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Regular Articles

Osmotic stress adaptation of *Paracoccidioides lutzii*, Pb01, monitored by proteomics

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ABSTRACT

The ability to respond to stressful conditions is essential for most living organisms. In pathogenic organisms, this response is required for effective transition from a saprophytic lifestyle to the establishment of pathogenic interactions within a susceptible host. Hyperosmotic stress has been used as a model to study signal transduction and seems to cause many cellular adaptations, including the alteration of protein expression and cellular volume as well as size regulation. In this work, we evaluated the proteomic profile of *Paracoccidioides lutzii* Pb01 yeast cells during osmotic stress induced by potassium chloride. We performed a high accuracy proteomic technique (NanoUPLC-MS^E) to identify differentially expressed proteins during osmotic shock. The data describe an osmoadaptative response of this fungus when subjected to this treatment. Proteins involved in the synthesis of cell wall components were modulated, which suggested cell wall remodeling. In addition, alterations in the energy metabolism were observed. Furthermore, proteins involved in amino acid metabolism and hydrogen peroxide detoxification were modulated during osmotic stress. Our study suggests that *P. lutzii* Pb01. presents a vast osmoadaptative response that is composed of different proteins that act together to minimize the effects caused by osmotic stress.

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1. Introduction

To achieve optimal growth rates, microorganisms must have balanced internal conditions. Fluctuations in the external environment can result in a variety of cellular disorders that can promote damage to the internal environment. To avoid this imbalance, the cells must develop adaptive mechanisms that control internal homeostasis (Gasch and Werner-Washburne, 2002). To establish infection, pathogenic fungi must adapt to their environment. In addition to virulence factor production, fungi, such as *Candida albicans*, require adaptation to stress conditions to establish a successful infection (Brown et al., 2014). The *Candida albicans* stress response is considered to be a fitness attribute by acting against a wide variety of stressors, including oxygen and nitrogen reactive species, heat shock, and osmotic and cationic stressors (Fradin et al., 2005; Lorenzen et al., 2004; Rubin-Bejerano et al., 2003). While this response contributes to virulence, it is not directly involved with the host-pathogen interaction.

Osmotic shock can change intracellular turgor pressure (Hohmann, 2002b). Most cells respond to high osmolality by producing and accumulating low molecular weight metabolites, which help to restore the osmotic balance (Burg and Ferraris, 2008). It is commonly accepted that the accumulation of glycerol is the primary and fastest mechanism for adaptation to osmotic stress (Hohmann, 2002a; Klipp et al., 2005; Muzzey et al., 2009). For example, KCl exposure imposes osmotic and cationic stress that leads to a loss of turgor pressure (Kuhn and Klipp, 2012), which mediates the activation of target genes including those

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encoding glycerol biosynthesis enzymes (Smith et al., 2004). The accumulation of glycerol occurs concomitantly with the change in intracellular osmolarity, contributing to the ability of the cell to resist hyperosmotic shock (Blomberg and Adler, 1989). Hyperosmotic shock in yeast specifically induces transcription of the glycerol-3-phosphate dehydrogenase gene (Gpd1p) that encodes a key enzyme for glycerol synthesis (Brewster et al., 1993). In bacteria and fungi, the high osmolarity glycerol (HOG) MAP kinase pathway triggers these responses. In *S. cerevisiae*, Sln1p and Sho1p are sensors of the two upstream branches controlling HOG pathway (Maeda et al., 1993; Van Wuytswinkel et al., 2000). Transcriptional analysis revealed that a Sho1p homolog was induced in *Paracoccidioides* sp. in the presence of human blood and plasma, suggesting that yeast cells can trigger an adaptive response to osmotic stress under these conditions (Bailão et al., 2006, 2007).

Paracoccidioidomycosis (PCM) is an important human systemic mycosis with a broad distribution in Latin America. The disease is caused by dimorphic fungi belonging to the genus *Paracoccidioides* that consists of a complex of phylogenetic species (Matute et al., 2006; Teixeira et al., 2009). This disease is transmitted by inhalation of infective airborne conidia or mycelia fragments, which convert to the pathogenic yeast phase in the lungs of the host (San-Blas et al., 2002). *Paracoccidioides* sp. is a pathogen able to infect numerous sites within the human body. The infection occurs primarily in the lungs, from where the fungus can disseminate via the bloodstream and/or the lymphatic system to all parts of the body, leading to the disseminated form of PCM (Franco, 1987).

Proteomic approaches in *Paracoccidioides* sp. have been successfully employed by our group in several studies, including those focusing on environmental stresses, such as the fungal response to iron and zinc deprivation, oxidative and nitrosative stresses (Grossklaus et al., 2013; Parente et al., 2011, 2013, 2015), and hypoxia (Lima et al., 2014), as well as evaluating the response to macrophage infection (Parente-Rocha et al., 2015). In this present study, we mapped the proteome of *Paracoccidioides lutzii* Pb01 yeast cells exposed to KCl for 6 h. Upon the induction of osmotic stress, yeast cells had alterations in protein levels of key enzymes involved in several metabolic pathways. Our data suggest a reduction in the expression of glycolytic enzymes, while the expression of enzymes related to gluconeogenesis was increased. The expression of enzymes for amino acid metabolism was also altered, suggesting decreases in tyrosine and alanine degradation, and cysteine and glycine biosynthesis. In addition, enzymes related to leucine, valine and isoleucine degradation were induced. These data suggest a potential cell wall remodeling mechanism that occurred when enzymes belonging to the glucan biosynthesis pathway were induced, while chitin biosynthesis enzymes were repressed. In summary, *P. lutzii* Pb01 yeast cells showed several metabolic changes revealing adaptive mechanisms developed by this pathogen to overcome the damage caused by osmotic stress.

2. Material and methods

2.1. Microorganism, osmotic stress treatment and viability analysis

The osmotic stress response was investigated in *P. lutzii* Pb01 yeast cells, (ATCC MYA – 826). Fungal cells were collected from stationary 7-day-old cultures in Fava Neto's medium [0.3% (w/v) protease peptone, 1% (w/v) peptone, 0.5% (w/v) meat extract, 0.5% (w/v) yeast extract, 4% (w/v) glucose, 0.5% (w/v) NaCl, pH 7.2], washed in PBS and exposed for 6 h to 0.1 M KCl in the same medium. In the same way, control cells were obtained without KCl treatment. The stress condition used in the majority of experiments (0.1 M KCl) was chosen due to the results of cell viability assays using different concentrations (0.1 and 0.2 M) of stressor. We tested for viability by assessing membrane integrity, and dead cells were marked with propidium iodide as previously described (Grossklaus et al., 2013).

2.2. Quantification of transcripts by RT-qPCR

Total RNA from *P. lutzii* Pb01 yeast cells with and without KCl-treatment was extracted using the Trizol Reagent (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions. The High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) was used to synthesize the first-strand cDNA from 1 μg of RNA. The cDNA samples were diluted 1:5 in water, and RT-qPCR was performed using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) in the Step One Plus PCR System (Applied Biosystems, Foster City, CA, USA). Data normalization was performed by measuring the transcriptional levels of the gene encoding α-tubulin in each set of RT-qPCR experiments. Samples of each cDNA were pooled and serially diluted 1:5 to generate a relative standard curve. Relative expression levels of the genes of interest were calculated using the standard curve method for relative quantification (Bookout et al., 2006). Statistical comparisons were performed using Student's *t*-test, and samples with p-values < 0.05 were considered statistically significant. The oligonucleotides used in RT-qPCR experiments were designed to span an exon-exon junction (Table S1).

2.3. Protein sample preparation, data acquisition and processing, and protein identification by mass spectrometry analysis

P. lutzii Pb01 cells were grown in Fava-Nettós medium with or without 0.1 M KCl for up to 6 h. The cells were then centrifuged at 1500*g*, resuspended in 50 mM ammonium bicarbonate, pH 8.5, and lysed using glass beads under strong agitation by a bead beater (BioSpec, Oklahoma, USA) in 5 cycles of 30 s, while on ice. The cell lysate was centrifuged at 10,000*g* for 15 min at 4 °C and the protein content in supernatant was quantified (Bradford, 1976). The samples were analyzed using nanoscale liquid chromatography coupled with tandem mass spectrometry. Aliquots (50 μg) were prepared for NanoUPLC-MS^E as previously described with modifications (Murad and Rech, 2012; Murad et al., 2011). Briefly, 50 mM ammonium bicarbonate containing 25 μl of RapiGEST™ (0.2% v/v) was added to the samples (Waters Corp, Milford, MA). The solution was maintained at 80 °C for 15 min. Following, a 100 mM DTT solution was added and samples were incubated for 30 min at 60 °C. The alkylating agent iodoacetamide (300 mM) was added, followed by sample incubation in a dark room for 30 min. The digestion step was performed using trypsin 50 ng/μl (Promega, Madison, WI, USA). The sample was vortexed slightly and digested at 37 °C overnight. Following the digestion, 10 μl of 5% (v/v) trifluoroacetic acid was added and samples were incubated at 37 °C for 90 min. The samples were centrifuged at 18,000*g* at 6 °C for 30 min, and the supernatant was transferred to a recovery vial (Waters Corp). MassPREP Digestion Standard [rabbit phosphorylase B (PHB)] (Waters Corp) was used as internal standard at a final concentration of 150 fmol μl^{-1}. The buffering solution of 20 mM ammonium formate was used to increase the pH.

The nanoscale LC separation of tryptic peptides was carried out on a Waters nanoAcquity UPLC device with 2D technology as previously described (Murad and Rech, 2012), with modifications. The first fractionation of the peptides was performed by first-dimension chromatography using the XBridge BEH 130 C18 NanoEase column (5 μm, 300 μm × 50 mm; Waters, USA). The first-dimension solvent A was ammonium formate (20 mM, pH 10.0) and solvent B was acetonitrile (ACN). All fractions obtained were trapped onto a nanoAcquity UPLC Symmetry C18 trap column

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