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Specific stress-induced storage of trehalose, glycerol and D-arabitol in response to oxidative and osmotic stress in *Candida albicans*

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ABSTRACT

Candida albicans exponential yeast cells are able to face environmental challenges by mounting a rapid and efficient "general stress response". Here we show that one of the main components of this response consists of the intracellular protective accumulation of the non-reducing disaccharide trehalose and two polyols, glycerol and D-arabitol, an accumulation that occurs in a stress-specific dependent manner. Thus, oxidative exposures promoted a marked increase in both trehalose and D-arabitol in the wild type strain, RM-100, whereas the glycerol content remained virtually unaffected with respect to basal levels. In contrast, osmotic challenges induced the significant storage of glycerol accompanied by minor changes, or even a slight drop, in the intracellular content of trehalose and D-arabitol. We examined the hypothetical role in this process of the MAP kinase Hog1, which regulates the protective responses in *C. albicans* against both oxidative and osmotic stress. Interestingly, unlike glycerol synthesis, the stress-induced trehalose accumulation was always Hog1-independent, whereas the ability to synthesize D-arabitol was only partially dependent on a functional Hog1 pathway.

1. Introduction

Being lower unicellular eukaryotes, yeasts are particularly sensitive to nutrient availability and to sudden and unforeseen fluctuations in their physical environment. Accordingly, these organisms have evolved a set of elaborate mechanisms that ensure rapid and efficient responses to counteract potentially harmful external injuries (termed as "stress") [1,2]. The existence of one sole "General stress response" has been demonstrated in budding (*Saccharomyces cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeasts and a large body of evidence also supports its presence in *Candida albicans* and some other pathogenic fungi. This defensive mechanism, which is crucial for survival, is triggered by a wide range of stress conditions and also guarantees cross-protection against further exposure to different unrelated stress treatments [3,4]. However, this common signalling stress-responsive pathway seems to be adapted to the specific ecological niches and biological activities played by each particular organism [2].

In a number of yeast species, one of the main defensive elements developed to counteract environmental challenges is the rapid endogenous synthesis of compatible solutes, including the non-reducing disaccharide trehalose and a set of polyols, namely glycerol, D-arabitol, sorbitol or mannitol [5–7]. Together with their intrinsic protective function as osmolytes, these polyols may also play other important metabolic roles, for instance as carbon sources, redox regulators or virulence factors in *in vivo* host-pathogen interactions [7–9].

C. albicans remains the most prevalent infectious fungus in humans and, consequently, systemic candidiasis represent a serious public health problem [10]. In this opportunistic pathogen yeast, the Hog1 MAP kinase plays a crucial role in cell protection against oxidative and osmotic aggressions as well as to thrive other physiological processes, which include cell wall biosynthesis, dimorphic conversion or virulence [4,11,12]. In turn, Hog1 is known to be required for endogenous glycerol production, but its involvement in the synthesis of other protective compounds; e.g. D-arabitol or trehalose seems more controversial [7,13]. Using an easy and reliable HPLC procedure, we demonstrate the selective and differential intracellular accumulation of protective solutes in *C. albicans*, which is specifically dependent on the type of stress applied (oxidative agents and salt). In addition, whereas osmotic stress-induced glycerol storage is strictly Hog1-dependent, the trehalose and D-arabitol synthesis induced by oxidative exposures is, at least in part, Hog1-independent.

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2. Materials and methods

2.1. Yeast strains and growth conditions

RM-100 (*ura3Δ::imm*^{434}/*ura3Δ::imm*^{434}*his1Δ::hisG*/*his1Δ::hisG-URA3-hisG*) was used as parental strain. The isogenic homozygous *hog1Δ*/*hog1Δ* mutant was obtained as reported elsewhere [11].

Unless otherwise stated, yeast cell cultures were grown at 37 °C with shaking in a medium consisting of 2% peptone, 1% yeast extract and 2% glucose (YPD). Strains were maintained at 4 °C by periodic subculturing on solid YPD. Usually, preinoculated overnight cultures were harvested, resuspended in fresh YPD and incubated further until they reached exponential phase (OD_{600} = 0.8–1.0). The growth was monitored turbidimetrically by measuring the OD_{600} of cultures or by direct cell counting with a haemocytometer; at least 200 cells were counted for each determination.

2.2. Oxidative and osmotic stress treatments

Cultures were grown in YPD and harvested in exponential phase (OD_{600} = 0.8–1.0) and then divided into several identical aliquots, which were treated with the indicated concentrations of oxidant agents (H_2O_2 and menadione) and salt (NaCl), or maintained without any exposure as a control and immediately incubated at 37 °C for one hour.

Viability was determined after appropriate dilution of the samples with sterile water by triplicate plating on solid YPD and further incubation for 48 h. Between 30 and 300 colonies were counted per plate. Survival was normalized to control samples (100% viability).

2.3. Extraction of trehalose and polyols and HPLC determinations

To measure the endogenous levels of trehalose and the polyols, D-arabitol and glycerol, accumulated during stress treatments, duplicate cell samples (20–30 mg, wet weight) were washed, resuspended in 1 ml water (milliQ) and boiled for two periods of 30 min with occasional shaking. The supernatants collected by centrifugation were frozen (−20 °C) until analysis.

HPLC analysis was performed using an Aminex HPX-87H column with a micro-guard cartridge (Bio Rad). Supernatants (20 l) were loaded and high-purity 1.5 mM sulphuric acid was utilised as eluent at a constant flow of 0.6 ml/min. Detection was carried out by RID. Analytical grade samples containing trehalose (Merck), glycerol and D-arabitol (Sigma) were loaded as internal standards, the corresponding retention times are shown in Fig. 2. For quantification of the osmolytes, a reference pattern with several concentrations of each compound was introduced. Prior to direct injection for HPLC analysis, the unfrozen supernatants were centrifuged (16,500×g, 5 min) to remove insoluble solids. In addition, trehalose was also measured with commercial trehalase (Sigma), following the procedure described elsewhere [14] and glycerol was also estimated with a commercial kit (Roche Diagnostics) following the manufacturer's manual.

3. Results and discussion

3.1. Effect of oxidative and osmotic stress on cell survival

Unlike the budding yeast *S. cerevisiae*, in which cell viability is very sensitive to treatment with mild doses of oxidants (e.g. 1–5 mM H_2O_2) [15,16], several independent genetic backgrounds of the dimorphic yeast *C. albicans* exhibit a noticeable intrinsic resistance to oxidative stress (10–25 mM H_2O_2 or 20 mM menadione, (MD) [2,6,14]. This might reflect its natural habitat as an opportunistic pathogen of human and warm-blooded organisms as hosts [17]. We have previously shown that the addition of acute (non-physiological) concentrations of oxidant compounds (50–100 mM H_2O_2 or 40 mM MD) is life-threatening for *C. albicans*, in which they cause a high degree of cell death [6,14]. In this infective yeast, the MAP kinase Hog1 pathway plays an essential role in the control of important physiological processes, including the protective responses against acute oxidative or osmotic stressors [11,12,14].

Nevertheless, the generally accepted importance of certain defensive responses against potentially lethal oxidative challenge could be questioned, since aerobic organisms are able to deal with the oxygen free radicals (ROS) generated inside the cell because of their respiratory metabolism and can withstand the damage provoked by external agents, with no irreversible degree of cell killing [1,11]. Therefore, the experimental application of potentially lethal oxidant challenges is precluded and other alternative conditions should be chosen that will allow evaluation of the induced protective responses against oxidative stress while having minor effects on cell survival. Here, we have designed a sublethal stress treatment consisting of exposure to a non-radical compound (5 mM H_2O_2) and to a generator of superoxide anions (10 mM MD), both of which cause a tolerable toxicity.

The results presented in Fig. 1 show that the survival of YPD-grown exponential blastoconidia from the parental strain RM-100 only suffered a minor loss after exposure to these oxidative challenges. This sensitive phenotype was much more striking in an isogenic homozygous *hog1*/*hog1* mutant (Fig. 1). An approximately 10-fold reduction in the doses of both oxidant agents was required to achieve to almost totally abolish the negative effect on cell viability, the percentage of survival being consistently lower in *hog1* cultures ([14], results not shown). In contrast to oxidative challenges, drastic osmotic exposures (1.0 M NaCl) provoked a slightly higher decrease in cell viability in RM-100 cells, but the homozygous *hog1Δ*/*hog1* mutant cells showed a marked phenotype of hypersensitivity to salt (Fig. 1). Our data provide additional support concerning the inability of different *hog1* backgrounds to efficiently overcome environmental aggressions [4,11,14].

3.2. Trehalose and polyols synthesis in response to stress

As a key marker of the general stress response in *C. albicans*, the intracellular accumulation of a set of protective molecules was

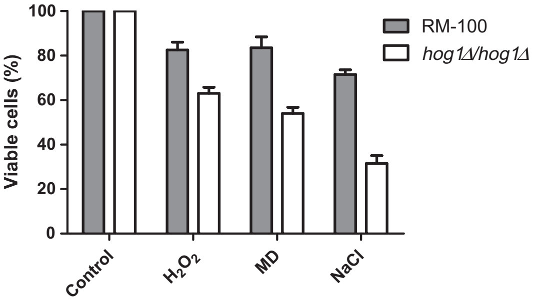

Fig. 1. Percentage of cell survival following different stress treatments in the *C. albicans* wild type RM-100 strain (parental) and its congenic *hog1Δ*/*hog1Δ* mutant, deficient in Hog1 MAP kinase pathway. Exponential YPD-grown cultures were adjusted to a cell density of 1.0 × 10^6–1.3 × 10^6 cells/ml and subjected to the following stress challenges for 1 h: 5 mM H_2O_2, 10 mM MD and 1 M NaCl. Identical, untreated samples were maintained at 37 °C as a control (panel A). Viability data were normalized with regard to the control measurement (100%) The experiment was repeated three times with consistent results and the values shown are the mean ± SD of two independent measurements. The distinction between the mean values shown are the mean ± SD.