, e1002585. doi:10.1371/journal.ppat.1002585.

Feng, B., Wang, X., Hauser, M., Kaufmann, S., Jentsch, S., Haase, G., et al. (2001). Molecular Cloning and Characterization of WdPKS1, a Gene Involved in Dihydroxynaphthalene Melanin Biosynthesis and Virulence in Wangiella (Exophiala) dermatitidis. *Infect. Immun.* 69, 1781–1794. doi:10.1128/IAI.69.3.1781-1794.2001.

Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., et al. (2014). Pfam: the protein families database. *Nucleic Acids Res.* 42, D222-30. doi:10.1093/nar/gkt1223.

Fleck, C. B., Schöbel, F., and Brock, M. (2011). Nutrient acquisition by pathogenic fungi: Nutrient availability, pathway regulation, and differences in substrate utilization. *Int. J. Med. Microbiol.* 301, 400–407. doi:10.1016/j.ijmm.2011.04.007.

Franzen, A. J., Cunha, M. M. L., Batista, E. J. O., Seabra, S. H., De Souza, W., and

Rozental, S. (2006). Effects of tricyclazole (5-methyl-1,2,4-triazol[3,4] benzothiazole), a specific DHN–melanin inhibitor, on the morphology ofFonsecaea pedrosoi conidia and sclerotic cells. *Microsc. Res. Tech.* 69, 729–737. doi:10.1002/jemt.20344.

Franzen, A. J., Cunha, M. M. L., Miranda, K., Hentschel, J., Plattner, H., da Silva, M. B., et al. (2008). Ultrastructural characterization of melanosomes of the human pathogenic fungus Fonsecaea pedrosoi. *J. Struct. Biol.* 162, 75–84. doi:10.1016/j.jsb.2007.11.004.

Franzen, A. J., Souza, W., Farina, M., Sales Alviano, C., and Rozental, S. (1999). Morphometric and densitometric study of the biogenesis of electron-dense granules in Fonsecaea pedrosoi. *FEMS Microbiol. Lett.* 173, 395–402. doi:10.1111/j.1574-6968.1999.tb13531.x.

Gadd, G. M., and de Rome, L. (1988). Biosorption of copper by fungal melanin. *Appl. Microbiol. Biotechnol.* 29, 610–617. doi:10.1007/BF00260993.

Giles, S. S., and Czuprynski, C. J. (2004). Extracellular calcium and magnesium, but not iron, are needed for optimal growth of Blastomyces dermatitidis yeast form cells in vitro. *Clin. Diagn. Lab. Immunol.* 11, 426–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15013999 [Accessed July 30, 2018].

Glass, K., Rengifo, R., Porrka, F., and Simon, J. D. (2014). Probing the surface calcium binding sites of melanosomes using molecular rulers. *J. Phys. Chem. B* 118, 14110–4. doi:10.1021/jp505384j.

Gorbushina, A. A., Kotlova, E. R., and Sherstneva, O. A. (2008). Cellular responses of microcolonial rock fungi to long-term desiccation and subsequent rehydration. *Stud. Mycol.* 61, 91–97. doi:10.3114/SIM.2008.61.09.

Gostinčar, C., Grube, M., and Gunde-Cimerman, N. (2011). Evolution of fungal pathogens in domestic environments? *Fungal Biol.* 115, 1008–18. doi:10.1016/j.funbio.2011.03.004.

Gough, J., Karplus, K., Hughey, R., and Chothia, C. (2001). Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. *J. Mol. Biol.* 313, 903–919. doi:10.1006/jmbi.2001.5080.

Großhans, H., and Filipowicz, W. (2008). The expanding world of small RNAs. *Nature* 451, 414–416. doi:10.1038/451414a.

Grumbt, M., Monod, M., Yamada, T., Hertweck, C., Kunert, J., and Staib, P. (2013). Keratin Degradation by Dermatophytes Relies on Cysteine Dioxygenase and a Sulfite Efflux Pump. *J. Invest. Dermatol.* 133, 1550–1555. doi:10.1038/jid.2013.41.

Gümral, R., Özhak-Baysan, B., Tümgör, A., Saraçlı, M. A., Yıldıran, Ş. T., Ilkit, M., et al. (2015). Dishwashers provide a selective extreme environment for human-opportunistic yeast-like fungi. *Fungal Divers.* doi:10.1007/s13225-015-0327-8.

Gümral, R., Tümgör, A., Saraçlı, M. A., Yıldıran, Ş. T., Ilkit, M., de Hoog, G. S., et al. (2014). Black Yeast Diversity on Creosoted Railway Sleepers Changes with Ambient Climatic

Conditions. *Microb. Ecol.* 68, 699–707. doi:10.1007/s00248-014-0459-5.

Gunde-Cimerman, N., Zalar, P., De Hoog, S., Plemenitaš, A., Hoog, S., and PlemenitaÅ¡, A. (2000). Hypersaline waters in salterns - Natural ecological niches for halophilic black yeasts. *FEMS Microbiol. Ecol.* 32, 235–240. doi:10.1016/S0168-6496(00)00032-5.

Haarmann, T., Machado, C., Lübbe, Y., Correia, T., Schardl, C. L., Panaccione, D. G., et al. (2005). The ergot alkaloid gene cluster in Claviceps purpurea: Extension of the cluster sequence and intra species evolution. *Phytochemistry* 66, 1312–1320. doi:10.1016/j.phytochem.2005.04.011.

Haas, B. J., Delcher, A. L., Mount, S. M., Wortman, J. R., Smith, R. K., Hannick, L. I., et al. (2003). Improving the Arabidopsis genome annotation using maximal transcript alignment assemblies. *Nucleic Acids Res.* 31, 5654–66. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=206470&tool=pmcentrez&rendertype=abstract [Accessed November 13, 2015].

Haas, B. J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P. D., Bowden, J., et al. (2013). De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat. Protoc.* 8, 1494–512. doi:10.1038/nprot.2013.084.

Hawksworth, D. L. (1991). The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycol. Res.* 95, 641–655. doi:10.1016/S0953-7562(09)80810-1.

Heinekamp, T., Thywißen, A., Macheleidt, J., Keller, S., Valiante, V., and Brakhage, A. A. (2013). Aspergillus fumigatus melanins: interference with the host endocytosis pathway and impact on virulence. *Front. Microbiol.* 3, 440. doi:10.3389/fmicb.2012.00440.

Heymann, P., Ernst, J. F., and Winkelmann, G. (2000). Identification and substrate specificity of a ferrichrome-type siderophore transporter (Arn1p) in Saccharomyces cerevisiae. *FEMS Microbiol. Lett.* 186, 221–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10802175 [Accessed March 6, 2018].

Hiruma, M., Kawada, A., Ohata, H., Ohnishi, Y., Takahashi, H., Yamazaki, M., et al. (1993). Systemic phaeohyphomycosis caused by Exophiala dermatitidis. *Mycoses* 36, 1–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8316255 [Accessed March 6, 2018].

Hölker, U., Bend, J., Pracht, R., Tetsch, L., Müller, T., Höfer, M., et al. (2004). Hortaea acidophila, a new acid-tolerant black yeast from lignite. *Antonie Van Leeuwenhoek* 86, 287–294. doi:10.1007/s10482-005-0101-0.

Hoog, G. S. de, and Guarro, J. (1995). Atlas of clinical fungi. *Atlas Clin. fungi.* Available at: https://www.cabdirect.org/cabdirect/abstract/19951202596 [Accessed July 25, 2018].

Hoog, G. S. de, Queiroz-Telles, F., Haase, G., Fernandez-Zeppenfeldt, G., Angelis, D. A., H. G. Gerrits van den Ende, A., et al. (2000). Black fungi: clinical and pathogenic approaches. *Med. Mycol.* 38, 243–250. doi:10.1080/mmy.38.s1.243.250.

Horré, R., Schaal, K. P., Siekmeier, R., Sterzik, B., de Hoog, G. S., and Schnitzler, N. (2004). Isolation of fungi, especially Exophiala dermatitidis, in patients suffering from cystic fibrosis. A prospective study. *Respiration.* 71, 360–6. doi:10.1159/000079640.

Howard, R. J., and Ferrari, M. A. (1989). Role of melanin in appressorium function. *Exp. Mycol.* 13, 403–418. doi:10.1016/0147-5975(89)90036-4.

Huang, Y., Busk, P. K., Herbst, F.-A., and Lange, L. (2015). Genome and secretome analyses provide insights into keratin decomposition by novel proteases from the non-pathogenic fungus Onygena corvina. *Appl. Microbiol. Biotechnol.* 99, 9635–9649. doi:10.1007/s00253-015-6805-9.

Huang, Y., Li, Y., Hu, Z., Yue, X., Proetto, M. T., Jones, Y., et al. (2017). Mimicking Melanosomes: Polydopamine Nanoparticles as Artificial Microparasols. *ACS Cent. Sci.* 3, 564–569. doi:10.1021/acscentsci.6b00230.

Hurbain, I., Romao, M., Sextius, P., Bourreau, E., Marchal, C., Bernerd, F., et al. (2018). Melanosome Distribution in Keratinocytes in Different Skin Types: Melanosome Clusters Are Not Degradative Organelles. *J. Invest. Dermatol.* 138, 647–656. doi:10.1016/J.JID.2017.09.039.

Inglis, D. O., Binkley, J., Skrzypek, M. S., Arnaud, M. B., Cerqueira, G. C., Shah, P., et al. (2013). Comprehensive annotation of secondary metabolite biosynthetic genes and gene clusters of Aspergillus nidulans, A. fumigatus, A. niger and A. oryzae. *BMC Microbiol.* 13, 91. doi:10.1186/1471-2180-13-91.

Isola, D., Zucconi, L., Onofri, S., Caneva, G., de Hoog, G. S., and Selbmann, L. (2015). Extremotolerant rock inhabiting black fungi from Italian monumental sites. *Fungal Divers.* doi:10.1007/s13225-015-0342-9.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–21. doi:10.1126/science.1225829.

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30, 1236–40. doi:10.1093/bioinformatics/btu031.

Jung, N.-Y., and Kim, E. (2014). Cerebral phaeohyphomycosis: a rare cause of brain abscess. *J. Korean Neurosurg. Soc.* 56, 444–7. doi:10.3340/jkns.2014.56.5.444.

Kaufman, G., Berdicevsky, I., Woodfolk, J. A., and Horwitz, B. A. (2005). Markers for Host-Induced Gene Expression in Trichophyton Dermatophytosis. *Infect. Immun.* 73, 6584–6590. doi:10.1128/IAI.73.10.6584-6590.2005.

Keller, S., Macheleidt, J., Scherlach, K., Schmaler-Ripcke, J., Jacobsen, I. D., Heinekamp, T., et al. (2011). Pyomelanin formation in Aspergillus fumigatus requires HmgX and the transcriptional activator HmgR but is dispensable for virulence. *PLoS One* 6, e26604. doi:10.1371/journal.pone.0026604.

Kendrick, B. (2000). *The fifth kingdom*. 3rd ed. Focus Publishing.

Khaldi, N., Seifuddin, F. T., Turner, G., Haft, D., Nierman, W. C., Wolfe, K. H., et al. (2010). SMURF: Genomic mapping of fungal secondary metabolite clusters. *Fungal Genet. Biol.* 47, 736–741. doi:10.1016/j.fgb.2010.06.003.

Köster, J., and Rahmann, S. (2012). Snakemake--a scalable bioinformatics workflow engine. *Bioinformatics* 28, 2520–2. doi:10.1093/bioinformatics/bts480.

Koziel, J., and Potempa, J. (2013). Protease-armed bacteria in the skin. *Cell Tissue Res.* 351, 325–37. doi:10.1007/s00441-012-1355-2.

Lamoth, F., and Kontoyiannis, D. P. (2018). The Candida auris Alert: Facts and Perspectives. *J. Infect. Dis.* 217, 516–520. doi:10.1093/infdis/jix597.

Lau, C.-C., Sun, T., Ching, A. K. K., He, M., Li, J.-W., Wong, A. M., et al. (2014). Viral-human chimeric transcript predisposes risk to liver cancer development and progression. *Cancer Cell* 25, 335–49. doi:10.1016/j.ccr.2014.01.030.

Lebecque, P., Leonard, A., Huang, D., Reychler, G., Boeras, A., Leal, T., et al. (2010). Exophiala (Wangiella) dermatitidis and cystic fibrosis - Prevalence and risk factors. *Med. Mycol.* 48 Suppl 1, S4-9. doi:10.3109/13693786.2010.495731.

Lechenne, B., Reichard, U., Zaugg, C., Fratti, M., Kunert, J., Boulat, O., et al. (2007). Sulphite efflux pumps in Aspergillus fumigatus and dermatophytes. *Microbiology* 153, 905–913. doi:10.1099/mic.0.2006/003335-0.

Lenardon, M. D., Munro, C. A., and Gow, N. A. R. (2010). Chitin synthesis and fungal pathogenesis. *Curr. Opin. Microbiol.* 13, 416–23. doi:10.1016/j.mib.2010.05.002.

Li, D. M., and de Hoog, G. S. (2009). Cerebral phaeohyphomycosis—a cure at what lengths? *Lancet Infect. Dis.* 9, 376–383. doi:10.1016/S1473-3099(09)70131-8.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–9. doi:10.1093/bioinformatics/btp352.

Li, J., Liang, X., Wei, Y., Liu, J., Lin, F., and Zhang, S.-H. (2015). An ATP-dependent protease homolog ensures basic standards of survival and pathogenicity for Magnaporthe oryzae. *Eur. J. Plant Pathol.* 141, 703–716. doi:10.1007/s10658-014-0572-9.

Li, L., Chen, O. S., Ward, D. M., and Kaplan, J. (2001). CCC1 Is a Transporter That Mediates Vacuolar Iron Storage in Yeast. *J. Biol. Chem.* 276, 29515–29519. doi:10.1074/jbc.M103944200.

Li, X. Q., Guo, B. L., Cai, W. Y., Zhang, J. M., Huang, H. Q., Zhan, P., et al. (2016). The role of melanin pathways in extremotolerance and virulence of Fonsecaea revealed by de novo assembly transcriptomics using illumina paired-end sequencing. *Stud. Mycol.* 83,

1–18. doi:10.1016/j.simyco.2016.02.001.

Liang, D., Zhou, H., Zhang, P., Chen, Y.-Q., Chen, X., Chen, C.-L., et al. (2002). A novel gene organization: intronic snoRNA gene clusters from Oryza sativa. *Nucleic Acids Res.* 30, 3262–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12136108 [Accessed July 27, 2018].

Liao, Y., Smyth, G. K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–30. doi:10.1093/bioinformatics/btt656.

Lin, X., Chacko, N., Wang, L., and Pavuluri, Y. (2015). Generation of stable mutants and targeted gene deletion strains in Cryptococcus neoformans through electroporation. *Med. Mycol.* 53, 225–34. doi:10.1093/mmy/myu083.

López-Ribot, J. L., Alloush, H. M., Masten, B. J., and Chaffin, W. L. (1996). Evidence for presence in the cell wall of Candida albicans of a protein related to the hsp70 family. *Infect. Immun.* 64, 3333–40. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=174226&tool=pmcentrez&rendertype=abstract [Accessed January 7, 2016].

Lorenz, M. C., Bender, J. A., and Fink, G. R. (2004). Transcriptional Response of Candida albicans upon Internalization by Macrophages. *Eukaryot. Cell* 3, 1076–1087. doi:10.1128/EC.3.5.1076-1087.2004.

MacDiarmid, C. W., Gaither, L. A., and Eide, D. (2000). Zinc transporters that regulate vacuolar zinc storage in Saccharomyces cerevisiae. *EMBO J.* 19, 2845–2855. doi:10.1093/emboj/19.12.2845.

Martinez-Lopez, R., Park, H., Myers, C. L., Gil, C., and Filler, S. G. (2006). *Candida albicans* Ecm33p Is Important for Normal Cell Wall Architecture and Interactions with Host Cells. *Eukaryot. Cell* 5, 140–147. doi:10.1128/EC.5.1.140-147.2006.

Matos, T., de Hoog, G. S., de Boer, A. G., de Crom, I., and Haase, G. (2002). High prevalence of the neurotrope Exophiala dermatitidis and related oligotrophic black yeasts in sauna facilities. *Mycoses* 45, 373–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12421284 [Accessed July 6, 2015].

Matsumoto, T., Matsuda, T., McGinnis, M. R., and Ajello, L. (1993). Clinical and mycological spectra of Wangiella dermatitidis infections*. *Mycoses* 36, 145–155. doi:10.1111/j.1439-0507.1993.tb00743.x.

Mayer, F. L., Wilson, D., and Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence* 4, 119–128. doi:10.4161/viru.22913.

Mayer, V. E., Nepel, M., Blatrix, R., Oberhauser, F. B., Fiedler, K., Schönenberger, J., et al. (2018). Transmission of fungal partners to incipient Cecropia-tree ant colonies. *PLoS One* 13, e0192207. doi:10.1371/journal.pone.0192207.

Mercer, T. R., Dinger, M. E., and Mattick, J. S. (2009). Long non-coding RNAs: insights into functions. *Nat. Rev. Genet.* 10, 155–159. doi:10.1038/nrg2521.

Mirastschijski, U., Impola, U., Karsdal, M. A., Saarialho-Kere, U., and Agren, M. S. (2002). Matrix metalloproteinase inhibitor BB-3103 unlike the serine proteinase inhibitor aprotinin abrogates epidermal healing of human skin wounds ex vivo. *J. Invest. Dermatol.* 118, 55–64. doi:10.1046/j.0022-202x.2001.01652.x.

Moreno, L. F., Ahmed, A. A. O., Brankovics, B., Cuomo, C. A., Menken, S. B. J., Taj-Aldeen, S. J., et al. (2018a). Genomic Understanding of an Infectious Brain Disease from the Desert. *G3: Genes|Genomes|Genetics* 8, g3.300421.2017. doi:10.1534/g3.117.300421.

Moreno, L. F., Vicente, V. A., and de Hoog, S. (2018b). Black yeasts in the omics era: Achievements and challenges. *Med. Mycol.* 56, 32–41. doi:10.1093/mmy/myx129.

Morens, D. M., Folkers, G. K., and Fauci, A. S. (2004). The challenge of emerging and re-emerging infectious diseases. *Nature* 430, 242–249. doi:10.1038/nature02759.

Muszewska, A., Stepniewska-Dziubinska, M. M., Steczkiewicz, K., Pawlowska, J., Dziedzic, A., and Ginalski, K. (2017). Fungal lifestyle reflected in serine protease repertoire. *Sci. Rep.* 7, 9147. doi:10.1038/s41598-017-09644-w.

Myoken, Y., Sugata, T., Fujita, Y., Kyo, T., Fujihara, M., Katsu, M., et al. (2003). Successful treatment of invasive stomatitis due to Exophiala dermatitidis in a patient with acute myeloid leukemia. *J. Oral Pathol. Med.* 32, 51–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12558959 [Accessed July 27, 2018].

Nagano, Y., and Nagahama, T. (2012). Fungal diversity in deep-sea extreme environments. *Fungal Ecol.* 5, 463–471. doi:10.1016/J.FUNECO.2012.01.004.

Nakaar, V., Samuel, B. U., Ngo, E. O., and Joiner, K. A. (1999). Targeted Reduction of Nucleoside Triphosphate Hydrolase by Antisense RNA Inhibits Toxoplasma gondii Proliferation. *J. Biol. Chem.* 274, 5083–7. doi:10.1074/jbc.274.8.5083.

Nelson, K. E., Fleischmann, R. D., DeBoy, R. T., Paulsen, I. T., Fouts, D. E., Eisen, J. A., et al. (2003). Complete genome sequence of the oral pathogenic Bacterium porphyromonas gingivalis strain W83. *J. Bacteriol.* 185, 5591–601. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12949112 [Accessed July 27, 2018].

Nguyen, S., Baker, K., Padman, B. S., Patwa, R., Dunstan, R. A., Weston, T. A., et al. (2017). Bacteriophage Transcytosis Provides a Mechanism To Cross Epithelial Cell Layers. *MBio* 8, e01874-17. doi:10.1128/mBio.01874-17.

Novak Babič, M., Zalar, P., Ženko, B., Džeroski, S., and Gunde-Cimerman, N. (2016). Yeasts and yeast-like fungi in tap water and groundwater, and their transmission to household appliances. *Fungal Ecol.* 20, 30–39. doi:10.1016/j.funeco.2015.10.001.

Noverr, M. C., Cox, G. M., Perfect, J. R., and Huffnagle, G. B. (2003). Role of PLB1 in

pulmonary inflammation and cryptococcal eicosanoid production. *Infect. Immun.* 71, 1538–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12595473 [Accessed July 27, 2018].

Ogura, T., and Wilkinson, A. J. (2001). AAA+ superfamily ATPases: common structure--diverse function. *Genes Cells* 6, 575–97. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11473577 [Accessed August 29, 2016].

Onofri, S., de la Torre, R., de Vera, J.-P., Ott, S., Zucconi, L., Selbmann, L., et al. (2012). Survival of Rock-Colonizing Organisms After 1.5 Years in Outer Space. *Astrobiology* 12, 508–516. doi:10.1089/ast.2011.0736.

Paolo, W. F., Dadachova, E., Mandal, P., Casadevall, A., Szaniszlo, P. J., Nosanchuk, J. D., et al. (2006). Effects of disrupting the polyketide synthase gene WdPKS1 in Wangiella [Exophiala] dermatitidis on melanin production and resistance to killing by antifungal compounds, enzymatic degradation, and extremes in temperature. *BMC Microbiol.* 6, 55. doi:10.1186/1471-2180-6-55.

Park, K. Y., Kim, H. K., Suh, M. K., and Seo, S. J. (2011). Unusual presentation of onychomycosis caused by Exophiala (Wangiella) dermatitidis. *Clin. Exp. Dermatol.* 36, 418–419. doi:10.1111/j.1365-2230.2010.03977.x.

Patel, A. K., Patel, K. K., Darji, P., Singh, R., Shivaprakash, M. R., and Chakrabarti, A. (2013). *Exophiala dermatitidis* endocarditis on native aortic valve in a postrenal transplant patient and review of literature on *E. dermatitidis* infections. *Mycoses* 56, 365–372. doi:10.1111/myc.12009.

Pedersen, O. A., and Langvad, F. (1989). Exophiala psychrophila sp. nov., a pathogenic species of the black yeasts isolated from farmed Atlantic salmon. *Mycol. Res.* 92, 153–156. doi:10.1016/S0953-7562(89)80004-8.

Peluso, R., Haase, A., Stowring, L., Edwards, M., and Ventura, P. (1985). A Trojan Horse mechanism for the spread of visna virus in monocytes. *Virology* 147, 231–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2998068 [Accessed May 29, 2018].

Peres da Silva, R., Puccia, R., Rodrigues, M. L., Oliveira, D. L., Joffe, L. S., César, G. V, et al. (2015). Extracellular vesicle-mediated export of fungal RNA. *Sci. Rep.* 5, 7763. doi:10.1038/srep07763.

Phan, Q. T., Myers, C. L., Fu, Y., Sheppard, D. C., Yeaman, M. R., Welch, W. H., et al. (2007). Als3 Is a Candida albicans Invasin That Binds to Cadherins and Induces Endocytosis by Host Cells. *PLoS Biol.* 5, e64. doi:10.1371/journal.pbio.0050064.

Pihet, M., Carrere, J., Cimon, B., Chabasse, D., Delhaes, L., Symoens, F., et al. (2009). Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. *Med. Mycol.* 47, 387–97. doi:10.1080/13693780802609604.

Plaza Reyes, A., and Lanner, F. (2017). Towards a CRISPR view of early human development: applications, limitations and ethical concerns of genome editing in human embryos. *Development* 144, 3–7. doi:10.1242/dev.139683.

Poyntner, C., Blasi, B., Arcalis, E., Mirastschijski, U., Sterflinger, K., and Tafer, H. (2016). The Transcriptome of Exophiala dermatitidis during Ex-vivo Skin Model Infection. *Front. Cell. Infect. Microbiol.* 6, 136. doi:10.3389/fcimb.2016.00136.

Poyntner, C., Mirastschijski, U., Sterflinger, K., and Tafer, H. (2018a). Transcriptome Study of an Exophiala dermatitidis PKS1 Mutant on an ex Vivo Skin Model: Is Melanin Important for Infection? *Front. Microbiol.* 9, 1457. doi:10.3389/fmicb.2018.01457.

Poyntner, C., Prem, M., Mann, O., Blasi, B., and Sterflinger, K. (2018b). Selective screening: isolation of fungal strains from contaminated soils in Austria. *Die Bodenkultur* 68, 157–169. Available at: https://content.sciendo.com/abstract/journals/boku/68/3/article-p157.xml [Accessed August 28, 2018].

Prenafeta-Boldú, F. X., Ballerstedt, H., Gerritse, J., and Grotenhuis, J. T. C. (2004). Bioremediation of BTEX hydrocarbons: effect of soil inoculation with the toluene-growing fungus Cladophialophora sp. strain T1. *Biodegradation* 15, 59–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14971858.

Prenafeta-Boldú, F. X., Summerbell, R., and Sybren de Hoog, G. (2006). Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard? *FEMS Microbiol. Rev.* 30, 109–30. doi:10.1111/j.1574-6976.2005.00007.x.

Puig, S., Lee, J., Lau, M., and Thiele, D. J. (2002). Biochemical and genetic analyses of yeast and human high affinity copper transporters suggest a conserved mechanism for copper uptake. *J. Biol. Chem.* 277, 26021–30. doi:10.1074/jbc.M202547200.

Pulschen, A. A., Rodrigues, F., Duarte, R. T. D., Araujo, G. G., Santiago, I. F., Paulino-Lima, I. G., et al. (2015). UV-resistant yeasts isolated from a high-altitude volcanic area on the Atacama Desert as eukaryotic models for astrobiology. *Microbiologyopen* 4, 574–88. doi:10.1002/mbo3.262.

Queiroz-Telles, F., de Hoog, S., Santos, D. W. C. L., Salgado, C. G., Vicente, V. A., Bonifaz, A., et al. (2017). Chromoblastomycosis. *Clin. Microbiol. Rev.* 30, 233–276. doi:10.1128/CMR.00032-16.

Quinlan, A. R., and Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–2. doi:10.1093/bioinformatics/btq033.

Quinn, J. J., and Chang, H. Y. (2015). Unique features of long non-coding RNA biogenesis and function. *Nat. Rev. Genet.* 17, 47–62. doi:10.1038/nrg.2015.10.

R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.r-project.org/.

Rawlings, N. D., Waller, M., Barrett, A. J., and Bateman, A. (2014). MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* 42, D503-9. doi:10.1093/nar/gkt953.

Revankar, S. G., Patterson, J. E., Sutton, D. A., Pullen, R., and Rinaldi, M. G. (2002).

Disseminated phaeohyphomycosis: review of an emerging mycosis. *Clin. Infect. Dis.* 34, 467–76. doi:10.1086/338636.

Revankar, S. G., and Sutton, D. A. (2010). Melanized fungi in human disease. *Clin. Microbiol. Rev.* 23, 884–928. doi:10.1128/CMR.00019-10.

Revankar, S. G., Sutton, D. A., and Rinaldi, M. G. (2004). Primary Central Nervous System Phaeohyphomycosis: A Review of 101 Cases. *Clin. Infect. Dis.* 38, 206–216. doi:10.1086/380635.

Robertson, K. L., Mostaghim, A., Cuomo, C. a, Soto, C. M., Lebedev, N., Bailey, R. F., et al. (2012). Adaptation of the black yeast Wangiella dermatitidis to ionizing radiation: molecular and cellular mechanisms. *PLoS One* 7, e48674. doi:10.1371/journal.pone.0048674.

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–40. doi:10.1093/bioinformatics/btp616.

Rodrigues, M. L., and Albuquerque, P. C. (2018). Searching for a change: The need for increased support for public health and research on fungal diseases. *PLoS Negl. Trop. Dis.* 12, e0006479. doi:10.1371/journal.pntd.0006479.

Saier, M. H., Tran, C. V, and Barabote, R. D. (2006). TCDB: the Transporter Classification Database for membrane transport protein analyses and information. *Nucleic Acids Res.* 34, D181-6. doi:10.1093/nar/gkj001.

Samarsky, D. A., Schneider, G. S., and Fournier, M. J. (1996). An essential domain in Saccharomyces cerevisiae U14 snoRNA is absent in vertebrates, but conserved in other yeasts. *Nucleic Acids Res.* 24, 2059–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8668536 [Accessed July 27, 2018].

Sánchez, M., and Colom, F. (2010). Extracellular DNase activity of Cryptococcus neoformans and Cryptococcus gattii. *Rev. Iberoam. Micol.* 27, 10–13. doi:10.1016/j.riam.2009.11.004.

Santiago-Tirado, F. H., and Doering, T. L. (2017). False friends: Phagocytes as Trojan horses in microbial brain infections. *PLoS Pathog.* 13, e1006680. doi:10.1371/journal.ppat.1006680.

Sav, H., Ozakkas, F., Altınbas, R., Kiraz, N., Tümgör, A., Gümral, R., et al. (2016). Virulence markers of opportunistic black yeast in *Exophiala*. *Mycoses* 59, 343–350. doi:10.1111/myc.12478.

Schaal, J. V, Leclerc, T., Soler, C., Donat, N., Cirrode, A., Jault, P., et al. (2015). Epidemiology of filamentous fungal infections in burned patients: A French retrospective study. *Burns* 41, 853–63. doi:10.1016/j.burns.2014.10.024.

Schaller, M., Korting, H. C., Schäfer, W., Bastert, J., Chen, W., and Hube, B. (1999).

Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol. Microbiol.* 34, 169–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10540295 [Accessed July 27, 2018].

Schnitzler, N., Peltroche-Llacsahuanga, H., Bestier, N., Zündorf, J., Lütticken, R., and Haase, G. (1999). Effect of melanin and carotenoids of Exophiala (Wangiella) dermatitidis on phagocytosis, oxidative burst, and killing by human neutrophils. *Infect. Immun.* 67, 94–101. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9864201 [Accessed December 23, 2015].

Science News Staff (2015). And Science's 2015 Breakthrough of the Year is... | Science | AAAS. Available at: http://www.sciencemag.org/news/2015/12/and-science-s-2015-breakthrough-year [Accessed July 24, 2018].

Seiberg, M., Paine, C., Sharlow, E., Andrade-Gordon, P., Costanzo, M., Eisinger, M., et al. (2000a). The protease-activated receptor 2 regulates pigmentation via keratinocyte-melanocyte interactions. *Exp. Cell Res.* 254, 25–32. doi:10.1006/excr.1999.4692.

Seiberg, M., Paine, C., Sharlow, E., Eisinger, M., Shapiro, S. S., Andrade-Gordon, P., et al. (2000b). Inhibition of Melanosome Transfer Results in Skin Lightening1. *J. Invest. Dermatol.* 115, 162–167. doi:10.1046/j.1523-1747.2000.00035.x.

Selbman, L., de Hoog, S., Mazzaglia, A., Friedmann, E., and Onofri, S. (2005). Fungi at the edge of life: cryptoendolithic black fungi from Antarctic desert - Semantic Scholar. *Stud. Mycol.* 51, 1–32. Available at: https://www.semanticscholar.org/paper/Fungi-at-the-edge-of-life%3A-cryptoendolithic-black-Selbmann-Hoog/9da00baf0e869edcad106d9bfe27c7bc1f3c9a21 [Accessed May 17, 2018].

Selbmann, L., de Hoog, G. S., Zucconi, L., Isola, D., and Onofri, S. (2014a). "Black Yeasts in Cold Habitats," in *Cold-adapted Yeasts* (Berlin, Heidelberg: Springer Berlin Heidelberg), 173–189. doi:10.1007/978-3-642-39681-6_8.

Selbmann, L., Zucconi, L., Isola, D., and Onofri, S. (2014b). Rock black fungi: excellence in the extremes, from the Antarctic to space. *Curr. Genet.* doi:10.1007/s00294-014-0457-7.

Seyedmousavi, S., Netea, M. G., Mouton, J. W., Melchers, W. J. G., Verweij, P. E., and de Hoog, G. S. (2014). Black Yeasts and Their Filamentous Relatives: Principles of Pathogenesis and Host Defense. *Clin. Microbiol. Rev.* 27, 527–42. doi:10.1128/CMR.00093-13.

Sharlow, E. R., Paine, C. S., Babiarz, L., Eisinger, M., Shapiro, S., and Seiberg, M. (2000). The protease-activated receptor-2 upregulates keratinocyte phagocytosis. *J. Cell Sci.* 113 (Pt 17), 3093–101. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10934047 [Accessed May 29, 2018].

Song, Y., Laureijssen-van de Sande, W. W. J., Moreno, L. F., Gerrits van den Ende, B., Li, R., and de Hoog, S. (2017). Comparative Ecology of Capsular Exophiala Species Causing Disseminated Infection in Humans. *Front. Microbiol.* 8, 2514. doi:10.3389/fmicb.2017.02514.

Sterflinger, K. (1998). Temperature and NaCl-tolerance of rock-inhabiting meristematic fungi. *Antonie Van Leeuwenhoek* 74, 271–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10081587 [Accessed December 19, 2013].

Sterflinger, K. (2005). "Black Yeasts and Meristematic Fungi: Ecology, Diversity and Identification," in *Biodiversity and Ecophysiology of Yeasts The Yeast Handbook*, eds. D. G. Péter and P. C. Rosa (Berlin/Heidelberg: Springer-Verlag), 501–514. doi:10.1007/3-540-30985-3_20.

Sterflinger, K., Tesei, D., and Zakharova, K. (2012). Fungi in hot and cold deserts with particular reference to microcolonial fungi. *Fungal Ecol.* 5, 453–462. doi:10.1016/j.funeco.2011.12.007.

Sudhadham, M., de Hoog, G. S., Menken, S. B. J., Gerrits van den Ende, A. H. G., and Sihanonth, P. (2010). Rapid screening for genotypes as possible markers of virulence in the neurotropic black yeast Exophiala dermatitidis using PCR-RFLP. *J. Microbiol. Methods* 80, 138–142. doi:10.1016/J.MIMET.2009.11.007.

Sudhadham, M., Prakitsin, S., Sivichai, S., Chaiyarat, R., Dorrestein, G. M., Menken, S. B. J., et al. (2008). The neurotropic black yeast Exophiala dermatitidis has a possible origin in the tropical rain forest. *Stud. Mycol.* 61, 145–55. doi:10.3114/sim.2008.61.15.

Sun, J. N., Solis, N. V., Phan, Q. T., Bajwa, J. S., Kashleva, H., Thompson, A., et al. (2010a). Host cell invasion and virulence mediated by Candida albicans Ssa1. *PLoS Pathog.* 6, e1001181. doi:10.1371/journal.ppat.1001181.

Sun, J. N., Solis, N. V., Phan, Q. T., Bajwa, J. S., Kashleva, H., Thompson, A., et al. (2010b). Host Cell Invasion and Virulence Mediated by Candida albicans Ssa1. *PLoS Pathog.* 6, e1001181. doi:10.1371/journal.ppat.1001181.

Supek, F., Bošnjak, M., Škunca, N., and Šmuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One* 6, e21800. doi:10.1371/journal.pone.0021800.

Susan Aldrich (1999). Alexander Fleming Discovery and Development of Penicillin - Landmark - American Chemical Society. *Am. Chem. Soc. Int. Hist. Chem. Landmarks*. doi:https://doi.org/10.2307/3561468.

Taborda, C. P., da Silva, M. B., Nosanchuk, J. D., and Travassos, L. R. (2008). Melanin as a virulence factor of Paracoccidioides brasiliensis and other dimorphic pathogenic fungi: a minireview. *Mycopathologia* 165, 331–9. doi:10.1007/s11046-007-9061-4.

Taff, H. T., Nett, J. E., Zarnowski, R., Ross, K. M., Sanchez, H., Cain, M. T., et al. (2012). A Candida Biofilm-Induced Pathway for Matrix Glucan Delivery: Implications for Drug Resistance. *PLoS Pathog.* 8, e1002848. doi:10.1371/journal.ppat.1002848.

Takaya, A., Suzuki, M., Matsui, H., Tomoyasu, T., Sashinami, H., Nakane, A., et al. (2003). Lon, a stress-induced ATP-dependent protease, is critically important for systemic Salmonella enterica serovar typhimurium infection of mice. *Infect. Immun.* 71, 690–6. doi:10.1128/IAI.71.2.690-696.2003.

Taylor, D. L., Herriott, I. C., Stone, K. E., McFarland, J. W., Booth, M. G., and Leigh, M. B. (2010). Structure and resilience of fungal communities in Alaskan boreal forest soilsThis article is one of a selection of papers from The Dynamics of Change in Alaska's Boreal Forests: Resilience and Vulnerability in Response to Climate Warming. *Can. J. For. Res.* 40, 1288–1301. doi:10.1139/X10-081.

Tedersoo, L., Bahram, M., Polme, S., Koljalg, U., Yorou, N. S., Wijesundera, R., et al. (2014). Global diversity and geography of soil fungi. *Science (80-.).* 346, 1256688–1256688. doi:10.1126/science.1256688.

Teixeira, M. M., Moreno, L. F., Stielow, B. J., Muszewska, A., Hainaut, M., Gonzaga, L., et al. (2017). Exploring the genomic diversity of black yeasts and relatives (Chaetothyriales , Ascomycota). *Stud. Mycol.* 86, 1–28. doi:10.1016/j.simyco.2017.01.001.

Tesei, D., Marzban, G., Marchetti-Deschmann, M., Tafer, H., Arcalis, E., and 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.

Tesei, D., Tafer, H., Poyntner, C., Piñar, G., Lopandic, K., and Sterflinger, K. (2017). Draft Genome Sequences of the Black Rock Fungus *Knufia petricola* and Its Spontaneous Nonmelanized Mutant. *Genome Announc.* 5, e01242-17. doi:10.1128/genomeA.01242-17.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., et al. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* 7, 562–78. doi:10.1038/nprot.2012.016.

Tudzynski, P., Hölter, K., Correia, T., Arntz, C., Grammel, N., and Keller, U. (1999). Evidence for an ergot alkaloid gene cluster in Claviceps purpurea. *Mol. Gen. Genet.* 261, 133–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10071219 [Accessed July 30, 2018].

Turcotte, B., Liang, X. B., Robert, F., and Soontorngun, N. (2010). Transcriptional regulation of nonfermentable carbon utilization in budding yeast. *FEMS Yeast Res.* 10, 2–13. doi:10.1111/j.1567-1364.2009.00555.x.

Upadhyay, S. K., Mahajan, L., Ramjee, S., Singh, Y., Basir, S. F., and Madan, T. (2009). Identification and characterization of a laminin-binding protein of Aspergillus fumigatus: extracellular thaumatin domain protein (AfCalAp). *J. Med. Microbiol.* 58, 714–722. doi:10.1099/jmm.0.005991-0.

Usaite, R., Jewett, M. C., Oliveira, A. P., Yates, J. R., Olsson, L., and Nielsen, J. (2009). Reconstruction of the yeast Snf1 kinase regulatory network reveals its role as a global energy regulator. *Mol. Syst. Biol.* 5, 319. doi:10.1038/msb.2009.67.

Vasse, M., Voglmayr, H., Mayer, V., Gueidan, C., Nepel, M., Moreno, L., et al. (2017). A phylogenetic perspective on the association between ants (Hymenoptera: Formicidae) and black yeasts (Ascomycota: Chaetothyriales). *Proceedings. Biol. Sci.* 284, 20162519. doi:10.1098/rspb.2016.2519.

Walton, F. J., Idnurm, A., and Heitman, J. (2005). Novel gene functions required for

melanization of the human pathogen Cryptococcus neoformans. *Mol. Microbiol.* 57, 1381–96. doi:10.1111/j.1365-2958.2005.04779.x.

Wang, L., Park, H. J., Dasari, S., Wang, S., Kocher, J.-P., and Li, W. (2013). CPAT: Coding-Potential Assessment Tool using an alignment-free logistic regression model. *Nucleic Acids Res.* 41, e74. doi:10.1093/nar/gkt006.

Wang, X., Wang, W., Lin, Z., Wang, X., Li, T., Yu, J., et al. (2014). CARD9 mutations linked to subcutaneous phaeohyphomycosis and TH17 cell deficiencies. *J. Allergy Clin. Immunol.* 133, 905–8.e3. doi:10.1016/j.jaci.2013.09.033.

Wang, Y., Aisen, P., and Casadevall, A. (1995). Cryptococcus neoformans melanin and virulence: mechanism of action. *Infect. Immun.* 63, 3131–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7622240 [Accessed December 1, 2017].

Washietl, S., Findeiss, S., Müller, S. A., Kalkhof, S., von Bergen, M., Hofacker, I. L., et al. (2011). RNAcode: robust discrimination of coding and noncoding regions in comparative sequence data. *RNA* 17, 578–94. doi:10.1261/rna.2536111.

Washietl, S., Hofacker, I. L., Lukasser, M., Hüttenhofer, A., and Stadler, P. F. (2005a). Mapping of conserved RNA secondary structures predicts thousands of functional noncoding RNAs in the human genome. *Nat. Biotechnol.* 23, 1383–1390. doi:10.1038/nbt1144.

Washietl, S., Hofacker, I. L., and Stadler, P. F. (2005b). Fast and reliable prediction of noncoding RNAs. *Proc. Natl. Acad. Sci. U. S. A.* 102, 2454–9. doi:10.1073/pnas.0409169102.

Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H. U., Bruccoleri, R., et al. (2015). antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* 43, W237–W243. doi:10.1093/nar/gkv437.

Webster, J., and Weber, R. W. S. (2007). *Introduction to Fungi*. 3rd ed. Cambridge: Cambridge University Press.

Weete, J. D., Abril, M., and Blackwell, M. (2010). Phylogenetic distribution of fungal sterols. *PLoS One* 5, e10899. doi:10.1371/journal.pone.0010899.

Weiberg, A., Wang, M., Lin, F.-M., Zhao, H., Zhang, Z., Kaloshian, I., et al. (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* 342, 118–23. doi:10.1126/science.1239705.

WHO | Mycetoma, chromoblastomycosis and other deep mycoses (2018). *WHO*. Available at: http://www.who.int/neglected_diseases/diseases/mycetoma-chromoblastomycosis-deep-mycoses/en/index1.html [Accessed July 26, 2018].

Wilson, R. A., and Talbot, N. J. (2009). Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. *Nat. Rev. Microbiol.* 7, 185–195. doi:10.1038/nrmicro2032.

Winnenburg, R., Urban, M., Beacham, A., Baldwin, T. K., Holland, S., Lindeberg, M., et al. (2008). PHI-base update: additions to the pathogen host interaction database. *Nucleic Acids Res.* 36, D572-6. doi:10.1093/nar/gkm858.

Woo, P. C. Y., Ngan, A. H. Y., Tsang, C. C. C., Ling, I. W. H., Chan, J. F. W., Leung, S.-Y., et al. (2013). Clinical spectrum of exophiala infections and a novel Exophiala species, Exophiala hongkongensis. *J. Clin. Microbiol.* 51, 260–7. doi:10.1128/JCM.02336-12.

Woollons, A., Darley, C. R., Pandian, S., Arnstein, P., Blackee, J., and Paul, J. (1996). Phaeohyphomycosis caused by Exophiala dermatitidis following intra-articular steroid injection. *Br. J. Dermatol.* 135, 475–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8949449 [Accessed July 27, 2018].

Wu, H., Downs, D., Ghosh, K., Ghosh, A. K., Staib, P., Monod, M., et al. (2013). Candida albicans secreted aspartic proteases 4-6 induce apoptosis of epithelial cells by a novel Trojan horse mechanism. *FASEB J.* 27, 2132–44. doi:10.1096/fj.12-214353.

Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J., Dong, S., et al. (2011). KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res.* 39, W316-22. doi:10.1093/nar/gkr483.

Ye, X., and Szaniszlo, P. J. (2000). Expression of a Constitutively Active Cdc42 Homologue Promotes Development of Sclerotic Bodies but Represses Hyphal Growth in the Zoopathogenic Fungus Wangiella (Exophiala) dermatitidis. *J. Bacteriol.* 182, 4941–4950. doi:10.1128/JB.182.17.4941-4950.2000.

Young, E. T., Dombek, K. M., Tachibana, C., and Ideker, T. (2003). Multiple Pathways Are Co-regulated by the Protein Kinase Snf1 and the Transcription Factors Adr1 and Cat8. *J. Biol. Chem.* 278, 26146–26158. doi:10.1074/jbc.M301981200.

Yu, J.-H., and Keller, N. (2005). Regulation of Secondary Metabolism in Filamentous Fungi. *Annu. Rev. Phytopathol.* 43, 437–458. doi:10.1146/annurev.phyto.43.040204.140214.

Yurlova, N. A., and de Hoog, G. S. (2002). Exopolysaccharides and capsules in human pathogenic *Exophiala* species. *Mycoses* 45, 443–448. doi:10.1046/j.1439-0507.2002.00807.x.

Zakharova, K., Marzban, G., de Vera, J.-P., Lorek, A., and Sterflinger, K. (2015). Protein patterns of black fungi under simulated Mars-like conditions. *Sci. Rep.* 4, 5114. doi:10.1038/srep05114.

Zakharova, K., Tesei, D., Marzban, G., Dijksterhuis, J., Wyatt, T., and Sterflinger, K. (2013). Microcolonial fungi on rocks: a life in constant drought? *Mycopathologia* 175, 537–47. doi:10.1007/s11046-012-9592-1.

Zalar, P., Novak, M., de Hoog, G. S., and Gunde-Cimerman, N. (2011). Dishwashers--a man-made ecological niche accommodating human opportunistic fungal pathogens. *Fungal Biol.* 115, 997–1007. doi:10.1016/j.funbio.2011.04.007.

Zeng, J. S., Sutton, D. A., Fothergill, A. W., Rinaldi, M. G., Harrak, M. J., and de Hoog, G. S. (2007). Spectrum of clinically relevant Exophiala species in the United States. *J. Clin. Microbiol.* 45, 3713–20. doi:10.1128/JCM.02012-06.

Zhang, Y., Ge, X., Yang, F., Zhang, L., Zheng, J., Tan, X., et al. (2015). Comparison of non-canonical PAMs for CRISPR/Cas9-mediated DNA cleavage in human cells. *Sci. Rep.* 4, 5405. doi:10.1038/srep05405.

Zhao, H., and Eide, D. J. (1997). Zap1p, a metalloregulatory protein involved in zinc-responsive transcriptional regulation in Saccharomyces cerevisiae. *Mol. Cell. Biol.* 17, 5044–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9271382 [Accessed July 27, 2018].

Zhao, J., Zeng, J., de Hoog, G. S., Attili-Angelis, D., and Prenafeta-Boldú, F. X. (2010). Isolation and identification of black yeasts by enrichment on atmospheres of monoaromatic hydrocarbons. *Microb. Ecol.* 60, 149–56. doi:10.1007/s00248-010-9651-4.

Zheng, H., Chatfield, C. H., Liles, M. R., and Cianciotto, N. P. (2013). Secreted Pyomelanin of Legionella pneumophila Promotes Bacterial Iron Uptake and Growth under Iron-Limiting Conditions. *Infect. Immun.* 81, 4182–4191. doi:10.1128/IAI.00858-13.

Zupančič, J., Novak Babič, M., Zalar, P., and Gunde-Cimerman, N. (2016). The Black Yeast Exophiala dermatitidis and Other Selected Opportunistic Human Fungal Pathogens Spread from Dishwashers to Kitchens. *PLoS One* 11, e0148166. doi:10.1371/journal.pone.0148166.

7 Curriculum vitae

PERSONAL INFORMATION

Name: Caroline Poyntner **email:** caroline.poyntner@boku.ac.at

Date of birth: 06.10.1987 **phone:** +43 680 2314883

Nationality: Austria **languages**: German (native), English, French

EDUCATION AND RESEARCH EXPERIENCE

Since 2014: **PhD candidate** working group Sterflinger, VIBT EQ Extremophile Center University of Natural Resources and Life Sciences, Vienna, Austria
Research focus: Investigation of infections mechanisms and stress resistance of the black yeast *Exophiala dermatitidis* on a transcriptome level
Tutor basic mycology course

2011-2014 **Masterstudies** Biotechnology
Masterthesis "Screening of extremotolerant fungi for bioremediation", **Award MA Wasser, Vienna** 2014, data published: Blasi et al. 2016 (see list of publications)

2006-2011 **Bachelorstudies** Food- and Biotechnology Bachelorthesis "Untersuchung der fungiziden Wirkung eines *Isatis tinctoria* Extraktes"

2009 **Erasmus stay** Kuopio, Finland (6 moths)

2011-2013 **Student assistant** in various Drosophila projects, BOKU Vienna, supervisor: Nancy Stralis-Pavese; **student assistant** in project: Degradation by yeasts in biogas tanks, BOKU Vienna, supervisor: Katja Sterflinger; **student assistant** in organization of the IBBS-15 congress in Vienna;

2010-2011 **Erasmus internship** (3 months): "EMS genetic screen for identifying host genes required to maintain gut immune tolerance to commensal microbiota in Drosophila flies", IBDML Marseille, supervisor: Karine Narbonne

2007 Internship QC Biomin (2 months)

8 List of publications, presentations and posters

8.1 Publications presented in this thesis:

Poyntner, C., Mirastschijski, U., Sterflinger, K. and Tafer, H. (2018): Transcriptome Study of an Exophiala dermatitidis PKS1 Mutant on an ex Vivo Skin Model: Is Melanin Important for Infection? Frontiers in Microbiology, Fungi and their interactions. 2018; 9: 1457.

Poyntner, C., Blasi, B., Arcalis, E., Mirastschijski, U., Sterflinger, K. and Tafer, H. (2016): The Transcriptome of Exophiala dermatitidis during Ex-vivo Skin Model Infection. Frontiers in Cellular and Infection Microbiology 2016 Oct 24; 6:136.

8.2 Authored/coauthored publications during period of the Dissertation:

Poyntner, C., Prem, M., Mann, O., Blasi, B. and Sterflinger, K. (2018): Selective screening: isolation of fungal strains from contaminated soils in Austria. Die Bodenkultur: Journal of Land Management, Food and Environment 68, 3, 157–169

Sterflinger K., Blasi B., Poyntner C., Tesei D., Tafer H. (2018): Klein, stark, schwarz: Pilze als Modelle für Stresstoleranz und als Herausforderung für die Medizin. Biospektrum (in press).

Sigmund, G., Poyntner, C., Piñar, G., Kah, M. and Hofmann, T. (2018): Influence of compost and biochar on microbial communities and the sorption/degradation of PAHs and NSO-substituted PAHs in contaminated soils. Journal of Hazardous Materials. 345,107–113.

Blasi, B., Tafer, H., Kustor, C., Poyntner, C., Lopandic, K. and Sterflinger, K. (2017): Genomic and transcriptomic analysis of the toluene degrading black yeast Cladophialophora immunda. Scientific Reports. 7,11436.

Tesei, D., Tafer, H., Poyntner, C., Piñar, G., Lopandic, K. and Sterflinger, K. (2017): Draft Genome Sequences of the Black Rock Fungus *Knufia petricola* and Its Spontaneous Nonmelanized Mutant. Genome Announcements. 5,e01242-17

Blasi, B., Poyntner, C., Rudavsky, T., Prenafeta-Boldú, F.X., Hoog, S. De, Tafer, H. and Sterflinger, K. (2016): Pathogenic Yet Environmentally Friendly? Black Fungal Candidates for Bioremediation of Pollutants. Geomicrobiology journal. 33,308–317.

Sterflinger, K., Lopandic, K., Blasi, B., Poynter, C., de Hoog, S. and Tafer, H. (2015): Draft Genome of Cladophialophora immunda, a Black Yeast and Efficient Degrader of Polyaromatic Hydrocarbons. Genome Announcements. 3,e01283-14.

Tafer, H., Lopandic, K., Blasi, B., Poyntner, C. and Sterflinger, K. (2015): Draft Genome Sequence of Exophiala mesophila, a Black Yeast with High Bioremediation

8.3 Conference contributions

Oral Presentations

C. Poyntner, U. Mirastschijski, H. Tafer, K. Sterflinger: Transcriptomic studies of *Exophiala dermatitidis* - wild type and CRISPR-Cas9 generated white mutant - during *ex-vivo* skin model infections (4.-5.7.2018) Workshop Group Black Yeasts, Amsterdam, The Netherlands

C. Poyntner, U. Mirastschijski, B. Blasi, H. Tafer, K. Sterflinger: *Exophiala dermatitidis*: stress resistance and pathogenicity (15.-18.4.2018) Annual Conference 2018 of the Association for General and Applied Microbiology (VAAM), Wolfsburg, Germany, (travel grant VAAM)

Caroline Poyntner, Barbara Blasi, Francesc Prenafeta, Katja Sterflinger: Screening of extremotolerant fungi for the bioremediation of hydrocarbon contaminated sites (12-17.4.2015), European Geoscience Union General Assembly 2015, Vienna Austria, Pico Presentation

Poster Presentations

C. Poyntner, G. Piñar, H. Tafer, K. Sterflinger: Environmental stress resistance of extremophilic fungi: genomic and transcriptomic study (12-17.8.2018), 17th International Symposium on Microbial Ecology, Leipzig, Germany

C. Poyntner, B. Blasi, H. Tafer, K. Sterflinger: Non-coding RNAs in black-yeasts (2-4.5.2016), 11. Microsymposium Small RNAs IMBA, Vienna, Austria

C. Poyntner, H. Tafer, U. Mirastschijski, E. Arcalis, K. Sterflinger: Monitoring of skin infection by *Exophiala dermatitidis* using transcriptomics (14-16. 4. 2016), DK RNA Retreat, Pamhagen, Austria

C. Poyntner, H. Tafer, U. Mirastschijski, E. Arcalis, K. Sterflinger: The transcriptome of *Exophiala dermatitidis* during artificial skin infection (8-10. 9.2016), 50. Wissenschaftliche Tagung der Deutschsprachigen Mykologischen Gesellschaft e. V., Essen, Germany

C. Poyntner, H. Tafer, B. Blasi, U. Mirastschijski, E. Arcalis, K. Sterflinger: The transcriptome of *Exophiala dermatitidis* during artificial skin infection (13-14.10.2016), Ion World Tour, Vienna User Meeting, Austria

Attended Workshops and Symposia:

Workshop Group Black Yeasts, (15-17 September 2016) Viterbo , Italy
Symposium on Extracellular Vesicles in Inflammation (24.6. 2016), Krems, Austria
Workshop Group Black Yeasts, (20. 4.2015) Utrecht, The Netherlands
High-Throughput and Single Cell Approaches in Infection Biology Workshop (22-23.10.2015) ,Munich, Germany
Ion RNA library construction training at Life Tech (15-17.7.14) Darmstadt, Germany
Project management seminar, Peter Birnstingl (8.-10.11.2017)

Supervised Bachelor theses:

Max Prem, Lisa Berdorfer, Katharina Martinu and Tamara Rudavsky, were cosupervised during their labwork for their bachelor thesis.

Awards/travel grants:

AKGL Boku travel grant 2018
VAAM travel grant 2018
FEMS travel grant 2018
Award MA Wasser Vienna 2014

adapted from: J. Cziches (1987)