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ARTICLE

In vitro response of clinical isolates of *Candida* species to oxidative stress

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ABSTRACT: (*In vitro* response of clinical isolates of *Candida* species to oxidative stress). Pathogenic species of *Candida* differ in many aspects, including their clinical prevalence, virulence, and profile of antifungal resistance. One of the causes of these differences is possibly related to the differential capacity of these species to deal with oxidative stress. In this study, we compared clinical isolates of eight species of *Candida* with respect to their oxidative stress resistance *in vitro*, oxidative damage induction, and antioxidant enzymes. Intraspecific and interspecific variation was observed. In accord with data previously obtained from laboratory isolates, the results here indicate that *C. albicans*, *C. glabrata* and *C. krusei* have a more effective antioxidant system, and that *C. dubliniensis*, *C. famata* and *C. guilliermondii* are highly sensitive to oxidative stress. *C. parapsilosis* and *C. tropicalis* have intermediate resistance profiles. The stronger antioxidant system of some species may enable them to cause systemic infections or to resist antifungals.
Key words: *Candida* species, oxidative stress, antioxidant defenses.

RESUMO: (Resposta *in vitro* de isolados clínicos de espécies de *Candida* ao estresse oxidativo). Espécies patogênicas de *Candida* diferem em muitos aspectos, incluindo sua prevalência clínica, virulência, e perfil de resistência a antifúngicos. Uma das causas destas diferenças é possivelmente relacionada com a capacidade diferencial de estas espécies lidarem com o estresse oxidativo. Neste estudo, comparamos isolados clínicos particulares de oito espécies de *Candida* com respeito a sua resistência ao estresse oxidativo *in vitro*, indução de dano oxidativo e enzimas antioxidantes. Foram observadas variações intraespecíficas e interespecíficas. De acordo com dados previamente observados de isolados de laboratório, os resultados aqui indicam que *C. albicans*, *C. glabrata* e *C. krusei* possuem um sistema antioxidante mais efetivo, e que *C. dubliniensis*, *C. famata* e *C. guilliermondii* são altamente sensíveis ao estresse oxidativo. *C. parapsilosis* e *C. tropicalis* possuem perfis de resistência intermediários. O sistema antioxidante mais potente de algumas espécies possivelmente habilita as mesmas a causar infecções sistêmicas ou resistir a antifúngicos.
Palavras-chaves: Espécies de *Candida*, Estresse oxidativo, Defesas antioxidantes.

INTRODUCTION

The clinical spectrum of infections caused by species of *Candida* ranges from benign colonization of the skin and mucosal surfaces to mucocutaneous forms of candidiasis and systemic infections (Netea *et al.* 2008, Favalessa *et al.* 2010). Sobel (2010) pointed out that, for more than a decade, multiple epidemiological studies have indicated that *Candida* species are the fourth most common cause of nosocomial bloodstream infection worldwide; emphasis was given to the continued extremely high mortality associated with candidemia, which approaches 35% in the United States (Carlisle *et al.* 2009, Sobel 2010).

Some factors involved in the development of candidiasis are: mucosal and cutaneous barrier disruption, neutrophil dysfunction (quantitative and qualitative), metabolic disorders, and extremes of age (< 1 and > 70 yr) (Pfaller & Diekema 2007). AIDS patients, organ transplant recipients, cancer patients receiving chemotherapy, recipients of artificial joints and prosthetic devices, and other immunocompromised individuals are particularly susceptible to candidiasis (Carlisle *et al.* 2009).

Although *Candida albicans* remains the most important human fungal pathogen because of its frequency of isolation (do Couto *et al.* 2011), and the amount of morbidity and mortality that it causes, the relative prevalence of species of *Candida* has changed (Sobel 2010). Horn *et al*. (2009) described a higher incidence of non-*albicans* species (54.4%) than *C. albicans* (45.6%) in 2019 candidemia cases. *C. tropicalis* and *C. parapsilosis* are highly important in the hospital environment (Storti *et al.* 2012). The decline in relative occurrence of *C. albicans* has largely been the result of an increased proportion of *C. glabrata* (Sobel 2010).

The initial host response to *Candida* is through recognition by innate immune cells (especially dendritic cells, macrophages, and neutrophils) and subsequent phagocytosis and elimination (Brown 2005). Professional phagocytes destroy pathogens in part through reactive oxygen species (ROS), including the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical

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($OH^{\cdot}$), generated directly or indirectly by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase (Flannagan *et al.* 2009). The importance of ROS in pathogen elimination is highlighted by individuals with mutations that cause partial or total inactivation of NADPH oxidase, and who as a consequence suffer from chronic granulomatous disease. This disease is characterized by severe, recurrent, life-threatening infections. In addition, incontrovertible evidence of the antimicrobial roles of $O_2^{\cdot-}$ and H_2O_2 has been established from the results of targeted deletion of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) (Wysong *et al.* 1998, Martchenko *et al.* 2004).

Butler *et al*. (2009) showed recently that there are significant expansions of gene families related to virulence in pathogenic *Candida* species. The occurrence of genes involved in stress responses is also variable among species. Previous studies have also demonstrated differences among *Candida* species in their capacity to overcome ROS toxicity (Tosello *et al.* 2007, Cuéllar-Cruz *et al.* 2008). Our previous results also revealed some differences in responses to oxidative stress (Abegg *et al.* 2010). Here, we analyzed *in vitro* oxidative stress responses of clinical isolates of eight important pathogenic *Candida* species.

MATERIALS AND METHODS

Yeast Strains, Media, and Culture Conditions

Yeast isolates studied were: *C. albicans* 1 (isolate from a nosocomial patient), *C. albicans* 51 (orotracheal tube of an AIDS patient), *C. dubliniensis* 23 and *C. dubliniensis* 25 (both from the oropharynx of AIDS patients), *C. famata* 1 and *C. famata* 24 (both clinical isolates from nosocomial patients), *C. glabrata* 1, *C. glabrata* 75, and *C. glabrata* 118 (all obtained from catheter tips), *C. guilliermondii* 73 (clinical isolate from a nosocomial patient) and *C. guilliermondii* 6260 (American Type Culture Collection - ATCC isolate from a patient with bronchomycosis), *C. krusei* 1 and *C. krusei* 2 (both isolated from skin lesions of diabetic patients), *C. parapsilosis* 81 and *C. parapsilosis* 115 (both isolated from patients with onychomycosis) and *C. tropicalis* 1 (isolated from an oral granuloma), and *C. tropicalis* 55 and *C. tropicalis* 56 (both clinical isolates from nosocomial patients). The maintenance of the yeasts on solid yeast extract-peptone-dextrose (YPD) medium (4-8 °C) and species identification of the isolates by means of morphological and biochemical tests were based on Kurtzman *et al.* (2011).

Viable cells obtained from yeast cultivated on solid YPD medium were grown in liquid YPD medium on an orbital shaker (30 °C, 100 rpm) until exponential growth was reached (OD_{600nm} = 1.5-1.6). Cells were then washed and diluted to OD_{600nm} = 0.15 in fresh liquid YPD for use. To obtain cell-free extracts, cells were lysed by disruption with glass beads and then centrifuged (10 min, 8,000 g) to remove debris.

Oxidant Sensitivity Assays

Disk diffusion tests

For disk diffusion tests, sterile 6-mm diameter filter paper disks were used. Each disk was impregnated with 5 µl of 7.5 M H_2O_2 (freshly opened bottles were always used), 0.5 M paraquat (1,1V-dimethyl-4,4V-bipyridinium dichloride hydrate, 95% purity) or 0.3 M menadione (2-methyl-1,4-naphthoquinone, vitamin K3, 95% purity). Diluted yeast cells (OD_{600nm} = 0.15) were plated onto YPD solid agar by means of sterile cotton swabs and incubated at 30 ºC for up to 72 h with the disks. Halos of growth inhibition were measured with a micrometer (Lamarre *et al.* 2001).

Spot tests

For spot tests, initial cell suspensions were diluted up to 10^{-4} in sterile water and spotted (5 µl) onto YPD agar plates containing 12 mM H_2O_2. After 72 h at 30 ºC the growth of each isolate was examined and the plates were photographed (Abegg *et al.* 2010).

MDA Determination

Malondialdehyde (MDA) was measured by HPLC using a method described by Karatepe (2004). The chromatograms were monitored at 250 nm.

Protein Carbonyl Groups

The protein carbonyl groups were quantified by a method employing 2,4-dinitrophenylhydrazine (DNPH) (Levine *et al.* 1990) as described elsewhere (Abegg *et al.* 2010).

Enzymatic Assays

For enzymatic assays, cell suspensions were treated with 0.5 mM H_2O_2 (final concentration) or left untreated, and incubated for 1 h, 30 ºC, 100 rpm on a rotary platform. Cells were then washed, lysed and centrifuged. Clarified supernatants were used in enzymatic assays.

Catalase (EC 1.11.1.6) activity was determined by monitoring the removal of H_2O_2 as proposed by Aebi (1984).

Glutathione peroxidase (GPx - EC 1.11.1.9) activity was assayed using 10 µl samples of the supernatants with the RANSEL commercial kit (Randox® Laboratories, Crumlin, UK). Superoxide dismutase (EC 1.15.1.1) activity was measured in 10 µl samples of cell supernatant using the RANSOD commercial kit (RANSOD SD 125, Randox® Laboratories, Crumlin, UK) according to the manufacturer's protocol.

Total Protein Content

To normalize the results, the total protein content of the cell extracts was quantified using the Bradford method (1976).

Data Analysis

Results are expressed as means ± S.D. Statistical analysis was performed using PASW 18.0 software (SPSS, Chicago, IL, USA). To compare variables between

groups, a one-way ANOVA was performed, followed by the *post-hoc* test of Tukey. Untreated samples were compared with treated samples by Student's *t*-test. Correlations were determined by Spearman rank correlation coefficient (rho). Values of $P \leq 0.05$ were considered statistically significant. In the figures and Table 1, statistics have been omitted to facilitate interpretation.

RESULTS

Disk diffusion and spot tests were used to compare the relative sensitivity of the isolates to oxidants (Fig. 1 and Fig. 2). The *C. albicans* and *C. krusei* 2 isolates were significantly more resistant to menadione than the *C. dubliniensis* and *C. guilliermondii* isolates. The *C. glabrata* isolates were significantly more resistant to H_2O_2 than were *C. dubliniensis* 23 and *C. guilliermondii* 6260. The *C. albicans* isolates were significantly more resistant to paraquat than were *C. famata* 1 and *C. guilliermondii* 6260 (Fig. 1).

Spots obtained without oxidative treatment and with 12 mM H_2O_2 YPD plates are shown in Figure 2a and 2b, respectively. The *C. dubliniensis*, *C. famata* and *C. guilliermondii* isolates were the most sensitive to H_2O_2. The other species were more resistant and grew better under H_2O_2 stress (Fig. 2b).

MDA levels were determined as a means to evaluate the lipid peroxidation index, and the degree of protein damage was assessed by measurement of protein carbonylation. The *C. parapsilosis* and *C. tropicalis* isolates showed the highest MDA levels. MDA levels in the *C. parapsilosis* and *C. tropicalis* isolates were significantly higher than in the *C. krusei* isolates. The clinical *C. guilliermondii* isolates showed carbonyl levels that were significantly higher than in the *C. albicans* isolates.

CAT, GPx, and SOD activities were determined with and without oxidative treatment (0.5 mM H_2O_2) (Table 1). Oxidative treatment increased CAT activity in all species tested. *C. albicans*, *C. glabrata* and *C. krusei* demonstrated the highest CAT activity. We found a reduction in GPx activity in most of the isolates under the conditions employed. SOD activity either was increased or was not significantly altered in most of the isolates (Student *t*-test; $P > 0.05$) (Table 1). The *C. parapsilosis* isolates had the highest SOD activity, followed by the *C. glabrata* isolates.

Table 1. Antioxidant enzyme activities in *Candida* extracts.

Strain	Treatment	GPx (U/mg ± SD)	SOD (U/mg ± SD)	Catalase (U/g ± SD)
C. albicans 1	Without H_2O_2	359.6±12.8	5.3±0.6	529.5±43.2
	0.5 mM H_2O_2	99.9±5.9	6.7±0.2	7253.5±539.9
C. albicans 51	Without H_2O_2	351.2±9.7	7.7±0.2	294.2±43.5
	0.5 mM H_2O_2	74.9±11.8	7.1±0.3	3681.3±622.5
C. dubliniensis 23	Without H_2O_2	80.9±12.9	11.45±1.0	282.4±15.9
	0.5 mM H_2O_2	99.8±7.4	7.3±2.7	1511.5±356.3
C. dubliniensis 25	Without H_2O_2	204.4±22.3	7.2±0.3	154.2±19.8
	0.5 mM H_2O_2	121.8±25.3	5.7±0.1	158.9±43.4
C. famata 1	Without H_2O_2	177.5±32.7	6.1±0.2	326.7±31.7
	0.5 mM H_2O_2	58.5±17.8	6.0±0.2	4867.8±656.3
C. famata 24	Without H_2O_2	249.3±45.9	4.6±0.5	308.8±102.9
	0.5 mM H_2O_2	65.2±11.9	4.1±0.1	2228.6±335.6
C. glabrata 1	Without H_2O_2	154.41±34.5	3.4±0.2	3554.78±69.5
	0.5 mM H_2O_2	126.7±12.8	3.5±0.2	8286.5±260.9
C. glabrata 75	Without H_2O_2	219.7±88.8	2.8±0.3	749.5±177.9
	0.5 mM H_2O_2	198.9±18.7	7.7±0.1	5868.8±1082.6
C. glabrata 118	Without H_2O_2	332.8±104.5	8.9±0.3	520.2±104.0
	0.5 mM H_2O_2	78.0±17.8	12.3±0.4	2130.0±134.6
C. guilliermondii 73	Without H_2O_2	396.0±88.4	5.6±0.2	123.9±56.7
	0.5 mM H_2O_2	276.5±35.6	4.7±0.2	1409.6±282.4
C. guilliermondii 6260	Without H_2O_2	397.9±28.9	10.0±0.2	147.8±29.6
	0.5 mM H_2O_2	239.8±65.7	6.8±1.9	2921.6±177.0
C. krusei 1	Without H_2O_2	249.6± 13.7	4.1±0.5	159.4±21.4
	0.5 mM H_2O_2	128.7±11.9	6.6±1.0	5702.0±691.2
C. krusei 2	Without H_2O_2	480.5±22.6	9.9±1.1	203.6±26.2
	0.5 mM H_2O_2	376.8±67.8	6.8±0.8	4357±568.8
C. parapsilosis 81	Without H_2O_2	413.9±6.8	11.3±2.6	236.4±81.9
	0.5 mM H_2O_2	120.8± 39.9	14.8±2.2	3032.8±772.5
C. parapsilosis 115	Without H_2O_2	275.3±77.9	7.1±0.1	732.8±60.2
	0.5 mM H_2O_2	121.3±15.3	13.5±1.0	2347.9±133.8
C. tropicalis 1	Without H_2O_2	356.9±38.8	7.0±0.4	257.1±17.3
	0.5 mM H_2O_2	54.1±9.9	6.9±0.1	1433±98.5
C. tropicalis 55	Without H_2O_2	422.9±61.9	12.4±0.9	65.2±18.4
	0.5 mM H_2O_2	114.3± 6.9	15.1±1.7	1266±83.2
C. tropicalis 56	Without H_2O_2	288.2±31.3	4.0±0.49	296.5±52.3
	0.5 mM H_2O_2	140.4±7.6	3.3±0.5	1891.0±78.8

Cultures of *Candida* species were grown aerobically at 30 ºC in YPD medium at 100 rpm until early exponential phase. Cultures were then either exposed to H_2O_2 or left untreated, and then frozen at -80 ºC. Cells were harvested and extracts were prepared and assayed as described in Materials and Methods. Values reported are the means of three determinations.

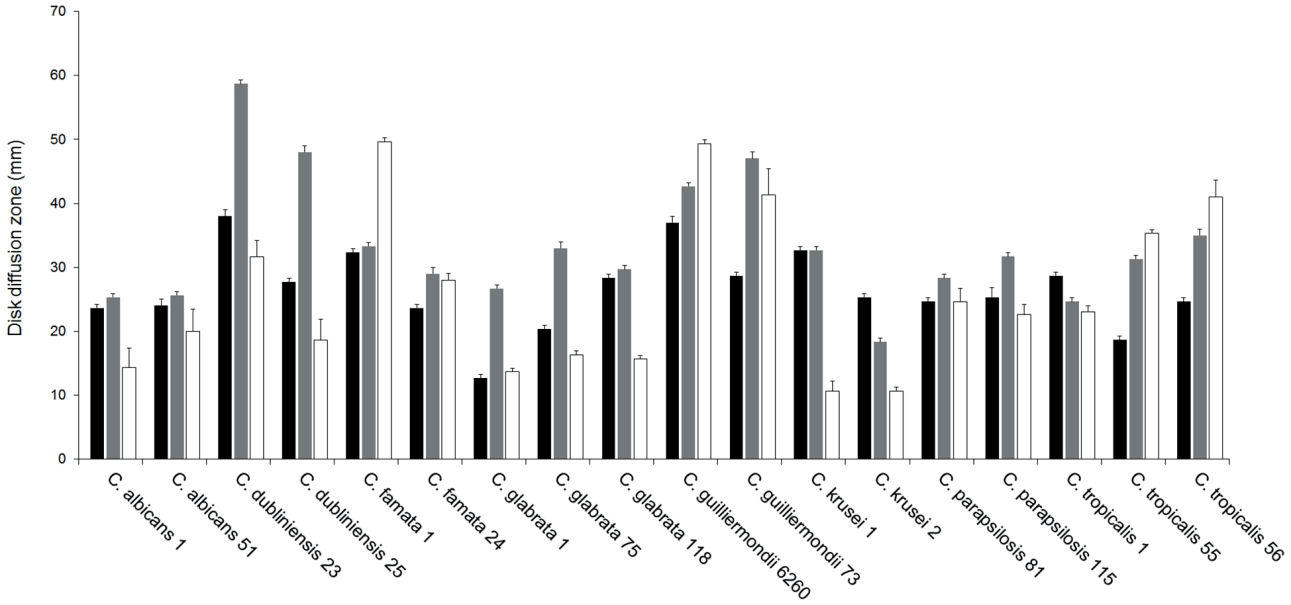

Figure 1. Results of disk diffusion assay. The diameters of the zones of inhibition of growth around filter disks on YPD agar were determined. The following quantities of oxidant solutions were used per disk: 5 µl of 7.5 M H_2O_2 (black bars), of 0.3 M menadione (gray bars) and of 0.5 M paraquat (white bars). Data represent the mean ± S.D. from three independent experiments.

DISCUSSION

In this study, we tested the *in vitro* oxidative stress response of clinical isolates of *Candida* species. The disk diffusion test is widely employed to evaluate the sensitivity of microorganisms to oxidative agents (Lamarre *et al.* 2001, Sampaio *et al.* 2009). In agreement with the present results, Sampaio *et al*. (2009) also found significant differences in the susceptibility patterns of *C. albicans* to various stress agents. These authors found inhibition halos ranging from 30-35 mm using 10 µl of 8.8 M H_2O_2, compared to the 20-23 mm halos with 5 µl of 8.8 M H_2O_2, observed in this study. They also found halos ranging from 20-30 mm using 10 µl of 0.5 M menadione, compared to the 20 mm halos with 5 µl of 0.3 M menadione, observed in this study (Fig. 1).

Our results for growth of *C. albicans* under 12 mM H_2O_2 stress were equivalent to those under 8 mM H_2O_2 stress obtained by Walia and Calderone (2008).

Taken together, the results of the sensitivity assays generally agree with our previous observation that *C. albicans*, *C. glabrata* and *C. krusei* are particularly resistant to oxidative stress, that *C. parapsilosis* and *C. tropicalis* possess an intermediate degree of resistance, and that *C. dubliniensis*, *C. famata* and *C. guilliermondii* exhibit more sensitivity to *in vitro* oxidative stress (Abegg *et al.* 2010). Costa-de-Oliveira *et al.* (2012) also observed that *C. krusei* isolates showed a high degree of resistance to oxidative stress, and these investigators found the presence of an alternative oxidase (AOX) in *C. krusei*, which may be related to this resistance.

MDA and lipid peroxidation assays were conducted under mild oxidative stress (0.5 mM H_2O_2) according to the method of Srinivasa *et al.* (2012) (Fig. 3a and 3b). MDA levels did not correlate with carbonyl levels. However, carbonyl levels correlated with the sensitivity of the isolates in disk diffusion tests (rho = 0.424, 0.300, and 0.411 for menadione, H_2O_2, and paraquat, respectively). The production of MDA depends on the availability of substrates, which are usually membrane polyunsaturated fatty acids (PUFA) (Antunes *et al.* 1996). *C. albicans* possesses membrane PUFA (Murayama *et al.* 2006), but little is known about the PUFA content in other pathogenic *Candida* species. The increase in CAT activity following oxidative treatment was higher than our previous observation (Abegg *et al.* 2010), and may contribute to the higher resistance to oxidative stress observed in species such as *C. albicans*, *C. glabrata* and *C. krusei*, which demonstrated higher activity (Table 1). Similar increases in CAT activity with oxidative stress have been observed previously (Jamieson *et al.* 1996, González-Párraga *et al.* 2003, Tosello *et al.* 2007).

Kusch *et al*. (2007) observed an up-regulation in CAT and GPx proteins after treatment with 1 mM H_2O_2. However, Yang *et al*. (2009), investigating the influence of culture conditions on GPx production in *C. albicans*, observed a slight reduction in GPx activity using 0.1 mM H_2O_2 and a slight elevation using 1 mM H_2O_2. This seems to demonstrate that, in addition to the culture conditions, the different concentrations of the oxidant employed strongly influenced the antioxidant activity of GPx (Jamieson *et al.* 1996, González-Párraga *et al.* 2003).

The possible inhibition of SOD activity under mild oxidative stress is in agreement with the results of Gunasekaran *et al*. (1998). These authors found a slight reduction in SOD activity using 1 mM H_2O_2 (from 15.05 to 12.46 U/mg protein), and an 83.6% reduction using 50 mM H_2O_2. Pedreño *et al*. (2006) observed a reduction in SOD activity in yeast treated with 5 mM H_2O_2. However, Tosello *et al*. (2007) described a twofold increase in SOD activity in *C. albicans* and *C. dubliniensis* isolates

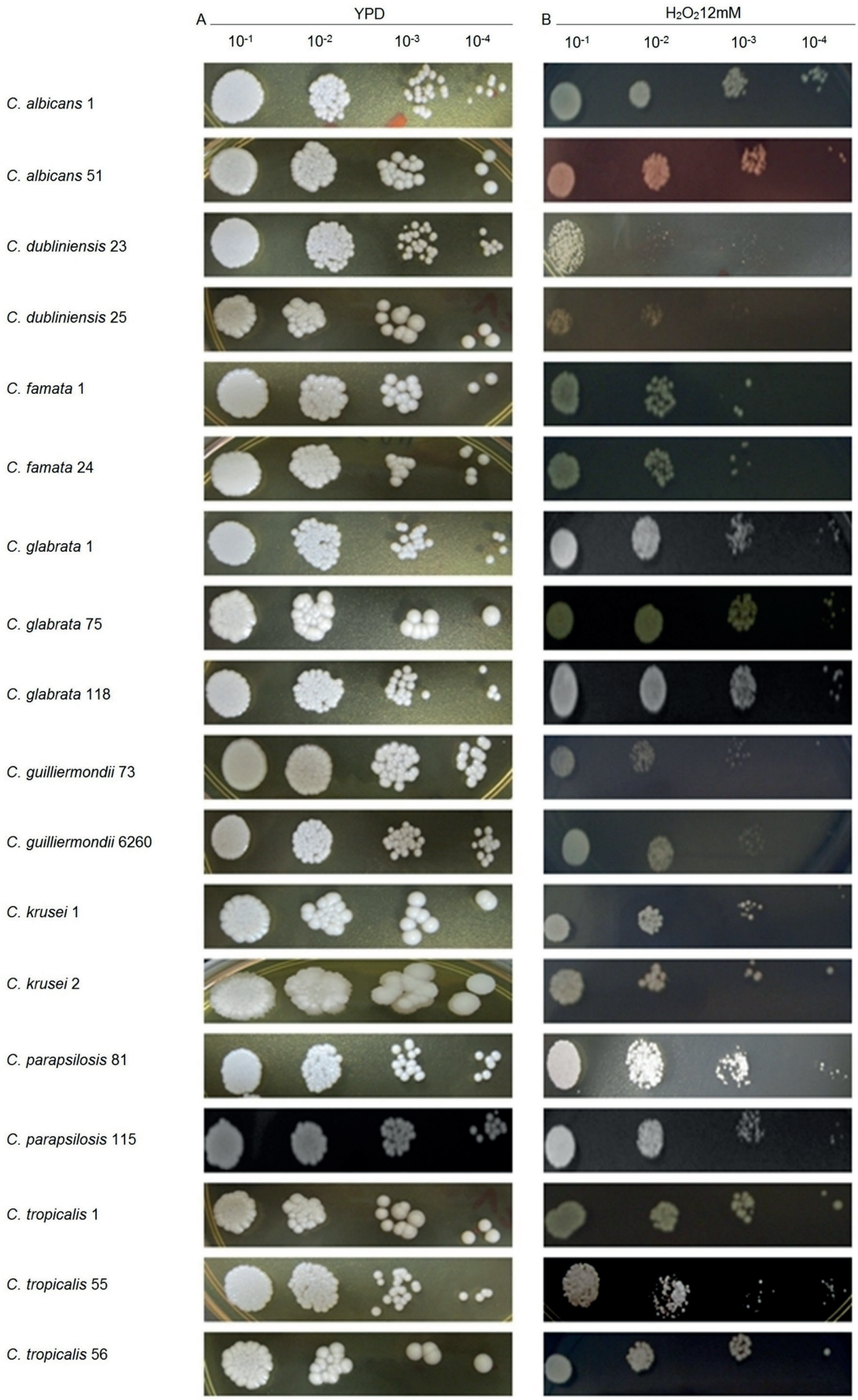

Figure 2. Resistance to H_2O_2 of *Candida* species clinical isolates during logarithmic growth. Mid-log phase cultures of *Candida* species were diluted to OD600 nm = 0.15. Dilutions up to 10-4 in sterile water were spotted (5 µL) onto YPD agar plates without oxidant (A) or onto YPD agar plates containing 12 mM H_2O_2 (B). Plates were incubated at 30 °C for up to 72 h and photographed.

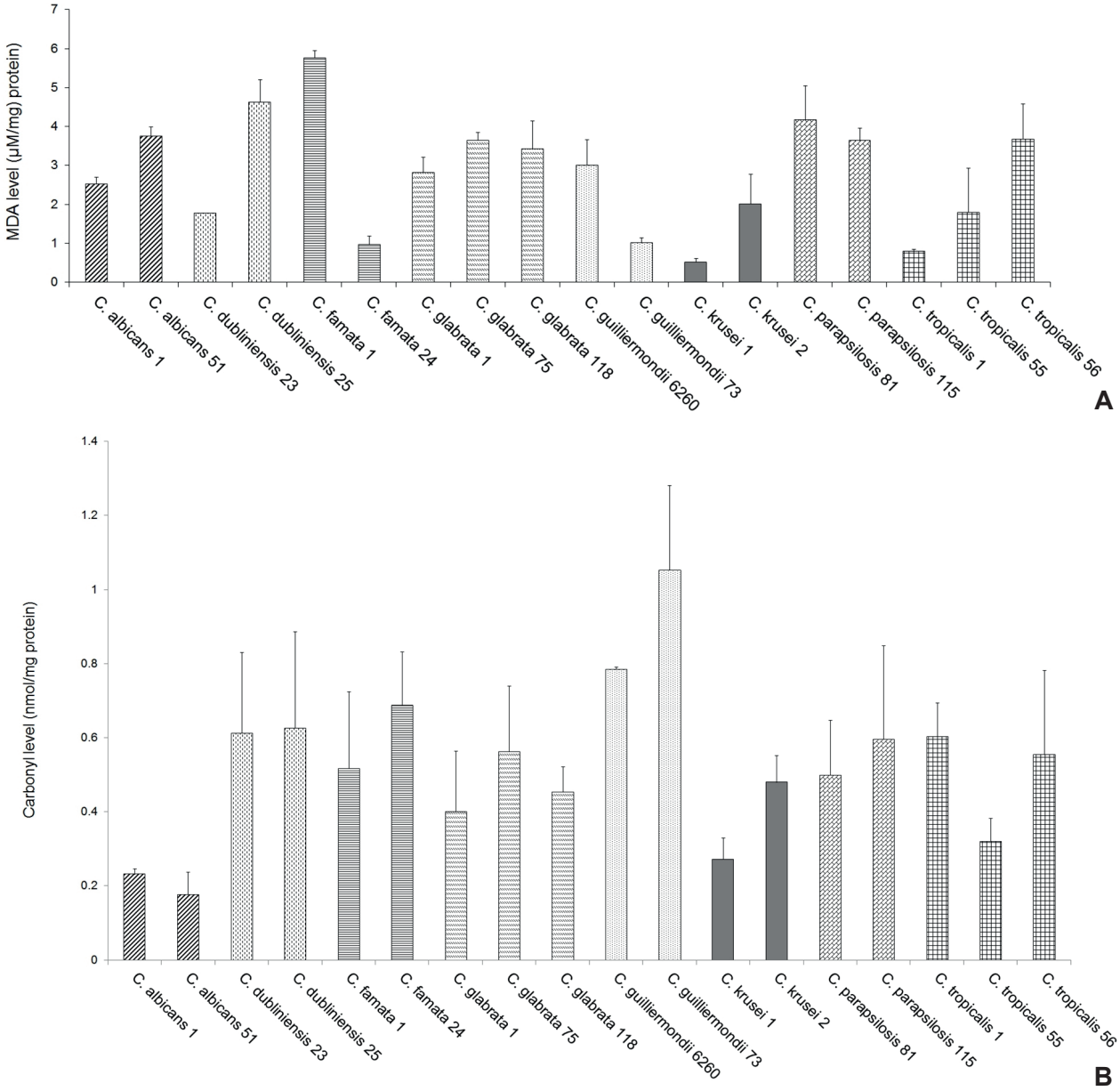

Figure 3. Lipid peroxidation and protein carbonylation in *Candida* species. Cells in exponential phase were treated with 0.5 mM H2O2 for 1 h at 30°C in YPD medium. MDA levels (A) were measured by HPLC, and protein damage (B) was measured by carbonyl assay as described in Materials and methods. Data represent the mean ± S.D. from three independent experiments.

treated with 0.4 mM H_2O_2. Fekete *et al*. (2007) observed SOD activities ranging from 3.7 to 11 U/mg protein in untreated and treated (1 to 6 mM of tert-butyl hydroperoxide - t-BOOH) cells of *C. albicans*. Gyetvai *et al*. (2007) observed similar SOD activities in untreated cells of *C. albicans*. In general, the SOD activities reported here agree with those found in other studies.

Brown *et al*. (2009) noted that *in vitro* experimental data on nitrosative and oxidative stress responses must be interpreted with caution. Stress-resistance mechanisms other than scavenging of ROS by redox-reactive molecules may be used by *Candida* species. This was proposed by Wellington *et al*. (2009), who observed that *C. albicans* and *C. krusei* were highly effective in suppressing ROS production by phagocytes through a mechanism that was thought to be independent of ROS scavenging. Also, yeast cells are probably exposed simultaneously to combinations of different stresses in their natural environments rather than to individual stresses in isolation. Kaloriti *et al.* (2012) compared combinatorial stress responses in *C. glabrata* and *C. albicans*, showing that combined stresses, particularly combinations of osmotic plus oxidative and oxidative plus nitrosative stress, are especially potent against these two species. Brown *et al.* (2014) recently reviewed the multiple and complex mechanisms that *C. albicans* cells employ in stress adaptation. The antioxidant enzymatic activities determined in the present study may be an important aspect of the stress response.

Suppression of ROS production was less effective in *C. glabrata* and *C. tropicalis* than in other *Candida* species (MacCallum 2008, Wellington *et al.* 2009). This suggests

that certain *Candida* species use additional mechanisms to subvert the toxic effects of phagocytes. Recently, Bruce *et al.* (2011) identified and characterized a novel response regulator in *C. albicans*, termed Crr1 (*Candida* Response Regulator 1), that is not conserved in *S. cerevisiae* or *S. pombe*. They demonstrated that Crr1 is specifically involved in the response of *C. albicans* to hydrogen peroxide stress, but not to other oxidizing agents or a range of other stress conditions.

Haynes (2001) posed some questions yet to be answered on the subject: "Why is *C. albicans* a more prevalent pathogen than other *Candida* species? Is it more widespread and the prevalence is just a reflection of this, or does *C. albicans* have a different repertoire of virulence determinants compared to other *Candida* species that allow it to be a *better* pathogen?" Our view is that species such as *C. albicans* and *C. glabrata* have a larger repertoire of virulence determinants despite the fact that, for example, *Candida tropicalis* and *Candida parapsilosis* are good producers of acid protease and phospholipase B, and species with a stronger antioxidant capacity are better prepared to cause disseminated infections. *C. albicans*, *C. glabrata* and *C. krusei* probably developed multiple evasion mechanisms in order to survive in the host.

Considering that, *in vivo*, *C. albicans* (Brown *et al.* 2014) and other pathogenic *Candida* species occupy complex and dynamic host niches characterized by alternative carbon sources and simultaneous exposure to combinations of stresses, different aspects of *in vivo* pathogenicity such as adhesion, biofilm formation and cell morphology are likely to be closely related to the cell antioxidant capacity.

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