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Moonlight-like proteins of the cell wall protect sessile cells of *Candida* from oxidative stress

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ABSTRACT

Biofilms of *Candida* species are associated with high morbidity and hospital mortality. *Candida* forms biofilms by adhering to human host epithelium through cell wall proteins (CWP) and simultaneously neutralizing the reactive oxygen species (ROS) produced during the respiratory burst by phagocytic cells. The purpose of this paper is to identify the CWP of *Candida albicans*, *Candida glabrata, Candida krusei* and *Candida parapsilosis* expressed after exposure to different concentrations of H_2O_2 using a proteomic approach. CWP obtained from sessile cells, both treated and untreated with the oxidizing agent, were resolved by one and two-dimensional (2D-PAGE) gels and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Some of these proteins were identified and found to correspond to moonlighting CWP such as: (i) glycolytic enzymes, (ii) heat shock, (iii) OSR proteins, (iv) general metabolic enzymes and (v) highly conserved proteins, which are up- or down-regulated in the presence or absence of ROS. We also found that the expression of these CWP is different for each *Candida* species. Moreover, RT-PCR assays allowed us to demonstrate that transcription of the gene coding for Eno1, one of the moonlight-like CWP identified in response to the oxidant agent, is differentially regulated. To our knowledge this is the first demonstration that, in response to oxidative stress, each species of *Candida*, differentially regulates the expression of moonlighting CWP, which may protect the organism from the ROS generated during phagocytosis. Presumptively, these proteins allow the pathogen to adhere and form a biofilm, and eventually cause invasive candidiasis in the human host. We propose that, in addition to the antioxidant mechanisms present in *Candida*, the moonlighting CWP also confer protection to these pathogens from oxidative stress.

1. Introduction

Opportunistic pathogenic fungi of the *Candida* genus are the leading cause of invasive candidiasis (IC) in immunocompromised and hospitalized patients [1–4]. *Candida albicans* is by far the most commonly identified fungus in patients with candidiasis. However, in recent years, due to indiscriminate use of broad-spectrum antibiotics combined with immunosuppressive therapies, there has been an increase in the number of infections due to non-*C. albicans Candida* (NCAC) species, such as *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis* [4–14]. The first event in the onset of IC is the adhesion of the yeast to the cells, tissues and medical devices implanted in the human host with subsequent biofilm formation [15–22]. In recent decades, biofilms have been considered as important virulence factors as sessile cells exhibit high resistance to antifungal treatments and host defense mechanisms [3,4,23–32]. This resistance prevents the patient from responding adequately to antifungals, leading to high morbidity and hospital mortality [3,4,33,34]. During biofilm formation, *Candida* first adheres to the medical device and/or the patient's cells through the cell wall proteins (CWP) [19,35–37]. Thus, these proteins are of particular interest because of their immediate exposure and interaction with the host cells, including reactive oxygen species (ROS) generated during the respiratory burst by the phagocytic cells [38–40]. It has been reported that some CWP elicit an immune response and alter with morphological state [39–41]. We have a special interest in the interaction between the CWP and the medical device to form the biofilm, particularly in the identification and understanding of the nature and function of these

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proteins in sessile cells of *Candida* species in response to oxidative stress (OSR). The fact that *Candida* is able to colonize the human host while resisting the ROS suggests that IC involves an interplay of several, closely related virulence factors: adhesion, biofilm formation and oxidative stress (OS). Here, we identified CWP of sessile cells of *C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis* in OSR. To achieve biofilm formation, *C. albicans*, *C. krusei* and *C. parapsilosis* were grown in 2% YPD while *C. glabrata* was grown in 0.2% YNB, all for 48 h a 28 °C [42]. Sessile cells of the four species were then mechanically detached from the Petri dish, cell suspensions were adjusted to an OD_{600nm} 0.5 and exposed to different concentrations of H_2O_2 (0, 50 and 150 mM for *C. albicans* and *C. glabrata*, and 0, 300 and 1500 mM for *C. krusei* and *C. parapsilosis*). CWP were obtained from sessile cells, both treated or not with the oxidizing agent, and were resolved in uni- and two-dimensional (2D-PAGE) gels and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. This approach allowed us to detect and identify thirty different protein spots, fourteen out of which were differentially expressed in the four species of *Candida*. Some of them were found to correspond to moonlighting CWP including: (i) glycolytic enzymes such as triosephosphate isomerase (Tpi1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), fructose-bisphosphate aldolase (Fba1), phosphoglycerate kinase (Pgk), phosphoglycerate mutase (Gpm1), pyruvate kinase (Pk) and enolase (Eno1); (ii) heat shock proteins such as Ssa1 or Hs71 of the Hsp70 family; (iii) OSR proteins such as peroxisomal catalase (Cta1); (iv) other metabolic enzymes such alcohol dehydrogenase (Adh), acetyl-CoA hydrolase (Ach1) and 6-phosphogluconate dehydrogenase, decarboxylating 1 (Gnd1, Dor14) and pryruvate decarboxylase (Pdc1, Pdc11); and (v) highly conserved proteins like actin (Act1). RT-PCR assays allowed us to observe that transcription of the gene coding for Eno1, one of the identified moonlight-like CWP, is differentially regulated. Altogether these results indicate that, in response to OS, sessile cells of *Candida* differentially regulate the expression of CWP, protecting the organism from the ROS generated during phagocytosis. This response allows cells to adhere, form the biofilm and cause IC in the human host.

Additionally, data also allow us to propose that, in addition to the antioxidant mechanisms present in *Candida*, moonlighting CWP types also confer protection to these pathogens from ROS. Altogether, these mechanisms facilitate colonization of the patient by the pathogen. Our group is currently working on this hypothesis.

To our knowledge this is the first report where the CWP of sessile cells of four species of *Candida* have been identified in OSR. These clinically relevant results show that there appears to be an association between several virulence factors, including OSR, adhesion and biofilm formation. This is promising in terms of development of vaccines specific to each of these pathogens, which would help to reduce the high mortality of hospitalized and immunocompromised infected with *Candida*.

2. Materials and methods

2.1. Strains and culture conditions

The strains of *C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis* used in this study are clinical isolates from the collection of Department of Microbiology, ENCB-IPN, México, and were kindly donated by Prof. M. A. Martínez Rivera. Yeast strains were cultured on yeast peptone (YP; yeast extract, 1%; peptone, 2%) or amino acid-free yeast nitrogen base (YNB). YP and YNB media were supplemented with 2% and 0.2% glucose, respectively (Sigma–Aldrich, USA) [42] and 2% agar was added to solidify the media [43].

2.2. Biofilm formation

We have previously set up the *in vitro* conditions for the formation of biofilms of all *Candida* species used in this study [42]. However, the formation of the biofilm in 96 well boxes provides only small amounts of protein that were insufficient for this study. We therefore modified the method by increasing the surface area of biofilm formation using Petri dishes as described above. All four *Candida* species were grown in YPD for 48 h at 28 °C. To promote biofilm formation, yeasts were harvested by low-speed centrifugation, resuspended in 100 mL of medium (*C. albicans*, *C. krusei* and *C. parapsilosis* in YPD 2% and *C. glabrata* in YNB 0.2%) to a cell density of OD_{600nm} 1.0 [42]. Aliquots of 30 mL were taken from each sample, placed in 18-cm Petri dishes (Nunc, Nalgene) and incubated for 48 h at 37 °C. After biofilm formation, planktonic cells were discarded by washing the dish four times with sterile 10 mM calcium chloride ($CaCl_2$, Sigma–Aldrich) prepared in phosphate-buffered saline, pH 7.2 (PBS, Sigma–Aldrich). Sessile cells (biofilm) were detached from the dish using a metal spatula and an aliquot (100 μL) of the suspension was used to determine metabolic activity with XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (Sigma–Aldrich). This compound is reduced by mitochondrial dehydrogenase to a water-soluble formazan that results in a colorimetric change [42,44]. Accordingly, 100 μL of XTT-menadione (0.1 mg/mL XTT, 1 μM menadione, Sigma–Aldrich) were added to each well and plates were incubated at 37 °C. After 105 min, the XTT-derived formazan was measured at 492 nm using a microtiter plate reader (Varioskan® Flash, Thermo Scientific). The same assay was used to determine the viability of sessile cells after exposure to H_2O_2 (data not shown, but described in reference [42]).

2.3. Susceptibility to H_2O_2

Sessile cells (50 mL; OD_{600nm} 0.5) were exposed to increasing concentrations of H_2O_2 (0, 50 and 150 mM for *C. albicans* and *C. glabrata*, and 0, 300 and 1500 mM for *C. krusei* and *C. parapsilosis*), and incubated with shaking at 28 °C. After 90 min, the cell density was re-adjusted to OD_{600nm} 0.5 and used to obtain the CWP. Experiments were carried out in triplicate.

2.4. Extraction of CWP from sessile cells

Sessile cells of the four species of *Candida* treated with different concentrations of the oxidizing agent were centrifuged at 1300 × *g* for 5 min and the supernatant was carefully discarded. The cell pellet was resuspended in 2 mL of cold sterile water containing 1 mM phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteases and acetylcholinesterase [45,46], and cells were disrupted with an ultrasonic homogenizer (Fisher Scientific) at 80% of pulser, 30 W, for alternate periods of sonication (30 s) and cooling until 3 min of breakage was completed. The lysate was centrifuged at 4 °C for 10 min at 18,000 × *g*, the supernatant was discarded and the CW pellet was washed exhaustively with cold sterile water containing 1 mM PMSF until a clear supernatant was obtained [45,47]. To extract the CWP, the washed CW were resuspended in 2% SDS and 5% β-mercaptoethanol and boiled for 4 min. After removing the insoluble material by centrifugation at 6800 × *g* for 10 min, the supernatant containing the soluble proteins was collected. Ionic contaminants were removed from the CWP preparation by selective precipitation. Detergents, lipids and phenolic compounds were removed using the ReadyPrep 2-D cleanup kit (Bio-Rad) and protein concentration was determined by the DC method (Bio-Rad).