Superoxide dismutases and glutaredoxins have a distinct role in the response of *Candida albicans* to oxidative stress generated by the chemical compounds menadione and diamide

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To cope with oxidative stress, Candida albicans *possesses several enzymes involved in a number of biological processes, including superoxide dismutases (Sods) and glutaredoxins (Grxs). The resistance of* C. albicans *to reactive oxygen species is thought to act as a virulence factor. Genes such as* SOD1 *and* GRX2*, which encode for a Sod and Grx, respectively, in* C. albicans *are widely recognised to be important for pathogenesis. We generated a double mutant, Δ*grx2/sod1*, for both genes. This strain is very defective in hyphae formation and is susceptible to killing by neutrophils. When exposed to two compounds that generate reactive oxygen species, the double null mutant was susceptible to menadione and resistant to diamide. The reintegration of the* SOD1 *gene in the null mutant led to recovery in resistance to menadione, whereas reintegration of the* GRX2 *gene made the null mutant sensitive to diamide. Despite having two different roles in the responses to oxidative stress generated by chemical compounds,* GRX2 *and* SOD1 *are important for* C. albicans *pathogenesis because the double mutant Δ*grx2/sod1 *was very susceptible to neutrophil killing and was defective in hyphae formation in addition to having a lower virulence in an animal model of systemic infection.*

Key words: *Candida albicans* - superoxide dismutases - glutaredoxins - phagocytosis - virulence

Candida albicans is a diploid yeast belonging to the human normal microbiota and may occur as a commensal organism of the oral cavity, gastrointestinal tract and vagina of healthy individuals (Colombo et al. 2007, Southern et al. 2008, Chaves et al. 2012). Several factors may trigger its transition from commensalism to infection. As a consequence, this harmless yeast may become pathogenic, predominantly depending on the host's immune status. Immunocompromised hosts, diabetic patients and pregnant women are the populations that are the most susceptible to candidiasis (Colombo et al. 2007).

Despite the fact that the human host plays the major role in the establishment of successful candidiasis, the fungus also possesses attributes of virulence. For *C. albicans*, its widely advocated virulence traits include the ability to adhere to host epithelial cells, which is the first step in the acquisition and maintenance of a stable yeast population in the oral cavity, the secretion of hydrolytic enzymes (proteinases and phospholipases), the ability to grow with different morphologies (blastoconidia, pseudo hyphae and true hyphae), i.e., because filamentation appears to be related to tissue invasion, biofilm formation and its resistance to the oxidative burst generated inside phagocytic cells (Karkowska-Kuleta et al. 2009, Chaves et al. 2012).

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In addition to combating reactive oxygen species (ROS) from their own metabolism, pathogenic organisms also react against the primary oxidative burst generated by macrophages and polymorphonuclear neutrophils (PMNs), which utilise the nicotinamide adenine dinucleotide phosphate oxidase present in the membrane to produce the superoxide anion (O_2^-) (Battistoni et al. 1998, Lamarre et al. 2001).

The role of the innate immune response in *C. albicans* is related to its recognition by dendritic cells, macrophages and neutrophils. This process is mediated by pattern recognition receptors involved in phagocytosis and the clearance of the pathogen (Gow et al. 2007, Taylor et al. 2007), such as Toll-like receptors and lectin receptors. These receptors recognise the pathogen-associated molecular patterns that are commonly found in a wide range of pathogens, but not in the mammalian host (Nicola et al. 2008). In response to ROS produced by phagocytic cells, *C. albicans* possesses several enzymes involved in a number of biological processes, such as superoxide dismutases (Sods), catalases (Cats), thioredoxin (Trx) peroxidases (Prxs) and glutaredoxins (Grxs) (Chaves et al. 2007, Tosello et al. 2007).

Several genes involved in the oxidative stress response in *C. albicans* have been recognised as virulence factors. For example, the disruption of the *CAT1* gene in this yeast led to an increased sensitivity to hydrogen peroxide (H_2O_2) and an attenuation of virulence in an animal model (Wysong et al. 1998).

In addition, *C. albicans* possesses six different genes that encode for Sods. These enzymes possess antioxidant properties that catalyse the dismutation of O_2^- into molecular oxygen and H_2O_2, which can then be detoxified by

Cat (Chaves et al. 2007), thereby scavenging the toxic effects of O_2 (Frohner et al. 2009). These Sods include cytoplasmic Sod1 and Sod3, mitochondrial Sod2 and the cell surface GP-anchored Sod4, Sod5 and Sod6 (Martchenko et al. 2004, Frohner et al. 2009, Bink et al. 2011).

The cytosolic Cu/Zn SOD encoded by *SOD1* is important for virulence in *C. albicans*. The *C. albicans* Δ*sod1* mutant has been shown to be attenuated in virulence in an animal model of systemic infection (Hwang et al. 2002, Chaves et al. 2007) and more susceptible to macrophage (Hwang et al. 2002) and PMN killing (Chaves et al. 2007). The mitochondrial Sod2 of *C. albicans* has been ruled out as a putative virulence factor, but the Δ*sod2* mutant is more sensitive to heating, ethanol and high concentrations of salts, indicating no direct role in virulence, but a function in different environmental stresses (Hwang et al. 2003).

SOD5 expression is increased in *C. albicans* yeast cells exposed to PMNs (Fradin et al. 2005) and during morphological change from yeasts to hyphae (Nantel et al. 2002). However, while *sod1*Δ is hypersensitive to killing by a macrophage cell line in vitro (Hwang et al. 2002), the same was not observed for *sod5*Δ (Martchenko et al. 2004), indicating that the virulence role of Sod5 may be due to a resistance to different phagocytic cells.

Frohner et al. (2009) recently demonstrated that the Sod4 and Sod5 produced by innate immune response cells are extremely important as ROS scavengers. When both the *SOD4* and *SOD5* genes were disrupted in *C. albicans,* the double mutant generated (Δ*sod4*/s*od5*) was extremely susceptible to in vitro macrophage killing (Frohner et al. 2009).

Grx are cytosolic enzymes that act as thiol oxidoreductases and are responsible for the reduction of protein disulphides or glutathione (GSH)-protein mixed disulphides. This reaction also involves cysteine residues from the enzyme's active site (Herrero et al. 2008). Chaves et al. (2007) demonstrated that a mutant lacking the *GRX2* gene, which encodes a Grx in *C. albicans*, was deficient in hyphae formation and more susceptible to PMN killing. This Δ*grx2* mutant was also attenuated in virulence in a murine model of systemic infection and susceptible to menadione, but resistant to diamide.

Srinivasa et al. (2012) recently characterised a Prx in *C. albicans* that reduces both H_2O_2 and tert-butyl hydroperoxide (t-BOOH). The deletion of the *PRX* gene led to a mutant that was virtually insensitive to H_2O_2, diamide and menadione, but susceptible to low concentrations of t-BOOH, indicating different roles for genes related to oxidative stress and induced by chemical compounds.

Some genes related to oxidative stress are also regulated during hyphae formation and appear to be somehow related to *C. albicans* morphogenesis (Hwang et al. 2002, Nantel et al. 2002, Martchenko et al. 2004, Chaves et al. 2007). In addition, Schroter et al. (2000) analysed the amount of ROS produced when cells were incubated in 10% serum. Those authors found that ROS were produced in large amounts in a strain that formed true hyphae. Recently, it has been demonstrated that hyphal differentiation is induced by a subtoxic concentration of exogenous H_2O_2 in *C. albicans* (Nasution et al. 2008).

Younes et al. (2011) reported that the *HWP2* gene, which is involved in morphogenesis, adherence and biofilm formation in *C. albicans*, is also required to tolerate oxidative stress because the mutant Δ*hwp2* is susceptible to H_2O_2 compared with the wild-type strain.

As mentioned, previous studies have demonstrated that the *SOD1* gene and *GRX2* gene play a role in the virulence of *C. albicans* (Hwang et al. 2002, Chaves et al. 2007). When separately disrupted, these genes led to an attenuation of virulence in an animal model of systemic infection. Furthermore, the disruption of these genes, particularly the *GRX2* gene, appears to influence morphogenetic development. Other phenotypic changes were found between the single mutants Δ*sod1* and Δ*grx2*, such as different responses to oxidative stress induced by menadione and diamide induction. We hypothesised that the disruption of two of the oxidative stress genes would alter virulence and/or interfere with other *C. albicans* phenotypic properties. Therefore, we generated the double mutant Δ*sod1*/*grx2* and re-integrated each of these genes separately, creating the Δ*sod1*/*grx2*+*GRX2* and Δ*sod1*/*grx2*+*SOD1* reintegrant strains.

MATERIALS AND METHODS

Strains, media and culture conditions - All *C. albicans* strains used and constructed in this study are listed in Table I. These strains were stored at -80ºC in 20% (v:v) glycerol and later subcultured on Sabouraud agar. The following media were routinely used to grow the fungi: nutrient broth + glucose + YE (NGY) broth: 1 g/L neopeptone (Difco, Detroit, MI, USA), 4 g/L glucose and 1 g/L yeast extract; yeast extract-peptone-dextrose (YPD) broth: 10 g/L yeast extract, 20 g/L glucose and 20 g/L mycological peptone (Oxoid, Basingstoke, UK); SD medium: 6.7 g/L yeast nitrogen base with ammonium sulphate and without amino acids (Difco) and 20 g/L glucose, with 25 µg/mL uridine added as required.

Construction of the grx2Δ null mutant and reintegrant strain - All primers used in this study are listed in Table II. To make the double mutant strains, the *GRX2* and *SOD1* genes were disrupted using the ura-blaster method (Fonzi & Irwin 1993). To achieve this objective, the *GRX2* gene was disrupted in the Δ*sod1* mutant. The Ura-Δ*sod1* mutant was kindly provided by Dr Sa-Ouk Kang (Laboratory of Biophysics, Seoul National University, Republic of Korea). The *GRX2* disruption cassette pGC4, which was previously generated (Chaves et al. 2007), was released by digestion with *Hin*dIII and *Ban*II and *GRX2* was disrupted by sequential rounds of transformation into strain Δ*sod1*. The *URA3* marker was recycled by selection on SD medium plus 5-fluoroorotic acid (1 mg/mL) and uridine (50 µg/mL). Gene disruption was confirmed by polymerase chain reaction (PCR). To avoid potential problems associated with ectopic expression of *URA3* (Brand et al. 2004), the Ura-Δ*sod1*/*grx2* mutant was transformed with *Stu*I-digested CIp10 plasmid (Murad et al. 2000), thus ensuring *URA3* expression at the neutral *RPS1 locus* (orf19.3002).

As a control, each of these genes was then individually reintegrated. The Δ*sod1*/*grx2*+*GRX2* mutant was con-

TABLE I

Candida albicans mutant strains

Strain	Parent strain	Genotype	Source
NGY 152	CAI4	As CAI4 but *RPS1*/*rps*1Δ::CIp10	Brand et al. (2004)
CH 104	CH 103	As CAI4 but *sod1*Δ::hisG/*sod1*Δ::hisG	Hwang et al. (2002)
GCY 209	CH 104	As CAI4 but *sod1*Δ::*hisG/sod1*Δ::*hisG, GRX2/grx2*Δ::*hisG-URA3-hisG*	This paper
GCY 210	GCY 209	As CAI4 but *sod1*Δ::*hisG/sod1*Δ::*hisG, GRX2/grx2*Δ::*hisG*	This paper
GCY 211	GCY 210	As CAI4 but *sod1*Δ::*hisG/sod1*Δ::*hisG, GRX2/grx2*Δ::*hisG*, *RPS1*/*rps1*Δ::CLp10	This paper
GCY 212	GCY 210	As CAI4 but *sod1*Δ::*hisG*/*sod1*Δ::*hisG, grx2*Δ::*hisG/grx2*Δ::*his-URA3-hisG*	This paper
GCY 213	GCY 212	As CAI4 but *sod1*Δ::*hisG/sod1*Δ::*hisG, grx2*Δ::*hisG/grx2*Δ::*hisG*	This paper
GCY 214	GCY 213	As CAI4 but *sod1*Δ::*hisG/sod1*Δ::*hisG, grx2*Δ::*hisG/grx2*Δ::*hisG, RPS1/rps1*Δ::CIp10	This paper
GCY 215	GCY 213	As CAI4 but *sod1*Δ::*hisG/sod1*Δ::*hisG, grx2*Δ::*hisG/grx2*Δ::*hisG, RPS1/rps1*Δ::CIp10-*GRX2*	This paper
GCY 216	GCY 213	As CAI4 but *sod1*Δ::*hisG/sod1*Δ::*hisG, grx2*Δ::*hisG/grx2*Δ::*hisG, RPS1/rps1*Δ::CIp10-SOD1	This paper

TABLE II

Primers used in this study

Primer	Sequence (5'-3')	Target for amplification
GRX2SF	GAGAAAGATCGTGGATTTGG	*GRX2* screening forward in the upstream region
GRX2SR	CAATACCATTCTGACCAACC	*GRX2* screening reverse in the downstream region
RP10-GS	TACATTCCTACTCCGTTCG	*RP1* sequence in CAI4 genome
CIp-10	GATATCGAATTCACGCGTAG	CIp10 plasmid
SOD1SF	AGATCTGCAACAACCAATAGGTAAACGC	*SOD1* promoter + Bg II restriction site
SOD14 rev	AGATCTGGTTTAGGCTTAAGCTGTAG	*SOD1* terminator + Bg II restriction site

structed by transforming the Δ*sod1*/*grx2* mutant with the reintegrant cassette pGC6, which was previously generated (Chaves et al. 2007). The pGC6 plasmid insert was subcloned into the *Not*I site of CIp10. The resulting plasmid was digested with *Stu*I and then transformed into the Ura-*Δsod*1/*grx2* null mutant. To generate the Δ*sod1*/*grx2*+*SOD1* mutant, a new reintegrant cassette, pGC8, was created. Therefore, the *SOD1* open-reading frame plus 938 bp of its promoter and 404 bp of its terminator was amplified by PCR (primers SOD1SF and SOD14 rev) and the product was cloned into pGEM-T Easy (Promega Ltd, Southampton, UK). The plasmid insert was subcloned into *the Not*I site of CIp10 and the resulting plasmid was digested with *Stu*I and transformed into the Ura-*Δsod*1/*grx2* null mutant.

C. albicans growth rate determination - Growth rates were measured in duplicate. A volume from an overnight culture in NGY was transferred to YPD broth or RPMI-1640 (Gibco, Paisley, UK) to give an initial optical density $(OD)_{600}$ nm = 0.05 and the culture was incubated at 37ºC with rotation at 200 rpm in a gyratory shaker. Growth was measured using the OD_{600} nm vs. a medium blank. Maximum growth rates were determined from the logarithms of values taken in the exponential phase.

Tests for hyphal formation - For the induction of hyphal formation on solid media, the cells were grown in NGY, centrifuged and washed three times in water. From a suspension adjusted to 10^9 cells/mL, 5 µL was spotted on the surface of Spider (Liu et al. 1994) and GlcNAc agars (20 g/L *N*-acetyl-D-glucosamine, 6.7 g/L yeast nitrogen base, 16 g/L micro agar) in triplicate. The plates were incubated for seven days at 30ºC.

For tests in liquid media, an adaptation of Odds et al. (2000) was used. Therefore, the cells were grown, washed and standardised to an initial concentration of 10^6 cells/mL in 10% foetal calf serum (FCS), YPD + 10% FCS and GlcNAc pre-warmed to 37ºC and incubated at the same temperature with gyratory shaking (9,200 rpm). After 1 and 3-h incubation periods, culture samples were mixed with an equal volume of 10% formaldehyde to arrest further development. The 1-h sample was examined microscopically to determine the percentage of cells bearing evaginations. The 3-h sample was examined to approximate the mean morphology index (MI) (Merson-Davies & Odds 1989), in which a value close to 1 indicates a population of spheroidal yeast cells and value close to 4 indicates a population of true hyphal cells. Values between 1-4 indicate mixed or pseudohyphal morphologies (Chaves et al. 2007).

Sensitivity of C. albicans to oxidative stress inducers - The method of Izawa et al. (1995) was used with some modifications to determine the sensitivity of *C. albicans* to oxidative stress inducers. Yeast cells grown in NGY were standardised to 2×10^7 cells/mL. Five-microlitres volumes of a 10-fold dilution series prepared from this suspension were spotted on the surface of YPD agar plates containing concentrations (from 0.5-2.5 mM) of menadione and diamide. The plates were incubated for 48 h at 30ºC. Alternatively, 50-µL volumes of NGY-grown yeast cells were transferred to 5-mL YPD broth with and without the addition of menadione or diamide (0.5-2.0 mM) and incubated in a rotator wheel for 16 h at 30ºC. The OD_{600} nm was determined for the control and test tubes. Sensitivity to the compounds was measured by examining growth in the presence of compounds as a percentage of control growth.

The susceptibility of C. albicans killing by PMN - PMN freshly isolated from blood samples of healthy volunteers on the day of the experiment (Fradin et al. 2005) was suspended in Eagle's minimal essential medium + 20 mM HEPES (pH 7.2) and standardised to 8×10^5 PMN/mL. *C. albicans* cells grown overnight in NGY were centrifuged, washed three times in saline and resuspended at 5×10^6 yeast cells/mL in HEPES-buffered Eagle's minimal essential medium containing one-tenth of a volume of fresh human plasma. Equal volumes of PMN and yeast cell suspensions were mixed and incubated at 37ºC for 1 h with rotation at 50 rpm. Control suspensions contained *C. albicans* without PMN. The suspensions were centrifuged and the pellets were resuspended in water to lyse the PMN. After three cycles of washing and resuspension, viable *C. albicans* cell counts were determined by plating a 10-fold dilution series on YPD agar. Yeast cell susceptibility was determined as the mean difference between viable counts in the presence and absence of PMN. The assays were performed in triplicate (Chaves et al. 2007).

Virulence of C. albicans in the murine IV challenge model - Immunocompetent female BALB/c mice with a weight range from 17-23 g were supplied with food and water *ad libitum*. The mice were intravenously inoculated with *C. albicans* strains that had been grown overnight in NGY, washed and resuspended in saline. The inoculum was standardised to allow for an injection of 2-3 × 10^4 colony-forming unit/g mouse body weight. The mice were examined daily. Animals that displayed a loss of body weight > 20% or signs of serious illness were humanely terminated and survival curves were performed.

Statistical analysis - Means ± standard deviations were determined from the results of at least three independent experiments. Differences between values for phenotypic tests were analysed using Student's *t* test. Animal survival data were compared with Kaplan-Meier/LogRank statistics. $p < 0.05$ were considered significant for all comparisons.

RESULTS

Gene disruptions and reintegrations - The Δ *sod1*/*grx2* mutant (GCY 213) was successfully generated by deleting 1,583 bp from the central region of the *GRX2* gene in the Δ *sod1* mutant (GCY 200) using the "Ura-blasting" technique (Fonzi & Irwin 1993) (Fig. 1A). The *URA3* gene was reintegrated at the neutral *RPS1 locus* to avoid ectopic *URA3* expression problems (Brand et al. 2004), creating the mutant GCY 214. The *GRX2* gene and *URA3* were integrated at the *RPS1* l*ocus* of the Δ *sod1*/*grx2* null mutant to generate the Δ *sod1*/*grx2* + *GRX2* reintegrant (GCY 215) (Fig. 1B). In the same manner, the *SOD1* gene and *URA3* were integrated at the *RPS1 locus* of the Δ *sod1*/*grx2* null mutant to generate the Δ *sod1*/*grx2* + *SOD1* reintegrant (GCY 216) (Fig. 1C).

Virulence phenotypic properties of the mutants in vitro - The growth rates of the yeast cell null double mutant GCY 214 and the *GRX2* and *SOD1* reintegrant strains (GCY 215 and GCY 216, respectively) were similar to that of the wild-type, NGY 152.

Hyphae formation of the mutants on solid and liquid media - The colony morphologies on Spider, YPD + 20% serum and GlcNAc agars were slightly different for the double mutant compared with NGY 152. Specifically on the Spider medium, the mutants formed a more wrinkled and less filamentous form (Fig. 2). During the first hour of incubation, the double mutant Δ*sod1*/*grx2* (GCY 214) and double mutant reintegrant strains Δ*sod1*/*grx2*+*GRX2* and Δ*sod1*/*grx2*+*SOD1* (GCY 215 and GCY 216, respectively) demonstrated an even more noticeable delay in their ability to evaginate than the single mutants Δ*sod1* and Δ*grx2* (previously generated) (Chaves et al. 2007). The heterozygote for the double mutant Δ*sod1*/*grx2/GRX2* (GCY 211), double mutant GCY 214 and re-

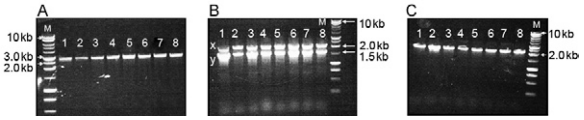

Fig. 1: *Candida* colony diagnostic polymerase chain reaction. A: generation of Δ*sod1/grx2* null mutant strain (demonstrated with GRX2SF and GRXSR primers). All colonies represent transformants after post-5FOA step (expected band size 2.6 kb); B: generation of a *GRX2* reintegrant in the Δ*sod1/grx2* background checked with GRX2 SF and GRX2 SR primers [x: *RPS1 locus* allele (1.9 kb); y: *GRX2* disrupted alleles (1.5 kb)]; C: generation of a SOD1 reintegrant in the Δ*sod1/grx2* background checked with SOD1 SF and SOD 14 rev primers (expected band size 2.1 kb); M: 1 kb ladder.

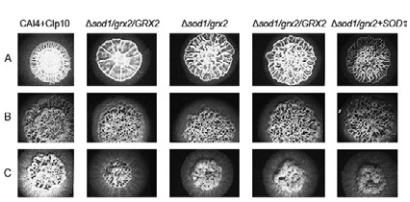

Fig. 2: hyphae formation on solid media. A: Spider medium; B: yeast extract-peptone-dextrose + 10% serum; C: GlcNac. Plates were incubated in triplicates at 30ºC for seven days.

integrant strains GCY 215 and GCY 216 did not evaginate during the first hour of incubation in the YPD + 10% serum. When grown in 10% serum in water (the most potent germ tube inducer), all of the strains had fewer than 20% evaginating cells, a very different result compared with the control strain NGY 152, in which nearly 100% of the cells evaginated during the first hour of incubation. Evagination in GlcNAc was also less evident in the double mutant strain series than the single mutants and both had fewer cells evaginating than the control strain NGY 152. The disruption of both genes appeared to have a stronger effect on early germ tube formation compared with when only one gene of each was disrupted (Fig. 3).

After 3 h of incubation, the MI showed noticeable differences for the double mutant strain series. The mean MI was less than 2.0 for all strains in the YPD + 10% serum and the double mutant GCY 214 had an MI close to 1.0, indicating a lack of ability to form hyphae under this condition. All of the mean MIs were significantly lower than the control strain NGY 152 ($p < 0.05$). In addition, the MI was lower for the double mutant strain series compared with NGY than the single mutants when the cells were induced to form hyphae in the presence of 10% serum (Chaves et al. 2007). The double mutant strain series formed fewer true hyphae, with a mean MI in the range of 3.5-3.7. Although this difference was not large, it was still statistically significant ($p < 0.05$). When cells were grown in GlcNac, the mean MI was approximately 2.0 or below for all of the double mutant strain series (Fig. 4).

Sensitivity to oxidative stress inducers - The heterozygous GCY 211, double mutant GCY 214 and *GRX2* reintegrant GCY 215 were sensitive to menadione. The reintroduction of the *SOD1* gene restored resistance to this chemical compound in the *SOD1* reintegrant strain, GCY 216 (Fig. 5). Disruption of one copy of the *GRX2* gene in the single Δ*sod1* mutant (GCY200) did not lead to any difference because the heterozygous strain Δ*sod1*/*grx2/GRX2* (GCY 211) was still sensitive to 2.5 mM diamide. Following the same trend, as previously observed (Chaves et al. 2007), the knockout of both copies of the *GRX2* gene (generating GCY 214) led to a phenotype resistant to diamide. The reintroduction of one copy of *GRX2* (generating GCY 215; Δ*sod1*/*grx2*+*GRX2*) led this double mutant reintegrant to become sensitive again, similar to the heterozygous Δ*sod1*/*grx2/GRX2* (GCY 211). In the same manner, the introduction of the *SOD1* gene, creating Δ*sod1*/*grx2*+*SOD1* (GCY 2160), maintained the same diamide-resistant phenotype when both copies of the *GRX2* genes were disrupted (Fig. 6). We reproduced the same results obtained for the tests in liquid media by using agar plates with different concentrations of menadione and diamide (Fig. 7).

PMN killing assays of the mutants - Interactions with human peripheral PMNs revealed that the double mutant strain series was more susceptible to killing than the control strain NGY 152. After an incubation period of 1 h at 37ºC in the presence (test) or absence of PMNs (control) and incubating the *C. albicans* cells at 30ºC on YPD agar plates, the heterozygous double mutant strain Δ*sod1*/*grx2/GRX2* (GCY 211), double mutant null Δ*sod1*/*grx2* (GCY 214) and Δ*sod1*/*grx2*+*SOD1* (GCY 216) reintegrant strains were more susceptible to PMN killing

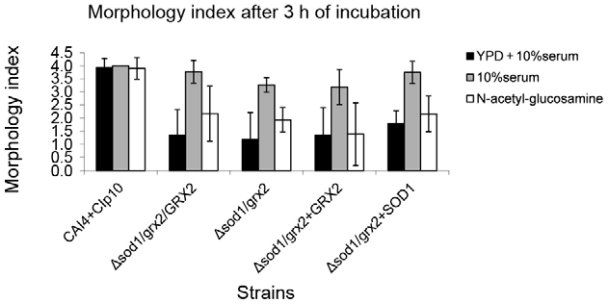

Fig. 4: morphology index (MI) the oxidative stress double mutant strain series cells after 3 h of incubation in liquid media at 37ºC, 200 rpm. Bars represent variation on MI of cells. For all the mutants in all conditions tested, $p < 0.05$ relative to CAI4+CIp10. YPD: yeast extract-peptone-dextrose.

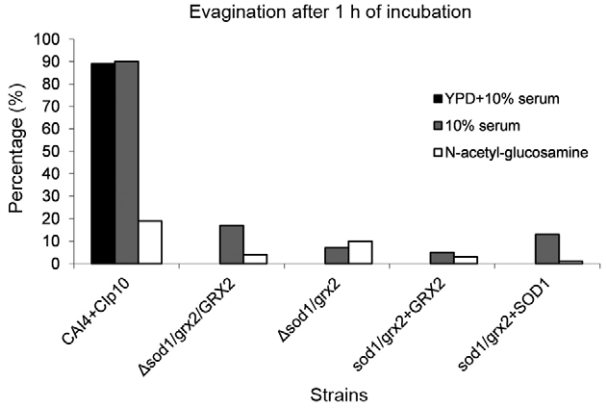

Fig. 3: percentage of evagination in the oxidative stress double mutant strain series cells after 1 h of incubation in liquid media at 37ºC, 200 rpm. YPD: yeast extract-peptone-dextrose.

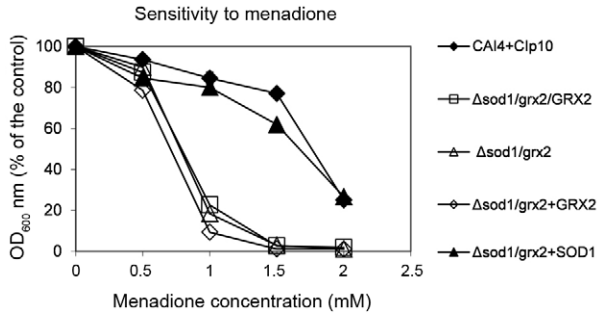

Fig. 5: sensitivity to menadione in liquid medium (0.5-2.0 mM). The tubes were incubated at 30ºC in a rotator wheel for 16 h. Growth optical density (OD) 600 nm was expressed as a percentage of control growth. Bars represent standard deviations for three independent experiments.

($p < 0.05$). However, only the Δ*sod1*/*grx2*+*GRX2* (GCY 215) mutant cells were less efficiently killed by the PMNs after 1 h (Fig. 8). This number is closer to the findings for the single mutant killings (Chaves et al. 2007).

Virulence of the mutants in an animal model of systemic infection - The virulence of the double mutant strain series did demonstrate some degree of attenuation by disruption of the two genes related to oxidative stress in *C. albicans*. Survival curves demonstrated that the double null mutant Δ*sod1*/*grx2* (GCY 214) and reintegrant strain Δ*sod1*/*grx2*+*SOD1* (GCY 216) were the most attenuated in virulence (40% and 50% survivors at the end of the experiment, respectively; $p < 0.05$). The reintegrant strain Δ*sod1*/*grx2*+*GRX2* (GCY 215) had a survival curve comparable to the control strain NGY152, demonstrating some degree of regaining virulence when the *GRX2* gene was reintegrated (Fig. 9).

DISCUSSION

In the present study, we evaluated the role of two different genes related to oxidative stress in *C. albicans*. The first, the *GRX2* gene, encodes a Grx and the other, the *SOD* gene, encodes a copper and zinc-containing SOD. We hypothesised that the disruption of two genes involved in oxidative stress would lead to a strain with an increased attenuation in virulence and higher degree of susceptibility to chemical compounds that induce oxidative stress.

The ability to combat ROS is vital for microbial pathogenesis to either cope with ROS generated as a consequence of their own metabolism or escape from human phagocytic cells (Cabiscol et al. 2000, Lamarre et al. 2001, Hwang et al. 2002, 2003, Martchenko et al. 2004, Gonzalez-Parraga et al. 2005). Some chemical compounds have been used to induce oxidative stress in microorganisms, such as menadione and diamide.

Oxidative stress may be generated by the addition of menadione (2-methylnaphthalene-1,4-dione), a polycyclic aromatic ketone commonly used in yeast studies (Grant 2001, Osorio et al. 2003), which accepts an electron from a respiratory carrier and transfers it to molecular oxygen (a redox cycling agent), stimulating superoxide formation without inhibiting the respiratory chain (Halliwell & Gutteridge 2007). Diamide is a thiol-specific oxidant that can readily oxidise GSH, leading to molecular cross-linking (Hwang et al. 2003, Herrero et al. 2008).

As previously observed by Hwang et al. (2002) and then by Chaves et al. (2007), the Δ*sod1* mutant is sensitive to menadione. The Δ*grx2* mutant is less sensitive to this chemical compound than the mutant Δ*sod1* and very resistant to diamide (Chaves et al. 2007). The idea of specific roles for oxidative stress genes in *C. albicans* was further reinforced when the double mutant strain

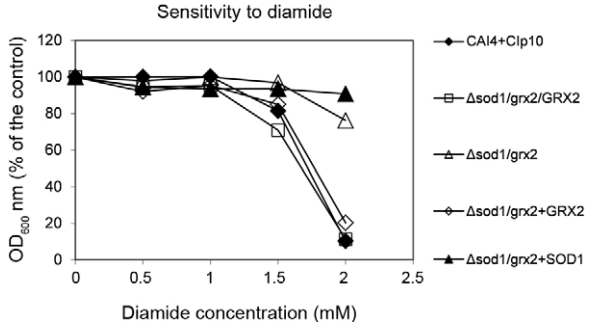

Fig. 6: sensitivity to diamide in liquid medium (0.5-2.0 mM). The tubes were incubated at 30ºC in a rotator wheel for 16 h. Growth optical density (OD) 600 nm was expressed as a percentage of control growth. Bars represent standard deviations for three independent experiments.

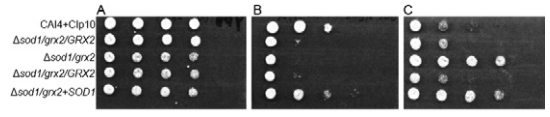

Fig. 7: sensitivity to diamide in liquid medium (0.5-2.0 mM). The tubes were incubated at 30ºC in a rotator wheel for 16 h. Growth optical density (OD) 600 nm was expressed as a percentage of control growth. Bars represent standard deviations for three independent experiments.

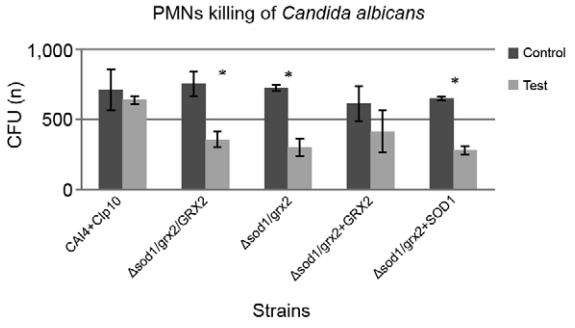

Fig. 8: oxidative stress double mutant strain series killing by polymorphonuclear neutrophils (PMNs). Cells were incubated in triplicate for 1 h in the presence of PMNs and colony-forming unit (CFU) were determined after three days of incubation of viable cells. Reduction in mean CFU of yeasts exposed to PMNs was expressed as a percentage of mean CFU for control suspensions.

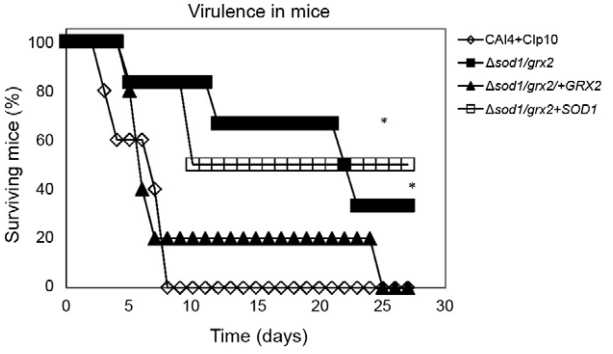

Fig. 9: survival of groups of six mice challenged IV with 5.7 x 104 colony-forming unit/g of *Candida albicans* null mutant strains. All the strains except Δ*sod1*/*grx2*+*GRX2* were attenuated in virulence when compared to the control strain CAI4+CIp10. Asterisks mean $p < 0.05$.

series was tested for susceptibility to oxidative stress generators in the present study. When one copy of *GRX2* was disrupted in the Δ*sod1* background, generating the heterozygous strain Δ*sod1*/*grx2*/*GRX2* (GCY 211), this strain remained sensitive to menadione. When both copies of *GRX2* were disrupted, generating the double mutant Δ*sod1*/*grx2* (GCY 214), the phenotype remained the same. Furthermore, the reintegration of one copy of *GRX2*, generating the Δ*sod1*/*grx2*+*GRX2* (GCY 215) mutant, had no effect, confirming that the response of *GRX2* is minimal when the stress generated is due to the generation of intracellular O_2^- (menadione). This hypothesis was conclusive because the reintegration of only one copy of *SOD1* (generating the Δ*sod1*/*grx2*+*SOD1* mutant) conferred resistance to menadione.

Another completely different response was observed in the double mutant strain series when they were grown with diamide. The deletion of one copy of *GRX2* in the Δ*sod1* background (generating the Δ*sod1*/*grx2*/*GRX2* mutant) was insufficient to lead to resistance to diamide. The deletion of two copies of the gene, generating the double mutant Δ*sod1*/*grx2* (GCY 214), led to resistance, as was observed for the Δ*grx2* mutant (Chaves et al. 2007). This result confirms that *SOD1* plays no role in combating the oxidative stress generated by diamide because the reintroduction of *GRX2* (generating the Δ*sod1*/*grx2*+*GRX2* mutant) again rendered this mutant strain sensitive to diamide. The Δ*sod1*/*grx2*+*SOD1* mutant was resistant to diamide, indicating that the reintroduction of *SOD1* did not have an effect, confirming again that *GRX2* and not *SOD1* is involved in the resistance to the oxidative stress generated by diamide. Distinct roles for menadione and diamide in generating oxidative stress have also been observed with *Aspergillus nidulans* (Pocsi et al. 2005).

Herrero et al. (2008) demonstrated that Sods are particularly related to ROS detoxification and Grx in the control of the redox state of protein sulfhydryl groups in *Saccharomyces cerevisiae*. Furthermore, the *Candida* genome database (candidagenome.org/) currently describes four Grx (*GRX1*, *GRX2*, *GRX3* and *GRX5*) and two thioredoxins (*TRX1* and *TRX2*) for this species, further reinforcing that resistance to diamide is related to a compensatory mechanism for other Grx (or even thioredoxins) in *C. albicans* because these other genes might be up-regulated to act as thiol oxidoreductases responsible for reducing the protein disulphides or GSH-protein mixed disulphides generated during diamide-induced oxidative stress. Conversely, Luikenhuis et al. (1998) found different functions for defences against diamide in *S. cerevisiae* that were dependent on the growth phase. In exponential phase cells, the lack of both Grx resulted in a resistance to diamide, whereas in stationary phase cells, the deletion of *GRX1* alone led to resistance, suggesting that a complexity of mechanisms for different Grx in *C. albicans* may exist.

Regarding hyphae formation, the double mutant Δ*sod1*/*grx2* had a markedly lower mean MI when grown in YPD + 10% serum, 10% serum and GlcNac. Gonzales-Parraga et al. (2005) investigated a possible relationship between levels of GSH and oxidised GSH (GSSH) and germ tube formation induced by serum in *C. albicans*, specifically during the first hour of incubation. The levels of GSH decreased, while the levels of GSSH remained unmodified. Grx are responsible for reducing GSSH to GSH. Therefore, the lack of Grx (due to *GRX2* disruption) and GSSH accumulation may partially explain the inability to form hyphae in the double mutant, i.e., due to ROS accumulation. *SOD5* has been demonstrated to relate to hyphae formation by transcript profiling; i.e., this gene is up-regulated during the process of hyphae formation (Nantel et al. 2002) Furthermore, Martchenko et al. (2004) demonstrated with Northern analysis that *SOD5* expression during hyphae formation is due to the presence of serum. *SOD1* is also transcribed in the presence of serum (Lamarre et al. 2001), which could explain why a double mutant for two oxidative stress genes (*GRX2* and *SOD1*) is defective in hyphae formation.

We also observed that our double mutant Δ*sod1*/*grx2* is very susceptible to PMN killing. Conversely, both genes were up-regulated in the presence of PMNs (Fradin et al. 2005). However, in addition to *SOD1* and *GRX2* appearing to play different and specific roles in response to the oxidative stress generated by the chemical inducers menadione and diamide, *GRX2* also appears to play a major role in surviving PMN killing.

The double mutant strain series was unexpectedly not more attenuated in mouse virulence than the single mutants generated by Chaves et al. (2007). However, the double null mutant Δ*sod1*/*grx2* and the Δ*sod1*/*grx2*+*SOD1* reintegrant were still attenuated in virulence, confirming the importance of oxidative stress genes for virulence in mice. Surprisingly, Δ*sod1*/*grx2*+*GRX2*, which was also more resistant to PMN killing, regained some degree of virulence in mice, reinforcing the role of this gene in *C. albicans* pathogenesis.

The double mutant generated here, Δ*sod1*/*grx2*, had a defect in hyphae formation and was susceptible to PMN killing, corroborating the hypothesis that both genes are important for *C. albicans* virulence. Further studies are required to elucidate the specific role of the importance of this complex antioxidant system and its relation to *C. albicans* virulence.

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