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 eukaryotes (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 OD₆₀₀ 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* DH5 α 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 *SOD1* 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 *SOD1* using the URA-blaster technique (Fonzi & Irwin, 1993), the oligonucleotide primers 5'-GTTCATT-TTGAACAAGAA-3' and 5'-GCCAATGACACCACAAGC-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 *SOD1* within pCH101. The resulting plasmid, pCH102, was cut with *Apa*I/*Sac*I, to remove the vector, and the disrupted *SOD1* 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 *SOD1* locus was verified by Southern blot analysis. Spontaneous 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 *SOD1* into the genome of *C. albicans*, an *Xba*I/*Sac*I-digested URA3 fragment from pURA3 (Huh *et al.*, 2001) was inserted into the *Xba*I/*Eco*RV sites of pSOD1, which was constructed through the ligation of a 2.8 kb *Eco*RI-digested genomic DNA fragment containing the *SOD1* coding region into pBluescript KS(+) (Stratagene). The resulting plasmid, pCH103, was linearized by digestion at the unique *Hpa*I 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 *SOD1* 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'-tetramethylethylenediamine 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 electroblotting 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 (OD₆₀₀) 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

Strain/plasmid	Genotype and description	Source/reference
<i>C. albicans</i>		
SC5314	Wild-type isolate	Fonzi & Irwin (1993)
CA14	$\Delta ura3::imm434/\Delta ura3::imm434$	Fonzi & Irwin (1993)
CH101	$\Delta ura3::imm434/\Delta ura3::imm434$	This work
CH102	$\Delta sod1::hisG-URA3-hisG/SOD1$ $\Delta ura3::imm434/\Delta ura3::imm434$	This work
CH103	$\Delta ura3::imm434/\Delta ura3::imm434$ $\Delta sod1::hisG/\Delta sod1::hisG-URA3-hisG$	This work
CH104	$\Delta ura3::imm434/\Delta ura3::imm434$ $\Delta sod1::hisG/\Delta sod1::hisG$	This work
CH104R	$\Delta sod1::hisG/\Delta sod1::hisG::SOD1::URA3$	This work
Plasmid		
p5921	<i>hisG-URA3-hisG</i> in pUC18	Fonzi & Irwin (1993)
pURA3	URA3 in pGEM-T Easy	Huh <i>et al.</i> (2001)
pSOD1	2·8 kb <i>Eco</i> RI-digested genomic <i>SOD1</i> in pBluescript KS(+)	This work
pCH101	0·4 kb PCR fragment of <i>SOD1</i> in pGEM-T Easy	This work
pCH102	<i>sod1::hisG-URA3-hisG</i> in pGEM-T Easy	This work
pCH103	URA3 in pSOD1	This work

doubling time of the cultures during the exponential phase was determined by using the formula $\ln 2 \times t / (\ln b - \ln a)$, 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 (2×10^7 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 resuspended at 1×10^7 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 (1×10^5 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 $\pm$ 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 (1×10^6 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

Fig. 1. Disruption of *SOD1* in *C. albicans*. (a) Schematic representation of the disruption strategy used. The restriction map of the *SOD1* locus and insertion of the *hisG*–*URA3*–*hisG* cassette at the indicated *KpnI* site in the *SOD1*-coding sequence are shown. E, *EcoRI*; K, *KpnI*; H, *HpaI*. (b) Southern blot analysis with the sequences bracketed in (a) used as probes (shown at right of image). The DNA digested with *EcoRI* was from CAI4 (lane 1), CH101 (lane 2), CH102 (lane 3), CH103 (lane 4), CH104 (lane 5) or CH104R (lane 6).

Fig. 2. Verification of the disruption of *SOD1* by Western blot analysis (a) and by activity staining (b). SOD activity was revealed by staining the 7.5% native polyacrylamide gel with nitro blue tetrazolium and riboflavin. A sample (50 µg) of the crude extract from exponentially growing cells of CAI4 (lane 1), CH101 (lane 2), CH102 (lane 3), CH103 (lane 4), CH104 (lane 5) or CH104R (lane 6) was loaded into the wells.

activity staining (Fig. 2b), respectively. To investigate whether wild-type *SOD1* could directly restore the phenotypes of a *sod1* mutant, one copy of *SOD1* was re-introduced into the *sod1/sod1* mutant strain (CH104); the correct re-insertion of *SOD1* was confirmed by

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 (μ) 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.*

Fig. 3. Defective hyphal growth of the *C. albicans* *sod1/sod1* mutant. Each strain was grown on solid Spider medium and photographed after growth for 5 days at 37 °C. Bar, 5 mm and applies to each panel.

Fig. 4. Effect of oxidants on the *C. albicans* *sod1/sod1* mutant. (a) Survivabilities of the wild-type CA14 (●) and *sod1/sod1* CH104 (○) *Ura*[−] strains after treatment with menadione and hydrogen peroxide. The survival of the strains was measured as described in Methods; survival is expressed as a percentage of the number of colonies grown from the treated sample divided by the number of colonies grown from the (untreated) control sample. Data shown represent the mean $\pm$ SE of three independent experiments. (b) Effect of menadione on the growth of *C. albicans*. Wild-type SC5314 (●), *sod1/sod1* CH103 (○) and revertant CH104R (▲) *Ura*⁺ cells were grown in YPG broth containing 60 μ g menadione ml^{−1}. Aliquots from the growing cultures were taken at various times and growth was determined as a measure of the OD₆₀₀ value. For culturing on solid medium, cells of SC5314 (lane 1), CH103 (lane 2) and CH104R (lane 3) were serially diluted and then spotted onto YPG plates containing 0.3 mM menadione.

albicans 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 μ M) for 1 h in YPG medium. As with cells of the wild-type strain CA14, cells of the *sod1/sod1* mutant CH104 showed an adaptive response when treated with 25 μ M

Fig. 5. Effect of *SOD1* disruption on the adaptation of *C. albicans* cells to menadione. Cells of *C. albicans* growing exponentially in YPG medium were treated with 0, 0.2, 0.4 and 0.6 mM menadione. To induce a sufficient adaptive response, the cells were pre-treated with 25 μ M menadione for 1 h. ●, CA14; ○, pre-treated CA14; ▲, CH104; △, pre-treated CH104.

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 $\pm$ SE from three independent experiments.

Growth characteristic	Strain				
	Ura ⁻		Ura ⁺		
	CAI4	CH104	SC5314	CH103	CH104R
Doubling time (h)	2.32 $\pm$ 0.15	2.76 $\pm$ 0.24	2.10 $\pm$ 0.15	2.30 $\pm$ 0.08	2.27 $\pm$ 0.03
Growth yield (OD ₆₀₀)	6.48 $\pm$ 0.53	4.43 $\pm$ 0.16	8.46 $\pm$ 0.47	7.10 $\pm$ 0.20	7.35 $\pm$ 0.28

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 (△).

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).

Fig. 7. Virulence assay. BALB/c mice were inoculated with 1×10^6 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 end-point 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 $39.7 \pm 8.1\%$, 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*

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, 1995) 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 erythroascorbic 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 *C. albicans* to resist the oxidant-mediated killing actions of the host immune system. Another possible explanation for the attenuated virulence of the *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 *C. albicans* cells can only utilize this limited nutrient via a *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 *sod1/sod1* and wild-type or revertant pathogenic 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 *C. albicans* from yeast-like to hyphal growth and its virulence. The positive control of hyphal development in *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 *C. albicans* on Spider medium (Liu *et al.*, 1994). The *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 *C. albicans* *sod1/sod1* cells still form hyphae when grown in liquid culture and in response to serum (like the *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 *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 *C. albicans* and provide important clues as to how the antioxidant enzymes of fungal pathogens function in the infection process.

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