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Glycerol metabolism genes in *Aureobasidium pullulans* and *Aureobasidium subglaciale*

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ABSTRACT

Intracellular glycerol accumulation is one of the main fungal adaptations to osmotic and also cold stress. We investigated the management of glycerol metabolism in polyextremotolerant black yeasts *Aureobasidium pullulans* and *Aureobasidium subglaciale*. We show that increased salinity (5 % and 10 %; w/v), but not cold (10 °C) trigger intracellular glycerol accumulation. The transcriptional response of the genes involved in glycerol synthesis, degradation and import, to increased salinity, low temperature or a combination of both was analysed with real-time PCR. Each of the two species contains an NAD^+-dependent glycerol-3-phosphate dehydrogenase, a glycerol-3-phosphate phosphatase, a mitochondrial glycerol-3-phosphate dehydrogenase, two copies of a glycerol kinase, and more than ten copies of major facilitator superfamily transporters similar to glycerol proton symporters. Similarly to glycerol accumulation itself, transcriptional response to hypersaline stress was larger compared to low temperature stress and was more consistent in *A. pullulans* compared to *A. subglaciale*, reflecting the different stress tolerance and ecological strategy of each species.

Introduction

Certain fungi can thrive in some of the most extreme environments on our planet (Gostinčar *et al.* 2010). Such lifestyle is enabled by a complex combination of molecular, physiological, and morphological adaptations, the role of many of which is still poorly understood (Gostinčar *et al.* 2011; Gostinčar *et al.* 2015). One of the most prevalent adaptations is the synthesis and accumulation of small protective compounds (Gostinčar *et al.* 2012), glycerol being the best known and arguably the most common. Glycerol plays a crucial role as a compatible solute (Hohmann 2002; Lenassi *et al.* 2011), contributing to the maintenance of a proper intracellular osmotic pressure and thus preventing plasmolysis in environments with high concentration of solutes (e.g. salts or sugars) or during desiccation. Glycerol also acts as a cryoprotectant during freezing (Jin *et al.* 2005) and in yeast cells it accumulates even upon cooling to temperatures well above freezing (Panadero *et al.* 2006).

Glycerol concentration management is best studied in *Saccharomyces cerevisiae*. In this yeast, as in other fungi, glycerol is accumulated upon hyperosmotic stress as the most important osmoprotectant solute (Saito & Posas 2012). Sudden drops in turgor pressure, such as those caused by an increase of the external osmolyte concentration, activate the high-osmolarity glycerol (HOG) response pathway, a branched mitogen-activated protein kinase signal-transduction system, which mediates the fungal cellular adaptation to several types of

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stress (Hohmann 2002). HOG in turn induces a broad transcriptional response including genes involved in glycerol production (Hohmann 2002, 2015). Glycerol accumulation increases intracellular osmolarity which leads to the restoration of the turgor pressure (Saito & Posas 2012). Activation of the HOG pathway was traditionally regarded as a specific response to hyperosmotic stress (Hohmann 2002), but was later discovered to be responsive also to a number of non-osmotic stresses, including a downshift in temperature (Aguilera *et al.* 2007; Hayashi & Maeda 2006; Panadero *et al.* 2006). The enzymes directly responsible for the synthesis of glycerol, i.e. glycerol-3-phosphate dehydrogenase (Gpd1) and glycerol-3-phosphatases (Gpp1 and Gpp2), are upregulated upon osmostress and shift to low temperature (Panadero *et al.* 2006). The key factor in integrating the sensing mechanism of cold and hyperosmolarity might be the decreased fluidity state of the cell membrane (Hayashi & Maeda 2006; Panadero *et al.* 2006). In support of this explanation it was found that the HOG pathway is also activated by membrane-rigidifier agents such as dimethylsulfoxide (Hayashi & Maeda 2006). Studies in *S. cerevisiae* have demonstrated that cold exposure limits its respiratory capacity which results in an "overflow metabolism" that leads to NADH accumulation, as well as to the formation of ethanol and glycerol (Ballester-Tomas *et al.* 2017). Much of the damage to cells during freezing/thawing is a result of osmotic shrinkage and glycerol accumulation provides cryoprotection (Aguilera *et al.* 2007; Panadero *et al.* 2006).

In *S. cerevisiae* the major pathway for glycerol production starts with the NADH-dependent reduction of glycolytic dihydroxyacetone phosphate to L-glycerol-3-phosphate by a cytosolic NAD^+-dependent glycerol-3-phosphate dehydrogenase (Gpd), after which L-glycerol-3-phosphate is dephosphorylated to glycerol by a glycerol 3-phosphatase (Gpp). Gpd is encoded by the two isogenes *GPD1* and *GPD2*, while Gpp is encoded by the isogenes *GPP1* and *GPP2*. Homologues of these genes have also been characterized in several other yeasts and filamentous fungi. Among dissimilative glycerol pathways the widespread among fungi is the aerobic degradation via the intermediate L-glycerol-3-phosphate. It involves a glycerol kinase encoded by *GUT1* and a FAD-dependent glycerol-3-phosphate dehydrogenase located at the outer surface of the inner mitochondrial membrane and encoded by *GUT2* (Klein *et al.* 2017; Nevoigt & Stahl 1997).

Besides the regulation of enzymes involved in glycerol synthesis and degradation, the intracellular concentration of glycerol is also influenced by its export and import (Hohmann 2002). The key protein mediating glycerol export triggered by hypoosmotic shock is Fps1, a member of the aquaporin family of transmembrane channels. On the other hand import is facilitated by Stl1, a major facilitator superfamily (MFS) transporter protein, the expression of which is strongly induced by Hog1 upon osmotic stress, leading to glycerol accumulation by importing it from the environment (Saito & Posas 2012).

While the ascomycetous black yeast *Aureobasidium pullulans* (de Bary) G. Arnaud (*Dothideales*, *Pezizomycotina*) is not one of the most extremophilic fungi known, it is remarkable for the wide variety of extremes it can tolerate. It has been suggested that this polyextremotolerant character is behind the ubiquitous distribution of the species (Gostinčar *et al.* 2014; Gostinčar *et al.* 2015). The fungus is frequently found in moderately osmotic environments, on plants (Grube *et al.* 2011), in coastal hypersaline water (Gunde-Cimerman *et al.* 2000), glacial ice (Zalar *et al.* 2008), in food (Pitt & Hocking 1999), various indoor habitats (Kaarakainen *et al.* 2009; Lotrakul *et al.* 2009), on degrading polyurethane and PVC plastics (Shah *et al.* 2008) and in many other unusual habitats. *Aureobasidium pullulans* is also well known for its biotechnological potential, among other things for the production of the polysaccharide pullulan (Cheng *et al.* 2011) and for its biocontrol use in agriculture (Sharma *et al.* 2009). It also produces a large spectrum of extracellular enzymes (Molnarova *et al.* 2013) and an antifungal peptide aureobasidin A (Takesako *et al.* 1991). While closely related and phenotypically relatively similar, *Aureobasidium subglaciale* (Zalar, Gostincar, Gunde-Cimerman) on the other hand has a much more restricted distribution. Its known habitats are limited to just a few cold polar environments (Gostinčar *et al.* 2014; Zalar *et al.* 2008). This ecological difference is reflected in some of the traits of these species: *A. pullulans* tolerates higher concentrations of NaCl than other related species of the same genus and *A. subglaciale* is characterised by its psychrotolerant nature (Zalar *et al.* 2008). The genomes of both species have been sequenced and analysed recently (Gostinčar *et al.* 2014).

Glycerol management in *Aureobasidium* spp. is not well researched. One published study observed an increase in the intracellular glycerol concentration in response to salt stress, but not to heat stress in *A. pullulans* (Managbanag & Torzilli 2002), while cold stress was not studied. Along with the compatible solute response, cells of *A. pullulans* appear to rigorously manage the intracellular concentrations of alkali-metal cations even in hypersaline conditions (Kogej *et al.* 2005).

It is now generally accepted that the production of large quantities of intracellular glycerol is beneficial for survival in both hypersaline and cold conditions. However, glycerol metabolism and transport are energetically demanding processes, which have to be carefully managed (Oren 1999). Understanding the mechanisms behind these processes should help us better understand the polyextremotolerant nature of the *Aureobasidium* spp., yeast with increasing role in biotechnology and agriculture. This led us to investigate the transcriptional response of glycerol metabolism and transport genes in *A. pullulans* and *A. subglaciale* subjected to high salinity and low temperatures.

Materials and methods

Phylogenetic analyses

To identify the genes putatively involved in glycerol metabolism in *Aureobasidium pullulans* and *Aureobasidium subglaciale*, as well as to infer their phylogeny, the proteins similar to *Saccharomyces cerevisiae* Gpd1, Gpp1, Gut1, Gut2, and Stl1 were identified in the predicted proteomes of *A. pullulans* (http://genome.jgi.doe.gov/Aurpu_var_pul1/Aurpu_var_pul1.home.html and GenBank: AYEO00000000), and *A. subglaciale* (http://genome.jgi.doe.gov/Aurpu_var_sub1/Aurpu_var_sub1.home.html and GenBank: AYYB00000000) by blastp, included in standalone blast 2.6.0+ (Altschul *et al.* 1997). The sequences

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