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Oxidative stress response to menadione and cumene hydroperoxide in the opportunistic fungal pathogen *Candida glabrata*

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Candida glabrata *is an opportunistic fungal pathogen that can cause severe invasive infections and can evade phagocytic cell clearance. We are interested in understanding the virulence of this fungal pathogen, in particular its oxidative stress response. Here we investigated* C. glabrata, Saccharomyces cerevisiae *and* Candida albicans *responses to two different oxidants: menadione and cumene hydroperoxide (CHP). In log-phase, in the presence of menadione,* C. glabrata *requires Cta1p (catalase), while in a stationary phase (SP), Cta1p is dispensable. In addition*, C. glabrata *is less resistant to menadione than* C. albicans *in SP. The* S. cerevisiae *laboratory reference strain is less resistant to menadione than* C. glabrata *and* C. albicans*; however* S. cerevisiae *clinical isolates (CIs) are more resistant than the lab reference strain. Furthermore,* S. cerevisiae *CIs showed an increased catalase activity. Interestingly, in SP* C. glabrata *and* S. cerevisiae *are more resistant to CHP than* C. albicans *and Cta1p plays no apparent role in detoxifying this oxidant.*

Key words: *Candida glabrata* - menadione - cumene - *CTA1* - *Candida albicans* - *Saccharomyces cerevisiae*

Reactive oxygen species (ROS), including superoxide ion, hydrogen peroxide and hydroxyl radicals, are normal by-products of aerobic respiration. ROS can damage all biomolecules, but cells have developed enzymatic (catalases, superoxide dismutases and peroxidases) and non-enzymatic (glutathione and thioredoxin) mechanisms for keeping ROS levels low. Phagocytic cells, which are the first line of defence against fungal infections, generate ROS in order to eliminate invading pathogens (Mansour & Levitz 2002). Interestingly, pathogens have co-opted these well-conserved antioxidant mechanisms to evade phagocyte defences, thus survival and persistence are ensured (Thorpe et al. 2004, Temple et al. 2005).

Candida glabrata is an opportunistic fungal pathogen that can cause severe invasive infections and recent surveys show that *C. glabrata* is one of the most frequently isolated species in hospital acquired disseminated infections (Trick et al. 2002). *C. glabrata* virulence factors have only recently begun to be identified (Kaur et al. 2005). *C. glabrata* has a well-defined oxidative stress response and we have previously found that *C. glabrata* can withstand very high concentrations of H_2O_2 relative to that of *Saccharomyces cerevisiae* and even *Candida albicans* (Cuéllar-Cruz et al. 2008). Resistance of cells to H_2O_2 in stationary phase (SP) is dependent on the concerted role of Yap1, Skn7 and Msn4, well-conserved stress-related transcription factors. In log-phase cells (LP) *C. glabrata* adapts to high levels of H_2O_2 and this adaptive response is dependent on Yap1 and Skn7 and partially on Msn2 and Msn4. The single catalase gene *Cta1* is absolutely required for resistance to H_2O_2 in vitro, however in a mouse model of systemic infection the catalase is dispensable. A strain lacking *Cta1* showed no effect on virulence (Cuéllar-Cruz et al. 2008).

In this report, to better understand the oxidative stress response in *C. glabrata*, we screened the resistance of *C. glabrata* to two different oxidants: menadione and cumene hydroperoxide (CHP). We asked whether *C.g.*Cta1p is important for this response. We compared the resistance to these oxidants with the distantly related fungal opportunistic pathogen *C. albicans* and the non-pathogenic and closely related yeast *S. cerevisiae*. For *S. cerevisiae* we used a laboratory reference strain and two clinical isolates (CIs). In addition, we determined catalase activity in these strains.

MATERIALS AND METHODS

Strains - All strains used in this study are summarised in Table.

Media - Yeast media were prepared as described (Ausubel et al. 2001) and 2% agar was added for plates. YPD media contained yeast extract (10g/L) and peptone (20g/L), supplemented with 2% glucose.

Menadione and CHP sensitivity assays - All the starting overnight cultures of *C. glabrata*, *C. albicans* and *S. cerevisiae* were grown in YPD for 36 h at 30ºC. Menadione and CHP (88%) were purchased from Sigma-Aldrich. All liquid cultures and plates were incubated at 30ºC. For sensitivity assays for LP cells overnight cultures were diluted in fresh rich media, YPD, in such a

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way that cells went through seven doublings (D) to reach OD$_{600nm}$ 0.5. The cultures were divided, exposed to different menadione or CHP concentrations and incubated with shaking for 3 h. After the treatment the oxidant was removed and the cultures were adjusted when needed to O.D.$_{600nm}$ 0.5, serially diluted in 96-well plates and each dilution was spotted onto YPD plates and incubated at 30ºC. Each dilution had the same amount of cells.

For SP cells, 36 h cultures were diluted to O.D.$_{600nm}$ 0.5 with spent media from the same culture. The cells were divided into aliquots and treated with menadione or CHP at different concentrations for 3 h at 30ºC. After the treatment, the oxidant was removed and the cultures remained at the same O.D.$_{600nm}$ 0.5. The cultures were diluted in 96-well plates and spotted onto YPD plates.

All manipulations for these assays were performed in our 30ºC temperature-controlled room to prevent abrupt changes in temperature because it has been reported that cold shock has an impact on oxidative stress resistance in *S. cerevisiae*. The experiments were repeated three times.

Preparation of cell-free extracts and catalase activity assay - The cells were grown for 48 h in YPD containing 2% glucose and were harvested by centrifugation at 800 *g* for 10 min. Cells were washed twice with distilled water and resuspended in lysis buffer [potassium phosphate (pH 7.0) supplemented with SIGMA*FAST* protease inhibitor from SIGMA] and 0.5 g of glass beads (SIGMA). Cells were frozen and thawed two times and the mixture was vortexed 4-6 times with 1 min cooling on ice. The lysate was centrifuged for 20 min at 4ºC to remove cell debris and glass beads. The supernatant was used for enzymatic activity. Protein quantification was determined by Bradford assay (Bradford 1976). Bovine serum albumin from Sigma was used as standard. Catalase activity was determined in cell-free extracts by a spectrophotometric method that measures the breakdown of H_2O_2 by catalase (Aebi 1984). Assays were performed four times. The catalase activity was normalised to total protein from the lysate and expressed as units per mg of protein. One unit is defined as the amount of catalase required for degradation of 1.0 µmol H_2O_2 per min.

RESULTS

C. glabrata resistance to menadione - In order to understand the oxidative stress response in *C. glabrata*, we decided to assess its resistance in LP cells to menadione, a cytotoxic quinone that generates superoxide. The cells were grown and exposed to menadione (Fig. 1). *C. glabrata* BG14 (wt, reference strain) grows in up to 0.2 mM menadione. However, the *cta1*Δ strain, which lacks the single catalase gene, showed reduced resistance to menadione (Fig. 1A). We then assessed whether *C. glabrata* cells are more resistant to menadione in SP (Fig. 1). Both strains BG14 and *cta1*Δ exhibited increased resistance to menadione to the same extent (Fig. 1B).

C. albicans, S. cerevisiae and C. glabrata resistance to menadione in SP - Once we had established *C. glabrata* resistance to menadione in SP and that Cta1 was dispensable, we decided to compare its resistance with a *C. albicans* strain (an opportunistic fungal pathogen), a *S. cerevisiae* lab reference strain (non-pathogenic) and two *S. cerevisiae* CIs in SP. The cells were grown and exposed to menadione (Fig. 2). *C. albicans,* which is distantly related to *C. glabrata,* is more resistant to menadione than *C. glabrata* (Fig. 2). *C. albicans* can grow in up to 1.2 mM menadione while *C. glabrata* can only grow in up to 0.8 mM (Fig. 2). *S. cerevisiae* lab reference strain W303 showed resistance only up to 0.2 mM menadione. However, both *S. cerevisiae* CIs (YJM128 and YJM336) showed increased resistance to menadione. These strains were able to grow in the presence of 0.6 mM menadione (Fig. 2).

C. albicans, S. cerevisiae and C. glabrata resistance to CHP in SP - We decided to determine the resistance of these fungal strains to CHP, an organic hydroperoxide, in SP. The cells were grown and exposed to CHP (Fig. 3). Both strains, BG14 and *cta1*Δ, were resistant to 0.4 mM CHP (Fig. 3) and more resistant than *C. albicans* strain CA5, which showed sensitivity to 0.4 mM (Fig. 3). Interestingly, the non-pathogenic *S. cerevisiae* strain W303 and both CI strains, YJM128 and JYM336, showed almost the same resistance at 0.4 mM CHP as *C. glabrata*. At this concentration, *C. albicans* strain CA5 is completely sensitive (Fig. 3).

TABLE

Strains used in this study

	Strain	Parent	Genotype	Reference
Saccharomyces cerevisiae strain	W303		*MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 ade2-1 ade3::hisG*	McDonald et al. (1997)
	YJM128		Clinical isolate	Clemons et al. (1994)
	YJM336		Clinical isolate	Clemons et al. (1994)
Candida albicans strain	CA5		Clinical isolate	Laboratory collection
Candida glabrata strains	BG2		Clinical isolate (strain B)	Fidel et al. (1996)
	BG14	BG2	*ura3*Δ::Tn*903* G418^R Ura$^-$ strain used in this study	Cormack and Falkow (1999)
	CGM295 *cta1*Δ	BG14	*ura3*Δ::Tn*903* G418^R *cta1*Δ:: *hph* Hyg^R	Cuéllar-Cruz et al. (2008)

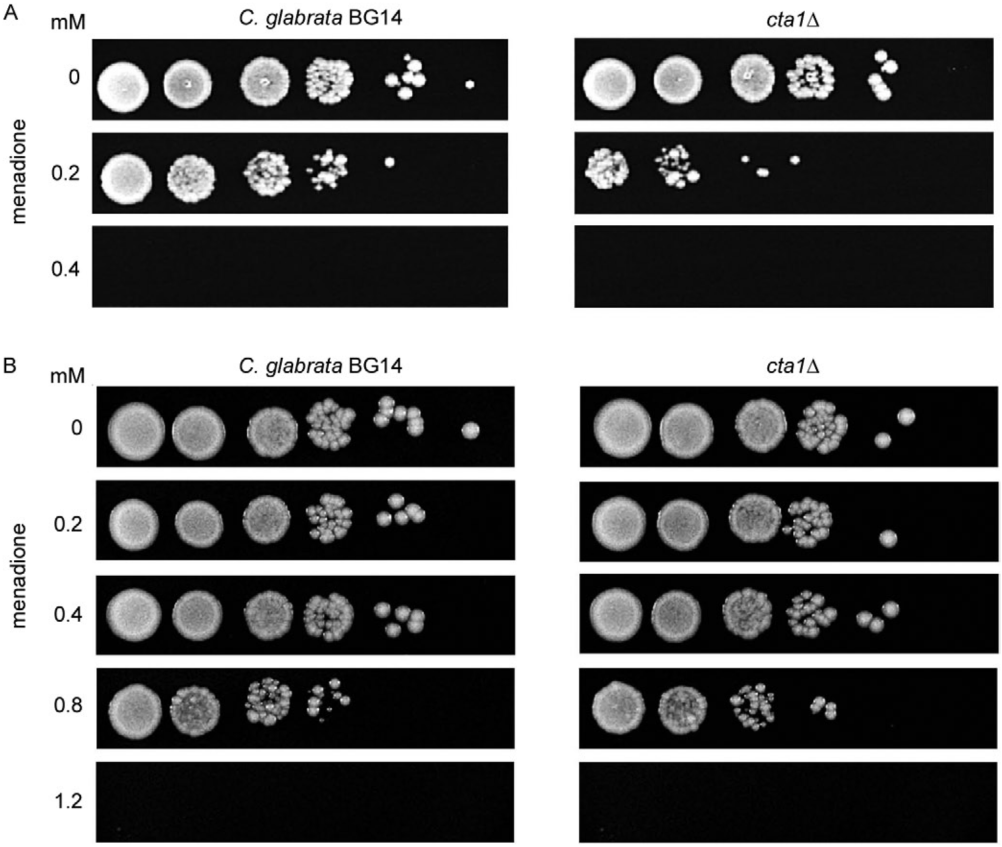

Fig. 1: *Candida glabrata* resistance to menadione. A: for log-phase cells LP sensitivity assay, saturated cultures of *C. glabrata* strains BG14 (wt) and CGM295 (*cta1*Δ) were diluted into fresh media (YPD) so that cells would reach an OD_{600nm} 0.5 after seven doublings at 30ºC. *C. glabrata* BG14 and CGM295 cultures were divided and exposed to 0, 0.2 and 0.4 mM of menadione. After the treatment, the cultures were adjusted when needed to $O.D._{600nm}$ 0.5, serially diluted and each dilution spotted into YPD plates, assuring that same amount of cells is plated. Plates were incubated at 30ºC; B: for stationary phase sensitivity assay, saturated cultures of *C. glabrata* strains BG14 (wt) and CGM295 (*cta1*Δ) were diluted to $O.D._{600nm}$ 0.5 with spent media from the same culture. The cells were divided into aliquots and treated for 3 h with menadione at different concentrations: 0, 0.2, 0.4, 0.8 and 1.2 mM. After the treatment, the cultures remained at $O.D._{600nm}$ 0.5, oxidant was removed and cells were diluted and spotted onto YPD plates. Plates were incubated at 30ºC.

C. glabrata, C. albicans and S. cerevisiae catalase activity - We have previously shown in plate assays that *C. glabrata* is more resistant to H_2O_2 than *C. albicans* and *S. cerevisiae* (Cuéllar-Cruz et al. 2008). Therefore, we decided to assay catalase activity in these strains. Surprisingly, *C.g.*B14, *C.a.*CA5 and *S.c.*W303 showed almost no difference in catalase activity (Fig. 4). As expected, the *C. glabrata* strain *C.g.cta1*Δ that lacks catalase showed no activity. However, *S. cerevisiae* CIs showed increased catalase activity, consistent with the fact that these *S. cerevisiae* CIs are more resistant to H_2O_2 than *C.a.*CA5 and *S.c.*W303 (Cuéllar-Cruz et al. 2008) (Fig. 4).

DISCUSSION

Pathogens are able to evade oxidative killing by phagocytic cells by using the well conserved enzymatic and non-enzymatic mechanisms that keep ROS, generated naturally by aerobic respiration, at low levels (Gonzalez-Parraga et al. 2003). In this way, pathogens can survive and persist in their host. The oxidative stress response for *C. glabrata* has only recently been described (Chen et al. 2007, Cuéllar-Cruz et al. 2008, Roetzer et al. 2008). *C. glabrata* is extremely resistant to very high levels of H_2O_2 and can evade phagocytic cell clearance (Kaur et al. 2007, Cuéllar-Cruz et al. 2008). *C. glabrata* has one catalase (Cta1p) that is required to respond to oxidative stress generated by H_2O_2 (Cuéllar-Cruz et al. 2008). Furthermore, the well-conserved transcription factors Yap1, Skn7, Msn2 and Msn4 coordinate, in part, the oxidative stress response in *C. glabrata* (Chen et al. 2007, Cuéllar-Cruz et al. 2008, Roetzer et al. 2008). In this report, we investigated the natural resistance of *C. glabrata, C. albicans* and *S. cerevisiae* to menadione and CHP. In addition, we measured their catalase activity in SP. Fig. 1A shows that LP cells of *C.g.*BG14 (wt) can resist up to 0.2 mM menadione and that this resistance is dependent on Cta1. Menadione is a cytotoxic quinone that generates superoxide and it has been shown that exposure to menadione induces the expression of *S.c.* and *S.p.Cta1* (Nakagawa et al. 1995, Osorio et al. 2003). This result suggests that the superoxide generated by menadione is dismutated to H_2O_2, which is then reduced by the

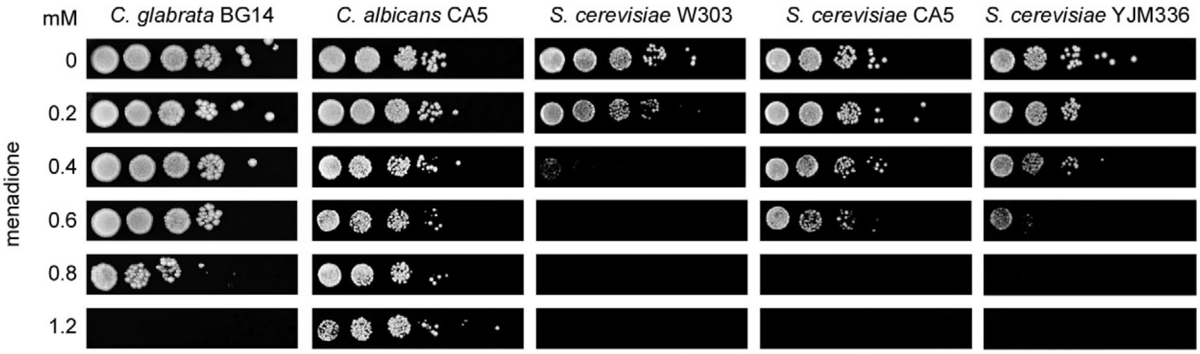

Fig. 2: *Candida glabrata*, *Candida albicans* and *Saccharomyces cerevisiae* stationary phase resistance to menadione. Saturated cultures of *C. glabrata* strain BG14, *C. albicans* strain CA5, *S. cerevisiae* strains W303, CIs YJM128 and YJM336 were treated as described in Fig. 1B.

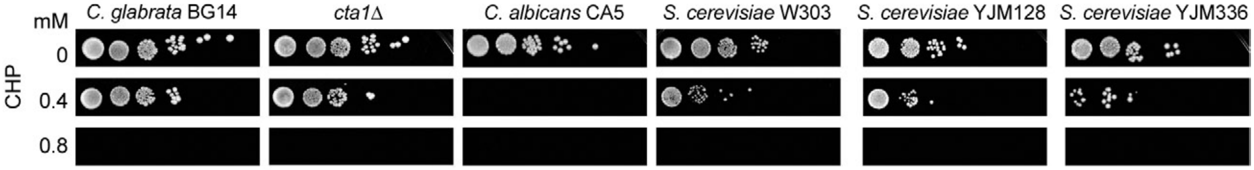

Fig. 3: *Candida glabrata*, *Candida albicans* and *Saccharomyces cerevisiae* stationary phase resistance to cumene hydroperoxide (CHP). Saturated cultures of *C. glabrata* strains BG14 and CGM295 (*cta1*Δ), *C. albicans* strain CA5, *S. cerevisiae* strains W303, CIs YJM128 and YJM336 were grown and treated as in Fig. 2, but cultures were expose to CHP at 0, 0.4 and 0.8 mM.

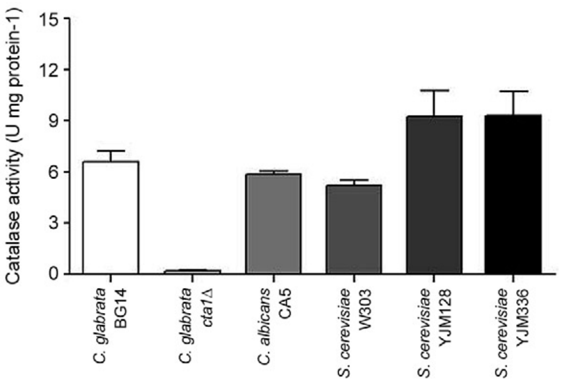

Fig. 4: *Candida glabrata*, *Candida albicans* and *Saccharomyces cerevisiae* catalase activity. *C. glabrata* strains BG14 and CGM295 (*cta1*Δ), *C. albicans* strain CA5, *S. cerevisiae* strains W303, CIs YJM128 and YJM336 were grown for 48 h (stationary phase) in YPD containing 2% glucose and cell extracts were prepared. The catalase activity was normalized to total protein from the lysate and expressed as units per mg of protein. One unit is defined as the amount of catalase required for degradation of 1.0 µmol H_2O_2 per min. Data is presented as the mean ± S.D. of four independent experiments.

catalase. In SP the resistance to menadione is increased and there is no difference in resistance between BG14 and the strain lacking the catalase (*cta1*Δ) (Fig. 1B). However, the increased resistance in SP is not surprising. It has previously been shown that SP cells of different pathogens are more resistant to different stresses including oxidative stress (Cyrne et al. 2003). Interestingly, Cta1 is dispensable in SP and this suggests that other genes are compensating for the lack of Cta1. However, this phenomenon appears to occur only in SP. In LP these genes may be silent. Other elements in SP that could be playing important roles in detoxifying oxidative stress are superoxide dismutases, glutathione, glutathione peroxidases and thioredoxins (Jamieson 1992, Jamieson et al. 1994, Stephen & Jamieson 1996, Zadzinski et al. 1998). In any case, the regulatory network of the oxidative stress response in SP in *C. glabrata* is an important aspect that requires further studies.

We compared the resistance to menadione in SP between *C. glabrata* and the closely related non-pathogenic yeast *S. cerevisiae* and the distantly related opportunistic fungal pathogen *C. albicans*. As shown in Fig. 2, *C. albicans* strain *C.a.*CA5 is more resistant to menadione than *C. glabrata* and *S.c.*W303 is even more sensitive. The enzymatic removal of superoxide ions is carried out by the enzyme superoxide dismutase. Thus, the resistance to menadione shown by *C. albicans* could be explained, in part, by the fact that *C. albicans* has six superoxide dismutases genes, *SOD1*, *SOD2*, *SOD3*, *SOD4*, *SOD5* and *SOD6*. It has been shown that *C.a.SOD1*, *C.a.SOD2* and *C.a.SOD3* are important for protection in LP and SP and that *C.a.SOD1* is important for virulence (Lamarre et al. 2001, Hwang et al. 2002, Martchenko et al. 2004). On the other hand, *C. glabrata* possesses only two superoxide dismutase genes, *C.g.SOD1* and *C.g.SOD2*, which are orthologs of *S. cerevisiae S.c.SOD1* and *S.c.SOD2* (Longo et al. 1996, Pereira et al. 2003) and *C. albicans C.a.SOD2* and *C.a.SOD3*. Analysis of the amino acid sequence homology between *C. glabrata*, *S. cerevisiae* and

C. albicans superoxide dismutases showed that *C.g.*SODs are more closely related to *S.c.*SODs than to *C.a.*SODs [*C.g.*Sod1p (CAGL0C04741g) 83% identical/7% similar to *S.c.*Sod1p; *C.g.*Sod2p (CAGL0E04356g) 70% identical/11% similar to *S.c.*Sod2p, 58% identical/14% similar to *C.a.*Sod2p; 54% identical/13% similar to *C.a.*Sod3p]. Interestingly, both *S.c.* CIs YJM128 and YJM336 are more resistant to menadione than W303 (Fig. 2) and it has been shown that these CIs are also more resistant to H_2O_2 than W303 reference strain (Cuéllar-Cruz et al. 2008). These results support the idea that pathogens need to have a proper response to oxidative damage in order to survive within the host (Clemons et al. 1994, McCullough et al. 1998, Cassone et al. 2003, Munoz et al. 2005). These CIs could have acquired an increased expression/activity of SOD and multiple drug resistant genes and/or reduced permeability.

We investigated the natural resistance of *C. glabrata* to CHP, an organic hydroperoxide. As shown in Fig. 3, *C. glabrata* is more resistant than *C. albicans*. *S. cerevisiae* (W303 and CIs) are almost as resistant as *C. glabrata*. The oxidative stress generated by CHP is removed in part by glutathione peroxidases and glutaredoxins. One likely explanation of the difference in resistance between *C. glabrata and S. cerevisiae* and *C. albicans* could be gene dosage. *S. cerevisiae* possesses two phospholipid hydroperoxide glutathione peroxidase genes, *GPX1* and *GPX2*, eight glutaredoxin genes, *GRX 1-8*. *C. glabrata* possesses five glutaredoxin genes, *GRX1 -5 and* four glutathione peroxidase genes, *GPX1-4*. Whereas *C. albicans* possesses only three glutaredoxin genes, *GRX1-3*.

Analysis of the catalase activity (Fig. 4) showed almost no difference between *C. glabrata*, *C. albicans* and *S. cerevisiae*, whereas *C. glabrata* has been shown to be more resistant to H_2O_2 than *C. albicans* and *S. cerevisiae* (Cuéllar-Cruz et al. 2008). This result indicates that there must be additional elements in *C. glabrata* that determine the increased resistance. Interestingly, *S. cerevisiae* CIs showed increased catalase activity. This result suggests that *S. cerevisiae* CIs could have acquired a more robust response to oxidative stress in order to evade the attack and elimination by phagocytic cells. It would be interesting to determine if *Cta1* expression is upregulated and/or the activity of Cta1 is increased in *S. cerevisiae* CIs.

Currently, we are making knockout mutants of *C. glabrata SOD*, *GPX* and *GRX* genes in order to evaluate their role in the response to menadione and CHP. Furthermore, we are determining whether expression of *S. cerevisiae Cta1* is modified in CIs.

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