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Copper overload in *Paracoccidioides lutzii* results in the accumulation of ergosterol and melanin

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ABSTRACT

Paracoccidioidomycosis is a highly prevalent systemic mycosis in Latin America, caused by fungi of the genus *Paracoccidioides*. Copper is essential for eukaryotes and bacteria. This micronutrient is used in many vital biochemical processes, although metal excess levels can be toxic for organisms. Pathways underlying copper overload are poorly understood in members of the *Paracoccidioides* complex. The responses of *Paracoccidioides lutzii* yeast cells to copper overload were here evaluated. The results showed that under copper overload, cells presented a dark brown pigment, identified as melanin. Proteomic analyses identified mainly the accumulation of proteins related to amino acids metabolism, ergosterol synthesis and melanin production, suggesting that *P. lutzii* responds to copper overload by changing aspects of its metabolism and also plasma membrane and cell wall remodeling. Proteomic data were confirmed by biochemical analysis.

1. Introduction

The ability of copper ions to cycle between an oxidized (Cu^{2+}) and reduced (Cu^{+}) state is exploited by many enzymes to catalyze biochemical processes including aerobic respiration, superoxide detoxification, and iron acquisition (Nevitt et al., 2012). Copper (Cu) interacts with many other chemical compounds such as sulfur, oxygen, nitrogen and is a cofactor responsible for modifying the structure of many proteins, participating in many biochemical events (Linder, 1991). The capacity of free Cu to catalyze the transformation of a superoxide radical anion to the hydroxyl radical makes copper a potentially toxic metal, affecting DNA, lipid and proteins (Lalioti et al., 2009). More recently, it has been suggested that that Cu^{+} toxicity in bacterial cells is significantly related to its interference in iron-sulfur (Fe-S) clusters (Macomber et al., 2007; Macomber and Imlay, 2009).

Copper can be an important resource in elimination strategies against certain microorganisms by host cells (Hodgkinson and Petris, 2012). The release of copper by macrophages generates an environment that is hostile for many pathogens (Langfelder et al., 2003). On the other hand, some pathogens have implemented a robust molecular mechanism for Cu resistance (Festa and Thiele, 2012). Studies with the model yeast *Saccharomyces cerevisiae* have provided significant insights into the intricacies of Cu mediated metal detoxification. Under high copper conditions, *S. cerevisiae* repressed some proteins that absorb Cu, and activated the detoxification system, to protect cells from Cu toxicity (Dong et al., 2013). In this organism, Ace1, which is a Cu responsive transcription factor, regulates the expression of genes related to Cu detoxification, such as those encoding metallothioneins (MTs) Cup1 and Crs5, and Cu/Zn SOD1 (Beaudoin and Labbe, 2001). MTs are metal-ion-detoxification proteins, which scavenge cytosolic Cu to avoid metal cytotoxicity (Bittel et al., 2000). *Cryptococcus neoformans*, utilizes primarily MTs (Cmt1 and Cmt2), to manage Cu toxicity, and their loss leads to decreased fungal pathogenicity in a mouse model. *In vivo*, live imaging of *C. neoformans*–infected mice has revealed a high copper environment at the initial site of infection in the lungs, presumably within the phagosomes of resident alveolar macrophages (Ding et al.,

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2013). Copper-specific induction of MT1 and MT2 in the lungs is critical for *C. neoformans* survival in a pulmonary infection model. Of special note, in both, *S. cerevisiae* and *C. neoformans,* Fe-S cluster-containing proteins and the machinery for Fe-S assembly are targets for Cu toxicity, and consequently a novel step of protection against Cu stress. *C. neoformans* responded to high levels of copper by activating the expression of ATM1, which encodes a mitochondrial ABC transporter which mobilizes a precursor for Fe-S biogenesis to the cytosol (Garcia-Santamarina et al., 2017).

Copper detoxification in *Candida albicans*, in addition to MTs (Cup1/Crd2), presents Crp1, which is a P type ATPase plasma membrane protein, involved in Cu export outside of the cell (Riggle and Kumamoto, 2000; Weissman et al., 2000). Also, Sur7 was identified as a Cu detoxification protein, present on the cell surface; this component of the detoxification machinery is required for virulence in a mouse model of infection (Douglas et al., 2012). Additionally, two studies have demonstrated that in both, *Aspergillus nidulans* and *Aspergillus fumigatus*, the transcription factor, AceA, and CrpA, a P type ATPase, are required for tolerance of high Cu (Wiemann et al., 2017; Antsotegi-Uskola et al., 2019).

Copper is a cofactor for key enzymes involved in melanin formation (Walton et al., 2005), which is a high molecular weight multifunctional polymer negatively charged and hydrophobic which has been reported as a "fungal armor" protecting fungi from adverse environmental conditions. Studies have demonstrated that fungi that have melanin are more resistant to extreme temperatures, heavy metal toxicity, oxidant-mediated damages, UV-light, hydrolytic enzymes and antimicrobial drugs (Fogarty and Tobin, 1996; Garcia-Rivera and Casadevall, 2001; Gomez et al., 2001; Dadachova et al., 2007).

The genus *Paracoccidioides* comprises thermo-dimorphic fungi that cause paracoccidioidomycosis, the most prevalent systemic mycosis of Latin America (Restrepo and Tobon, 2005). These microorganisms are endemic in areas going from Mexico to Argentina (Brummer et al., 1993; Restrepo-Moreno, 2003). Members of the *Paracoccidioides* complex, grow in the soil as mycelium and in the yeast form in the host. The infection produced by members of the *Paracoccidioides* complex occurs by inhalation of mycelia/conidia present in the soil. The ability to undergo morphogenetic transition from mycelium to yeast cells is a virulence attribute and this process is essential for infection (Rooney and Klein, 2002).

Mechanisms of Cu detoxification in members of the *Paracoccidioides* complex have not been described. In order to explore processes associated with Cu detoxification, we investigated the proteome profile of yeast cells in one member of the genus, *Paracoccidioides lutzii*, a specie with high prevalence in Brazil. Notably, *Paracoccidioides* spp. conidia and yeast cells synthesize melanin and this characteristic is associated with a reduction in phagocytosis and increased survival of yeast cells in macrophages and stress oxidative protection (Gomez et al., 2001). Increased survival of melanized yeast cells of *Paracoccidioides* spp. can be due to the fact that melanization protects the fungus from damage caused by free radicals derived from nitrogen or oxygen and increases the resistance to H_2O_2 and hypochlorite (Silva et al., 2009). Using proteomic analyses, we demonstrated that *P. lutzii* metabolic adaptation to copper overload includes induction of enzymes associated with the metabolism of amino acids, enhanced melanization of yeast cells, and increased ergosterol biosynthesis.

2. Material and methods

2.1. Paracoccidioides lutzii growth

All experiments used *P. lutzii* yeast cells (ATCC MYA-826). The fungus was maintained in the yeast form at 36 °C, by sub culturing every 3 days in Fava-Netto medium [1% (w/v) peptone; 0.3 % (w/v) proteose peptone; 0.5 % (w/v) yeast extract ; 0.5 % (w/v) NaCl; 0.5 % (w/v) meat extract; 4% (w/v) glucose; 1 % (w/v) agar, pH 7.2] (Fava Netto, 1961).

2.2. Determination of Cu overload

Paracoccidioides lutzii yeast cells were grown in liquid BHI (Brain Hearth Infusion) medium for 3 days at 36 °C with rotation of 150 rpm. In order to determine the effect of copper on *P. lutzii* growth, these cells were collected, washed with PBS (phosphate-buffered saline) and then spotted in McVeigh/Morton (MVM) modified solid medium (Restrepo and Jimenez, 1980) supplemented with 0.06, 50 or 100 μM of copper sulfate (Merck, Millipore, USA) at density 10^6 to 10^3; 36 °C for 7 days; in triplicates. In parallel, growth in liquid medium was also performed to determine cell viability. Yeast cells were submitted to grown in liquid McVeigh/Morton (MVM) also supplemented with 0.06, 50 or 100 μM of copper sulfate, in triplicates. Posteriorly, cells were collected at 0, 3, 6, 24 and 48 h of incubation and viability was analyzed by using a Guava easyCheck kit for Flow Cytometry (Guava, Merck, Millipore, USA; Ref. Nº: 4500-0025). A total of 1000 events were obtained for each sample. The Guava software version 1.3 was used to analyze the data (Guava ViaCount Assay). In brief, the assay is based on membrane permeability of 2 DNA-binding dyes: propidium iodate (PI) and LDS751. First, cells go through a blue laser (488 nm wavelenght) which excitates fluorescently labeled cells. The fluorescent dye PI is used to detect dead cells by the photomultiplier tube 1 (PM1) which detected 680/30 nm emission waves (red color); the fluorescent dye LDS751 is used to detect live cells by the photomultiplier tube 2 (PM2) at 583/26 nm emission waves (orange color). The LDS751 dye enters all cells, binds to the DNA, and fluoresces at its characteristic wavelength; the PI enters only dead cells, binds to the DNA, and fluoresces at a different wavelength. The data are expressed in percentage. Low PM1 and high PM2 values indicate high percentage of viable cells.

2.3. Analysis of gene expression by RT-qPCR

The yeast cells of *P. lutzii* were grown in liquid MVM medium containing 0.06 μM copper sulfate (control), 50 μM and 100 μM copper sulfate (copper overload) for 6 h. The cells were collected, washed with PBS 1X and then RNA was extracted using Trizol (TRI reagent, Sigma Aldrich; Ref. Nº: 93289) as manufacture instructions in biological triplicates. For the synthesis of cDNA, the commercial system High Capacity RNA-to-cDNA kit (Applied Biosystems; Ref. Nº: 4387406) was employed. Prior to qPCR reactions, the cDNAs were diluted (1:5) by adding water. Then, in triplicates, qPCR reactions were performed using SYBR green PCR master mix (Applied Biosystems; Ref. Nº: 4309115) in an Applied Biosystems Step One real-time Plus PCR system (Applied Biosystems). Oligonucleotides were designed, as following: Copper transporter 3 (PAAG_05251.1; *ctr3*); and *alpha*-tubulin (PAAG_12506.2, *tub*), the last used as the reference gene. The choice of the normalizing gene was evaluated by NormFinder Visual Basic application for Microsoft Excel (Andersen et al., 2004). The oligonucleotides used in the reactions have the following sequences: *ctr3*-S, 5'-GCCTGCTTCATTTCCTCATCG-3'; *ctr3*-AS, 5'-GGGTCGGGAGACTCAAGCG-3'; *tub*-S, 5'-ACAGTGCTTGGGAACTATACC-3'; *tub*-AS, 5'-GGGACATATTTGCCACTGCC-3'. Expression levels were calculated based on standard curve method for relative quantification (Bookout et al., 2006). The results were analyzed by the Student's t-test, considering significant differences when $p \leq 0.05$.

2.4. Protein extraction and digestion

Yeast cells were obtained in the following experimental conditions: 0.06 μM copper sulfate (control) and copper overload (100 μM). Cells were collected by centrifugation at 5000 × *g* for 15 min and washed twice with PBS. Then, the cells were suspended in Tris-Ca buffer (2 mM $CaCl_2$, 20 mM Tris-HCl, pH 8.8) added of 1 % (v/v) protease inhibitors (GE Healthcare Life Sciences; Ref. Nº: 80650123), and disrupted in the

presence of glass beads, using a beadbeater apparatus (BioSpec, Oklahoma, USA) in 5 cycles of 30 s on ice, as described (Lima et al., 2014). The cell lysates were centrifuged at 10,000 × *g* for 15 min at 4 °C until no pellet formation was observed. The concentration of protein extract was quantified according to Bradford (Bradford, 1976).

Protein digestion (tryptic digestion) was performed as described (Murad et al., 2011; Murad and Rech, 2012), with some modifications. For digestion, 300 µg of protein was used and surfactant RapiGest SF™ [0.4 % (v/v); Waters Corp, Milford, MA; Ref. Nº: 186001861] was added for 15 min at 80 °C. Then, 0.2 mM dithiothreitol - DTT (GE Healthcare; Ref. Nº: 17131802) was mixed followed by incubation at 60 °C for 30 min. Then, 6 mM Iodacetamide (Sigma-Aldrich; Ref. Nº: I6125) was added, and the mixture was kept in the dark for 30 min, at room temperature. Additionally, 1 ng of trypsin (Promega, Madison, WI, USA; Ref. Nº: V5280) was added and the samples were incubated overnight, at 37 °C. After tryptic digestion, trifluoracetic acid (TFA) (Sigma-Aldrich; Ref. Nº: 302031) at a final concentration of 0.4 % (v/v) was added, followed by samples incubation at 37 °C for 90 min. After centrifugation at 18,000 x *g* at 6 °C, for 30 min, the digested peptides were vacuum dried and suspended in 80 µL ammonium formate (20 mM). The samples were spiked with 200 fmol/µL of MassPREP Digestion Standard rabbit phosphorylase B (PHB; Waters Corp., Milford, MA; Ref. Nº: 186002326) used as an endogenous control.

2.5. NanoUPLC-MS^E analysis and protein identification

Samples were applied to a Waters Total Recovery vial (Waters, USA; Ref. Nº: 86007197C) prior to NanoUPLC-MS^E analysis. Digested peptides mixtures were separated by ultra-performance liquid chromatography using the system NanoAcquity (Waters Corporation, Manchester, UK), with 2 D technology (Schenauer et al., 2012) with some modifications. The first dimension chromatography was carried out with the XBridge BEH 130 C18 NanoEase column (5 µm, 300 µm x50 mm; Waters, USA; Ref. Nº: 186003573). Ammonium formate (20 mM, pH 10.0) was the first-dimension solvent A. Acetonitrile (ACN) was the solvent B. An initial condition of running the samples at 2 µL per minute of 3% solvent B was used. Peptides were fractionated five times (F1-F5) by different solvent B concentration: F1- 10.8 % (v/v); F2-14 % (v/v); F3-16.7 % (v/v); F4-20.4 % (v/v); F5-65 % (v/v). The second dimension chromatography was carried out on a Nano Acquit UPLC 130 C18 (1.7 µm, 100 µm x100 mm, Waters, USA; Ref. Nº: 186007496), with 0.1 % (v/v) formic acid and 0.1 % formic acid diluted in acetonitrile (v/v; pH 2.4).

The raw data were processed by Protein Lynx Global Server (PLGS) version 2.4 (Waters Corp.) and searched against the *Paracoccidioides* genome databank (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). The search was based on taxonomy; a maximum of 1 missed cleavage by trypsin was allowed. For peptide identification, the mass error tolerance was under 50 ppm. A minimum of 1 matched peptide and 5 fragments was obtained in the identified proteins. A total of 4 % was the maximum false positive ratio admitted. The score provided by PLGS was used to define confidence level of the identification. The intensity of the three most intense tryptic peptides from the MassPREP Digestion Standard rabbit PHB, allowed the determination of protein abundance. The false positive rate (FPR) was set to 4% in at least two sample injections. Normalization was performed with ATP synthase subunit alpha [accession number PAAG_04820 from *Paracoccidioides lutzii*] (https://www.broadinstitute.org/fungal-genome-initiative/paracoccidioides-genome-project) that showed no significant difference in abundance in all injections (Pizzatti et al., 2012). Furthermore, only those proteins with a 1.2 cut off in a fold change were considered as significantly induced/ repressed.

2.6. Sterol quantification

The quantification of ergosterol was performed according to standard protocols (Arthington-Skaggs et al., 1999; Neto et al., 2014) with some modifications. Five grams of *P. lutzii* cells (cultivated in 0.06 µM and 100 µM copper sulfate-MVM medium/48 h/36 °C) were collected and washed 3 times with PBS. Then, the samples were resuspended in 5 mL of 25 % (v/v) alcoholic potassium hydroxide and samples were mixed for 2 min. Then, the suspension was incubated for 3 h at 85 °C in water bath. To extracted sterols, a total of 5 mL of n-heptane (Sigma-Aldrich; Ref. Nº: 1.04390) and 2 mL of sterile water was added to each tube, followed by vigorous mixing for 5 min. The samples were kept for 2 h at room temperature to allow the *n*-heptane phase separation. Next, 200 µL of the *n*-heptane layer (containing ergosterol) was transferred to 96-well plate in biological triplicates, to obtain 8 absorbance readings. Ergosterol content was calculated by the following equations: crystalline ergosterol = $[(Abs_{281.5}/290) \times F]$/wet cell weight; dihydroergosterol = $[(Abs_{230}/518) \times F]$/wet cell weight. The values of 290 and 518 were used to identify crystalline ergosterol and dihydroergosterol, respectively, and F refers to dilution factor.

2.7. Heterologous expression of the pCat

The peroxisomal catalase gene sequence used for heterologous expression was obtained from the *Paracoccidioides* Genomic Databank available by Broad Institute (https://www.broadinstitute.org/fungal-genome-initiative/paracoccidioides-genome-project). The empty pGEX-4T-3 vector (GE Healthcare; Ref. Nº: 27-154201) was used in construction of the pGEX-4T-3::*pCat* plasmid. Gene transcript of pCat was amplified with specific primers pCat-Forward (5'-CCCGGGTCGACTCG AGATGGACCCCGAGTCTTCC-3') and pCat-Reverse (5'- GATGCGGCCG CTCGAGTCACAGATGCGACTGTGG-3') and cloned into the *Xho*I site into the pGEX-4T-3 vector using In-Fusion® HD system (Takara Bio, USA; Ref. Nº: 638910). *Escherichia coli pLysS* competent cells were used. Then, thermal transformation was carried out using standard procedures. Transformed cells were cultured in LB medium plus ampicillin (0.1 µg/mL) for 16 h at 37 °C. Culture was diluted 100-fold and cultured with agitation at 37 °C until $O.D_{600}$ ~0.5. The recombinant protein induction was performed by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich; Ref. Nº: I6758) solution at final concentration of 0.5 mM by 3 h. pCat size and identity were evaluated using a 12 % SDS-PAGE and in-gel protein digestion followed by LC–MS/MS as described(Rezende et al., 2011).

2.8. Polyclonal antibodies production

Paracoccidioides peroxisomal Catalase recombinant protein was used to generate specific polyclonal antibodies in male BALB/c mice. The use of mice for antibody generation was approved by the animal committee at Universidade Federal de Goiás (Protocol number 092/17). pCat was extracted from the gel, which was disrupted with a glass grinder. Pre-immune sera were obtained, and then mice were immunized by intraperitoneal injection of 200 µg in-gel protein (in 100 µL of PBS). The animals were immunized again twice, at 15-days intervals, with the same amount of antigen. The obtained sera were aliquoted and stored at −20 °C.

2.9. Western blotting analysis

To evaluate the expression of peroxisomal catalase in *P. lutzii*, protein extract and the anti-pCatalase polyclonal antibodies (anti-pCat) were employed. The protein extract was obtained as described above. Forty micrograms of proteins were used, which were separated by 12 % SDS-PAGE gel. Subsequently, the gel containing the proteins was exposed to a nitrocellulose membrane to which the proteins were transferred for 16 h at 4 °C in buffer [0.3 % Tris base (w/v), 1.4 % (w/v)

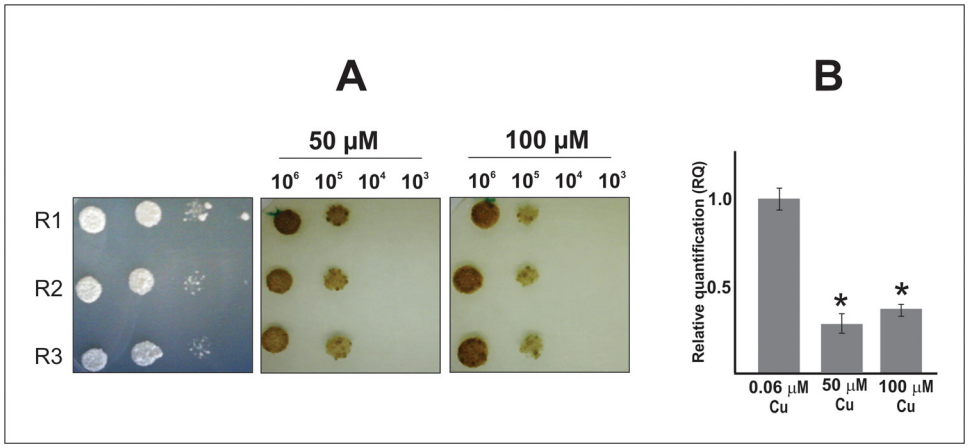

Fig. 1. Effect of Cu on *P. lutzii* growth and CTR3 transcript level. (A) *P. lutzii* yeast cells growth during Cu overload. *P. lutzii* yeast cells were serially diluted and dropped onto MVM agar plates containing 0.06 μM (control), 50 or 100 μM Cu sulfate, and grown at 36 °C, for 7 days. Experiments were performed in triplicates. **(B)** Transcript level of *P. lutzii* trans membrane Cu transporter CTR3 during Cu overload. *P. lutzii* yeast cells were incubated in control media or in media with Cu excess (50 and 100 μM Cu) for 6 h. Total RNAs were obtained and cDNAs were produced. Data were normalized using the constitutive gene for *alpha*-tubulin (GeneBank accession number PAAG_12506.2) as a reference gene. The data are presented with the mean and standard deviation of triplicates. Student-*t* test was applied to determine statistical difference. (*) Significantly different from the control, using the *p-value* ≤ 0.05.

glycine and 20 % (v/v) methanol]. The transfer efficiency was verified by staining the membranes with Rouge-Ponceau [0.2 % (w/v) Rouge-Ponceau and 3% (w/v) TCA], which allowed the visualization of the protein profiles. After this step, the membranes were incubated in blocking solution [0.1 % (v/v) Tween 20 and 2.5 % (w/w) skimmed milk powder in PBS] for 2 h at room temperature. After blocking, a buffer [PBS and 0.1 % (v/v) Tween 20] was used to wash the membranes for 3 times. Subsequently, the membrane was incubated with the polyclonal antibodies anti-pCat (1:150) and control was performed using polyclonal antibodies against triosephosphate isomerase (anti-TPI, 1:1000) (Pereira et al., 2007), for 1 h, at room temperature, under continuous stirring. For the western blot, the membrane was washed 3 times with buffer and subsequently incubated with anti-mouse secondary antibodies (1:20,000 diluted), for anti-pCat. Anti-rabbit secondary antibodies (1:30,000) were used for anti–TPI. Both incubations were performed for 1 h with stirring at room temperature and sheltered from light. After washing the membrane reactions were developed using BCIP/NBT substrate solution.

2.10. Melanin assays: dot blot analysis and melanin ghost analysis

For analysis of melanin production by yeast cells exposed to copper overload, dot blot analysis was performed. Cells were cultured in MVM liquid medium, with 0.06 μM or 100μM copper sulfate, for 7 days. Cells were centrifuged at 5000 *g* × for 5 min and after freezing in liquid nitrogen, were disrupted. The material was centrifuged at 12,000 × *g* for 20 min and the cellular extracts were vacuum spotted onto nitrocellulose membranes (*Hybond*™ ECL™; Ref. Nº: GERPN132D) at the concentration of 25 μg of proteins. After, membranes were blocked with milk powder (10 % w/v) in PBS plus Tween 20 (PBS-T) (0.1 % v/v), for 2 h, at room temperature. A murine monoclonal antibody to melanin (1:1000 diluted) (Nosanchuk et al., 1998) was applied to the membrane for 1 h at room temperature, followed by washing with PBS. Polyclonal antibodies to triose phosphate isomerase anti-TPI (1:1000) were used as control (Pereira et al., 2007). The membranes were incubated with secondary antibodies anti-mouse (1:5000), for the anti-melanin antibody, and anti-rabbit (1:30,000) for the anti-TPI, for 1 h at room temperature, and the reactive bands were revealed with BCIP/ NBT substrate solution.

To generate melanin particles ("melanin ghosts"), yeast cells of *P. lutzii* were incubated for 7 days in MVM medium with 0.6 μM or 100 μM copper sulfate at 36 °C with rotation of 150 rpm. The yeast cells were harvested after centrifugation, washed three times in PBS pH 7.2, and suspended in a solution of 1 M sorbitol and 0.1 M sodium citrate (pH 5.5). Cell-wall digestion was achieved by incubation of cells at 30 °C in 10 mg/mL of cell wall-lysing enzymes (from *Trichoderma harzianum*; Sigma Chemical Co.; Ref. Nº: L1412) for 1 h. Centrifugation at 2300 × *g* for 10 min was performed to collect protoplasts that were then washed with PBS, followed by incubation in 4 M guanidine thiocyanate for 1 h at 25 °C, under shaking. The digested material was washed three times with PBS, collected by centrifugation and boiled in 6 M hydrochloric acid for 1 h to hydrolyze cellular components associated with melanin. The debris was collected by centrifugation and washed exhaustively with PBS. This method isolates melanin ghosts of pigmented *Paracoccidioides* yeast cells (Gomez et al., 2001). Intact cells and melanin debris were observed in a Zeiss PrimoStar light microscope. At least 30 cells were measured with the software AxioVision after microscope image capture.

3. Results

3.1. Growth and viability of P. lutzii in Cu overload and analysis of ctr3 expression by RT-qPCR

We evaluated the effects of Cu excess on the growth and viability of *P. lutzii* yeast cells. For this, we initially grew the yeast cells in solid and liquid MVM medium with increasing concentrations of Cu (0.06, 50 and 100 μM). Yeast cells were plated in solid MVM media and incubated for 7 days. As depicted in Fig. 1A, a partial reduction in cell growth was observed in both conditions, 50 and 100 μM of Cu, and the cells presented a brown pigmentation when compared to control condition (0.06 μM of Cu). Similarly, melanization of *C. neoformans* is increased at high Cu concentrations (Mauch et al., 2013).

Cell viability was determined by flow cytometry. Yeast cells were collected at 0, 3, 6, 24 and 48 h in liquid medium. The results reveal that viability was not impacted over the 48-h interval independent of Cu concentration (Supplementary Fig. 1).

A previous study identified genes homologous to CTR3 in *Paracoccidioides* spp. (Silva et al., 2011). CTR3 encodes a transmembrane high affinity copper transporter (Labbe et al., 1997). Thus, the transcript levels of CTR3 (GeneBank accession number PAAG_05251.1) was quantified by RT-qPCR in yeast cells, in Cu overload. As depicted in Fig. 1B, the expression of CTR3 was significantly reduced in yeast cells in Cu overload. Based on these results, we determined that the concentration of 100 μM was reasonable to utilize in subsequent experiments.

3.2. Overview of proteomic analysis in yeast cells in Cu overload

The proteome of *Paracoccidioides* spp. has been extensively studied

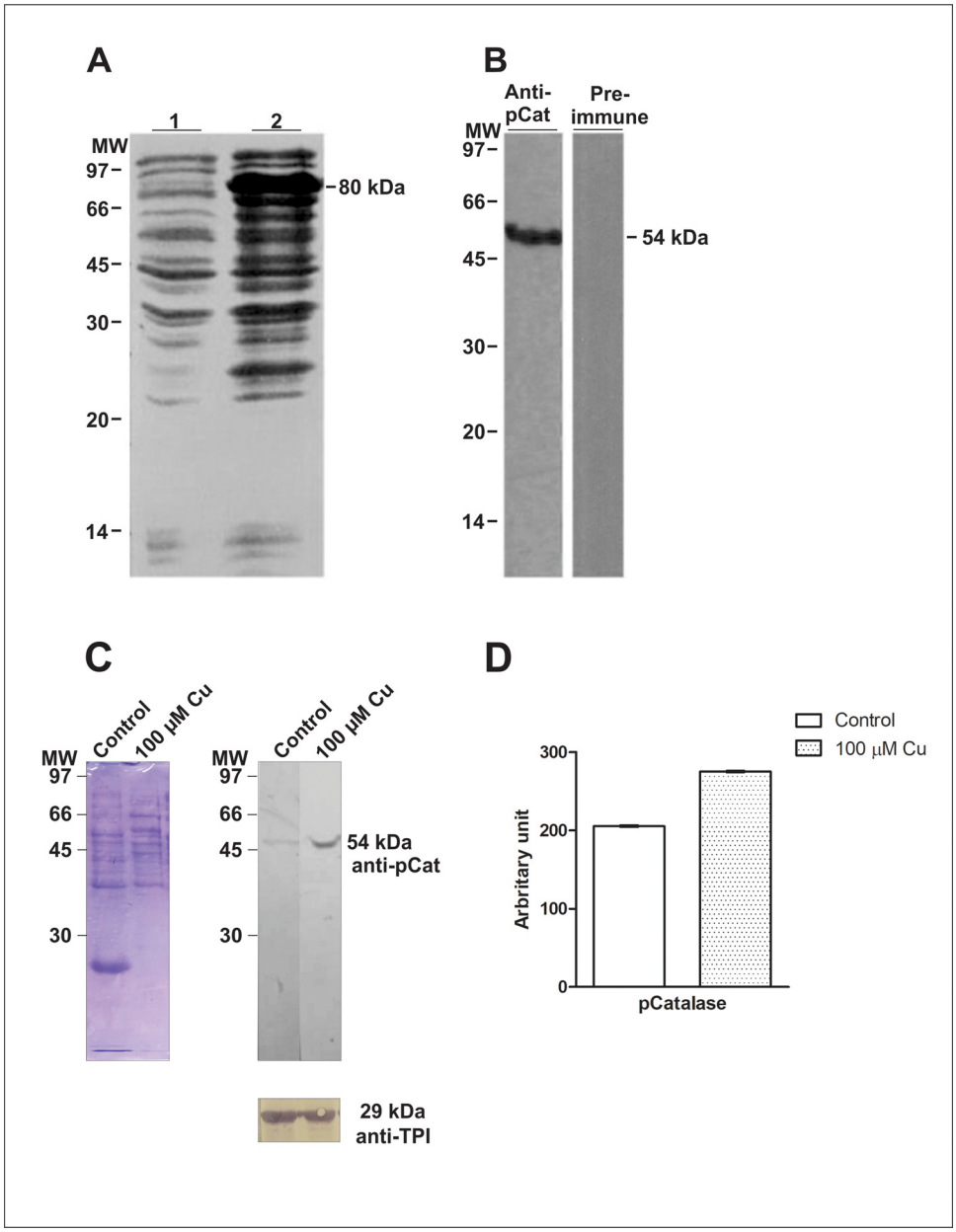

Fig. 2. Expression analysis of Catalase P (pCat) in *P. lutzii*. (A) Heterologous expression of pCat, and production of polyclonal antibodies. SDS-PAGE of pCat expression by *E. coli* pLysS (DE3) MW: molecular weight marker (kDa); 1, cell-free extract of *E. coli* before induction of IPTG; 2, cell-free extract of *E. coli* induced by 0.5 mM IPTG for 3 h; the induced recombinant protein has a molecular mass of 80 kDa. **(B) Western blot analysis.** Membranes containing cellular extract from *P. lutzii* were incubated with polyclonal antibodies to anti-pCat or pre-immune serum. The native protein shows a molecular mass of 54 kDa. **(C) Western blot analysis with protein extracts of *P. lutzii* yeast cells in Cu overload.** Protein extracts of *P. lutzii* cells (30 µg) were evaluated with polyclonal antibodies to pCatalase (anti-pCat) and anti-triosephosphate isomerase (anti-TPI) as control. Proteins were fractionated by 12 % SDS-PAGE and stained with Coomassie Blue (left panel) or transferred to nitrocellulose membranes (right panel). Membranes were incubated with the secondary polyclonal antibodies anti-mouse IgG for 1 h. The reaction was developed with BCIP/NBT. **(D) Quantification of peroxisomal catalase.** The pixels from images of Panel B were analyzed using ImageJ software and plotted in the graphic.

over the last five years (Bailao et al., 2014; Lima et al., 2015; Parente-Rocha et al., 2015; Araujo et al., 2017; Casaletti et al., 2017; de Oliveira et al., 2018). Here, a proteomic analysis of *P. lutzii* in Cu excess was performed. Processing of the resulting peptide and protein data were used to verify the quality of proteomic analysis. In addition to an acceptable false positive rate, the software provides additional parameters such as peptide detection type, mass accuracy and the dynamic range of proteomic analyzes (Supplementary Fig. 2).

Using a control condition (containing Cu 0.06 µM) for comparison to Cu overload (100 µM), we obtained a total of 3238 and 3014 peptides, respectively. Using the *P. lutzii* reversed sequence database, the false positive rate was calculated (0.37 and 0.39 % to control and copper excess samples, respectively). In this study, the NanoUPLC-MS^{E} analysis provided the identification of 372 proteins from both samples. The ATP synthase subunit alpha mitochondrial (GeneBank accession number PAAG_04820 from *Paracoccidioides* genome database (https://www.broadinstitute.org/fungal-genome-initiative/paracoccidioides-genome-project) was used as the normalizing protein, since it presented no significant difference in abundance in all injections (Pizzatti et al., 2012). A 1.2-fold change was applied as a threshold to determine positively and negatively expressed proteins. As result, 195 proteins were considered differentially expressed. From those, 103 were considered as up (Supplementary Table 1) and 92 as down (Supplementary Table 2) regulated proteins.

Enzymes involved in biosynthesis of methionine and glutamate were increased when *P. lutzii* cells were submitted to Cu excess, as determined by proteomic analysis, seen in Supplementary Table 1. For example, S adenosylmethionine synthase (PAAG_02901), homoserine dehydrogenase (PAAG_01991), cobalamin-independent synthase (PAAG_07626), aminomethyltransferase (PAAG_03045), all related to the biosynthesis of methionine were up regulated. Also were increased enzymes related to glutamate biosynthesis ornithine aminotransferase (PAAG_06431), delta-1-pyrroline-5-carboxylate dehydrogenase (PAAG_05253), glutaminase A (PAAG_02070) and glutamate carboxypeptidase (PAAG_08931), as depicted in Supplementary Table 1.

The biological category of cell rescue, defense, virulence and melanin biosynthesis had some representatives, as can be seen in Supplementary Table 1. The peroxisomal Catalase (pCat)

(PAAG_01454) was up regulated. The protein is responsible for detoxification of hydrogen peroxide (H_2O_2), whereby pCat reduces it to oxygen (O_2) and water (H_2O) (Ighodaro and Akinloye, 2017). Moreover, some heat shock proteins such as 30 kDa heat shock protein (PAAG_00871) and HSP90 (PAAG_05679) presented increase of expression (Supplementary Table 1). In addition, glutathione S-transferase Gst3 (PAAG_03931) and thioredoxin (PAAG_02364) were down regulated, as described in Supplementary Table 2, suggesting that Cu overload could disturb *P. lutzii* cell redox homeostasis.

For confirming proteomic data, we looked for the increase in peroxisomal catalase by obtaining the *P. lutzii* recombinant protein and polyclonal antibodies in mice, as can be seen in Fig. 2. After obtaining the recombinant pCat and polyclonal antibodies (Fig. 2 A and B), blotting assays confirmed that pCat was more accumulated in Cu overload compared to control (Fig. 2 C and D). The polyclonal antibodies directed to the triose phosphate isomerase (Pereira et al., 2007) were used as the loading control.

3.3. Copper overload induces melanin biosynthesis

As showed in Supplementary Table 1, there were increase in enzymes related to melanin production, such as fumarylacetoacetase (PADG_08163) and maleylacetoacetate isomerase (PADG_08162), which convert phenylalanine to L-Dopa and quinone oxidoreductase (PAAG_02382) and NADPH quinone oxidoreductase type IV (PAAG_12076) that convert dopaquinone in leukodopachrome, and in dopachrome, which is converted in melanin, as depicted in Fig. 3.

In order to validate high melanin production in yeast cells submitted to Cu overload, microscopy analysis and dot-blotting were performed. The presence of melanin and melanin ghosts were observed in yeast cells incubated in Cu overload as depicted in Fig. 4. Cells grown in culture medium supplemented with Cu^{2+} (100 μM) were darker than cells grown upon control conditions (Fig. 4 A and B). Moreover, cells grown in the presence of Cu excess (size range: 17.28 − 11.40 μm) were significantly smaller than control (size range: 19.77 − 14.61 μm), with mean cell diameters of 15.17 ± 0.46 and 16.82 ± 0.56 μm respectively (p value = 0.0317), as shown in Supplementary Fig. 3. Fig. 4 C depicts melanin ghosts recovered from cells grown in the presence of Cu excess. No melanin ghosts were retrieved from P. lutzii yeast cells grown in absence of Cu. Dot blot analysis demonstrate the higher accumulation of melanin in cells upon Cu overload (Fig. 4 D and E), confirming the role of Cu for melanin production.

3.4. Ergosterol accumulation in yeast cells in Cu overload

Phenylalanine and tyrosine metabolism can participate in ergosterol synthesis, component of the cell wall and plasma membrane. Enzymes related to sterol biosynthesis were up-regulated in *P. lutzii* yeast cells in Cu overload (Supplementary Table 1; Fig. 5). Enzymes that participate in cell wall and plasma membrane synthesis, including maleylacetoacetate isomerase (PAAG_08162), which converts 4-maleylacetoacetate to 4-fumarylacetoacetate, and fumarylacetoacetase (PAAG_08163), which cleaves 4-fumarylacetocetate to acetoacetate and fumarate were increased. Acetoacetate is converted in acetoacetyl-CoA, which participates in mevalonate pathway (Nelson and Cox, 2014; Miziorko, 2010). The mevalonate pathway produces isoprenoids precursors that, by catalysis by IPP isomerase (PAAG_03960), produce sterols using two enzymes, 3-beta-hydroxysteroide dehydrogenase (PAAG_04526) and esterase D (PAAG_05690). These enzymes were up regulated, which is consistent with the suggestion that Cu overload results in an increase in the content of ergosterol, as showed in Supplementary Table 1 and Fig. 5 A. In order to verify ergosterol accumulation during Cu overload, we performed sterol quantification, as presented in Fig. 5 B. Ergosterol presented a 2.7-fold increase in yeast cells in Cu overload in comparison to control, supporting our suggestion that *P. lutzii* yeast cells significantly remodels plasma membrane in response to Cu stress (Fig. 5 B).

4. Discussion

The mechanisms underlying Cu toxicity are poorly understood in fungi. Regarding to members of the *Paracoccidioides* complex, the knowledge concerning to Cu homeostasis and overload is still scarce. Due to the relevance of Cu detoxification by pathogens, we started our studies by investigating the responses of *P. lutzii,* a member of the *Paracoccidioides* genus, to Cu overload.

The genomes of members of the *Paracoccidioides* genus encode for two Cu transporters at the plasma membrane, named *CTR2* (PADG_04146), of low affinity and *CTR3* (PAAG_052251) of high affinity (Silva et al., 2011). We tested for Cu overload in yeast cells by measuring the transcript level of *ctr3* that at 100 u M of Cu presented reduction. Also, we verified that at this Cu concentration, the cells presented high viability and for that the experiments were conducted at this concentration. Our results showing the decreased expression of *CTR3* (Fig. 1) in yeast cells submitted to Cu overload are consistent with the findings in *S. cerevisiae*. *CTR1*, which is a Cu transporter, similarly showed decreased gene expression in Cu overload (Liu et al., 2012). The data therefore support the adaptability of *P. lutzii* to Cu overload, regulating genes related to Cu homeostasis, thereby enhancing its survival in this potentially toxic milieu.

Another meaningful finding was that yeast cells growing in high Cu concentration presented a brown pigment, presumably melanin.

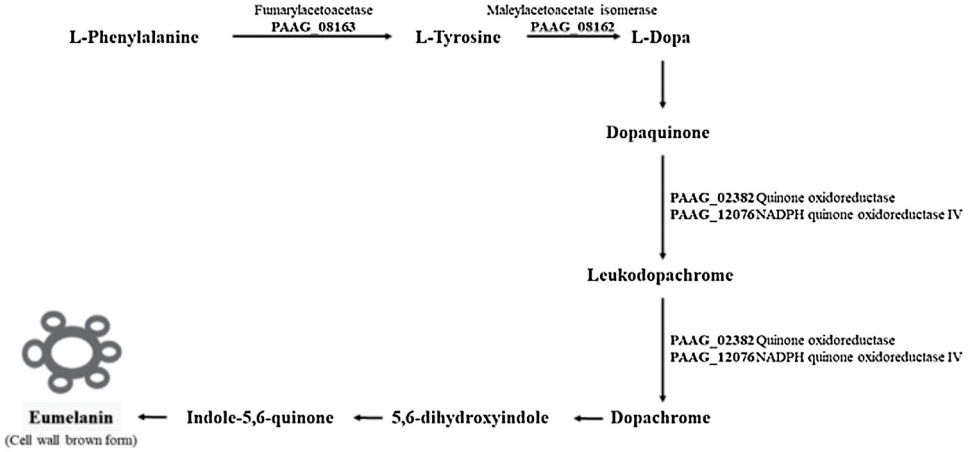

Fig. 3. Predicted pathway for melanin synthesis in *P. lutzii* cells in Cu overload. The pathway shows the enzymes identified in the proteome that participate in melanin synthesis in *P. lutzii*. The up-regulated enzymes from melanin synthesis are shown with names and accession numbers.

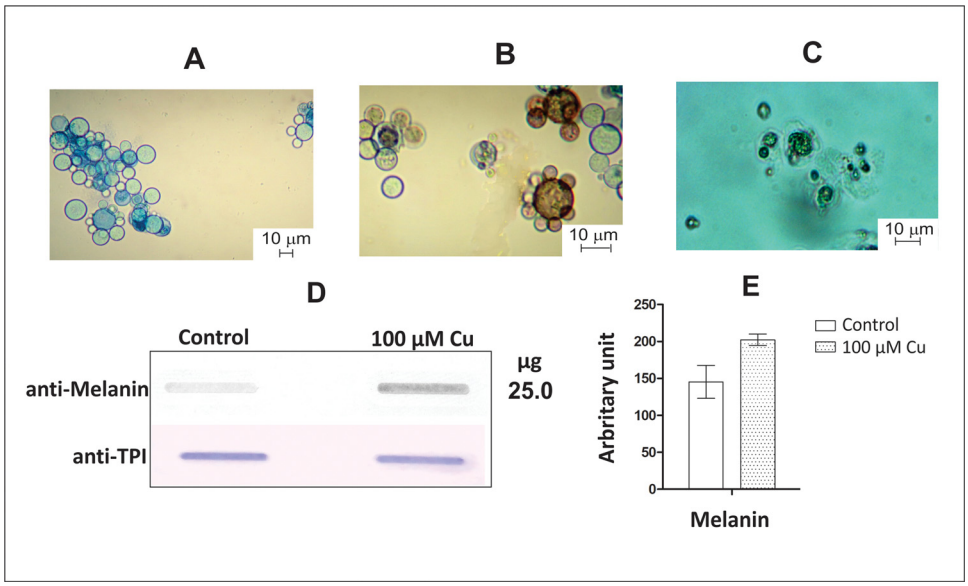

Fig. 4. Melanin accumulation in *P. lutzii* yeast cells in Cu overload. (A, B) *P. lutzii* cells visualized in optical microscope, with trypan blue dye (magnified 400 X). **(A)** Control *P. lutzii* cells. **(B)** *P. lutzii* cells in Cu overload. Yeast cells cultured with 100 µM of Cu sulfate present a brown pigment. **(C)** *P. lutzii* yeast cells producing melanin ghosts. **(D)** Dot blot analysis with protein extracts of *P. lutzii* yeast cells in copper overload. 25 µg of protein was used to adjust the amount of cellular debris. Protein extracts of yeast cells were evaluated with a monoclonal antibody to melanin; control was obtained with polyclonal antibodies against triosephosphate isomerase (anti-TPI). BCIP/NBT revealed the reaction. **(E)** Quantification of dot blot of D. The pixels from images of Panel B were analyzed using ImageJ software and plotted in the graphic.

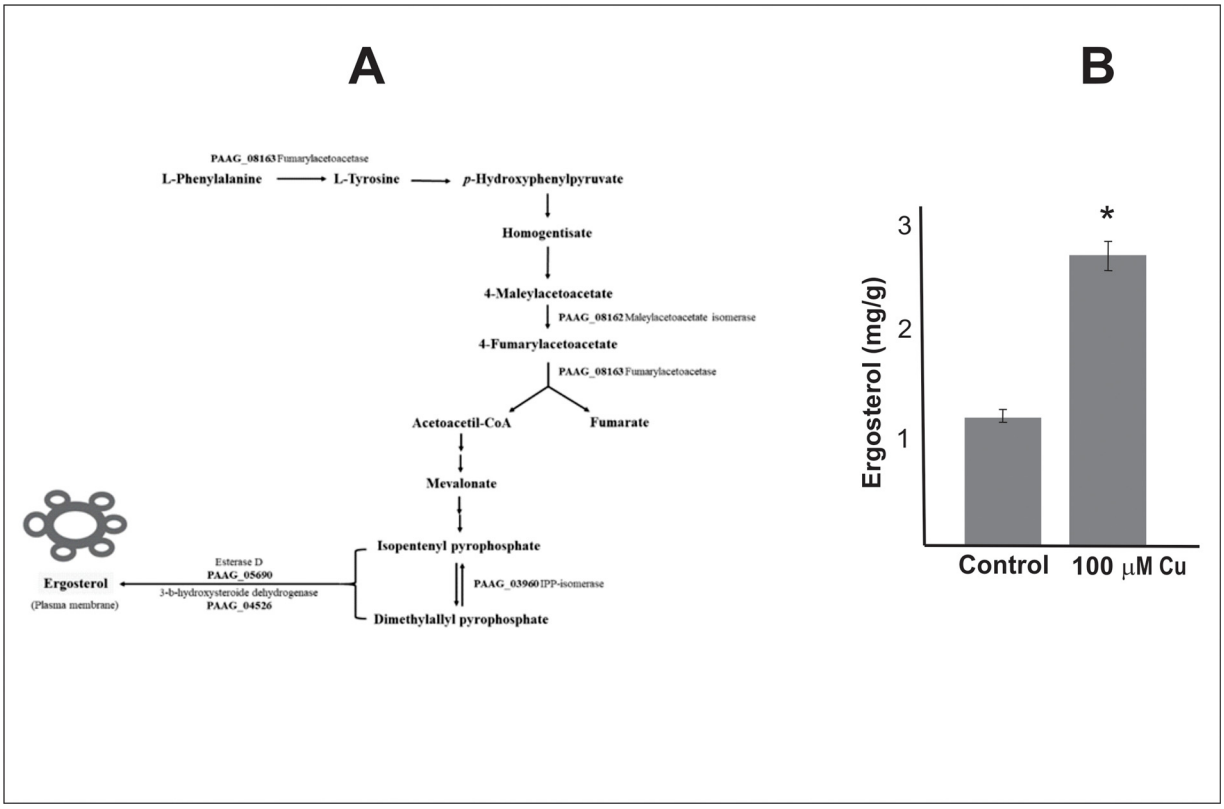

Fig. 5. Metabolism of ergosterol and accumulation in *P. lutzii* in Cu overload. (A) The data from proteomic analysis of *P. lutzii* cells during Cu overload was used to infer modulation of ergosterol metabolism in fungal cells. The enzymes up-regulated during Cu overload in *P. lutzii* are shown with names and accession numbers. **(B)** Ergosterol quantification in *P. lutzii* cells in Cu overload. * indicates statistical significance at p value ≤ 0.05.

Proteomic and biochemical assays confirmed this presumption (Figs. 3 and 4). Melanin enhances the virulence of many important human pathogenic fungi and it is typically located in the cell wall (Nosanchuk and Casadevall, 2003). There are two classic pathways of melanin synthesis in fungi: DHN pathway in which the precursor molecule is acetyl-CoA or malonyl-CoA and *via* L-3,4-dihyroxyphenylalanine (L-DOPA), for which there are two possible starting molecules, L-DOPA or tyrosine (Langfelder et al., 2003). Fungi may also produce alternative melanin pigments, like pyomelanin (Schmaler-Ripcke et al., 2009; Keller et al., 2011; Carreira et al., 2016. Pyomelanin is produced *via* degradation of L-tyrosine with oxidation and polymerization of the homogentisic acid (HGA) as the main intermediate (Keller et al., 2011) or by dopaquinone (Ito and Wakamatsu, 2003). In this study, we observed that *P. lutzii* yeast cells in copper overload had altered morphology and also accumulate a dark pigment in their cells, by melanin accumulation. Enzymes related to melanin synthesis were up regulated in yeast cells in copper overload when compared to control. Proteomic results suggest that melanin produced comes from classic L-DOPA and not from DHN or the pyomelanin pathway. In this study, we observed that *P. lutzii* yeast cells in Cu overload had altered morphology. These

changes facilitate evasion of the host's immune system and/or increased virulence in fungi (Hernandez-Chavez et al., 2017; Garcia-Carnero et al., 2020), including *Paracoccidioides* (Almeida et al., 2009; de Oliveira et al., 2015; Camacho and Nino-Vega, 2017). In addition, it was observed the melanin accumulation inside cells. This also could interfere on *Paracoccidioides* interaction with host. Pathogenic fungus as *Sporothrix schenckii* (Almeida-Paes et al., 2016) and *Aspergillus fumigatus* (Akoumianaki et al., 2016) produce melanin as the virulence factor. The melanin pigments were described in *Paracoccidioides* firstly by (Gomez et al., 2001). Melanized *P. brasiliensis* cells increase its resistance against phagocytosis. In addition, melanin can protect the fungus against injury caused by nitrogen or oxygen-derived free radicals, and drugs, like amphotericin B, ketoconazole, fluconazole, itraconazole (da Silva et al., 2006).

Amino acids metabolism supply precursors that feed relevant biochemical pathways for fungal cell maintenance as previously shown in *Paracoccidioides* spp. (Lima et al., 2015, 2014; Parente-Rocha et al., 2015). In addition to the synthesis of melanin from L-Dopa, the increase in enzymes concerned to the synthesis of methionine detected in the proteomic analysis suggests Cu buffering, preventing Cu toxicity in *P. lutzii.* Studies in *C. neoformans* demonstrated that absence of exogenous amino acids, in a strain deleted of the gene encoding *ATM1*, a mitochondrial ABC-transporter that mobilizes a precursor for Fe-S biogenesis, resulted in around 30-fold increase sensitivity do Cu stress (Garcia-Santamarina et al., 2017) evidencing the buffering capacity of amino acids to Cu excess. It is relevant to point the absence of genes encoding metalothioneins in the genome of *Paracoccidioides* (Silva et al., 2011) highlighting the relevance of alternative mechanisms for copying with Cu toxicity.

A high number of proteins that specifically participate in sterol biosynthesis were up regulated in *P. lutzii* during Cu overload exposure. Ergosterol is one of the most abundant lipid of fungal cell membrane and affects membrane rigidity, fluidity, and permeability (Abe and Hiraki, 2009). It is known that *S. cerevisiae* deleted of genes coding for ergosterol synthesis (especially Δ*erg2*) presents excessive incorporation of cations, such as Li^+ and Na^+. It is speculated that water molecules bound to membrane proteins could be extracted when cations are abundant. In this sense, the cells increase ergosterol content in the membrane to avoid membrane destabilization during incubation with high cation content. Corroborating this hypothesis, *Candida glabrata* cells incubated with fluconazole – an antifungal agent that inhibits ergosterol synthesis – presents increased susceptibility to cationic toxic compounds (Elicharova and Sychrova, 2014). In this sense, the ergosterol increased content in *P. lutzii* cells during Cu overload could enhance membrane stabilization and avoid higher cation incorporation.

5. Conclusions

In conclusion, the homeostasis of Cu in this pathogen has not previously been assessed. Here we report the response of *P. lutzii* to *in vitro* Cu overload. By using proteomics and biochemical assays, we obtained insights of how Cu excess can activate some pathways of detoxification and protection mechanisms. In response to Cu overload, *P. lutzii* modulates its metabolism synthesizing melanin, changing cell morphology, and remodeling plasma membrane, by synthesizing ergosterol. This is a pioneering study in the *Paracoccidioides* genus that demonstrates the remarkable and extensive adaptations of this thermally dimorphic fungus against the Cu overload.

Disclosures

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CRediT authorship contribution statement

Igor Godinho Portis: Conceptualization, Formal analysis, Methodology, Software, Validation, Writing - original draft, Writing - review & editing. **Patrícia de Sousa Lima:** Formal analysis, Methodology, Software, Validation, Writing - original draft. **Rodrigo Almeida Paes:** Formal analysis, Methodology, Validation, Writing - original draft. **Lucas Nojosa Oliveira:** Methodology, Validation. **Christie Ataídes Pereira:** Methodology. **Juliana Alves Parente-Rocha:** Methodology, Software, Validation, Writing - review & editing. **Maristela Pereira:** Validation, Investigation. **Joshua Daniel Nosanchuk:** Data curation, Validation, Writing - original draft, Investigation. **Célia Maria de Almeida Soares:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing, Investigation.

Declaration of Competing 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.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126524.

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