Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Candida albicans GRX2, encoding a putative glutaredoxin, is required for virulence in a murine model

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Genet. Mol. Res. 6 (4): 1051-1063 (2007)
Received May 10, 2007
Accepted October 11, 2007
Published November 27, 2007

ABSTRACT. Resistance of *Candida albicans* to reactive oxygen species is thought to enhance its virulence in mammalian hosts. Genes such as *SOD1*, which encodes the anti-oxidant, superoxide dismutase, are known virulence factors. We disrupted the gene *GRX2*, which encodes a putative glutathione reductase (glutaredoxin) in *C. albicans*, and we compared the mutant with an *sod1*Δ mutant. *In vitro*, the *grx2*Δ strain, but not the *sod1*Δ strain, was defective in hypha formation. The *grx2*Δ strain, but not *sod1*Δ, was significantly more susceptible to killing by neutrophils. When exposed to two compounds that generate reactive oxygen species, both mutants were susceptible to 1 mM menadione, but *grx2*Δ null alone was resistant to diamide. Both mutants were attenuated in a murine intravenous challenge model, and a *GRX2* reintegrant regained partial virulence. Emphasis on the putative function of products of genes such as *SOD1* and *GRX2* in resistance to oxidative stress may oversimplify their functions in the virulence process, since the *grx2*Δ strain also gave defective hypha formation. Both mutants were sensitive to menadione and were slow to form germ tubes, though growth rates matched controls once the lag phase was passed.

Key words: *Candida albicans*, Virulence, *GRX2*, Glutathione reductase

INTRODUCTION

Candida albicans is the most common cause of life-threatening, opportunistic fungal disease in severely debilitated or immunocompromised patients (Calderone, 2002). It is a diploid fungus that can assume different morphologies, from budding yeast cells through pseudohyphae to true hyphae, depending on environmental conditions (Berman and Sudbery, 2002). More than 70 genes have now been implicated as contributors to the virulence of *C. albicans* in experiments based on selective gene disruption and on virulence testing by intravenous challenge in experimental mice (Navarro-Garcia et al., 2001; Brand et al., 2004).

The anti-*Candida* role of the oxidative burst within phagocytic leukocytes has long been recognized, along with observations indicating that this fungus possesses mechanisms to resist reactive-oxygen species (Odds, 1988). The *C. albicans* genome includes a family of six superoxide dismutase (*SOD*) genes (Martchenko et al., 2004) with the potential to convert damaging superoxide radicals to hydrogen peroxide, which can be detoxified by catalase. The cytosolic Cu^{II}/Zn^{II} superoxide dismutases encoded by *SOD1* (Hwang et al., 2002) and *SOD5* (Martchenko et al., 2004) have been implicated as virulence factors in specific gene disruption studies. *SOD5* expression is increased in *C. albicans* yeast cells exposed to polymorphonuclear neutrophils (PMN) (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 found for *sod5*Δ (Martchenko et al., 2004), indicating that the virulence role of Sod5p may be due to resistance to PMN but not to macrophages. Mn^{II}-dependent mitochondrial Sod2p in *C. albicans* has been ruled out as a putative virulence factor (Hwang et al., 2003), and the virulence roles of *SOD3*, *SOD4* and *SOD6* have not yet been directly investigated.

Exposure of *C. albicans* cells to low levels of hydrogen peroxide leads to an increase in catalase, glutathione reductase and superoxide dismutase activity, while higher levels of H_2O_2 are associated only with an increase in glutathione reductase activity (Gonzalez-Parraga et al., 2003). Transcript profiling of H_2O_2-stressed *C. albicans* showed an increase in expression of *GRX1*, *GRX2* and *CTA1*, but not of *SOD2* (Enjalbert et al., 2003), matching the enzyme data. Disruption of the catalase-encoding gene *CTA1* generates a virulence-attenuated mutant (Wysong et al., 1988), and glutathione reductase activities have been repeatedly implicated as correlated not only with oxidative stress resistance but also with yeast-hypha morphogenesis (Gunasekaran et al., 1995), a property itself often regarded as a contributor to *C. albicans* virulence (Gow et al., 2002). However, a role for glutathione metabolism in *C. albicans* virulence has not been directly investigated. Glutaredoxins (glutathione-reductase enzymes) are ubiquitous cytosolic proteins that catalyze reduction of glutathione disulphide. Mammalian glutaredoxins are involved in a wide variety of cellular processes, including resistance to oxidative stress and growth control (Arnér and Holmgren, 2000); they may therefore play an adjunct role in defense against reactive oxygen species (ROS). They have been shown to play a role in combating oxidative stress in the erythrocytic stages of *Plasmodium falciparum* (Muller, 2004).

Like *Saccharomyces cerevisiae*, *C. albicans* possesses more than one gene homologue encoding glutaredoxin activity. One of these, presently named *TTR1*, is a homologue of *GRX2* in *S. cerevisiae*. Expression profiling shows that this gene is upregulated not only in *C. albicans* cells exposed to H_2O_2 (Enjalbert et al., 2003; Gonzalez-Parraga et al., 2003), but also in cells exposed to the inhibitor benomyl (Karababa et al., 2004). Other glutaredoxin homologues are not upregulated in response to such stresses. When Fradin et al. (2005) compared *C. albicans* gene

expression in response to PMN with whole blood depleted of PMN as a control, they found that expression of *GRX2* was more highly upregulated than that of *SOD1*, under both circumstances. We, therefore, decided to investigate this particular gene as a putative virulence factor, possibly involved in defense against oxidative stress. The gene name *TTR1* derives from the older yeast literature, from which the abbreviation related to thiol transferase and the name *TTR1* carried over into the *C. albicans* literature and genome databases (http://www.candidagenome.org/, http://genolist.pasteur.fr/CandidaDB/). However, available genomic information indicates at least three homologues of glutathione reductase in *C. albicans* bearing the characteristic CP*C motif, two of which (orf19.6059 and orf19.3920) are homologous to *S. cerevisiae GRX2*. We, therefore, chose to name the gene formerly described as *TTR1* (orf19.6059) as *CaGRX2*, since it is likely in the long term that *GRX*-based nomenclature will be used for the entire family of glutaredoxin-encoding genes. *GRX2* is an acknowledged synonym of *TTR1* (http://genolist.pasteur.fr/CandidaDB/).

We investigated the role of *GRX2* in *C. albicans* by specific gene disruption and characterization of the mutant. We found that the *grx2*Δ null mutant is attenuated in mouse virulence and is more readily killed by PMN *in vitro*. An *sod1*Δ mutant was susceptible to both menadione and diamide, which generate ROS *in vitro*; however, the *grx2*Δ null was menadione-susceptible and diamide-resistant, suggesting complexity in the cellular roles of individual anti-oxidant proteins.

MATERIAL AND METHODS

Strains, media and culture conditions

All *C. albicans* strains used and constructed in this study are listed in Table 1. They were stored at -80°C in 20% (v:v) glycerol and later subcultured on Sabouraud agar. The following media were used routinely to grow the fungi. NGY broth: 1 g/L Neopeptone (Difco, Detroit, MI, USA), 4 g/L glucose and 1 g/L yeast extract; 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), 20 g/L glucose, with 25 µg/mL uridine added as required.

Table 1. *Candida albicans* mutant strains.

Strain	Parent strain	Genotype	Source
CAI 4		*ura3*Δ::*imm434/ura3*Δ::*imm434*	Fonzi and Irwin, 1993
NGY152	CAI-4	As CAI-4 but *RPS1/rps1*Δ::CIp10	Brand et al., 2004
CH104	CH103	As CAI-4 but *sod1*Δ::*hisG/sod1*Δ::*hisG*	Hwang et al., 2002
GCY200	CH104	As CAI-4 but *sod1*Δ::*hisG/sod1*Δ::*hisG*, *RPS1/rps1*Δ::CIp10	Present study
CH204	CH203	As CAI-4 but *sod2*Δ::*hisG/sod2*Δ::*hisG*	Hwang et al., 2003
GCY201	CH204	As CAI-4 but *sod2*Δ::*hisG/sod2*Δ::*hisG*, *RPS1/rps1*Δ::CIp10	Present study
GCY202	CAI-4	As CAI-4 but *GRX2/grx2*Δ::*hisG-URA3-hisG*	Present study
GCY203	GCY202	As CAI-4 but *GRX2/grx2*Δ::*hisG*	Present study
GCY204	GCY203	As CAI-4 but *GRX2/grx2*Δ::*hisG*, *RPS1/rps1*Δ::CIp10	Present study
GCY205	GCY203	As CAI-4 but *grx2*Δ::*hisG/grx2*Δ::*hisG-URA3-hisG*	Present study
GCY206	GCY205	As CAI-4 but *grx2*Δ::*hisG/grx2*Δ::*hisG*	Present study
GCY207	GCY206	As CAI-4 but *grx2*Δ::*hisG/grx2*Δ::*hisG*, *RPS1/rps1*Δ::CIp10	Present study
GCY208	GCY206	As CAI-4 but *grx2*Δ::*hisG/grx2*Δ::*hisG*, *RPS1/rps1*Δ::CIp10-*GRX2*	Present study

Construction of the *grx2*Δ null mutant and reintegrant strain

All primers used in this study are listed in Table 2. The *GRX2* gene was disrupted by the ura-blaster method (Fonzi and Irwin, 1993). The regions of homology were amplified

by polymerase chain reaction (PCR) using the upstream primer pair GRX21 fwd and GRX22 rev, containing *Hin*dIII and *Sph*I restriction sites, respectively, and the downstream primer pair GRX23 fwd and GRX24 rev, containing *Asp*718 and *Ban*II restriction sites, respectively. The 460-bp upstream and 370-bp downstream products were cloned into the complementary restriction sites in pMB-7 (Fonzi and Irwin, 1993). The disruption cassette was released by digestion with *Hin*dIII and *Ban*II, and *GRX2* was disrupted by sequential rounds of transformation into strain CAI-4. 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 PCR. To avoid potential problems associated with ectopic expression of *URA3* (Brand et al., 2004), the Ura$^-$ *grx2*Δ mutant was transformed with *Stu*I-digested CIp10 plasmid (Murad et al., 2000), ensuring *URA3* expression at the neutral *RPS1* locus (orf19.3002). The Ura$^-$ *sod1*Δ and *sod2*Δ mutants (kindly provided by Dr. Sa-Ouk Kang from the Laboratory of Biophysics, Seoul National University, Republic of Korea) were also transformed with *Stu*I-digested CIp10.

Table 2. Primers.

Primer	Sequence (5' to 3')	Target for amplification
GRX21 fwd	AAGCTTCATGAGGGAGCAAATTTACC	*GRX2* upstream region (*Hin*dIII restriction site underlined)
GRX22 rev	GCATGCCCTTATTTGGAGAATCGTGG	*GRX2* upstream region (*Sph*I restriction site underlined)
GRX23 fwd	GGTACCCAATGTCGAACGTTGGAC	*GRX2* downstream region (*Asp*718 restriction site underlined)
GRX24 rev	GAGCTGCAACTCTACTTTCCATCTG	*GRX2* downstream region (*Ban*II restriction site underlined)
SOD1SF	AGATCTGCAACAACAATAGGTAAACGC	*SOD1* promoter (*Bgl*II restriction site underlined)
SOD14 rev	AGATCTGGTTTAGGCTTAAGCTGTAG	*SOD1* terminator (*Bgl*II restriction site underlined)
EFB1 fwd	ATTGAACGAATTCTTGGCTGAC	*EFB1* RT-PCR
EFB1 rev	CATCTTCTTCAACAGCAGCTTG	*EFB1* RT-PCR
GRX2 fwd	GATGTTTCGTACATTATTAACC	*GRX2* RT-PCR
GRX2SF	GAGAAAGATCGTGGATTTGG	*GRX2* reintegrant construction
GRX2 rev	GTCATCTAATTTGTCACTAGAC	*GRX2* RT-PCR

As a control, a reintegrant strain was constructed, in which the *GRX2* gene was introduced into the *grx2*Δ mutant. The *GRX2* open-reading frame plus 934 bp of its promoter and 546 bp of its terminator was amplified by PCR (primers GRX2SF and GRX24 rev), and the product cloned into pGEM-T Easy (Promega Ltd., Southampton, UK). The plasmid insert was subcloned into the *Not*I site of CIp10. The resulting plasmid was digested with *Stu*I and transformed into the Ura$^-$ *grx2*Δ null mutant.

RT-PCR for GRX2 expression

RNA was extracted by the method of Hayes et al. (2002), and multiplex semi-quantitative RT-PCR was done as previously described (Copping et al., 2005). Primers GRX2 fwd and GRX2 rev and EFB1 fwd and EFB1 rev were used to amplify *GRX2* and

EFB1 cDNA, respectively. *EFB1* contains an intron and was used as both a control for genomic DNA contamination and as an expression standard. PCR products were sampled after every two amplification cycles, from cycle 12 to cycle 28, and *GRX2* gene expression was estimated relative to *EFB1*.

Candida 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 $OD_{600\ nm} = 0.05$ and the culture incubated at 37°C rotated at 200 rpm in a gyratory shaker. Growth was measured by $OD_{600\ nm}$ versus a medium blank. Maximum growth rates were determined from the logarithms of values taken in the exponential phase.

Tests for hypha formation

For induction of hypha 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 30°C.

For tests in liquid media, cells were grown, washed and standardized to an initial concentration of 10^6 cells/mL in 10% fetal calf serum (FCS) or YPD + 10% FCS pre-warmed to 37°C and incubated at the same temperature, with gyratory shaking at 200 rpm. After 1- and 3-h incubation, samples of the cultures were mixed with an equal volume of 10% formaldehyde to arrest further development. The sample at 1 h was examined microscopically for determination of the percentage of cells bearing evaginations. The 3-h sample was examined for approximation of the mean morphology index (MI) (Merson-Davies and Odds, 1989), in which a value close to 1 indicates a population of spheroidal yeast cells and a value close to 4 indicates a population of true hyphal cells, with values between 1 and 4 indicating mixed or pseudohyphal morphologies (Odds et al., 2000).

Proteinase assay

Proteinase activity was determined by a method modified from MacDonald and Odds (1980). Fifty-microliter samples from NGY cultures were grown in 5 mL YCB + BSA medium (11.7 g/L Yeast Carbon Base [Difco]; 10 g/L glucose; 5 g/L bovine serum albumin, fraction V, Batch 08k0560 [Sigma]) rotated in a wheel angled 5° from the horizontal at 30°C for 72 h. Proteolytic activity was determined by measuring the increase in trichloroacetic acid soluble products absorbing at 280 nm in triplicate after 1-h incubation of the culture supernatant with BSA substrate at 37°C. Specific activity was expressed as OD_{280}/OD_{600} of the culture.

Phospholipase assay

Phospholipase activity was estimated by the egg yolk agar method (Price et al., 1982), with inocula prepared from overnight NGY cultures standardized to 2×10^5 cells/mL.

Candida albicans adhesion assay

The method of Kimura and Pearsall (1978) was used; *C. albicans* cells were grown overnight to stationary phase in SD at 37°C and were mixed with human buccal epithelial cells (HBEC) from healthy volunteers at a ratio of 1000 yeast cells per HBEC. The mixtures were incubated at 37°C for 45 min with shaking; then cells were filtered on polycarbonate (12 µm pores), Gram-stained and washed before transfer to a microscope slide. The number of *C. albicans* cells adhering to 100 HBEC was determined with the operator blinded to the nature of the material on the slide. Tests were done in triplicate.

Sensitivity of *Candida 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. Yeasts grown in NGY were standardized to 2 x 10^7 cells/mL. Five-microliter volumes of a 10-fold dilution series prepared from this suspension were spotted on the surface of YPD agar plates containing concentrations of menadione and diamide ranging from 0.5 to 2.5 mM. The plates were incubated for 48 h at 30°C. Alternatively, 50-µL volumes of NGY-grown yeasts were transferred to 5 mL YPD broth, with and without additions of menadione or diamide (0.5-2.0 mM), incubated in a rotator wheel for 16 h at 30°C and the $OD_{600\ nm}$ determined for control and test tubes. Sensitivity to the compounds was measured by examining growth in the presence of compounds as a percentage of control growth. Sensitivity to H_2O_2 was tested in the same way, except that incubation was continued for 3 h instead of 16 h.

Candida albicans killing by polymorphonuclear neutrophils

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

Virulence of *Candida albicans* in the murine IV challenge model

Animal experimentation was done under the terms of UK Home Office licenses for research on animals. Immunocompetent female BALB/c mice (Harlan Sera-lab, Loughborough, UK) with a weight range from 17-23 g were supplied with food and water *ad libitum*. Mice

were intravenously inoculated with *C. albicans* strains that had been grown overnight in NGY, washed and resuspended in saline. The inoculum was standardized to allow injection of 2-3 x 10^4 CFU/g mouse body weight. Mice were examined daily and animals showing a loss of body weight >20% or which showed signs of serious illness were humanely terminated and kidneys and brain removed aseptically for determination of fungal burdens by viable counting.

Statistical analysis

Means ± standard deviations were determined from the results of at least three independent experiments. Differences between values for phenotypic tests were analyzed by the Student *t*-test. Animal survival data were compared with Kaplan-Meier/LogRank statistics, and differences between tissue burden data with the Mann-Whitney U-test. P values <0.05 were considered to be significant for all comparisons.

RESULTS

Gene disruptions and reintegrations

GRX2/TTR1 was identified in the CandidaDB *C. albicans* genomic database as orf19.6059. BLAST searching revealed orf19.3920 as a close homologue, also annotated as encoding a putative glutaredoxin and even listed as a synonym of *GRX2/TTR1*. The nomenclature of the *GRX* gene family in *C. albicans* is presently a source of confusion, but our study was based on the sequence of orf19.6059, and primers for *GRX2* were designed according to this DNA sequence.

Table 1 details the mutants generated in this study. The *grx2*Δ mutant GCY206 was made by deletion of 1583 bp from the central region of the *GRX2* gene in *C. albicans* CAI-4 with the "Ura-blasting" technique (Fonzi and Irwin, 1993). The *URA3* gene was reintegrated at the neutral *RPS1* locus, to avoid ectopic *URA3* expression problems (Brand et al., 2004), creating mutant GCY207. The *GRX2* gene as well as *URA3* were integrated at the *RPS1* locus of the *grx2*Δ null mutant to make the *grx2*Δ + *GRX2* reintegrant GCY208. The *URA3* gene was also reintegrated at the *RPS1* locus in *sod1*Δ and *sod2*Δ mutants to create mutants GCY200 and GCY201, respectively.

Phenotypic properties of the mutants *in vitro*

Growth rates of yeast cells of all the null mutants and the *GRX2* reintegrant strain were essentially the same as for the wild-type NGY152 (Table 3). Putative virulence properties, such as proteinase, phospholipase activity and adhesion to HBEC were unaltered in the mutants and reintegrant strains. Colony morphologies on Spider and GlcNAc agars were not markedly different for the mutants when compared to NGY152.

Around 90% of NGY152 yeast cells had evaginated within 1 h in 10% FCS and YPD + FCS (Table 3). The *sod1*Δ and *sod2*Δ null mutants evaginated more slowly in YPD + FCS, but did so at the same rate in 10% serum. The *grx2*Δ mutant, GCY207, and the reintegrant, GCY208, were markedly delayed in evagination at 1 h, particularly in YPD + FCS.

After 3 h of incubation in 10% FCS, NGY152 cells formed true hyphae (mean MI = 4; Table 3) and the mean MI for the mutants similarly suggested formation of true hyphae in serum (Table 3). In YPD + FCS, while NGY152 cells continued to show a hyphal morphology (mean MI of 3.9), all mutants and the *GRX2* reintegrant had a lower mean MI, often with a large standard deviation (Table 3), suggestive of mixed morphologies with reduced proportions of true hyphae. The *grx2*Δ mutant, GCY207, was particularly deficient in hypha formation in YPD + FCS, with a mean MI of only 1.3, indicating growth mainly in the yeast form or as short pseudohyphae.

Table 3. Phenotypic properties of control and mutant *Candida albicans* strains.

Strain	Maximum growth rate/h		Evaginations from parent yeasts (%) after 1 h		Morphology index (mean ± SD) after 3 h		Killing by polymorphonuclear neutrophils (%)	
	YPD	RPMI 1640	YPD + FCS	10% FCS	YPD + FCS	10% FCS	Mean ± SD	P *vs* NGY152[a]
NGY152	0.85	0.69	89	90	3.9 ± 0.3	4.0 ± 0.0	16 ± 12	
GCY200 (*sod1*Δ)	0.84	0.73	20	97	2.5 ± 1.0	3.9 ± 0.4	25 ± 9	0.07
GCY201 (*sod2*Δ)	0.79	0.73	53	98	2.9 ± 1.0	4.0 ± 0.3	46 ± 6	0.0007
GCY207 (*grx2*Δ)	0.75	0.68	0	23	1.3 ± 0.5	3.9 ± 0.4	45 ± 11	0.0012
GCY208 (*grx2*Δ + *GRX2*)	0.88	0.63	4	14	1.9 ± 0.7	3.9 ± 0.3	25 ± 5	0.07

[a]Student *t*-test.

Effects of oxidative stress inducers

All mutants and the *GRX2* reintegrant were more susceptible than NGY152 to menadione (Figure 1A). GCY201 (*sod2*Δ) was the most susceptible to menadione. Agar plate tests gave essentially similar results (data not shown). A different pattern of susceptibility was observed with diamide (Figure 1B). In the presence of 2 mM diamide, GCY207 (*grx2*Δ) grew almost to control levels, while every other strain was inhibited. In plate tests, growth of GCY207 was unaffected even at 2.5 mM diamide, whereas NGY152 was completely inhibited at this concentration. All the strains were equally sensitive to H_2O_2, with growth reduced below 50% of control by 2.5 mM H_2O_2 after 3-h incubation (data not shown).

Effects of polymorphonuclear neutrophils

The *grx2*Δ and *sod2*Δ null mutants were significantly more susceptible to killing by PMN than NGY152 (Table 3) but the increase in killing measured for the *sod1*Δ null (GCY200) and the *GRX2* reintegrant (GCY208) was not significantly different from control values.

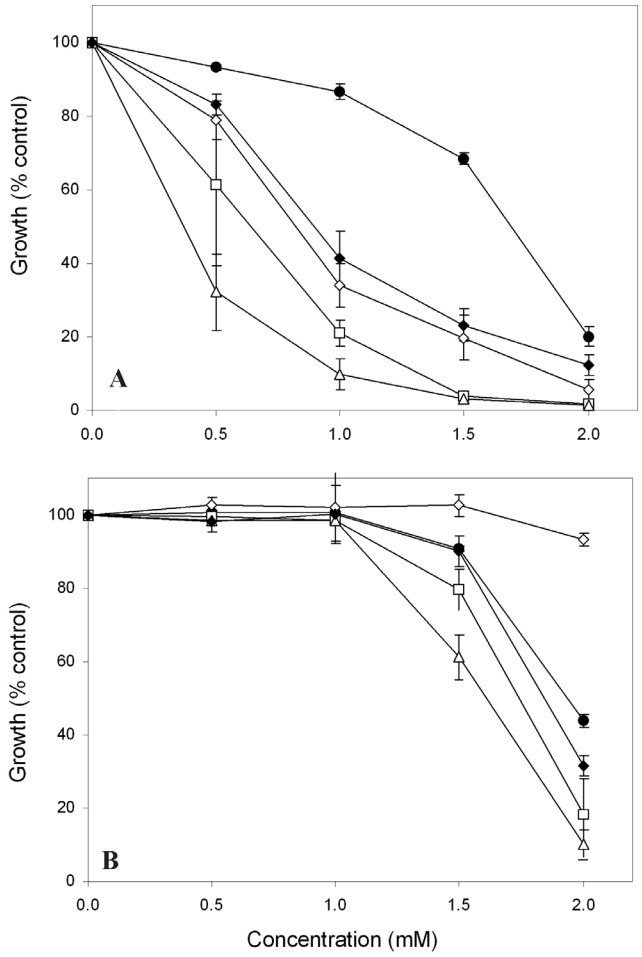

Figure 1. Dose-response curves showing susceptibility of *Candida albicans* strains to menadione (A) and diamide (B). Closed circles, NGY152; open triangles, GCY200 (*sod1*Δ); open squares, GCY201 (*sod2*Δ); open diamonds, GCY207 (*grx2*Δ); closed diamonds, GCY208 (*grx2*Δ + *GRX2*).

Virulence of mutants in the murine IV challenge model

Median survival times for animals infected with GCY200 (*sod1*Δ) or GCY207 (*grx2*Δ) was 28 days, as compared with five days for the control strain NGY152 (LogRank test; $P < 0.001$; Figure 2). The survival curve for the *GRX2* reintegrant strain GCY208 indicated some regain of virulence, but not to control levels ($P < 0.001$ relative to NGY152). Viable count data showed significantly reduced tissue burdens in both kidneys and brain for mice infected with null mutants GCY200 and GCY207. Kidney burdens were not significantly different from control for mice infected with the *GRX2* reintegrant GCY208, but brain burdens were significantly lower (Table 4).

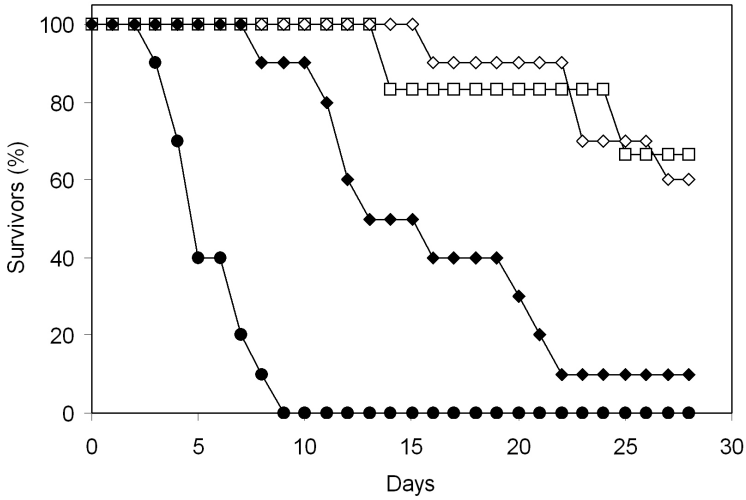

Figure 2. Survival of mice challenged intravenously with *Candida albicans* strains. Closed circles, NGY152; open squares, GCY200 (*sod1*Δ); open diamonds, GCY207 (*grx2*Δ); closed diamonds, GCY208 (*grx2*Δ + *GRX2*).

Table 4. Tissue burdens of mice infected with control and mutant strains of *Candida albicans*.

Strain	Log_{10} CFU/g tissue		
	Left kidney	Right kidney	Brain
NGY152 (N = 10)	7.0 ± 0.7	6.7 ± 0.5	4.9 ± 0.9
GCY200 (*sod1*Δ) (N = 6)	3.3 ± 2.5*	3.5 ± 2.1*	1.8 ± 1.0*
GCY207 (*grx2*Δ) (N = 9)	5.2 ± 2.0*	4.7 ± 1.7*	3.1 ± 1.0*
GCY208 (*grx2*Δ + *GRX2*) (N = 9)	6.3 ± 0.8	6.5 ± 0.3	3.2 ± 0.7*

*Indicates a significantly reduced burden relative to NGY152 (Mann-Whitney U-test, $P < 0.05$).

DISCUSSION

A possible role for *C. albicans GRX2* in virulence has been directly demonstrated in this study by the observations that survival was extended (Figure 2) and mean tissue burdens reduced (Table 4) in animals challenged with a *grx2*Δ mutant. However, reintegration of a single copy of *GRX2* in the null mutant partly, but not wholly, reversed virulence attenuation, and did not fully restore wild-type phenotypic properties *in vitro*. Similar inexplicable failures of reintegrants to regain full wild-type virulence have been noted for other genes, including some regarded as encoding virulence factors in similarly designed experiments (Hobson et al., 2004; Palmer et al., 2004; Tripathi et al., 2004; Bates et al., 2005) presumably because reintegration of only one copy of these genes is not enough for fully regaining of wild-type phenotype. We measured the level of expression of *GRX2* by semi-quantitative multiplex RT-PCR and found no difference between levels in the reintegrant, GCY208, and control, NGY152 (details not shown); therefore, expression level differences cannot account for the failure of the reintegrant to recover wild-type properties. The survival and burden data for the *grx2*Δ mutant were very similar to results for the *sod1*Δ mutant. We tested *sod1*Δ in this study with *URA3* reintegrated at the highly expressed *RPS1* locus

to avoid artefacts of *URA3* underexpression (Brand et al., 2004); the results confirm the original report of virulence attenuation in this mutant (Hwang et al., 2002).

Although the *grx2*Δ and *sod1*Δ nulls were attenuated in mouse virulence, their roles in the pathogenesis process cannot be similar. The *sod1*Δ mutant was unaffected in hypha formation in YPD + FCS and in 10% FCS alone, while the *grx2*Δ mutant had a delay in the evagination of daughter cell material under both conditions; there was a preponderance of yeast cells and short pseudohyphae in YPD + FCS (Table 3). Killing of the *grx2*Δ null by PMN was significantly increased over that of the control, while killing of the *sod1*Δ null was not. The *sod1*Δ null was inhibited by 1 mM menadione and 2 mM diamide, while the *grx2*Δ null was resistant to 2 mM diamide, indicating differences in the roles of *SOD1* and *GRX2* in combating oxidative stress.

Pereira et al. (2003) demonstrated that in *S. cerevisiae* the *sod2*Δ mutant is not sensitive, based on survival of the cells after exposure to menadione. They suggested that *SOD1* is sufficient to provide ROS tolerance in an *sod2*Δ mutant. Based on our study, together with the findings of Hwang et al. (2002, 2003), we infer that the role of *SOD* genes is different in *C. albicans* and *S. cerevisiae* because the *C. albicans sod2*Δ mutant is very sensitive to menadione, emphasizing a greater importance of this enzyme in ROS resistance to menadione-induced stress than in *S. cerevisiae*.

Susceptiblity of the *sod1*Δ mutant to diamide in *C. albicans* has not been investigated previously. Diamide is a thiol-specific oxidant that can readily oxidize glutathione (O'Brien, 1970; Kosower and Kosower, 1995). Therefore, a difference in diamide susceptibility between the *sod1*Δ mutant and the wild type is not expected.

We investigated the role of glutaredoxin in *C. albicans*. Apparently, menadione plays a minimal role in oxidative stress in the *grx2*Δ mutant. It is not surprising that the stress generated by menadione does not make *grx2*Δ very susceptible, because glutaredoxins do not interact directly with O_2^-.

The *C. albicans grx2*Δ mutant was very resistant to diamide. If this chemical is a rapid thiol oxidant, then this mutant should be sensitive rather than resistant to diamide. The resistance of the Δ*grx2* mutant that we found could be due to a compensatory mechanism of the other glutaredoxins present in *C. abicans*. The situation is clearly complicated, and extensive specific study may be required to evaluate the individual roles of all S-thiolation and oxidative-stress responses in *C. albicans*.

It is possible that the virulence attenuation found in the *grx2*Δ null was a consequence of changes in properties other than resistance to oxidative stress. With families of both *SOD* and *GRX* genes in *C. albicans* it is possible that redundancy allows other members of the family to take over the putative oxidative-stress resistance roles of *SOD1* or *GRX2*, when either is disrupted singly. Phenotypic behavior of the *sod2*Δ mutant closely followed that of the *sod1*Δ null, yet this mutant is not attenuated in virulence for mice (Hwang et al., 2003).

Attenuation in mouse virulence in the *grx2*Δ null might be related to its slower evagination in two growth media, and its defective hypha formation in YPD + FCS, as well as its susceptibility to neutrophil killing (Table 3). However, the *sod2*Δ null was also significantly more vulnerable than NGY152 to PMN killing, yet it is reportedly normally virulent for mice (Hwang et al., 2003). We did not directly retest the *sod2*Δ null for mouse virulence, since it is unethical to further expose animals to challenge with a strain already shown to possess wild-type virulence.

We have extended the rapidly growing list of genes whose disruption leads to virulence attenuation in the mouse challenge model. The differences in effects of diamide on *grx2*Δ and *sod1*Δ mutants suggest complex patterns of regulation of different *C. albicans* genes; emphasis of their properties in resistance to oxidative stress may oversimplify their virulence functions *in vivo*.

ACKNOWLEDGMENTS

We thank the Brazilian Ministry of Education (CAPES) for financial support to G.M. Chaves. S. Bates and D.M. MacCallum were supported by grants from the Wellcome Trust. We are grateful to Dr. Sa-Ouk Kang for the *sod1*Δ and *sod2*Δ strains.

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