The superoxide dismutases of *Candida glabrata* protect against oxidative damage and are required for lysine biosynthesis, DNA integrity and chronological life survival


IPICYT, División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, Camino a la Presa San José, no. 2055, Col. Lomas 4ª Sección, San Luis Potosí, San Luis Potosí 78216, Mexico

The fungal pathogen *Candida glabrata* has a well-defined oxidative stress response, is extremely resistant to oxidative stress and can survive inside phagocytic cells. In order to further our understanding of the oxidative stress response in *C. glabrata*, we characterized the superoxide dismutases (SODs) Cu,ZnSOD (Sod1) and MnSOD (Sod2). We found that Sod1 is the major contributor to total SOD activity and is present in cytoplasm, whereas Sod2 is a mitochondrial protein. Both SODs played a central role in the oxidative stress response but Sod1 was more important during fermentative growth and Sod2 during respiration and growth in non-fermentable carbon sources. Interestingly, *C. glabrata* cells lacking both SODs showed auxotrophy for lysine, a high rate of spontaneous mutation and reduced chronological lifespan. Thus, our study reveals that SODs play an important role in metabolism, lysine biosynthesis, DNA protection and aging in *C. glabrata*.

INTRODUCTION

*Candida glabrata* is an opportunistic fungal pathogen in immunocompromised individuals and is the second most commonly isolated *Candida* species from humans after *Candida albicans* (Kaur *et al.*, 2005). Together, these fungi are responsible for approximately 65–75 % of all systemic candidiasis (Pfaller & Diekema, 2007). Due to the increasing prevalence of *C. glabrata* (Kaur *et al.*, 2011), it is important to understand how *C. glabrata* cells counteract or evade the mammalian host defence systems.

As a successful pathogen, *C. glabrata* adheres to epithelial cells (Cormack *et al.*, 1999) and can invade into deeper tissues (Atanasova *et al.*, 2013; Jacobsen *et al.*, 2011). It has been shown in vitro that *C. glabrata* is recognized and ingested by macrophages (Kepper-Ross *et al.*, 2010), but can survive and replicate inside the phagosome (Kaur *et al.*, 2007). Recently, it has been shown that *C. glabrata* inhibits phagosome maturation and suppresses the production of the reactive oxygen species (ROS) generated by the oxidative burst of phagocytes (Seider *et al.*, 2011). These ROS include superoxide radical anion (O$_2^\cdot$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (HO$^\cdot$), which can damage all biomolecules.

Given that ROS are by-products of mitochondrial respiration, pathogens have antioxidant systems to counteract their deleterious effects, and use these antioxidant systems to evade being killed by phagocytes (Miller & Britigan, 1997). These systems include catalases, glutathione peroxidases, superoxide dismutases (SODs), and glutathione, which are effectors of the oxidative stress response (OSR). *C. glabrata* has a well-defined OSR and its high resistance to H$_2$O$_2$ requires the synthesis of the unique catalase Cta1. The expression of *CTA1* is controlled by Yap1 and Skn7 (Cuellar-Cruz *et al.*, 2008; Roetzer *et al.*, 2011). Interestingly, *CTA1* is dispensable in a mouse model of systemic infection, indicating the presence of additional detoxifying systems that could compensate for the lack of the catalase (Cuellar-Cruz *et al.*, 2008). Consistent with the role of glutathione in the OSR, cells lacking glutathione are sensitive to oxidative stress (Gutiérrez-Escobedo *et al.*, 2013).

One supplementary figure and five supplementary tables are available with the online Supplementary Material.

†Present address: Instituto de Salud Pública, Universidad Veracruzana, Xalapa, Veracruz 91190, Mexico.

Abbreviations: AAA, α-aminoadipate; BER, base excision repair; CLS, chronological lifespan; 5-FAA, 5-fluoroorotic acid; 5-FOA, 5-fluoroorotic acid; HU, hydroxyurea; LP, exponential phase; MD, menadione; MMS, methyl methane sulfonate; OSR, oxidative stress response; ROS, reactive oxygen species; SOD, superoxide dismutase; SP, stationary phase.
SODs are the first line of defence against oxidative stress, converting $O_2^-$ into $H_2O_2$ (Fridovich, 1995). The main targets of $O_2^-$ are the catalytic iron–sulfur clusters (4Fe–4S) of dehydratases (Flint et al., 1993). $O_2^-$ disrupts the function of these enzymes through the release of an iron atom, which is then available to participate in Fenton chemistry. Thus, the released iron atoms may promote DNA damage by increasing the amount of DNA-bound iron (Keyer & Imlay, 1996; Liochev & Fridovich, 1999).

Most eukaryotes contain two isoforms of SODs that differ in their metal ions, their protein folding, phylogenetic relation and localization (Fridovich, 1995). In Saccharomyces cerevisiae, Sod1 is a homodimeric enzyme with one zinc ion, one copper ion and one disulfide bond per subunit; it is highly abundant and is present in both the cytosol and the mitochondrial intermembrane space (Sturtz et al., 2001). In contrast, Sod2, localized in the mitochondrial matrix, is usually homotrameric with one Mn$^{2+}$ atom per subunit and is phylogenetically unrelated to Sod1 (Smith & Doolittle, 1992; Weisiger & Fridovich, 1973).

SODs contribute to the virulence of many pathogenic bacteria and fungal pathogens like C. albicans, Histoplasma capsulatum and Cryptococcus neoformans var. gattii (Hwang et al., 2002; Narasipura et al., 2003; Youseff et al., 2012); however, the role of the SODs in C. glabrata has been only partially analysed (Cuéllar-Cruz et al., 2009; Roether et al., 2011). C. glabrata can withstand twice the concentration of menadione [MD, an oxidant that generates $O_2^-$ through redox cycling (Hampsey, 1997)] compared with Sac. cerevisiae, but it is less resistant than C. albicans (Cuéllar-Cruz et al., 2009). Recently, it has been shown that a C. glabrata sod1Δ single mutant was highly sensitive to MD (Roether et al., 2011), and that the concomitant absence of Yap1 and Sod1 was detrimental to yeast survival in a primary mouse macrophage infection model (Roether et al., 2011). In this work, we are interested in understanding the role of the SODs in C. glabrata. Here we described the roles of SODs in virulence and in the general metabolism of C. glabrata. First, we found that Sod1 is cytoplasmic and Sod2 is a mitochondrial protein, and that Sod1 provides most of the SOD activity within the cell. We confirm that the sod1Δ single mutant is highly sensitive to superoxide and show that the double mutant, sod1Δ sod2Δ, is more sensitive to oxidants than the single mutants, sod1Δ and sod2Δ. SOD2 has a central role during cellular respiration because it is required for growth in non-fermentable carbon sources. Interestingly, C. glabrata cells lacking both SODs show auxotrophy for lysine, a high rate of spontaneous mutation and reduced chronological lifespan. Finally, we found that SODs are dispensable for colonization in a murine model of C. glabrata infection. Altogether our data demonstrate that SODs play an important role in the OSR and confer cellular protection against oxidative damage in C. glabrata.

**METHODS**

**Strains.** All strains used in this study are shown in Table S1 (available in the online Supplementary Material).

**Plasmids.** All plasmids used in this study are shown in Table S2.

**Primers.** All primers used for cloning are summarized in Table S3.

**Media and growth conditions.** Yeast media were prepared as described by Sherman et al. (1986), and 2% (w/v) agar was added to plates. YPD medium contained 10 g yeast extract $1^{-1}$ and 20 g peptone $1^{-1}$, and was supplemented when needed with 2% (w/v) glucose, 2% (w/v) glycerol or 2% (w/v) ethanol. Synthetic complete medium (SC) is composed of yeast nitrogen base (YNB) without ammonium sulfate, 5 g $(NH_4)_2SO_4$ $1^{-1}$, supplemented with 0.6% (w/v) Casamino acids and 2% (w/v) glucose. SC medium was supplemented with 25 mg uracil $1^{-1}$ or 1.1 g 5-fluoroorotic acid $1^{-1}$ (5-FOA; Toronto Research Chemicals) for 5-FOA plates, or 0.5 g 5-fluoroanthranilic acid $1^{-1}$ (5-FAA; Sigma Aldrich) for 5-FAA plates. Synthetic medium (SD) was a mixture of YNB without ammonium sulfate and 5 g $(NH_4)_2SO_4$ $1^{-1}$, supplemented with 2% (w/v) glucose and additionally, when needed, with 25 mg uracil $1^{-1}$ and/or 30 or 120 mg lysine $1^{-1}$, 60 mg leucine $1^{-1}$, 20 mg methionine $1^{-1}$ and/or 40 mg cysteine $1^{-1}$ (Sigma Aldrich). YPD plates were supplemented with either 100 mg nourseothricin $1^{-1}$ (Nat100) (Werner BioAgents) or 460 mg hygromycin B $1^{-1}$ (Hyg460) (A. G. Scientific), or with penicillin (100 U ml$^{-1}$) or streptomycin (100 mg l$^{-1}$) (Gibco-BRL). Bacterial medium was prepared as described by Ausubel et al. (2000), and 1.5% (w/v) agar was used for plates. Luria–Bertani (LB) medium contained 5 g yeast extract $1^{-1}$, 10 g bactopeptone $1^{-1}$ and 10 g NaCl $1^{-1}$, and was supplemented where needed with 50 mg carbenicillin $1^{-1}$ (Cb50) (Invitrogen). All plasmid constructs were introduced into Escherichia coli DH10 by electroporation (Ausubel et al., 2000) and plasmids were purified with the Qiagen Miniprep kit.

**Yeast transformation.** Yeast transformations with linear or supercoiled plasmid DNA were done as described by Castano et al. (2003).

**Sequence analysis.** The amino acid sequence homology analysis was done by CLUSTAL W alignment (Higgins et al., 1996) with the MacVector program (Accelrys), and the analysis of the mitochondria signal sequence was done with the MitoProtII- v1.101 program (Claros & Vincens, 1996).

**Plasmid and strain construction.** To construct the null mutant strains, the ORF of SOD1 and SOD2 was replaced with the nourseothricin resistance cassette (Nat). Briefly, we amplified the fragments with the NatMX4 cassette (flanked by two FRT direct repeats) by fusion PCR (Kuwayama et al., 2002). C. glabrata was transformed with the purified fusion PCR. Transformants were selected on YPD Nat100 plates. PCR analysis was performed to confirm the deletion and the correct replacement at the locus. To construct double mutants, we eliminated the nourseothricin marker on the sod1Δ or sod2Δ single mutants by expressing Flp1 recombinase from pMZ21 (Cuéllar-Cruz et al., 2008). Then, the sod1Δ and sod2Δ mutants were transformed with the fusion PCR SOD mutant construct. The SOD complementation plasmids were constructed by amplifying the SOD genes with their own promoters from the genomic DNA of BG14 strain. These fragments were cloned into pGRB2.0 (Zordan et al., 2013). To construct SOD–GFP fusions, a C-terminal segment of SOD1 or SOD2 without a stop codon was placed in phase with GFP. The translational fusion was followed by the 3' untranslated region UTR$_{C. glabrata}$ and a hygromycin resistance cassette, which were flanked by two FRT direct repeats. Downstream from the hygromycin cassette, the constructs contain a fragment of the 3' UTR of each gene (Orta-Zavalza et al., 2013). The BG14 parental strain was transformed with the translational fusions SOD1–GFP and SOD2–GFP and the transformants were selected on YPD Hyg460 plates. To place the SOD1–GFP and SOD2–GFP under their native 3' UTR, the GFP-tagged Hyg$^R$ strains were transformed with pMZ21-expressing...
FLP1 (Cue´llar-Cruz et al., 2008). To determine whether the tagged proteins were functional, we analysed their susceptibility to MD of the Sod1–GFP and Sod2–GFP in the sod1Δ sod2Δ background. Both GFP fusion constructs were verified by DNA sequencing. To overexpress the LYS genes, we amplified the ORFs of LYS4, LYS12, LYS20 and LYS21 from the BG14 genomic DNA and cloned them into pGRB2.2.

**Fluorescence microscopy.** Stationary phase (SP) cultures were grown in YPD at 30 °C. Cells (1 ml) were washed with PBS, resuspended in 0.5 ml PBS and incubated for 10 min with either 40 ng DAPI ml⁻¹, 500 ng DAPI ml⁻¹ for nuclei visualization or 40 nM MitoTracker™ to visualize the mitochondria. Cells were washed and analysed by direct fluorescence microscopy with an Axio Imager.M2 microscope (Carl Zeiss). Images were analysed with the software AxioVision v. 4.8.2.0 image browser.

**SOD activity assay.** SP cultures were grown at 30 °C in SC medium. Proteins were isolated by homogenizing the cells with glass beads in 0.3 ml lysis buffer [1× PBS supplemented with 1× general protease inhibitors (SigmaFAST)]. Protein content was determined by the Bradford assay (Fermentas) (Bradford, 1976). BSA (Sigma-Aldrich) was used as the standard.

**Zymograms for SOD activity.** Thirty to fifty micrograms of total protein was separated in 12 % (w/v) native polyacrylamide gels. Gels were run at 30 mA for 3 h. SOD activity was revealed by staining with nitro blue tetrazolium and riboflavin (SOD staining solution) in the polyacrylamide gel (Beauchamp & Fridovich, 1971).

**Menadione sensitivity assay.** Sensitivity to MD (Sigma-Aldrich) of exponential phase (LP) and SP cells was determined as previously described (Gutie´rrez-Escobedo et al., 2013). Plates were incubated for 48 h at 30 °C. The experiments were performed at least three times.

**Chroniclone lifespan.** Saturated cultures of C. glabrata Ura⁺ were grown in SD medium supplemented with 120 mg lysine 1⁻¹ for 48 h at 30 °C. Cells were washed and diluted into fresh SD + lysine (120 mg 1⁻¹) and incubated at 30 °C with constant shaking for 9 days. Samples were taken at days 3, 4, 5, 7 and 9. Cell density was adjusted to 2 × 10⁶ cells ml⁻¹ in YPD and 300 μl of the resulting suspension was taken to evaluate growth during a 24 h period at 30 °C in a Bioscreen C system. Viability was calculated from Bioscreen data as described previously (Gutiérrrez-Escobedo et al., 2013; Murakami et al., 2008).

**Mutation rate.** The mutation rate of the SOD mutants was estimated using the fluctuation assay (Lang & Murray, 2008). Briefly, 10 parallel cultures of the Ura⁺ Trp⁺ strains were grown in SD medium supplemented with lysine for 4 days at 30 °C. Tenfold serial dilutions of the cultures were plated on YPD for viable counts and on 5-FOA and 5-FAA plates to measure the 5-FOA⁻ and 5-FAA⁻ colony number. Mutation rates were calculated by the maximum-likelihood method using the web-based program Fluctuation AnaLysis Calculator (FALCOR; http://www.keshavsingh.org/protocols/FALCOR.html) (Hall et al., 2009). Independent 5-FOA⁻ or 5-FAA⁻ mutants were sequenced.

**Quantification of superoxide ion.** Quantification of superoxide ion was measured using the dihydroethidium probe in a microspectrofluorometric assay. Cells were grown in SD medium supplemented with lysine for 4 days at 30 °C. SP cultures were washed and resuspended in distilled water. Cells (4 × 10⁸) were harvested from triplicate independent cultures and incubated in 200 μM dihydroethidium solution for 10 min in the dark, and then washed with PBS solution. Fluorescence and absorbance were recorded (λex 485 nm, λem 595 nm). Fluorescence values were standardized using the fluorescence values of the unstained controls. The results are expressed in relative fluorescence units per absorbance unit (RFU ABS⁻¹).

**Hydroxyurea (HU) and methyl methanethiol sulfonate (MMS) sensitivity assays.** SP cultures were grown at 30 °C and serial dilutions were spotted on YPD or YPD containing HU or MMS at the indicated concentrations. Plates were incubated at 30 °C for 48 h.

**Murine disseminated candidiasis assay.** Eight- to nine-week-old BALB/c female mice were infected with 4 × 10⁵ cells in a volume of 100 μl by tail vein injection. All Ura⁺ strains were grown overnight in SC, and the cells were washed with 1× PBS and resuspended in 1 ml PBS to a concentration of 4 × 10⁶ cells ml⁻¹. The concentration of cells was determined by reading the OD₆₀₀, counting the cells in a haemocytometer and plating serial dilutions for counting c.f.u. Mice were kept in cages in groups of seven until they were euthanized in accordance with our ethics committee at day 7 of infection. Kidneys, livers and spleens were retrieved from the mice, and the organs were homogenized. Dilutions of the homogenates were plated onto YPD–penicillin–streptomycin plates. C.f.u. were counted the following day; geometric means were reported. Kruskal–Wallis test with Dunn’s post hoc was applied.

## RESULTS

**Sod1 is a cytoplasmic and Sod2 is a mitochondrial protein**

SODs are present in almost all organisms, and in the C. glabrata genome there are two SOD genes, CgSOD1 (CAGL0C04741g) and CgSOD2 (CAGL0E04356g), which are orthologues of Sac. cerevisiae ScSOD1 and ScSOD2 and share high similarity and conserved synteny. CgSod1 and ScSod1 are 91 % similar (84 % identical plus 7 % similar) and CgSod2 and ScSod2 are 81 % similar (70 % identical plus 11 % similar). In CgSod1 and CgSod2 the amino acid residues involved in metal ion coordination are conserved (Fink & Scandalios, 2002), and the first 24 amino acids of CgSod2 contain a putative targeting signal for mitochondria (Fig. S1). These analyses indicate that CgSod1 belongs to the Cu,ZnSOD family and CgSod2 to the MnSOD family.

To determine the cellular localization of Sod1 and Sod2, strains were constructed expressing functional translational fusions of Sod1–GFP and Sod2–GFP from their corresponding locus in the parental strain (BG14). Live yeast cells expressing Sod1–GFP or Sod2–GFP were stained with DAPI to reveal the nucleus (the large round structure) and mitochondrion (punctate structures), and with Mitotracker Red, specific for mitochondria. Sod1–GFP was uniformly localized in cytoplasm (Fig. 1a), whereas Sod2–GFP was present in discrete structures that co-localized with Mitotracker Red and DAPI (Fig. 1b–d). The dot with high signal intensity in Fig. 1d is an artefact. These results indicate that Sod1 is cytoplasmic whereas Sod2 is mitochondrial.

**Sod1 and Sod2 activity in C. glabrata**

To determine the contributions of Sod1 and Sod2 to total SOD activity, we constructed the single sod1Δ and sod2Δ mutants plus a sod1Δ sod2Δ double mutant and prepared zymograms with native extracts from these strains (Fig. 2). The parental strain revealed two bands
corresponding to Sod1 (lower band) and Sod2 (upper band). As expected, only one band was present in either sod1Δ or sod2Δ. No SOD activity bands were detected in the sod1Δ sod2Δ mutant (Fig. 2). Although the same protein concentrations were loaded on the gel, the Sod1 band was more enriched than the Sod2 band. Furthermore, we restored normal SOD activity in the SOD mutants by complementation with plasmids carrying SOD1, SOD2 or both SOD1 and SOD2. We conclude that C. glabrata has only two SOD genes and that Sod1 provides most of the SOD activity.

**SOD mutants of C. glabrata are sensitive to superoxide**

Recently, it was shown that SOD1 is required to protect against superoxide in C. glabrata (Roetzer et al., 2011); however, the role of both SODs in the OSR has not been characterized. We decided to analyse the susceptibility of the SP and LP cells of sod1Δ, sod2Δ and sod1Δ sod2Δ mutants to MD. In LP, the sod1Δ mutant was more sensitive to MD than the parental strain (Fig. 3a). In contrast, the sod2Δ mutant had no effect upon exposure to MD. The sod1Δ sod2Δ double mutant showed the same sensitivity to MD as the sod1Δ mutant (Fig. 3a). In SP, both the sod1Δ and sod2Δ single mutants behaved like the parental strain, whereas the sod1Δ sod2Δ mutant showed increased susceptibility (Fig. 3b), thus indicating functional redundancy between the SODs in this growth condition.

Given that SP cells readjust their metabolism from fermentation to respiration with more reliance on mitochondrial activity and consequently more production of ROS (Herker et al., 2004), we decided to evaluate the growth of the SOD mutants in ethanol or glycerol as carbon source, causing a metabolic shift from glycolysis to mitochondrial respiration (Rasmussen et al., 2003). As expected, there was a marked growth defect of the sod2Δ and sod1Δ sod2Δ mutant strains in YPG and YPE media (Fig. 3c). These data indicate that Sod2 is necessary to counteract the superoxide generated in the mitochondria when cells are under mitochondrial respiration.

**Lysine auxotrophy in the absence of SODs**

It has been shown that the Sac. cerevisiae sod1Δ mutant is auxotroph for lysine and requires cysteine, leucine and methionine for normal growth (Biliński et al., 1985). Hence, we wondered whether lysine auxotrophy was present in C. glabrata. We found that the parental strain and the sod2Δ mutant grew normally in SD (without the four amino acids) (Fig. 4, compare lanes 1 and 3 SD). This result indicates that sod2Δ does not cause auxotrophy. However, the sod1Δ mutant grew slowly on SD plates (Fig. 4, compare lanes 1 and 2 SD) and, interestingly, the sod1Δ sod2Δ mutant did not grow on SD plates (Fig. 4, lane 4 SD). Providing lysine to the SD plates restored the growth of sod1Δ and sod1Δ sod2Δ but not at the same rate as the parental strain (Fig. 4, compare lanes 2 and 4 SD + lysine). Complementation of sod1Δ or sod1Δ sod2Δ with pSOD1 (pMB101) restored growth on SD plates (Fig. 4, lanes 5 and 7 SD). In contrast, complementing the sod1Δ sod2Δ mutant with pSOD2 (pMB144) alone did not restore growth on SD (Fig. 4, lane 8 SD). As expected, complementing the sod1Δ sod2Δ mutant with pSOD1, sod2Δ (pMB127) restored growth on SD plates (Fig. 4, lane 9 SD). These results indicate that Sod1 is the main contributor to auxotrophy for lysine; however, there is a synergistic effect of both Sod1 and Sod2 in lysine auxotrophy. Surprisingly, pSOD2 did not complement lysine auxotrophy in the sod1Δ sod2Δ mutant. A simple explanation for this result is that in the sod1Δ sod2Δ mutant, which has a high rate of mutation (Fig. 7), a mutation was selected in pSOD2 and would explain the lack of complementation. In summary, our results suggest that SODs are involved in the metabolism of C. glabrata and highlight their importance, mainly of Sod1, in the maintenance of the α-aminoacidipate (AAA) pathway. This AAA pathway has been described for de novo lysine biosynthesis in fungi.
It has been proposed that deletion of SOD1 in Sac. cerevisiae increases superoxide damage and that the homoaconitase Lys4, the second enzyme of the AAA pathway, is a superoxide-labile target (Wallace et al., 2004). We therefore decided to determine whether CgLys4 (CAGL0K10978g) was the cause of lysine auxotrophy in the sod1Δ sod2Δ mutant. LYS4 was cloned downstream of the strong PGK1 promoter in the expression vector pGRB2.2. The resulting plasmid was introduced into the sod1Δ sod2Δ mutant but the overexpression of Lys4 did not restore growth of this mutant on SD plates (Fig. 5, lane 3 SD) or the slow growth of sod1Δ (data not shown). Similar results were obtained with overexpression of LYS12, LYS20 or LYS21 genes in the sod1Δ sod2Δ mutant (Fig. 5, lanes 4–6 SD). This result suggests that a different gene or more than one gene of the AAA pathway is responsible for lysine auxotrophy.

Fig. 2. SOD activity in C. glabrata. Protein extracts of SP cultures of C. glabrata Ura\(^+\) strains: parental (CGM1719), sod1Δ (CGM1721), sod2Δ (CGM1723), sod1Δ sod2Δ (CGM1725), sod1Δ pSOD1 (CGM1642), sod2Δ pSOD2 (CGM1651), sod1Δ sod2Δ pSOD1 (CGM1639), sod1Δ sod2Δ pSOD2 (CGM1653) and sod1Δ sod2Δ pSOD1,SOD2 (CGM1640) were obtained in native condition. After electrophoresis, SOD activity was observed as discoloured bands on a blue-stained background. See Methods.

Fig. 3. C. glabrata resistance to MD and growth on non-fermentable sources. Saturated cultures of C. glabrata strains parental (BG14), sod1Δ (CGM787), sod2Δ (CGM865) and sod1Δ sod2Δ (CGM937) were grown in YPD medium for 48 h at 30 °C. For LP, cells were diluted into fresh medium. LP (a) and SP (b) cultures were divided into aliquots and treated for 2 h with different amounts of MD. Cells were resuspended in distilled water, and serial dilutions were prepared and spotted on YPD agar plates. (c) SP cultures were washed and resuspended in distilled water. Serial dilutions were prepared and spotted on YPD (glucose), YPE (ethanol) or YPG (glycerol) agar plates. See Methods.

Fig. 4. Lysine auxotrophy in the sod1Δ sod2Δ double mutant. Saturated cultures of C. glabrata Ura\(^+\) strains parental (CGM1719), sod1Δ (CGM1721), sod2Δ (CGM1723), sod1Δ sod2Δ (CGM1725), sod1Δ pSOD1 (CGM1642), sod2Δ pSOD2 (CGM1651), sod1Δ sod2Δ pSOD1 (CGM1639), sod1Δ sod2Δ pSOD2 (CGM1653) and sod1Δ sod2Δ pSOD1,SOD2 (CGM1640) were grown in SD medium supplemented with lysine for 48 h at 30 °C. Cells were washed and resuspended in distilled water. Serial dilutions were prepared and spotted on SD agar plates with or without 30 μg ml\(^{-1}\) lysine. Plates were incubated for 72 h at 30 °C. See Methods.
The protective role of the SODs in Candida glabrata

Absence of SODs decreases chronological lifespan

The survival time of non-dividing yeast cells in late SP is known as the chronological lifespan (CLS) (Fabrizio & Longo, 2003). In SP, cells are under aerobic respiration and they accumulate superoxide anions that promote chronological ageing (Longo et al., 1999). Several lines of evidence indicate that the SODs are particularly important in eliminating mitochondrial superoxide and postponing ageing. In fact, SOD mutants of Sac. cerevisiae have shorter CLSs than wild-type cells (Longo et al., 1996). We decided to evaluate the role of SODs in the CLS of C. glabrata. Saturated cultures of C. glabrata strains: parental, sod1Δ, sod2Δ, sod1Δ sod2Δ, lys5A, sod1Δ pSOD1, sod2Δ pSOD2, sod1Δ sod2Δ pSOD1, sod1Δ sod2Δ pSOD2 or sod1Δ sod2Δ pSOD1 pSOD2 were grown in SD medium supplemented with lysine (120 μg ml\(^{-1}\)) for 72 h at 30 °C. At day 3, cells were not dividing, and samples were taken at days 3, 4, 5, 7 and 9. From these samples, surviving cells were grown in fresh YPD medium and viability was assessed in a Bioscreen C system (see Methods). Day 3 represented 100 % viability. We found that more than 80 % of the cells of all strains were viable at day 4 (Fig. 6 and Table S4). The parental, sod1Δ and reconstituted mutants (sod1Δ pSOD1, sod2Δ pSOD2, sod1Δ sod2Δ pSOD1, sod1Δ sod2Δ pSOD2 and sod1Δ sod2Δ pSOD1 pSOD2) showed similar rates of viability loss over the experiment. At day 9, between 25 and 50 % of the cells remained viable for parental, sod1Δ and the reconstituted mutants. In contrast, the sod2Δ mutant lost 90 % viability and the sod1Δ sod2Δ mutant lost almost all viability. Moreover, the survival defects of the SOD mutants (sod2Δ, sod1Δ sod2Δ) were completely reversed by complementing with plasmids expressing the deleted SOD gene (Fig. 6). We had expected that the sod2Δ mutant and sod1Δ sod2Δ pSOD1 would have the same loss of viability (12 %, see Table S4). Interestingly, however, we found that the sod1Δ sod2Δ pSOD1 mutant has the same viability as the parental strain (45 %). An important observation is that sod1Δ sod2Δ has 3 % viability whereas sod2Δ (SOD1) has 12 % viability, a fourfold increase. This indicates that SOD1 participates in chronological lifespan in addition to SOD2. A possible explanation for the increase in viability in the sod1Δ sod2Δ pSOD1 mutant is that, since there are more copies of pSOD1, this could be masking the lack of SOD2 and providing protection against oxidative stress. Interestingly, the lys5Δ mutant, which is auxotrophic for lysine, remained viable longer than the sod1Δ sod2Δ mutant (Fig. 6). This result suggests a crucial role for SOD2 in CLS and also indicates that factors different from lysine auxotrophy must be involved in loss of SP viability of the sod1Δ sod2Δ mutant.

Deletion of SODs causes accumulation of O\(_2^–\) and high rate of mutation

During the experiments with the sod1Δ sod2Δ mutant, we noticed that, after prolonged incubation on plates with or without lysine, spontaneous mutations appeared at a high frequency compared with the single mutants. These spontaneous mutations were flocculant upon growth to SP phase in YPD medium. These observations indicate that the sod1Δ sod2Δ mutant is genetically unstable, and it has...
been shown that mutations in SOD genes lead to increased rates of spontaneous mutation in *E. coli* (Farr et al., 1986) and *S. cerevisiae* (Gralla & Valentine, 1991). We then wondered whether SODs could be implicated in DNA protection against oxidative damage. First, we evaluated the spontaneous mutation rate of *S. cerevisiae* and *C. glabrata* cultures by measuring the frequency of mutants resistant to 5-FAA and 5-FOA. In principle, *S. cerevisiae* and *C. glabrata* are more sensitive to DNA damaging compounds like MMS and HU (Fig. 7d). In summary, all the above data show that increase in O$_2^••$ is a potential cause of DNA damage in SOD mutants and that SODs are required to protect DNA from damage. Moreover, given the kind of mutations found in the 5-FAA and 5-FOA experiments, the DNA repair system affected could be the base excision repair (BER) pathway (David et al., 2007).

**C. glabrata SODs are dispensable for colonization in a murine model of systemic infection**

SODs are well-recognized virulence factors for a number of bacterial and fungal pathogens. For example, Sod1 and Sod5 of *C. albicans*, Sod3 of *Histoplasma capsulatum* and Sod1 or Sod2 of *Cryptococcus neoformans* var. *gattii* are required for virulence in murine models of disseminated infection. We asked whether the SODs of *C. glabrata* play a role during disseminated infection. Groups of seven mice were infected by tail vein injection. Mice were euthanized

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**Fig. 7.** Mutator phenotype of SOD mutants. The Ura$^+$ parental strain (CGM139), sod1Δ (CGM1146), sod2Δ (CGM1131) and sod1Δ sod2Δ (CGM1133) were grown in SD medium supplemented with lysine for 4 days at 28 °C. Mutation rates, expressed as number of 5-FAA$^R$ mutants (a) and 5-FOA$^R$ mutants (b) per 10$^6$ cells were determined by fluctuation analyses and maximum-likelihood methods using data from multiple independent experiments. Error bars show 95% confidence intervals. *, P<0.05. (c) Superoxide levels were measured by dihydroethidium (DHE) in the Ura$^-$ strains parental (CGM1), sod1Δ (CGM787), sod2Δ (CM656) and sod1Δ sod2Δ (CM937) after 4 days in SP. The data represent the mean ± SD of triplicate cultures. *, P<0.05. (d) SP cultures of *C. glabrata* Ura$^+$ strains parental (CGM1719), sod1Δ (CM721), sod2Δ (CM1723) and sod1Δ sod2Δ (CM1725) were grown in YPD medium for 48 h at 30 °C. Cells were washed and resuspended in distilled water. Serial dilutions were prepared and spotted on YPD plates supplemented (as indicated) with different concentrations of MMS or HU.
on day 7 after infection, and c.f.u. from kidneys, spleens and livers were counted (see Methods). The mean numbers of c.f.u. of the parent and the four mutants recovered from the three organ types showed no significant difference (Kruskal–Wallis test with Dunn’s post hoc comparison) (Fig. 8). The results of these experiments indicate that C. glabrata SODs are dispensable in the murine disseminated-infection model.

**DISCUSSION**

SODs protect mitochondrial proteins and nuclear DNA from the deleterious effects of O$_2^*$ and its chain reactions. These findings support a model in which oxidative damage to biomolecules in the SOD mutants is superoxide-driven and is due mainly to iron release from the 4Fe-4S clusters. Moreover, SODs participate in the OSR and are associated with virulence in fungal pathogens like *C. albicans* or *H. capsulatum* (Hwang et al., 2002; Youseff et al., 2012). In this paper, we expand our knowledge about the roles of the superoxide dismutases in *C. glabrata*.

**Lysine auxotrophy and SODs**

The sod1Δ sod2Δ double mutant is auxotrophic for lysine (Fig. 4, lane 4 SD), indicating that SODs are involved in oxidative protection of the AAA pathway. Similar findings were observed in Sac. cerevisiae, C. albicans and Schizosaccharomyces pombe (Gralla & Kosman, 1992; Hwang et al., 2002; Mutoh et al., 2002), where lysine auxotrophy was detected in sod1Δ single mutants. Lysine auxotrophy in the sod1Δ single mutant of *Sac. cerevisiae* has been proposed to be caused by the inactivation of the 4Fe-4S cluster contained in the homoaconitase (encoded by the *LYS4* gene) by O$_2^*$ (Wallace et al., 2004). However, in Sch. pombe, the homocitrate synthase (HCS, the first enzyme of the AAA pathway) is the target for oxidative stress in the sod1Δ mutant (Kwon et al., 2006), and furthermore it was shown that the overexpression of this gene (*LYS4*, orthologue of *LYS20/LYS21* of *Sac. cerevisiae*) was sufficient to suppress the lysine requirement of the sod1Δ mutant. Interestingly, we found that lysine auxotrophy in the sod1Δ sod2Δ double mutant was mainly due to the absence of sod1Δ and that overexpression of any of the *LYS* genes could not suppress lysine auxotrophy (Fig. 5) in the sod1Δ sod2Δ mutant. One possibility is that the overexpression of *LYS4* was not sufficient to provide a functional protein in the sod1Δ sod2Δ mutant. The mechanism for lysine auxotrophy is still not clear. It is possible that, in *C. glabrata* *LYS4*, a different gene or more than one gene of the AAA pathway could be responsible for lysine auxotrophy. One study on aconitases in *Sac. cerevisiae* supports the second possibility. While Aco1 is essential for the tricarboxylic acid cycle (Gangloff et al., 1990), Aco2 specifically and exclusively contributes to lysine biosynthesis before the step reaction catalysed by Lys4 (Fazius et al., 2012); however, only the aco1Δ aco2Δ double mutant is an auxotroph for lysine (Fazius et al., 2012). Interestingly, *C. glabrata* sod1Δ sod2Δ mutant did not require methionine, cysteine or leucine for growth.

**Deletion of SODs causes accumulation of O$_2^*$ and increased risk of mutations**

*Sac. cerevisiae* and *E. coli* SOD mutants are more prone to mutations (Farr et al., 1986; Huang et al., 2003) and contain high levels of intracellular free iron, which is evidenced by their marked Fenton-dependent DNA damage (Srinivasan et al., 2000). Consistent with these observations, we showed that SOD mutants of *C. glabrata* exhibited not only high levels of O$_2^*$, but also sensitivity to DNA damage agents and a high mutation rate (Fig. 7). The base substitutions caused under oxidative stress were mainly single-base (Table S5) and these changes are repaired by the BER pathway, which is also the primary process for repair of oxidative damage of DNA in yeast (Bonatto, 2007; Huang et al., 2003; Slupphaug et al., 2003). Furthermore, a recent hypothesis proposes that SODs could act as sensors of intracellular O$_2^*$ by interacting with different DNA repair pathways and cell cycle checkpoints. In *Sac. cerevisiae*, Sod2 associates with Ogg1, the major 8-oxoguanine excision enzyme in the DNA BER pathway, and there is a direct association between Sod1 and Mec1 or Dun1 (Bonatto, 2007), where DNA-damaging agents (HU and MMS) activate the MEC1-dependent checkpoint response to oxidative damage, thus resulting in cell cycle arrest and activation of the DNA repair machinery (Branzei & Foiani, 2007). In addition to the MEC1 pathway, BER has also been implicated in the response to MMS-induced damage (Xiao et al., 1996). We hypothesized that this mutator phenotype

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**Fig. 8.** Virulence of *C. glabrata* SOD mutants. Groups of seven mice were infected with the *Ura*+ strains parental (CGM139), sod1Δ (CGM1146), sod2Δ (CGM1131) and sod1Δ sod2Δ (CGM1133) via tail vein injection. After 7 days, mice were euthanized and the numbers of c.f.u. from kidney, spleen and liver were determined. Individual points represent results for individual mice in groups of seven. Bars indicate the geometric mean for each group. *P*=0.05 (Kruskal–Wallis test with Dunn’s post-hoc comparison). See Methods.
in the SOD mutants could be caused by an increase in oxidative stress due to elevated levels of \( \text{O}_2^- \), thus compromising the cell cycle checkpoint and the DNA repair pathways. SODs could prevent the accumulation of DNA damage.

**Deletion of SOD2 is responsible for CLS reduction**

Cells lacking SODs in *E. coli*, *Saccharomyces cerevisiae* and mice accumulate signs of oxidative stress during the ageing process, demonstrating the crucial protective role of SODs in the lifespan of mutually distant organisms (Dukan & Nyström, 1999; Li et al., 1995; Longo et al., 1999). Here, we have demonstrated for the first time to our knowledge that SODs participate in the CLS of *C. glabrata*. We found that the SOD2 gene is mainly responsible for the reduction in CLS (Fig. 6). Given that (1) Sod2 is located in the mitochondria (Fig. 1), (2) the sod2Δ and sod1Δ sod2Δ mutants had elevated levels of \( \text{O}_2^- \) during SP (Fig. 7c), and (3) Sod2 is required during respiration (Fig. 3), we proposed that the early death of the sod1Δ sod2Δ double mutant during SP was due to \( \text{O}_2^- \)-related damage mainly in the mitochondria. These results are consistent with the findings in *Sac. cerevisiae* and *Cryptococcus neoformans var. gattii* (Longo et al., 1996; Narasipura et al., 2005) indicating that Sod2 activity is conserved among diverse fungal species.

**C. glabrata SODs are dispensable for colonization in a murine model of infection**

We assayed the SOD mutants in a murine model of systemic infection and provided evidence that there is no difference in colonization of the organs between SOD mutants and the parental strain (Fig. 8). This is surprising since previous observations have proposed SODs as virulence factors in bacteria and pathogenic yeasts (Hwang et al., 2002; Langford et al., 2002; Narasipura et al., 2003; Youseff et al., 2012). This evidence indicates that there are additional factors that compensate for the lack of SODs in vivo. An interestingly hypothesis is that the DNA mutator phenotype of SOD mutants could confer a survival advantage in the mouse model of infection. This finding creates the possibility that SOD mutants under oxidative stress might select mutations favouring survival and proliferation within the host. It would be interesting to assay whether SOD mutants that have passed through mice have increased virulence.

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