Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence

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Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is suspected to be one of the anti-oxidant enzymes and virulence determinants active in some pathogenic micro-organisms. To elucidate the role of Cu/ZnSOD in the major human fungal pathogen *Candida albicans*, its gene, designated *SOD1*, was disrupted by the URA-blaster technique. The resulting *sod1/sod1* mutant showed delayed hyphal growth on Spider medium but could still form hyphae on other solid media or in liquid media, particularly in response to serum. Moreover, the *sod1/sod1* mutant was more sensitive to menadione, a redox-cycling agent, than the isogenic wild-type strain, although it still showed an adaptive oxidative stress response. Furthermore, the *sod1/sod1* mutant cells exhibited slow growth in minimal medium when compared to the wild-type cells, but their growth was restored by the addition of lysine to the medium. Interestingly, *C. albicans* cells lacking Cu/ZnSOD showed increased susceptibility to macrophage attack and had attenuated virulence in mice. Thus, these results suggest that Cu/ZnSOD is required for the protection of *C. albicans* against oxidative stresses and for the full virulence of the organism to be expressed.

Keywords: *SOD1*, hyphal growth, lysine auxotrophy, candidiasis

INTRODUCTION

Although aerobic organisms obtain many benefits from dioxygen in their respiration and other functions, they often encounter highly toxic reactive oxygen species (ROS) such as superoxide radical anions, hydrogen peroxide and hydroxyl radicals. These ROS are a result of incomplete oxidation during aerobic metabolism. Therefore, many organisms have evolved that have anti-oxidants and anti-oxidant enzymes to protect themselves from highly toxic ROS (Fridovich, 1989, 1995).

Superoxide dismutase (SOD), an anti-oxidant enzyme, catalyses the dismutation of superoxide radical anions to dioxygen and hydrogen peroxide. Generally, SODs are categorized into four classes according to their metal cofactors – copper- and zinc-containing SOD (Cu/ZnSOD), manganese-containing SOD (MnSOD), iron-containing SOD (FeSOD) and nickel-containing SOD (Fridovich, 1989, 1995; Youn *et al*., 1996a, b). Cu/ZnSOD is found mostly in the cytosol (Fridovich, 1989, 1995) and mitochondria of eukaryotic cells (Okado-Matsumoto & Fridovich, 2001) and in the periplasmic space of some prokaryotes (Battistoni *et al*., 1998; Farrant *et al*., 1997; Wilks *et al*., 1998). This enzyme has been shown to play a role in protecting cells against oxygen toxicity (Fridovich, 1989, 1995) and to act as a major repository for copper ions in virtually all eukaryotic cells (Okado-Matsumoto & Fridovich, 2001) and in the periplasmic space of some prokaryotes (Battistoni *et al*., 1998; Farrant *et al*., 1997; Wilks *et al*., 1998). This enzyme has been shown to play a role in protecting cells against oxygen toxicity (Fridovich, 1989, 1995) and to act as a major repository for copper ions in virtually all eukaryotic cells (Culotta *et al*., 1995); however, the loss of Cu/ZnSOD activity has various pleiotropic consequences on organisms, which include slow growth, conditional auxotrophies and DNA damage (Fridovich,

Abbreviations: Cu/ZnSOD, copper- and zinc-containing SOD; FeSOD, iron-containing SOD; MnSOD, manganese-containing SOD; ROS, reactive oxygen species; SG, synthetic glucose; SGU, SG plus 25 µg uridine ml⁻¹; SOD, superoxide dismutase.
1989, 1995). For example, a Cu/ZnSOD-null yeast strain was shown to be oxygen-sensitive, hypermutable, auxotrophic for lysine and methionine and defective in sporulation (Liu et al., 1992). In some pathogenic organisms, Cu/ZnSOD has also been proposed as being a virulence determinant that could decompose the superoxide radical anions generated by phagocytic cells (Hong et al., 1992; Farrant et al., 1997; Wilks et al., 1998; Battistoni et al., 1998).

Candida albicans, the major fungal pathogen of humans, causes not only oral and vaginal thrush but also systemic or life-threatening infections in immunocompromised patients (Cutler, 1991; Coleman et al., 1993). A number of factors are known to be involved in the virulence of C. albicans, such as its adhesion to host tissues, its evasion of the host immune system, its secretion of protease and its reversible morphological conversion from yeast to hyphal growth (Cutler, 1991; Vázquez-Torres & Balish, 1997). Once C. albicans has infected a host, it inevitably encounters ROS produced by host phagocytes as well as ROS produced as a consequence of its own oxygen metabolism. Since phagocytic cells produce the superoxide radical anion, the first intermediate in the sequential univalent reduction of dioxygen, during the oxidative burst, the SODs of C. albicans are thought to play a protective role in this organism by suppressing oxidative killing by the infected host (Vázquez-Torres & Balish, 1997).

Although SODs are important anti-oxidant enzymes and have an additional hypothetical role in the virulence of pathogenic fungi (Hamilton & Holdman, 1999), there is no direct evidence that SODs are involved in the pathogenicity of C. albicans. We have previously reported the characterization of Cu/ZnSOD and its gene from C. albicans (Hwang et al., 1999). The present study shows that Cu/ZnSOD of C. albicans contributes to the protection of this organism against oxidative stresses and to the establishment of the full virulence of this organism. This is the first report to provide direct evidence that Cu/ZnSOD is involved in the virulence of pathogenic fungi.

METHODS
Organisms and culture conditions. C. albicans strains and plasmids used in this study are listed in Table 1. The strains were routinely cultured on YPG (1% yeast extract, 2% peptone, 2% glucose) medium at 28 °C. Cells carrying plasmids or disrupted genes were cultured in synthetic glucose (SG) medium containing 0.67% yeast/nitrogen base without amino acids (Difco), 2% glucose and appropriate supplements. Amino-acid requirements of the strains for growth were tested by seeding cells to an OD_{600} value of 0.01 in SG medium supplemented with various amino acids as needed. Solid media were prepared by adding 1.4% agar to liquid broth. Escherichia coli DH5α was used for most plasmid construction and maintenance, and cells of this strain were grown at 37 °C in Luria–Bertani medium supplemented with appropriate antibiotics.

DNA manipulation and analysis. Plasmid isolation, PCR, restriction-enzyme digestion, cloning and Southern blot hybridization were carried out by standard methods (Sambrook et al., 1989). Labelling of the DNA probes was performed by using the non-radioactive labelling and detection kit (Roche Molecular Biochemicals). The coding region of SODI in pCH101 was labelled by random-primed incorporation of digoxigenin-tagged dUTP.

Disruption and re-integration of C. albicans SOD1. To disrupt both alleles of SODI using the URA-blaste technique (Fonzi & Irwin, 1993), the oligonucleotide primers 5’-GTCTATTGTTGACCAAGA-3’ and 5’-GCCAGTACACACACGACTC-3’ were used. The resulting 402 bp PCR product was inserted into the pGEM-T Easy vector (Promega), yielding pCH101. A 4.1 kb hisG–URA3–hisG cassette from p5921 (Fonzi & Irwin, 1993) was then inserted into a portion of SODI within pCH101. The resulting plasmid, pCH102, was cut with ApaI/Sacl, to remove the vector, and the disrupted SODI gene was transformed into the Ura– C. albicans strain CA14 (Fonzi & Irwin, 1993). Ura– transformants were selected on an uracil-deficient medium and the integration of the hisG–URA3–hisG cassette into the SODI locus was verified by Southern blot analysis. Subsequent Ura– derivatives of the heterozygous disruptants were selected on SG medium containing 625 μg 5-fluoroorotic acid ml^{-1} and 100 μg uridine ml^{-1}. This procedure was repeated to delete the remaining functional allele of SOD1.

For re-integration of SODI into the genome of C. albicans, an XbaI/Scal-digested URA3 fragment from pURA3 (Huh et al., 2001) was inserted into the XbaI/EcoRV sites of pSOD1, which was constructed through the ligation of a 2.8 kb EcoRI-digested genomic DNA fragment containing the SODI coding region into pBluescript KS(+) (Stratagene). The resulting plasmid, pCH103, was linearized by digestion at the unique Hpal site upstream of the coding region of SOD1; the linearized plasmid was integrated into the SOD1 locus of the Ura– strain sod1∆/sod1. The targeted re-integration of SODI into the genome of sod1/sod1 was confirmed by PCR (data not shown) and Southern blot analysis.

Staining to detect SOD activity. SOD activity on a non-denaturing polyacrylamide gel was detected by negative staining (Manchenko, 1994). The gel was incubated in 50 mM phosphate buffer (pH 7.8) for 10 min, in nitro blue tetrazolium solution (1 mg ml^{-1}) for 10 min and then in 50 mM phosphate buffer (pH 7.8) containing 0.01 mg riboflavin ml^{-1} and 3.25 mg N,N,N′,N′-tetramethylthenediamine ml^{-1} for 10 min at room temperature with gentle shaking. Areas of SOD activity remained clear when the gel was exposed to the light.

Western blot analysis. The purified Cu/ZnSOD enzyme (Hwang et al., 1999) was isolated from a 12% SDS-polyacrylamide gel and then injected into a mouse (4-week-old ICR female). Boosting with purified Cu/ZnSOD was done twice, with a 2-week interval between each boost; the mouse was killed 10 days after the second boost. A total of 50 μg of cell-free extract of C. albicans was subjected to 12% SDS-PAGE. The protein was then transferred onto a nitrocellulose filter and electroblocting was done according to Bollag & Edelstein (1991). The signals were visualized by using a colorimetric detection kit (Roche Molecular Biochemicals), according to manufacturer’s instructions.

Determination of growth rate. To assess the yeast-phase growth rates, an overnight-grown culture of C. albicans was subcultured into YPG or SG media and incubated at 28 °C. The cell density (OD_{600}) of these cultures was measured after 4 h and then every 2 h up to the stationary phase. The
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype and description</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>Wild-type isolate</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CAI4</td>
<td>Δura3::imm34/Δura3::imm34</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CH101</td>
<td>Δura3::imm34/Δura3::imm34 Δsod1::hisG–URA3–bisG/SOD1</td>
<td>This work</td>
</tr>
<tr>
<td>CH102</td>
<td>Δura3::imm34/Δura3::imm34 Δsod1::hisG/SOD1</td>
<td>This work</td>
</tr>
<tr>
<td>CH103</td>
<td>Δura3::imm34/Δura3::imm34 Δsod1::hisG/Δsod1::hisG–URA3–bisG</td>
<td>This work</td>
</tr>
<tr>
<td>CH104</td>
<td>Δura3::imm34/Δura3::imm34 Δsod1::hisG/Δsod1::hisG</td>
<td>This work</td>
</tr>
<tr>
<td>CH104R</td>
<td>Δsod1::hisG/Δsod1::hisG::SOD1::URA3</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p5921</td>
<td>hisG–URA3–bisG in pUC18</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>pURA3</td>
<td>URA3 in pGEM-T Easy</td>
<td>Huh et al. (2001)</td>
</tr>
<tr>
<td>pSOD1</td>
<td>2·8 kb EcoRI-digested genomic SOD1 in pBluescript KSI(+)</td>
<td>This work</td>
</tr>
<tr>
<td>pCH101</td>
<td>0·4 kb PCR fragment of SOD1 in pGEM-T Easy</td>
<td>This work</td>
</tr>
<tr>
<td>pCH102</td>
<td>sod1::hisG–URA3–bisG in pGEM-T Easy</td>
<td>This work</td>
</tr>
<tr>
<td>pCH103</td>
<td>URA3 in pSOD1</td>
<td>This work</td>
</tr>
</tbody>
</table>

doubling time of the cultures during the exponential phase was determined by using the formula
\[ t = \frac{\ln 2 \times \ln (n_2 - n_1)}{\ln n_1}, \]
where \( t \) is the time period in hours, \( a \) is the cell density at the beginning of the time period, and \( b \) is the cell density at the end of the time period. To determine the extension rate of the hyphae, \( C. albicans \) cells were incubated in YPG containing 10% fetal bovine serum (FBS; Gibco-BRL) at 37 °C and the relative sizes of the \( C. albicans \) hyphae were measured every 1 h over a period of 4 h under a microscope.

**Assay for the resistance of \( C. albicans \) to oxidative stresses.** All experiments were done according to the method of Izawa et al. (1995) with some modifications. Cells were grown to mid-exponential phase (2 × 10⁵ cells ml⁻¹), harvested and re-suspended in 50 mM potassium phosphate buffer (pH 7·8), to obtain an initial OD₆₀₀ value of 0·1. To observe the sensitivity of the \( C. albicans \) cells to oxidants, various concentrations of menadione or hydrogen peroxide were added to the cell suspensions. After 1 h incubation at 28 °C, aliquots were taken from the cell suspensions, diluted appropriately in 50 mM potassium phosphate buffer (pH 7·8) and plated onto solid YPG medium. After 2 days incubation at 28 °C, the number of colonies on the plates was counted.

**Survival assay in macrophages.** To determine the survival rate of \( C. albicans \) cells exposed to macrophages (cell-line RAW264.7), we used an end-point dilution survival assay (Rocha et al., 2001). An aliquot (1 ml) of an overnight-grown culture of \( C. albicans \) cells was washed twice in PBS and re-suspended at 1 × 10⁷ cells ml⁻¹ in cold 10% heat-inactivated FBS. An aliquot of the suspension (50 μl) was added to 150 μl cold 10% heat-inactivated FBS in 96-well plates containing medium or only macrophages (1 × 10⁶ cells per well). After four-fold serial dilutions, the plates were incubated on ice for 30 min and subsequently for 24 h at 37 °C in 5% CO₂. Colonies were visualized with the use of an inverted-phase microscope (Zeiss Axiovert 100) at 40× magnification. Survival of \( C. albicans \) was expressed as a percentage of the number of colonies in the presence of macrophages divided by the number of colonies in the absence of macrophages. Data shown represent the mean ± se of three independent experiments.

**Virulence studies.** Inbred BALB/c mice (Seoul National University Laboratory Animal Center) weighing between 17 and 20 g were used to test the virulence of the different strains of \( C. albicans \). Two experiments were initiated by growing the \( C. albicans \) strains on YPG plates for 48 h at 28 °C, suspending the cells in PBS and adjusting the suspensions to the desired cell density (OD₆₀₀ = 0·5). The virulence of each \( C. albicans \) strain (i.e. SC5314, CH103 or CH104R) was tested by injecting 0·1 ml of the appropriate cell suspension (1 × 10⁶ cells) into five mice. The statistical analyses of the differences in survival between the paired groups were performed with the Kaplan–Meier log-rank test. A P value of 0·05 was taken to indicate statistical significance in the results.

**RESULTS**

**Disruption of SOD1 in \( C. albicans \)**

We have previously reported the characterization of Cu/ZnSOD and its gene from \( C. albicans \) (Hwang et al., 1999). To determine its role in \( C. albicans \), SOD1 (GenBank accession no. AF046872) was sequentially disrupted by using the URA-blaster technique (Fig. 1a). Homozygous disruption of the SOD1 gene was confirmed by PCR (data not shown) and Southern blot analysis (Fig. 1b). Our previous studies have shown that purified Cu/ZnSOD is 19·6 kDa in size after SDS-PAGE and that its activity appears in the lower of the two SOD bands detected after activity staining (Hwang et al., 1999; Rhie et al., 1999). Thus, the loss of Cu/ZnSOD can be confirmed by Western blot analysis (Fig. 2a) and...
Southern blot analysis (Fig. 1b), Western blot analysis (Fig. 2a) and activity staining (Fig. 2b).

**Determination of growth rate of C. albicans and its morphological phenotype**

When the doubling times ($t_d$) of yeast-phase growth of *C. albicans* were measured in YPG medium at 28 °C, there were no significant differences in the growth rate of the wild-type parental strain (SC5314; $t_d = 1.12 \pm 0.01$ h) and the growth rate of the homozygous sod1/sod1 mutant strain (CH103; $t_d = 1.19 \pm 0.03$ h). Moreover, both strains showed a similar extension rate ($\mu$) of hyphae in liquid YPG medium supplemented with 10% serum (SC5314, $\mu = 24.4 \pm 0.5$ µm h$^{-1}$; CH103, $\mu = 24.6 \pm 1.6$ µm h$^{-1}$).

To examine the effect of the sod1 mutation on the hyphal growth of *C. albicans*, Ura$^+$ prototrophs were grown on several solid or liquid media that induce hyphal growth, e.g. Spider medium (Liu et al., 1994), Lee’s medium (Lee et al., 1975), 10% serum, corn meal agar (Difco) containing 0.33% Tween 80 and RPMI 1640 medium (Gibco-BRL). Although closer examination of the sod1/sod1 strain revealed no clear differences in the morphological phenotypes of this strain when grown in/on the different media tested, the sod1/sod1 mutant did show significantly delayed hyphal growth on solid Spider medium. The sod1/sod1 mutant had a wrinkled colony phenotype when grown on Spider medium for 5 days at 37 °C, whereas wild-type and heterozygote cells formed extensive radial filaments when grown on this medium (Fig. 3). When grown on Spider medium the revertant strain (CH104R) regained the capacity to set up hyphal growth, consistent with that of the heterozygote strain grown under the same conditions (Fig. 3).

**Effects of oxidants on the sod1/sod1 mutant**

The defence mechanism of *C. albicans* against oxidants might be an important factor for survival of this yeast in host phagocytic cells. Thus, we tested whether disruption of SOD1 affected the survival of *C. albicans* cells in the presence of oxidants. For this purpose, exponentially growing wild-type CAI4 or sod1/sod1 CH104 cells were treated with various concentrations of menadione or hydrogen peroxide. After 2 days incubation on YPG medium, the number of viable cells was counted. As shown in Fig. 4(a), disruption of SOD1 made these cells more sensitive to menadione but not to hydrogen peroxide. Similarly, the Ura$^+$ sod1/sod1 strain CH103 showed decreased survivability in the presence of menadione (data not shown) and its growth was inhibited in presence of this oxidant when compared to wild-type SC5314 and revertant CH104R cells (Fig. 4b).

**Adaptation of the sod1/sod1 mutant to menadione**

An organism pre-treated with a sublethal dose of a particular stress can usually withstand subsequent treatments with higher doses of the same stress. As *C.
can adapt to oxidative stresses and the activity of Cu/ZnSOD has been shown to increase significantly (six-fold) upon exposure of the organism to menadione (Jamieson et al., 1996), the role of Cu/ZnSOD in the adaptation of C. albicans to menadione was tested. Exponentially growing cells of C. albicans were treated with a sublethal concentration of menadione (25 µM) for 1 h in YPG medium. As with cells of the wild-type strain CAI4, cells of the sod1/sod1 mutant CH104 showed an adaptive response when treated with 25 µM menadione (Fig. 5). Therefore, other anti-oxidant enzymes or factors other than Cu/ZnSOD are likely to be involved in the adaptive response of C. albicans to this oxidant.

Leaky lysine auxotrophy of the sod1/sod1 mutant

An E. coli strain with a double MnSOD/FeSOD mutation (sodA/sodB) and a Saccharomyces cerevisiae sod1 mutant strain have both been shown to exhibit aerobic auxotrophy when grown on minimal medium, although different factors influenced the auxotrophy observed in these two organisms. For its normal growth, the S. cerevisiae sod1 mutant requires lysine and either cysteine or methionine to be present in the growth medium (Chang et al., 1990; Liu et al., 1992), while the E. coli sodA/sodB mutant requires branched amino acids to be present in the growth medium (Fridovich, 1995). To examine whether a sod1 mutation would affect the growth of C. albicans in minimal medium, the doubling times and yields at stationary phase of the wild-type and sod1/sod1 strains of this organism were...
Table 2. Doubling times and yields of C. albicans wild-type, sod1/sod1 and revertant cells when grown in minimal medium

Doubling times were determined (as described in Methods) after the Ura- and Ura+ cells had been grown at 28 °C in SG or SGU medium, respectively. Growth yields were measured at OD600 after each strain had been grown to stationary phase in the aforementioned media. Data represent the mean ± se from three independent experiments.

<table>
<thead>
<tr>
<th>Growth characteristic</th>
<th>Strain</th>
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<tr>
<td></td>
<td>Ura-</td>
</tr>
<tr>
<td></td>
<td>CAI4</td>
</tr>
<tr>
<td>Doubling time (h)</td>
<td>2.32±0.15</td>
</tr>
<tr>
<td>Growth yield (OD600)</td>
<td>6.48±0.53</td>
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</table>

Fig. 6. ‘Leaky’ lysine auxotrophy of the sod1/sod1 mutant CH104. The wild-type CAI4 (●) and sod1/sod1 CH104 (○) cells were grown aerobically with shaking at 200 r.p.m. in SGU medium at 28 °C. The growth of each strain was determined as a measure of the OD600 value. To examine the amino acid requirements of the sod1/sod1 mutant, sod1/sod1 cells were also grown in SGU in the presence of 40 µg lysine ml⁻¹ (▲) or 20 µg methionine ml⁻¹ plus 20 µg cysteine ml⁻¹ (△).

Fig. 7. Virulence assay. BALB/c mice were inoculated with 1 x 10⁶ cells of SC5314 (●), CH103 (○) or CH104R (▲) in their tail veins and the survival of the animals was monitored. Curves represent the compiled results of two replicate experiments (five mice per group in each experiment).

Taken together, these results suggested that the C. albicans sod1/sod1 mutant had a ‘leaky’ lysine auxotrophy.

Survival of the sod1/sod1 mutant in macrophages

To examine the role of Cu/ZnSOD in the defence of C. albicans against the fungicidal mechanism of macrophages, the wild-type (SC5314), sod1/sod1 (CH103) and revertant (CH104R) strains were tested for their abilities to survive in the cell-line RAW264.7, by using the endpoint dilution survival assay (see Methods). When incubated with macrophages, the wild-type, sod1/sod1 and revertant strains survived to 55.4±9.6, 14.2±6.3 and 39.7±8.1%, respectively (values based on the number of colonies in the presence of macrophages divided by the number of colonies in the absence of macrophages). This result suggested that the sod1/sod1

monitored. As shown in Table 2, the sod1/sod1 mutant showed a slower growth rate and lower growth yield when grown in minimal medium compared to the wild-type and revertant cells. However, since the differences (if any) in the growth rates and yields of the different Ura+ strains were not that great, we used Ura- strains to investigate which amino acids were required for normal growth of the sod1/sod1 mutant. When grown in SGU medium cells of the sod1/sod1 mutant CH104 grew more slowly than those of wild-type CAI4; however, growth of the sod1/sod1 mutant was restored by the addition of lysine to the medium, but not by the addition of methionine and cysteine to the medium (Fig. 6).
mutant was significantly more susceptible to the fungicidal activity of macrophages than the wild-type and revertant strains.

**Virulence study**

To investigate the effect of the sod1 defect on the virulence of *C. albicans* in a BALB/c mouse model, wild-type, sod1/sod1 and revertant strains of *C. albicans* were intravenously injected into immunocompetent mice. Since the Ura− strains show decreased virulence, only isogenic Ura+ strains were used to infect the mice. All of the mice inoculated with the wild-type or revertant cells carrying SOD1 died within 9 days of being inoculated. However, the mice inoculated with the Cu/ZnSOD-deficient strain survived significantly longer than those inoculated with the wild-type parental or revertant strains in each of two separate experiments (Fig. 7). The survival differences between the sod1/sod1 and wild-type or revertant strains were significant (*P* < 0.05, according to the Kaplan–Meier log-rank test). This result indicated that Cu/ZnSOD contributes to the virulence of *C. albicans* in a mouse model of intravenous infection.

**DISCUSSION**

This study describes the effects of Cu/ZnSOD on the morphological phenotype, oxygen metabolism and virulence of *C. albicans*. To investigate the function of Cu/ZnSOD in *C. albicans*, we sequentially disrupted the gene encoding this enzyme, SOD1 (Fig. 1). The resulting sod1/sod1 mutant showed delayed hyphal growth on Spider medium (Fig. 3), although no clear differences in the morphological phenotypes of this strain were observed when it was grown in/on the other hypha-inducing media tested. Some reports suggest that Cu/ZnSOD may play a role in fungal differentiation; for example, sod1 mutants of *Neurospora crassa* or *S. cerevisiae* showed reduced mycelial growth or defective spore formation, respectively, when compared to the wild-types (Chary *et al.*, 1994; Liu *et al.*, 1992). Additionally, it has been reported that the Cu/ZnSOD activity of *C. albicans* increases during the morphological transition of the organism from yeast-like to hyphal growth (Gunasekaran *et al.*, 1998). Thus, our finding that disruption of SOD1 led to defective hyphal development by the resulting *C. albicans* mutant strain when grown on Spider medium reinforces the possibility that Cu/ZnSOD is likely to be involved in the morphogenesis of *C. albicans* under some conditions.

*C. albicans* sod1/sod1 cells had similar phenotypes to *S. cerevisiae* sod1 cells with respect to their sensitivity to increasing concentrations of menadione (Fig. 4), their slow aerobic growth in minimal medium (Table 2) and their ‘leaky’ lysine auxotrophy (Fig. 6). However, the sod1/sod1 mutant showed no auxotrophy for methionine or cysteine. The requirement of either methionine or cysteine to be present for the growth of *S. cerevisiae* sod1 cells is apparently not a true auxotrophy, but rather results from the metabolic consequences of sulfur assimilation, which increases in the absence of sulfur-containing amino acids (Chang *et al.*, 1990). Thus, the *C. albicans* sod1/sod1 cells examined here seem to be more resistant to attack by the superoxide radical anion-mediated sulfur radicals formed during sulfur assimilation than *S. cerevisiae* sod1 cells. Recently, it has been reported that *C. albicans* has an unusual cytoplasmic MnSOD that confers anti-oxidant protection to the organism during its growth phases (Lamarre *et al.*, 2001); this cytoplasmic MnSOD has also been found in a few other organisms, including unicellular green algae (Kitayama & Togasaki, 1993) and filamentous fungi (Diez *et al.*, 1998). Unlike the situation with the *S. cerevisiae* sod1 strain, the cytosolic MnSOD of *C. albicans*, possibly, relieves the detrimental effect of a sod1 defect in this organism. Therefore, it is plausible (as one of a number of explanations) that the *C. albicans* sod1/sod1 cells have a diminished requirement for the essential amino acids lysine, methionine or cysteine to be present in minimal medium when grown under aerobic conditions compared to that of *S. cerevisiae* sod1 cells. An *N. crassa* sod1 mutant did not show any auxotrophy when grown aerobically in minimal medium (Chary *et al.*, 1994). Taken together, these results indicate that across fungal species there might be, to varying extents, differences in the abilities of different species to resist toxic products derived from superoxide radical anions.

*C. albicans* is a member of the normal microflora of humans and does not usually cause disease in immunocompetent hosts. However, *C. albicans* causes serious diseases in immunocompromised individuals, such as patients suffering from leukaemia or diabetes, those that have undergone recent organ transplant and human-immunodeficiency-virus-infected individuals (Coleman *et al.*, 1993). For the removal of *C. albicans* from the infected host, infected host cells require the interaction of many different types of immune cells with several candidacidal mechanisms. Oxygen-dependent killing mechanisms are very important in the removal of *C. albicans* from the infected host; these include the superoxide radical anion, myeloperoxidase hydrogen peroxide/halide system and reactive nitrogen intermediate responses of host macrophages (Vázquez-Torres & Balish, 1997). Therefore, the anti-oxidant defence systems of *C. albicans* are assumed to be essential for this organism to resist the host immune response and for it to exhibit its full virulence. In agreement with this view, exogenous anti-oxidants have been shown to impair the killing of *C. albicans* cells by neutrophils (Wagner *et al.*, 1986) and a catalase-deficient (*Wysong et al.*, 1998) or erythrococacic acid-deficient *C. albicans* strain (Huh *et al.*, 2001) has been shown to be far less virulent for mice than the parental wild-type strain. The present study also shows that the Cu/ZnSOD-deficient strain used here has an increased susceptibility to fungicidal damage by macrophages and attenuated virulence in a mouse model for systemic candidiasis (Fig. 7). Considering the function of Cu/ZnSOD as an anti-oxidant enzyme, our
results and those of others suggest that Cu/ZnSOD may be essential for \textit{C. albicans} to resist the oxidant-mediated killing actions of the host immune system. Another possible explanation for the attenuated virulence of the \textit{C. albicans sod1/sod1} cells observed here is likely to involve lysine biosynthesis. Since host cells do not possess the ability to synthesize lysine, an essential nutrient, infecting \textit{C. albicans} cells can only utilize this limited nutrient via a \textit{de novo} pathway (Broquist, 1971). However, this possibility seems to be very unlikely because the differences, if any, in the doubling times and yields between \textit{sod1/sod1} and wild-type or revertant pathogenic \textit{Ura}^+ strains were very small when the strains were grown in minimal medium (Table 2). It is also well known that there is a strong correlation between the morphological transition of \textit{C. albicans} from yeast-like to hyphal growth and its virulence. The positive control of hyphal development in \textit{C. albicans} is signalled, to a certain extent, via the MAP kinase cascade to activate the transcription factor Cph1, whose deletion results in suppressed hyphal growth of \textit{C. albicans} on Spider medium (Liu et al., 1994). The \textit{cph1/cph1} mutant can develop hyphae when grown in serum or liquid media and is as virulent as the wild-type strain (Lo et al., 1997). Since \textit{C. albicans sod1/sod1} cells still form hyphae when grown in liquid culture and in response to serum (like the \textit{cph1/cph1} mutant cells), the delayed hyphal growth of this strain on Spider medium is unlikely to be the major cause of its attenuated virulence.

In conclusion, we have shown that Cu/ZnSOD of \textit{C. albicans} plays roles in the protection of this organism from oxidative stresses, in its defective hyphal development on Spider medium, in its survival in macrophages and in the establishment of its full virulence in a mouse infection model. Thus, our findings demonstrate that Cu/ZnSOD is involved in the virulence of \textit{C. albicans} and provide important clues as to how the antioxidant enzymes of fungal pathogens function in the infection process.

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**REFERENCES**


Roles of Cu/ZnSOD in Candida albicans

manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase. J Biol Chem 276, 43784–43791.


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