Biochemical and Biophysical Research Communications 430 (2013) 1334–1339

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Ruth Sánchez-Fresneda [a], José P. Guirao-Abad [a], Alejandra Argüelles [a], Pilar González-Párraga [a], Eulogio Valentín [b], Juan-Carlos Argüelles [a,*]

[a] Área de Microbiología, Facultad de Biología, Universidad de Murcia, E-30071 Murcia, Spain
[b] Departamento de Microbiología y Ecología, Universidad de Valencia, E-46100 Burjassot, Valencia, Spain

ARTICLE INFO

Article history:
Received 24 October 2012
Available online 20 December 2012

Keywords:
HPLC
Trehalose
Glycerol
D-Arabitol
Stress
Candida albicans

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.

* Corresponding author. Address: Area de Microbiología, Facultad de Biología, Universidad de Murcia, Campus de Espinardo, E-30071 Murcia, Spain. Fax: +34 868 88 39 63.
E-mail address: arguelle@um.es (J.-C. Argüelles).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.10.118

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

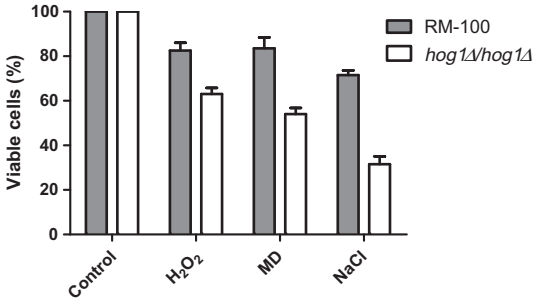

Fig. 1. Percentage of cell survival following different stress treatments in the *C. albicans* wild type RM-100 strain (parental) and its congenic *hog1Δ/hog1Δ* mutant, deficient in Hog1 MAP kinase pathway. Exponential YPD-grown cultures were adjusted to a cell density of 1.0×10^6–1.3×10^6 cells/ml and 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.

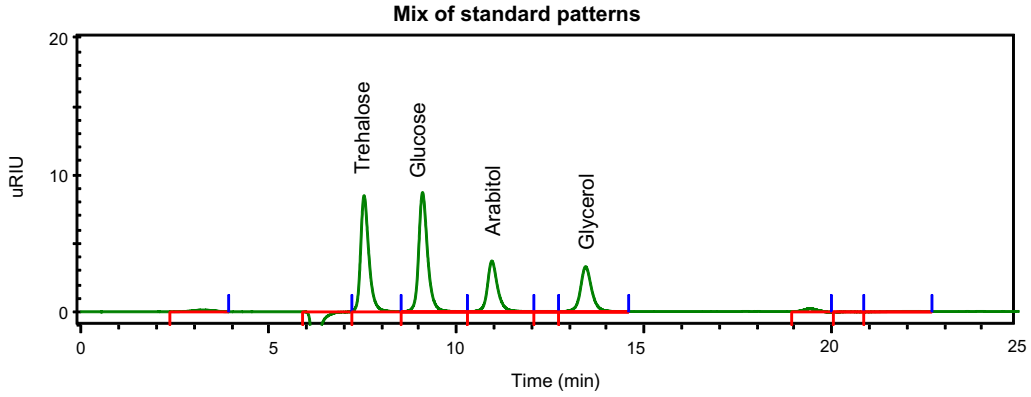

Fig. 2. HPLC analysis of a standard mixture containing commercial trehalose and the polyols D-arabitol and glycerol (analytical grade). A detailed report of the HPLC procedure is described in Section 2. The corresponding retention times (min.) were the following: trehalose (7.52), D-arabitol (10.93) and glycerol (13.43). Glucose (9.08), manitol and ethanol (not shown) were also resolved as single peaks. For quantitative measurements, several concentrations of each internal standard were loaded on the column.

determined alongside cell survival. For this purpose, we followed an experimental approach based on a reliable and highly-reproducible HPLC procedure that allows trehalose to be easily distinguished from glycerol (Fig. 2). The method also resolves as a single peak D-arabitol (retention time, 10.93; Fig. 2), which is another important osmolyte in *C. albicans* and other pathogenic yeasts [7,18]. Hypothetical contamination by glucose, resulting from glycogen or trehalose hydrolysis was excluded (Fig. 2), but in the tested experimental samples, another unknown compound eluted near D-arabitol (Figs. 2 and 3). As stated above, drastic oxidant concentrations (50 mM H_2O_2 or 40 mM MD) were not tested because they are known to cause severe cell mortality [6,14].

According to the results presented in Fig. 3, when RM-100 growing cells were subjected to external oxidative treatments with 5 mM H_2O_2 or 10 mM MD, they showed a significant synthesis of endogenous trehalose and an even more pronounced increase of D-arabitol (Fig. 3B and C) whereas the level of glycerol remained low or even decreased respect to control samples (Fig. 3B and C). Notably, the trehalose and D-arabitol increases promoted by MD were clearly higher compared than those recorded after hydrogen peroxide exposure (note the change of scale for *Y*-axis with MD; Fig. 3C). In turn, the osmotic/saline shock provoked by the addition of 1 M NaCl induced a minor drop in trehalose together with a much more marked reduction of the D-arabitol content (Fig. 3D), the opposite response to that recorded in the budding yeast *S. cerevisiae*, where trehalose accumulation in response to osmotic stimuli is a well established phenomenon [19]. This osmotic stress also triggered a 3-fold increase in the endogenous synthesis of glycerol (Fig. 3D) compared with the basal levels (Fig. 3A). These results strongly suggest that the intracellular accumulation of the protective osmolytes, trehalose, glycerol and D-arabitol, in *C. albicans* is regulated in a specific manner depending on the particular stress applied. Thus, trehalose is explicitly synthesized as a protectant against oxidative challenge and is irrelevant when the cells need to face sudden fluctuations of osmotic pressure [14]. The opposite mechanism seems to occur with glycerol responses (Fig. 3). Furthermore, our data also highlight the protective role of D-arabitol production in cells exposed to high temperatures or to H_2O_2, but not against NaCl or other saline treatments [7]. Quantitative data obtained from HPLC analysis and recalculated for trehalose and glycerol by means of enzymatic determinations are also presented in Table 1.

When similar experiments were carried out with exponential cells of the homozygous *hog1Δ/hog1* mutant, an oxidative stress-induced endogenous trehalose increase was recorded (Fig 4A and B). This response was more intense than that seen in parental cells was (Figs. 3 and 4, Table 1), which confirms that trehalose synthesis in *C. albicans* is independent of Hog1. As previously noted, the *hog1Δ* null mutant exhibited a higher basal level of the disaccharide [14] (Fig. 4, Table 1). However, in *hog1Δ* null cells, a marked rise of endogenous D-arabitol was also observed, which was clearly lower compared to the values recorded in parental cells (Figs. 3 and 4), although it should be noted that the D-arabitol basal level was also very low (compare Fig. 3A with Fig. 4A). As expected, in all the conditions examined, the intracellular content of glycerol was extremely low in *hog1Δ/hog1* cells (Fig. 4, Table 1), being almost at the limit of detection in the case of exposure to 10 mM MD (Fig. 4C, Table 1). Hence, while glycerol accumulation strictly requires a functional Hog1 pathway, as happens in *S. cerevisiae* [13,20], D-arabitol synthesis is partially independent of this MAP kinase [4,7,11,13]. As stated above, high concentrations of oxidants

Table 1
Effect of several oxidative and osmotic stress treatments on the intracellular synthesis of trehalose, D-arabitol and glycerol recorded in exponential cells of a parental strain (RM-100) and the isogenic Hog1-disrupted mutant (*hog1Δ/hog1Δ*). The cultures were grown on YPD, harvested in exponential phase (O.D. 0 0.8–1.0) and exposed to the indicated treatments for 1 h. A control sample was maintained at 37 °C. Trehalose and glycerol were measured following enzymatic protocols as reported in Section 2. D-arabitol values are expressed from quantitative control area recorded by the standard patterns measured in HPLC. The results are the average ± SD of three independent determinations.

Strain						
Treatment	RM-100			*hog1Δ/hog1Δ*		
	Trehalose[a]	D-arabitol[b]	Glycerol[b]	Trehalose[a]	D-arabitol[b]	Glycerol[b]
Control	4.1	52.8	12.6	5.9	7.3	1.9
5 mM H_2O_2	7.3	103.3	8.7	8.2	34.2	1.3
10 mM MD	13.59	125.7	1.1	25.2	67.9	<1.0
1 M NaCl	2.9	19.1	35.7	3.3	9.5	1.2

[a] nmoles/mg wet wt.
[b] μg/mg wet wt.

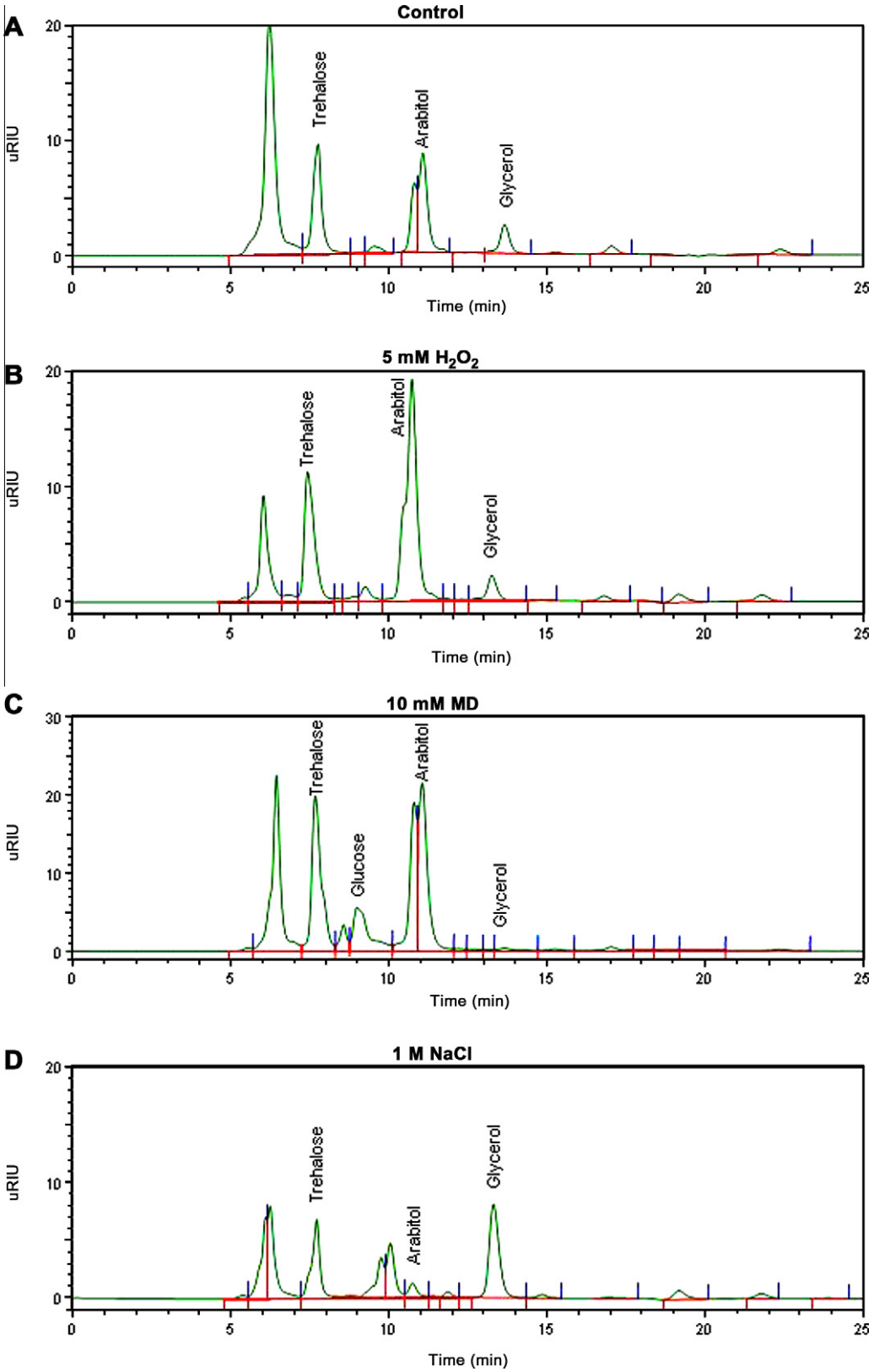

Fig. 3. Endogenous production of trehalose, D-arabitol and glycerol determined by HPLC in exponential cells of the wild type strain RM-100. Cultures were grown in YPD at 37 °C and subjected to the indicated stress exposures. The corresponding cell-free extracts were obtained and the content of trehalose and polyols analyzed by HPLC as reported in Section 2.

(50 mM H_2O_2 or 40 mM MD) were excluded because they cause severe cell mortality and would impede a rigorous HPLC analysis [14,21].

Taken together, these results support the existence in *C. albicans* of an elaborate and selective stress-responsive defensive mechanism, which controls the intracellular storage of the osmoprotec-

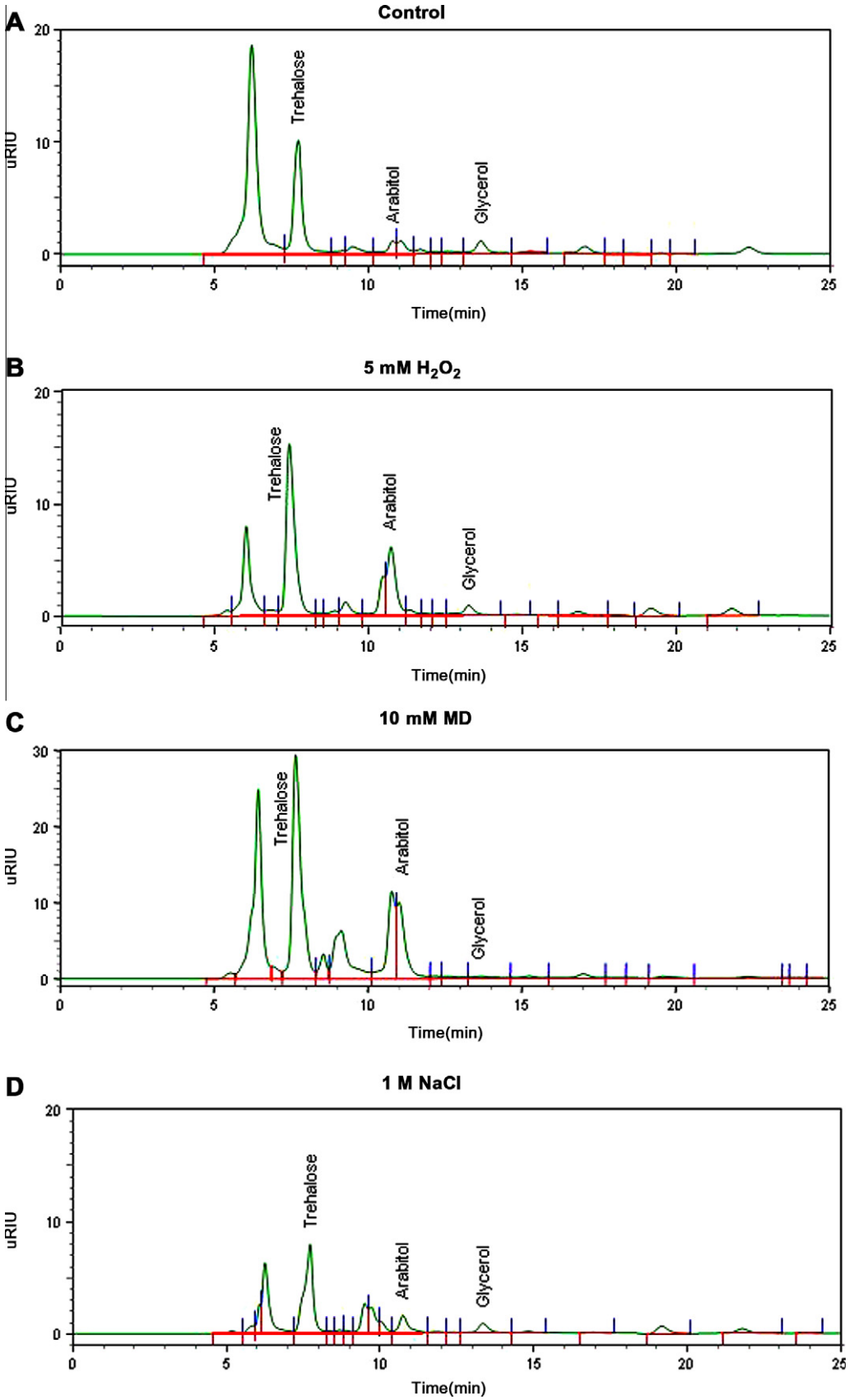

Fig. 4. HPLC analysis of the intracellular trehalose, D-arabitol and glycerol stored by the *hog1Δ* null mutant in response to oxidative and osmotic stress. The experimental conditions correspond to those reported in the Fig. 3.

tants, trehalose, glycerol and D-arabitol, as a function of the particular stress imposed. Thus, *C. albicans* exponential cells responded to oxidative exposure by increasing the intracellular concentration of trehalose and D-arabitol, whereas the levels of glycerol remain unaffected or even experienced a certain fell. The opposite metabolic situation occurred upon osmotic external stress. Concerning the regulatory pivotal role of the Hog1 pathway, our HPLC determinations clearly point to the need for glycerol synthesis, whereas D-arabitol appeared to have a partial Hog1-dependency and trehalose storage was totally independent of the Hog1 pathway.

Acknowledgments

We thank Dr. Jesus Plá for the kind provision of yeast strains used in this study and Dr. R. Alonso-Monge (Universidad Complutense, Madrid), for her critical reading of the manuscript. The experimental work was supported by Grants BIO-BMC 06/01-0003 from Dirección General de Investigación (Comunidad de Murcia, Spain) and UV-AE-10-24011 (University of Valencia, Spain). We are also indebted to the financial contract provided by Cespa, S.A. (Spain).

References

[1] P. Moradas-Ferreira, V. Costa, Adaptive response of the yeast *Saccharomyces cerevisiae* to reactive oxygen species: defences, damage and death, Redox Rep. 5 (2000) 277–285.
[2] P. González-Párraga, R. Sánchez-Fresneda, M. Martínez-Esparza, J.C. Argüelles, Stress responses in yeasts: what rules apply?, Arch Microbiol. 189 (2008) 293–296.
[3] M. Siderius, W.H. Mager, General Stress Response, Springer, New York, 1997.
[4] D.A. Smith, S. Nicholls, B.A. Morgan, A.J.B. Brown, J. Quinn, A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*, Mol. Biol. Cell 15 (2004) 4179–4190.
[5] L. Li, Y. Ye, L. Pan, Y. Zhu, S. Zheng, Y. Lin, The induction of trehalose and glycerol in *Saccharomyces cerevisiae* in response to various stresses, Biochem. Biophys. Res. Commun. 387 (2009) 778–783.
[6] F.J. Alvarez-Peral, O. Zaragoza, Y. Pedreño, J.C. Argüelles, Protective role of trehalose during severe oxidative stress caused by hydrogen peroxide and the adaptive oxidative stress response in *Candida albicans*, Microbiology 148 (2002) 2599–2606.
[7] G. Kayingo, B. Wong, The MAP kinase Hog1p differentially regulates stress-induced production and accumulation of glycerol and D-arabitol in *Candida albicans*, Microbiology 151 (2005) 2987–2999.
[8] R. Ansell, K. Granath, S. Hohmann, J.M. Thevelein, L. Adler, The two isoenzymes for yeast NAD-dependent glycerol-3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation, EMBO J. 16 (1997) 2179–2187.
[9] V. Chaturvedi, B. Wong, S.L. Newman, Oxidative killing of *Cryptococus neoformans* by human neutrophils. Evidence that fungal mannitol protects by scavenging reactive oxygen intermediates, J. Immunol. 156 (1996) 3836–3840.
[10] M.A. Pfaller, D.J. Diekema, Epidemiology of invasive candidiasis: a persistent public health problem, Clin. Microbiol. Rev. 20 (2007) 133–163.
[11] R. Alonso-Monge, F. Navarro-García, G. Molero, R. Diez-Orejas, M. Gustin, J. Pla, M. Sánchez, C. Nombela, Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*, J. Bacteriol. 181 (1999) 3058–3068.
[12] R. Alonso-Monge, F. Navarro-García, E. Román, A.I. Negredo, B. Eisman, C. Nombela, J. Plá, The Hog1 Mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*, Eukaryot. Cell 2 (2003) 351–361.
[13] C. San José, R. Alonso-Monge, R. Pérez-Díaz, J. Pla, C. Nombela, The mitogen-activated protein kinase homolog *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*, J. Bacteriol. 178 (1996) 5850–5852.
[14] P. Gonzalez-Parraga, R. Alonso-Monge, J. Pla, J.C. Argüelles, Adaptive tolerance to oxidative stress and the induction of antioxidant enzymatic activities in *Candida albicans* are independent of the Hog1 and Cap1-mediated pathways, FEMS Yeast Res. 10 (2010) 747–756.
[15] Y. Pedreño, J.V. Gimeno-Alcañiz, E. Matallana, J.C. Argüelles, Response to oxidative stress caused by H_2O_2 in *Saccharomyces cerevisiae* mutants deficient in trehalase genes, Arch. Microbiol. 177 (2002) 494–499.
[16] J.L. Parrou, M.A. Teste, J. François, Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose, Microbiology 143 (1997) 1891–1900.
[17] B. Enjalbert, D.M. McCallum, F.C. Odds, A.J.B. Brown, Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*, Infect. Immun. 75 (2007) 2143–2151.
[18] E.M. Bernard, K.J. Christiansen, S. Tsang, T.E. Kiehn, D. Armstrong, Rate of arabinitol production by pathogenic yeast species, J. Clin. Microbiol. 14 (1981) 189–194.
[19] E. Garre, R. Pérez-Torrado, J.V. Gimeno-Alcañiz, E. Matallana, Acid trehalase is involved in intracellular trehalose mobilization during postdiauxic growth and severe saline stress in *Saccharomyces cerevisiae*, FEMS Yeast Res. 9 (2009) 52–62.
[20] J. Albertyn, S. Hohmann, J.M. Thevelein, B. Prior, GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway, Mol. Cell. Biol. 14 (1994) 4135–4144.
[21] M. Martínez-Esparza, A. Tapia-Abellán, A. Vitse-Standaert, P. García-Peñarrubia, J.C. Argüelles, D. Poulain, T. Jouault, Glyconconjugate expression on the cell wall of *tps1/tps1* trehalose-deficient *Candida albicans* strain and implications for its interaction with macrophages, Glycobiology 21 (2011) 796–805.