INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

POSGRADO EN CIENCIAS EN BIOLOGIA MOLECULAR

"Las superóxido dismutasas de *Candida glabrata* participan en la respuesta a estrés oxidante y se requieren para la biosíntesis de lisina, la integridad del ADN y la supervivencia en fase estacionaria"

Tesis que presenta

Marcela Cecilia Briones Martín del Campo

Para obtener el grado de

Doctora en Ciencias en Biología Molecular

Director de la Tesis:

Dr. Alejandro De Las Peñas Nava

San Luis Potosí, S.L.P., febrero de 2015

IPICYT

Constancia de aprobación de la tesis

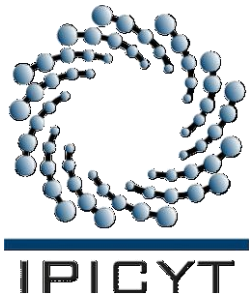
La tesis **"Las superóxido dismutasas de *C. glabrata* participan en la respuesta a estrés oxidante y se requieren para la biosíntesis de lisina, la integridad del ADN y la supervivencia en fase estacionaria"** presentada para obtener el Grado de Doctora en Ciencias en Biología Molecular fue elaborada por **Marcela Cecilia Briones Martín del Campo** y aprobada el **05 de febrero de 2015** por los suscritos, designados por el Colegio de Profesores de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C.

Dr. Alejandro De Las Peñas Nava, IPICYT
(Director de la tesis)

Dra. Ana Paulina Barba de la Rosa, IPICYT
(Asesor de la tesis)

Dr. J. Sergio Casas Flores, IPICYT
(Asesor de la tesis)

Dr. Roberto Sánchez Olea, UASLP
(Asesor externo de la tesis)



Créditos institucionales

Esta tesis fue elaborada en el Laboratorio de Microbiología Molecular de la División de Biología Molecular del Instituto Potosino de Investigación Científica y Tecnológica, A.C., bajo la dirección del Dr. Alejandro De Las Peñas Nava, apoyada por los proyectos CB-2010-153929 de CONACYT.

Durante la realización del trabajo la autora recibió una beca académica del Consejo Nacional de Ciencia y Tecnología con No. de registro 209276 y del Instituto Potosino de Investigación Científica y Tecnológica, A. C.

IPICYT

Instituto Potosino de Investigación Científica y Tecnológica, A.C.

Acta de Examen de Grado

El Secretario Académico del Instituto Potosino de Investigación Científica y Tecnológica, A.C., certifica que en el Acta 073 del Libro Primero de Actas de Exámenes de Grado del Programa de Doctorado en Ciencias en Biología Molecular está asentado lo siguiente:

En la ciudad de San Luis Potosí a los 17 días del mes de febrero del año 2015, se reunió a las 12:00 horas en las instalaciones del Instituto Potosino de Investigación Científica y Tecnológica, A.C., el Jurado integrado por:

Dr. J. Sergio Casas Flores	**Presidente**	**IPICYT**
Dr. Roberto Sánchez Olea	**Secretario**	**UASLP**
Dr. Alejandro De Las Peñas Nava	**Sinodal**	**IPICYT**
Dra. Ana Paulina Barba de la Rosa	**Sinodal**	**IPICYT**

a fin de efectuar el examen, que para obtener el Grado de:

DOCTORA EN CIENCIAS EN BIOLOGÍA MOLECULAR

sustentó la C.

Marcela Cecilia Briones Martín del Campo

sobre la Tesis intitulada:

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

que se desarrolló bajo la dirección de

Dr. Alejandro De Las Peñas Nava

El Jurado, después de deliberar, determinó

APROBARLA

Dándose por terminado el acto a las 13:45 horas, procediendo a la firma del Acta los integrantes del Jurado. Dando fe el Secretario Académico del Instituto.

A petición de la interesada y para los fines que a la misma convengan, se extiende el presente documento en la ciudad de San Luis Potosí, S.L.P., México, a los 17 días del mes de febrero de 2015.

Dr. Marcial Bonilla Marín
Secretario Académico

Mtra. Ivonne Lizette Cuevas Vélez
Jefa del Departamento del Posgrado

INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.
IPICYT
SECRETARIA ACADEMICA

Para Cecilia Martín del Campo

y Eduardo Guerrero Romero

"Gracias por ser, estar y simplemente por existir…"
Edukvo

Agradecimientos

Toda mi gratitud para el Dr. Alejandro De Las Peñas Nava y la Dra. Irene Castaño Navarro por su paciencia, confianza, e invaluable enseñanza. Son parte importante de mi vida.

A la Dra. Ana Paulina Barba de la Rosa, Dr. Roberto Sánchez Olea y al Dr. Sergio Casas Flores por sus acertadas opiniones, sugerencias y apoyo.

A la Dra. Ma. Guadalupe Gutiérrez Escobedo, la Dra. Olga Araceli Patrón Soberano y a Olivia Sánchez por su asistencia técnica.

A mis colegas Guadalupe Gutiérrez Escobedo, Jacqueline Juárez Cepeda, Emmanuel Orta Zavalza e Israel Cañas Villamar y por su invaluable amistad y por todos los momentos maravillosos que pasamos juntos en esta etapa.

A Gloria López por su amistad, consejos y apoyo.

A todos mis compañeros de laboratorio de Microbiología Molecular del IPICYT y amigos de generación.

Contenido

Resumen

Las superóxido dismutasas de *Candida glabrata* participan en la respuesta a estrés oxidante y se requieren para la biosíntesis de lisina, la integridad del ADN y la supervivencia en fase estacionaria

El hongo patógeno oportunista *Candida glabrata* tiene una respuesta al estrés oxidante bien definida. *C. glabrata* es extremadamente resistente a estrés oxidante e inclusive sobrevive dentro de las células fagociticas. Para entender la respuesta a estrés oxidante en *C. glabrata*, caracterizamos las superóxido dismutasas (SODs): la Cu/Zn-SOD (Sod1) y la MnSOD (Sod2). Las SODs catalizan la dismutación de superóxido en peróxido de hidrógeno y oxígeno. Encontramos que principalmente Sod1 contribuye al total de la actividad de SOD en esta levadura y se localiza en el citoplasma. En cambio, Sod2 es una proteína mitocondrial. Ambas SODs juegan un papel central en la respuesta a estrés oxidante, Sod1 es más importante durante el crecimiento fermentativo y Sod2 durante la respiración y el crecimiento en fuentes de carbono no fermentables. Es interesante notar que la cepa de *C. glabrata* carente de ambos genes Sod1 y Sod2 muestra auxotrofia a lisina, tiene alta tasa de mutación espontanea y una vida cronológica reducida. En consecuencia nuestro estudio revela que las SODs juegan un papel importante en el metabolismo, biosíntesis de lisina, protección al ADN y la vida cronológica de *C. glabrata*.

PALABRAS CLAVE. hongo, *Candida glabrata*, estrés, superóxido, daño oxidante, envejecimiento, lisina, menadiona.

Abstract

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

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

KEY WORDS. fungus, *Candida glabrata*, stress, superoxide, oxidative damage, aging, lysine, menadione.

1. Introducción

The superoxide dismutases in fungi.

Marcela Briones-Martin-del-Campo, Jacqueline Juárez-Cepeda, Javier Montalvo-Arredondo, Guadalupe Gutiérrez-Escobedo, Emmanuel Orta-Zavalza, Lina Riego, Irene Castaño and Alejandro De Las Peñas*

División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica (IPICYT), Camino a la Presa San José, #2055, Col. Lomas 4ª Sección. San Luis Potosí, San Luis Potosí 78216, México.
* Corresponding Author. E-mail: cano@ipicyt.edu.mx
+(52) 444 834 2038. Fax: +(52) 444 834 2010.

Keywords: SOD; superoxide dismutase; aging; fungi; pathogens; OSR; expression; DNA damage.

Introduction

During infection, opportunistic pathogenic fungi require a robust stress response to survive and adapt to the changing host environment. Moreover, they need to respond to the oxidative attack by phagocytes. Phagocytic cells are the first line of defense against fungal infections (Mansour & Levitz, 2002). These cells generate reactive oxygen species (ROS), which include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hypochlorite (OCl^-) and hydroxyl radical (HO•). These ROS can damage all biomolecules and destroy phagocytosed pathogens (Gonzalez-Parraga *et al.*, 2003).

In the presence of ROS, there is a reprograming of gene expression to respond and eliminate the oxidative stress; this has been called the Oxidative Stress Response (OSR). Pathogenic fungi antioxidant defense is fundamental in the resistance and adaptation to the host environment. The OSR in fungal pathogens eliminates ROS and is constituted by enzymatic [e.g., catalase, superoxide dismutase (SOD) and glutathione peroxidase] and non-enzymatic systems (e.g., glutathione). The OSR in fungal pathogens have been shown to be important for virulence.

SODs are central in the OSR. SODs are metalloenzymes that catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2. $O_2^{\bullet-}$ has a prominent role in oxidative stress and impacts the production of other ROS (Imlay & Fridovich, 1991). Due to its high attraction, $O_2^{\bullet-}$ oxidizes iron–sulphur ([Fe–S]) clusters and releases iron (Casteilla *et al.*, 2001; Flint *et al.*, 1993). Thus, the free iron is available to react with H_2O_2 and generate HO• by the Fenton reaction (Meneghini, 1997). These ROS react and damage lipids, proteins and DNA. ROS oxidation can lead to inactivation of enzymes, altered membrane composition, mutations, and ultimately to cell death. To counteract the effect of $O_2^{\bullet-}$, SODs regulate the cellular concentration of $O_2^{\bullet-}$ and protect cells against oxidative damage. The absence of the *SODs* can lead to

severe biological disorders. The physiological role of SODs of fungi is discussed here.

Structure and evolutionary relationship of the SOD families

SODs have been identified and characterized from a wide variety of organisms. Four families of SOD have been classified according to its protein structure and the metal used at the active site: the copper/zinc SODs (Cu/Zn-SOD), the manganese SODs (Mn-SOD), the iron SODs (Fe-SOD), and the niquel SODs (Ni-SOD).

Cu/Zn-SODs

The Cu/Zn-SODs are found as a homo-dimeric enzyme around of 32 kDa aproxicontaining one Cu and one Zn ion per subunit. The overall fold in each subunit is described as an eight-stranded antiparallel beta-barrel connected by three external loops. The metals are coordinated by one aspartate and six histidines (Tainer *et al.*, 1982). The two subunits are tightly associated by hydrophobic and electrostatic interactions. The binding of the metals allows the formation of an intramolecular disulfide bond between two conserved cysteine residues (Bordo *et al.*, 1994). The Zn ion and the disulfide bond are not directly involved in the catalytic reaction, but they are required for the stability of the active site (Getzoff *et al.*, 1983; Lamb *et al.*, 1999; Tainer *et al.*, 1983). The electrostatic loop is proposed to provide long- and short-range guidance for the superoxide substrate, thereby facilitating the remarkable kinetics of the SOD reaction (Getzoff *et al.*, 1992).

The Cu/Zn-SODs have a wide distribution among all organisms and evolved separately from the other SODs since they do not share sequence similarity (Smith & Doolittle, 1992). SODs are found in proteobacteria and eukaryotes. Many Gram-negative pathogenic bacteria have Cu/Zn-SODs in the periplasm (Lynch & Kuramitsu, 2000). In eukaryotes, Cu/Zn-SODs are present in animals, plants and fungi and apparently they absent in protists (Wilkinson *et al.*, 2006). The Cu/Zn-SODs have been studied from many different fungi like *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, *C. glabrata*, *Cryptococcus neoformans var. gattii*, *Histoplasma capsulatum*, *Neurospora crassa*, *Aspergillus fumigatus*, *Penicillium chrysogenum*, and others (Briones-Martin-Del-Campo *et al.*, 2014; Chary *et al.*, 1994; Diez *et al.*, 1998; Frohner *et al.*, 2009; Holbrook *et al.*, 2011; Holdom *et al.*, 2000; Narasipura *et al.*, 2003; Roetzer *et al.*, 2011).

Like most of the eukaryotic cells, fungi have Cu/Zn-SOD (product of the *SOD1* gene) mainly localized in the cytosol, and a small fraction present in the intermembrane mitochondrial space (IMS) (Fridovich, 1995)(McCord & Fridovich, 1969; Nedeva *et al.*, 2004; Sturtz *et al.*, 2001). Surprisingly in a recent study, Sod1 of the non-pathogenic fungus *S. cerevisiae* (*Sc*Sod1) was found in the nucleus in response to oxidative stress (Tsang *et al.*, 2014). In general, yeast like *S. cerevisiae*, *C. neoformans*, *S. pombe* (non-pathogenic fungus) and *C. glabrata* contain one Cu/Zn-SOD mainly localized in cytoplasm (Fig. 1) (Briones-Martin-Del-Campo *et al.*, 2014; Matsuyama *et al.*, 2006; Narasipura *et al.*, 2005). Others, like

C. albicans, have six SODs and four of these are Cu/Zn-SOD: the cytoplasmic *Ca*Sod1 and three additional extracellular Cu/Zn-SODs, *Ca*Sod4–6 (Fig. 1) (Hwang *et al.*, 2002; Martchenko *et al.*, 2004; O'Brien *et al.*, 2004). Interestingly, it has been recently reported that *Ca*Sod5 is a monomeric copper protein that lacks the zinc-binding site and the electrostatic loop element. Despite these structural deviations, *Ca*Sod5 still has the capacity to disproportionate $O_2^{\bullet-}$ (Gleason *et al.*, 2014b).

Two of four SODs in *H. capsulatum* are Cu/Zn-SOD, the intracellular *Hc*Sod1 and one extracellular *Hc*Sod3 (Fig. 1) (Holbrook *et al.*, 2011). *Coccidioides immitis* has a Cu/Zn-SOD (CIMG_06994, 5.88) highly related to *Hc*Sod3 of *H. capsulatum* (Holbrook *et al.*, 2011), and has a predicted signal sequence suggesting that it is secreted (Viriyakosol *et al.*, 2013). *A. fumigatus* has a cytoplasmic Cu/Zn-SOD (*Af*Sod1) (Lambou *et al.*, 2010). It has been suggested that the extracellular Cu/Zn-SOD of *A. fumigatus*, *C. albicans* (*Ca*Sod4 and *Ca*Sod5), and *H. capsulatum* (*Hc*Sod3) have a protective role from externally generated free radicals (Gleason *et al.*, 2014b; Hamilton *et al.*, 1996; Holbrook *et al.*, 2011).

Fe or Mn-SODs

Iron and manganese SODs exhibit a high degree of sequence and structure similarity, suggesting that these enzymes originate from a common ancestor (Wintjens *et al.*, 2004) unrelated to Cu/Zn-SODs (Smith & Doolittle, 1992). The Fe or Mn-SODs are present in all kingdoms including eubacteria and archeas. Obligate anaerobes contain Fe-SOD exclusively, cyanobacteria contain Fe-SOD and Mn-SOD, and facultative aerobes contain either Fe-SOD or Mn-SOD or both. Moreover, in eukaryotes, Mn-SOD and Fe-SOD are found in the mitochondria and in the chloroplast, and they show strong homology to the prokaryotic enzymes. These facts support the idea that they arise from prokaryotic endosymbionts (Grace, 1990). The Fe or the Mn-SOD monomer (45kDa approximately) can oligomerise to form a dimeric or tetrameric structure (Wintjens *et al.*, 2004). In eukaryotes, Mn-SODs are usually homo-tetrameric (Borgstahl *et al.*, 1992). Several residues are conserved for oligomerisation, metal specificity (Fe or Mn), and metal binding (Parker & Blake, 1988). Fungi contain Fe and/or Mn-SODs either in the cytoplasm or in the mitochondrial matrix. In *S. cerevisiae*, *C. neoformans*, *S. pombe* (non-pathogenic fungus) and *C. glabrata*, the Mn-SOD (product of *SOD2* gene) is a mitochondrial protein (Fig. 1) (Briones-Martin-Del-Campo *et al.*, 2014; Matsuyama *et al.*, 2006; Narasipura *et al.*, 2005). In *S. cerevisiae*, *Sc*Sod2 is synthesized in the cytosol and translocated to the matrix of the mitochondria, where it incorporates the Mn to form the active holo- enzyme (Luk *et al.*, 2005).

In *C. albicans*, two Mn-SODs are localized in the mitochondrial (*Ca*Sod2 homo-tetrameric) and in the cytosol (*Ca*Sod3 homo-dimeric) (Fig. 1) (Frealle *et al.*, 2005; Lamarre *et al.*, 2001; Rhie *et al.*, 1999). *A. fumigatus*, *Aspergillus nidulans*, *Candida famata*, *Candida lipolytica*, *Fusarium graminearum*, *Magnaporthe grisea*, *N. crassa*, and *Phaffia rhodozyma* have two Mn-SODs, one SOD predicted to be localized to the mitochondria, and the other SOD predicted to be in the cytosol (Frealle *et al.*, 2006; Lamarre *et al.*, 2001).

Ni-SODs

This family was first discovered in the bacteria *Streptomyces spp.* (Youn *et al.*, 1996) and orthologue genes have been identified in cyanobacteria (Eitinger, 2004). However, there is no evidence of Ni-SODs in fungi. These Ni-SODs are homo-hexamers of four-helix bundles with a total molecular weight of 80 kDa (Barondeau *et al.*, 2004). Each four-helix bundle binds a Ni ion.

Regulation of the SOD expression and activity in fungi

Given that the SODs are central in the OSR, it is not surprising that their transcription, synthesis and activity are tightly regulated. The transcriptional regulation of the SOD encoding genes has been shown to depend on the growth phase, the presence of oxidants, oxygen and nutrients. SOD regulation has been extensively studied in *S. cerevisiae*, where their activity increase during the diauxic shift and in the post diauxic phase under normal growth conditions (Costa *et al.*, 1997). However, during the post-diauxic phase, the *ScSOD1* expression is down-regulated (Costa *et al.*, 1997). Moreover, *ScSOD2* expression is up-regulated as cells enter to stationary phase (SP) in rich medium with glucose. This change in expression is due to the switch to a non-fermentable carbon source, nutrient limitation plus the effects of the heme activator protein (Hap) 2-3-4-5 complex. In addition, the *ScSOD2* promoter contains the stress-response elements (STRE) (Flattery-O'Brien *et al.*, 1997; Pinkham *et al.*, 1997). Thus, *ScSOD2* regulation involves oxidative stress sensing and the presence of oxygen during SP. Upon exposure to menadione (MD) and paraquat (PQ) in SP, both SODs (*ScSOD1* and *ScSOD2*) are induced (Cyrne *et al.*, 2003). *ScSOD1* is also induced by H_2O_2 and *ScSOD2* is induced upon exposure to ionizing radiation (Galiazzo & Labbe-Bois, 1993; Lee *et al.*, 2001). Recently, it has been shown that the ROS regulates *ScSOD*1 nuclear localization and acts as a nuclear transcription factor to regulate oxidative stress resistance (Tsang *et al.*, 2014).

Similar to *S. cerevisiae*, the *S. pombe SOD1* (*SpSOD1*) expression is down-regulated in SP, consistent with the decrease in enzymatic activity (Lee *et al.*, 2002). However, *SpSOD1* expression is induced in SP in the presence of H_2O_2 and MD. This induction is regulated by the transcription factors Pap1, Wis1, and Spc1 (Lee *et al.*, 2002). In addition, the expression of the *SpSOD1* gene is induced in the presence of metal ions including zinc (Tarhan *et al.*, 2007). *S. pombe SOD2* (*SpSOD2*) is induced in the presence of MD, H_2O_2, heat, high osmolarity, aluminum chloride, cadmium chloride, and manganese chloride. It is also induced by S-nitroso-N-acetylpenicillamine (SNAP) a NO-generating element. These suggest that oxidative stress, metals, and nitric oxide regulate the *SpSOD2* gene (Jeong *et al.*, 2001; Jung *et al.*, 2002).

The well-conserved Yap1 and Skn7 transcription factors have been shown to control the OSR in fungi like *S. cerevisiae*, *S. pombe*, and *C. glabrata* (Cuellar-Cruz *et al.*, 2008; Nikolaou *et al.*, 2009). Yap1 is a bZip transcription factor that contains cysteine rich domains in its N-and C-terminal portions. Yap1 controls the

OSR and accumulates transiently in the nucleus in response to oxidative stress, whereas Skn7 is an oxidative and cell wall stress-response transcription factor. In *S. cerevisiae*, both Yap1 and Skn7 control the expression of *ScSOD1* and *ScSOD2* (Lee *et al.*, 1999). In *S pombe* and *C. albicans*, *SOD1* expression is regulated by Yap1 (Pap1 in *S. pombe* and Cap1 in *C. albicans*) (Lee *et al.*, 2002; Lee *et al.*, 1999; Znaidi *et al.*, 2009).

CgSOD1 and *CgSOD2* are constitutively expressed even in the presence of oxidative stress, and their regulation is independent of CgYap1 and CgSkn7. Moreover, the expression of *CgSODs* is highly induced under glucose starvation (Roetzer *et al.*, 2011). However, our unpublished results suggest that CgMsn2 and CgMsn4 could be regulating the expression of *SOD1* during SP growth.

In *C. albicans*, *CaSOD1* is induced in LP but repressed in SP, whereas the expression of *CaSOD3* is strongly induced upon the entry and during SP (Lamarre *et al.*, 2001). This shows that *C. albicans* regulates in the opposite direction the expression of the *CaSOD1* and *CaSOD3* genes. *CaSOD1* expression can be induced by H_2O_2, MD, PQ, cholic acid, procaine and tocopherol (Gunasekaran *et al.*, 1998; Lamarre *et al.*, 2001). During the transition from yeast to hyphae, *CaSOD1* and *CaSOD5* expression is induced (Lamarre *et al.*, 2001; Martchenko *et al.*, 2004), but *CaSOD3* is repressed. In addition, *CaSOD5* transcription (Cu-only SOD) is up-regulated in several different conditions such as, osmotic or oxidative stress, switch to alkaline pH, non-fermentable or absence of carbon sources, response to neutrophils, and during an *in vivo* infection in mice (Fanning *et al.*, 2012; Fradin *et al.*, 2005; Martchenko *et al.*, 2004; Miramon *et al.*, 2012). *Ca*Sod5 was exclusively identified in the supernatants of hyphal cells whereas the *Ca*Sod4 enzyme was identified exclusively in the supernatants of yeast-form cultures (Rohm *et al.*, 2013).

The SOD activity of *C. neoformans var. neoformans* and var. *gattii* increases in culture supernatants during SP (Hamilton & Holdom, 1997). *CnSOD1* and *CnSOD2* expression is induced upon temperature shift from 30˚C to 37˚C (Narasipura *et al.*, 2005). However, no differences in *CnSOD1* expression were found in *C. neoformans* yeasts growing with pure oxygen, copper, *tert*-butyl hydroperoxide or PQ (Cox *et al.*, 2003).

The transcription of the extracellular *SOD3* gene of *H. capsulatum* is higher in the pathogenic yeast form than in the non-pathogenic mycelia. *HcSOD3* is expressed during *in vivo* infection consistent with a role in virulence (Holbrook *et al.*, 2011).

In the fungal pathogen *C. immitis*, the Cu/Zn-SOD is the orthologue of *SOD3* of *H. capsulatum* and is upregulated at day 2 and 8 in spherules phase. Presumably, this upregulation could protect the spherule against oxidative stress in the host. *C. immitis* also contains genes highly related to *A. fumigatus AfSOD2* and *AfSOD4* but neither of those is up- or downregulated (Viriyakosol *et al.*, 2013).

In *A. fumigatus*, the *AfSOD1* and *AfSOD2* are highly expressed in the conidia whereas *AfSOD3* is strongly expressed in mycelium. The *AfSOD4* is weakly expressed compared to other *SODs* (Lambou *et al.*, 2010). In *A. niger*, heat shock treatment significantly increased the levels of $O_2^{\bullet-}$, and concomitantly there is an increase in the levels of SODs (Abrashev *et al.*, 2005; Abrashev *et al.*, 2008).

SOD2 (Mn-SOD) in *Penicillium chrysogenum* has an increased expression after exposure to MD (Emri *et al.*, 1999). Furthermore, the SOD activity is increased by PQ treatment in fungi cultures of: *Mucor racemosus*, *Humicola lutea*, *Fusarium oxysporum*, *Alternaria solani*, *Cladosporium elatum*, *Penicillium chrysogenum*, *P. brevicompactum*, *P. claviforme*, *P. roquefortii*, *Aspergillus niger*, *A. argilaceum*, *and A. oryzae* (Angelova *et al.*, 2005).

SODs activation

The post-translation control of SODs involves the rapid conversion of an apo-inactive polypeptide to the enzymatically active SOD enzymes through the insertion of the metal ion cofactor. The Cu/Zn-SODs are activated through the controlled insertion of Cu and the formation of the disulfide bond. Most eukaryotic Cu/Zn-SODs can acquire copper by two different pathways, one dependent on the copper chaperone for SOD (Ccs1), and the other independent of Ccs1 (Carroll *et al.*, 2004; Rae *et al.*, 1999). To date, the Cu/Zn-SODs known to be fully dependent on Ccs1 are those from fungi such as *S. cerevisiae.* When the Ccs1 dependent pathway is used, copper is transported by the action of the copper transporters and Ccs1 (Portnoy *et al.*, 2001). The Ccs1 insert copper into a new or pre-existing *Sc*Sod1 apo-enzyme (Bartnikas & Gitlin, 2003; Schmidt *et al.*, 2000). The distribution of Ccs1 and Sod1 between the cytosol and the mitochondrial IMS depends on the Mia40/Erv1 disulfide relay system (Reddehase *et al.*, 2009). Mia40/Erv1 introduces a structural disulfide bond in Ccs1, which then shuttles the disulfide bond to Sod1 for full activation (Gross *et al.*, 2011; Kloppel *et al.*, 2011).

S. pombe and *C. albicans* also have a metallochaperone-like protein that is necessary to deliver copper in Sod1 (Gleason *et al.*, 2014a; Laliberte *et al.*, 2004). Loss of Ccs1 in *C. albicans* resulted in loss of *Ca*Sod1 activity. In contrast, *Ca*Sod5 activity is independent on the Ccs1. The *Ca*Sod5 is secreted as a disulfide-oxidized apo-protein and is activated by the capture of extracellular copper (Gleason *et al.*, 2014a; Gleason *et al.*, 2014b).

In the case *Sc*Mn-SODs, their maturation comprises the import to the mitochondria and the insertion of manganese. During *Sc*Sod2 synthesis, the ribosomes are juxtaposed to the outer mitochondrial membrane allowing the coupling of *Sc*Sod2 synthesis and mitochondrial import (Ginsberg *et al.*, 2003). Once imported into mitochondrial matrix, the polypeptide is fold into a stable quaternary tetramer and acquires the manganese through the metal transporter Smf2 (Cohen *et al.*, 2000; Portnoy *et al.*, 2000). A second transporter that affects the *Sc*Sod2 activation is Mtm1, a member of the mitochondrial carrier family of proteins located in the inner membrane of the mitochondria. The Mtm1 role could involve direct insertion of the manganese cofactor or maintaining *Sc*Sod2 in a conformation that is competent for metal activation (Luk *et al.*, 2005). Interestingly, *Sp*Sod2 is acetylated after import into mitochondria and its localization is not essential for the enzyme activity, but is crucial for OSR and growth under respiratory conditions (Takahashi *et al.*, 2011).

The Central Roles of SODs in Fungi

Oxidative and general stress response

SODs are essential components of the antioxidant defense system in fungi, providing protection against oxidative stress as a result of endogenous and exogenous ROS. The OSR of pathogenic fungi is extremely important because they are exposed not only to their own ROS (byproducts of aerobic respiration) but also to those produced by the respiratory burst by the phagocytic cells (Hampton *et al.*, 1998). The SODs play a central role in the OSR.

SOD null mutants exhibit increased sensitivity to MD, PQ and H_2O_2 in *S. cerevisiae*, *C. glabrata*, *S. pombe*, *C. neoformans*, *C. albicans*, *H. capsulatum*, *N. crassa*, and *Aspergillus fumigatus* (Bilinski *et al.*, 1985; Briones-Martin-Del-Campo *et al.*, 2014; Cyrne *et al.*, 2003; Giles *et al.*, 2005; Gralla & Valentine, 1991; Hwang *et al.*, 2003; Jeong *et al.*, 2001; Lambou *et al.*, 2010; Longo *et al.*, 1999; Manfredini *et al.*, 2004; Mutoh & Kitajima, 2007; Narasipura *et al.*, 2005) (See Table I). These data underscore the key role of the SODs in the antioxidant defense against drug-induced oxidative stress. For example, the response to oxidative stress generated by H_2O_2 and MD is largely dependent on Sod1 and also by the glutathione reductase in *S. pombe* (Lee *et al.*, 2002). In *S. cerevisiae*, the absence of SODs induces the glutathione peroxidase and glutathione levels in the defense against H_2O_2 during SP (Manfredini *et al.*, 2004). In *C. glabrata*, *SOD1* is required to protect against MD during LP and both SODs are necessary in protecting cells against to MD-induced stress during SP (Briones-Martin-Del-Campo *et al.*, 2014; Roetzer *et al.*, 2011).

Fungal pathogens are subjected to many others environment stresses such as nutrient limitations, osmotic changes, heat shock, drugs and pH change, and particularly during the host immune response. Several evidences show that SODs have a role not only in OSR but also in response to the presence of others kind of stresses. In *S. cerevisiae*, the *sod1*Δ mutant is sensitive to cell wall-perturbing agents, such as calcofluor white and congo red (Liu *et al.*, 2010). Moreover, the *sod1*Δ mutant is sensitive to osmotic stress showing increased oxidative damage. In anaerobic conditions, the presence of ascorbate, or glutathione alleviate the growth inhibition of *Scsod1*Δ (Koziol *et al.*, 2005). Unlike Sod1, which have the major role in OSR, Sod2 acts as a general defense agent protecting cells against multiple stresses. Previous studies revealed that the *sod2*Δ mutants of *S. cerevisiae*, *S. pombe*, *C. neoformans var. gattii*, *C. neoformans var. grubii*, and *C. albicans* are more sensitive to heat stress, high osmolarity, ethanol, or metalloid stress than the wild-type strain (Dziadkowiec *et al.*, 2007; Giles *et al.*, 2005; Hwang *et al.*, 2003; Hwang *et al.*, 2002; Jeong *et al.*, 2001; Narasipura *et al.*, 2005; Pereira *et al.*, 2001; Stoica *et al.*, 2011). In *A. fumigatus*, both *sod1*Δ and *sod2*Δ mutants show growth inhibition at high temperature whereas the *sod3*Δ mutant had only a slight growth delay at high temperature (Lambou *et al.*, 2010). These evidences confirmed the relationship between oxidative and other stress responses and the contribution of SODs to scavenge $O_2^{\bullet-}$ generated by the mitochondrial respiration.

Cellular growth, aminoacid metabolism, and respiration

Genetic analysis of SOD mutants have shown that the SODs are implicated in a variety of metabolic and growth defects related to oxidative damage even under non-stress conditions. Growth defects have been shown in SOD mutants under high oxygen environment, high temperatures, growth on non-fermentable carbon sources and in SP, and nutritional stress (see above). The role of SODs in metabolism has been well characterized in *S. cerevisiae.* The *sod1*Δ and *sod2*Δ mutants are highly sensitive to elevated oxygen concentrations (hyperoxia), but this phenotype disappears under anaerobic conditions, independent of the growth conditions (Gralla & Valentine, 1991; van Loon *et al.*, 1986).

In *S. cerevisiae* and *S. pombe*, the *sod1*Δ mutant causes severe growth defects even in rich media (Lee *et al.*, 2002; Longo *et al.*, 1996; Sehati *et al.*, 2011). In *C. glabrata*, the single *sod2*Δ and double *sod1*Δ *sod2*Δ mutants grow slow under aerobic conditions in rich medium but they can reach to the same cell density as the parental culture (Briones-Martin-Del-Campo *et al.*, 2014). In *C. albicans*, the *sod2*Δ/*sod2*Δ mutant (but not the *sod1*Δ/*sod1*Δ or *sod3*Δ/*sod3*Δ mutants) has a growth rate comparable to the wild-type in air. The same mutant shows normal growth under hyperoxic condition. Moreover, the *sod1*Δ/*sod1*Δ mutant showed retarded filamentation on Spider medium (filamentation-inducing media) (Hwang *et al.*, 2002). In contrast, the SODs do not appear to have any impact on the growth of *C. neoformans var. gattii* on YPD (yeast peptone dextrose media) in aerobic conditions (Narasipura *et al.*, 2005; Narasipura *et al.*, 2003).

In SP, cells readjust their metabolism from fermentation to respiration, and they utilize ethanol for energy. The mitochondrial activity increase and consequently there are more production of ROS (Herker *et al.*, 2004). The SOD mutants are characterized by their defects during aerobic respiration. In *S. cerevisiae*, it has been demonstrated that both *sod1*Δ and *sod2*Δ mutants show growth defects when grown on non-fermentable carbon sources such as ethanol or lactate (Gralla & Kosman, 1992); although, the *sod1*Δ can grow better on lactate and pyruvate, than on ethanol or glycerol (Longo *et al.*, 1996). Indeed, the *sod1*Δ mutant is unable to utilize ethanol, and cannot switch from glucose-based to ethanol-based growth (Sehati *et al.*, 2011).

Glucose has long been known to repress respiration in LP cells. Recently, it has been shown that Sod1 play a role in the respiratory repression in oxygen and glucose presence. *Sc*Sod1 physically interact with the Yck1, a casein kinase, which regulates glucose and amino acid sensing and respiration repression (Reddi & Culotta, 2013).

The *sod2*Δ mutants in the pathogenic fungi *C. neoformans*, *C. albicans*, and *C. glabrata* have similar growth defects when grown on ethanol, demonstrating the central role of SODs during cellular respiration (Briones-Martin-Del-Campo *et al.*, 2014; Hwang *et al.*, 2002; Narasipura *et al.*, 2005). In addition, *C. glabrata sod2*Δ mutant and the *sod1*Δ *sod2*Δ double mutant have a marked growth defect in the presence of glycerol as carbon source. Thus, *Cg*Sod2 is necessary to counteract the superoxide generated in the mitochondria when cells are under mitochondrial respiration (Briones-Martin-Del-Campo *et al.*, 2014).

Amino acid auxotrophies have been observed in yeast lacking *SOD1*, but

not observed in a *sod2*∆ mutant. In *S. cerevisiae* and *S. pombe,* the *sod1*Δ mutant exhibits auxotrophy for lysine, methionine and cysteine. These auxotrophies were restored by the addition of these amino acids to the medium (Gralla & Kosman, 1992; Lee *et al.*, 2002). Consistent with this phenotype, the deletion of the copper chaperone of Sod1, Ccs1, shows as well auxotrophy for methionine and lysine (Culotta, 2000). In *C. albicans*, the *sod1*Δ mutant exhibits only a slow growth in minimal medium without lysine, indicating a `leaky' lysine auxotrophy. However, this *sod1*Δ mutant does not show any kind of growth defects in absence of methionine or cysteine (Hwang *et al.*, 2002). In contrast, *sod1*Δ single mutants of *C. neoformans var. gattii* and the filamentous fungi *N. crassa*, does not show auxotrophies for lysine or methionine (Chary *et al.*, 1994; Narasipura *et al.*, 2003). On the other hand, *C. glabrata* shows that the *sod1*Δ mutant grows slowly in the absence of lysine, while the *sod2*Δ mutant has no growth defect in this medium. Interestingly, and unlike *S. cerevisiae*, only the *Cgsod1*Δ *Cgsod2*Δ double mutant is lysine auxotroph indicating that *Cg*SODs are functionally redundants (Briones-Martin-Del-Campo *et al.*, 2014). In summary, some fungi Sod1 enzymes are involved in the metabolism of aminoacids.

Studies have shown that the auxotrophies in the *sod1*Δ mutant in *S. cerevisiae* is due to the disruption of the biosynthetic pathways of lysine, methionine or cysteine by an altered redox environment. In the absences of *SOD1*, the cysteine and methionine requirement results from the decreased NADPH levels, and consequently blocking the first steps of the methionine biosynthesis pathway (Chang & Kosman, 1990; Slekar *et al.*, 1996). It has been shown that inactivation of coenzyme Q (Coq1) partially alleviates the lysine auxotrophy of a *sod1*Δ mutant in *S. cerevisiae*. The electron leakage from complex III via Coq1 is the main source of $O_2^{\bullet-}$, suggesting that *SOD1* does play a role in protect of lysine pathway from $O_2^{\bullet-}$ produced by mitochondrial respiration (Longo *et al.*, 1996). The $O_2^{\bullet-}$ oxidize 4Fe-4S clusters of different enzymes such as aconitase (Aco1, a dehydratase of Krebs cycle) (Flint *et al.*, 1993); thus, enzymes in the mitochondrial matrix with 4Fe-4S clusters are subject to reversible inactivation by $O_2^{\bullet-}$, resulting in loss of the labile iron atom, leaving a 3Fe-4S cluster (Flint *et al.*, 1993; Wallace *et al.*, 2004). In fungi, lysine is synthesized *de novo* by the alfa-aminoadipate (AAA) pathway (Zabriskie & Jackson, 2000), which contains an 4Fe-4S cluster enzyme, homoaconitase (*LYS4*), a likely target for inactivation by $O_2^{\bullet-}$. Thus, the lysine auxotrophy in the *sod1*Δ mutant results from the oxidation of the 4Fe-4S cluster of Lys4 resulting in a blockage of AAA pathway in *S. cerevisiae* (Wallace *et al.*, 2004). In contrast in *S. pombe*, it was found that the homocitrate synthase (HCS, first enzyme in the AAA pathway) is the target enzyme causing lysine auxotrophy in the *sod1*Δ mutant. Their overproduction is sufficient to suppress the lysine auxotrophy (Kwon *et al.*, 2006). In contrast in *C. glabrata*, the overexpression of *LYS4*, *LYS12*, *LYS20* or *LYS21* did not suppress the lysine auxotrophy in the *sod1*Δ *sod2*Δ double mutant. This suggests that a different gene or more than one gene of the AAA pathway is responsible for the lysine auxotrophy.

Several suppressors or multicopy suppressor overcoming the lysine auxotrophy in a *sod1*Δ mutant have been isolated in *S. cerevisiae*. These suppressors include the overexpression of *ATX1* (copper metallochaperone for ATPases), the overexpression of *ATX2* (Golgi membrane protein involved in

manganese homeostasis), a mutation in *PMR1* (Ca2+ ATPase involved in manganese homeostasis), and a mutation in *BSD2* (heavy metal ion homeostasis protein) (Lapinskas *et al.*, 1995; Lin & Culotta, 1995, 1996; Liu & Culotta, 1994). These suppressors act as metal-based mimics in the absence of *SOD1*, where the oxidative damage can be prevented through alterations in manganese and copper homeostasis.

Other suppressors called *seo,* (suppressors of endogenous oxidation) have been mapped in *SSQ1*, *JAC1*, *NFS1*, and *ISU1* genes. These genes encode mitochondrial proteins involved in iron-sulfur cluster assembly (Strain *et al.*, 1998). A recent study suggests that the *seo* mutants over-accumulate mitochondrial iron, increasing the expression of genes in the AAA pathway, perhaps through sensing of mitochondrial damage by the retrograde response (Jensen *et al.*, 2004).

DNA protection

The most consequential impact of SODs is DNA integrity. In different fungi, this has been demonstrated through analysis of SOD mutant phenotypes . In *S. cerevisiae*, the *sod1*Δ and *sod2*Δ mutants show high mutation frequency during SP and aerobic conditions (Gralla & Valentine, 1991; Longo *et al.*, 1999), though, *sod2*Δ show a reduced mutator phenotype than the *sod1*Δ mutant (Huang *et al.*, 2003). Sod1 and its chaperone Ccs1 were also identified as sensitive to DNA-damaging agents such as methyl methanesulfonate (MMS) and hydroxyurea (HU) (Carter *et al.*, 2005; Chang *et al.*, 2002). In *C. glabrata*, SOD mutants exhibited high mutation rate. The single mutants, *sod1*Δ and *sod2*Δ, show a twofold increase in the mutator phenotype than the parental strain, however the *sod1*Δ *sod2*Δ double mutant show a sevenfold increase in the mutator phenotype. Moreover, these mutants also show high levels of oxidative stress and sensitivity to the DNA damage agents (MMS and HU) (Briones-Martin-Del-Campo *et al.*, 2014). In the filamentous fungi *N. crassa*, the *sod1*Δ mutant exhibits an increased spontaneous mutation rate (Chary *et al.*, 1994).

Although DNA is an important target for ROS, it is likely that $O2^{\bullet -}$ cannot damage DNA directly (Bielski, 1985). Instead, $O2^{\bullet -}$ release an iron atom from [4Fe-4S] clusters, which is now available to participate in the Fenton reaction and produce HO• (Keyer & Imlay, 1996; Liochev & Fridovich, 1999). The HO• is a highly reactive oxidant which cannot diffuse far from its origin before it reacts. Thus DNA-bound iron is an especially favored target of damage through the Fenton reaction (Keyer *et al.*, 1995). It has been shown that SOD mutants of *S. cerevisiae* and *E. coli* contain high levels of intracellular free iron, suggesting the Fenton-dependent DNA damage (Keyer & Imlay, 1996; Srinivasan *et al.*, 2000). Thus, SODs prevent the accumulation of oxidative damage to DNA by avoiding the start of the Fenton reaction.

The oxidative damage may occur at the sugars or DNA bases moieties, thereby producing a broad spectrum of lesions. The predominant consequence of sugar damage is an eventual strand breakage and base release (Henle *et al.*, 1995). Relative to the other DNA bases moieties, guanine is particularly vulnerable to oxidation mediated by ROS. The most thoroughly examined guanine oxidation product is 7,8-dihydro-8-oxoguanine (8-oxoG) (Neeley & Essigmann, 2006). The 8-

oxoG is prone to mispair with adenine residues, leading to an increased frequency of spontaneous GC to TA transversion. Although oxidative damage could potentially increase all types of base pair mutations, in the *Scsod1*Δ mutant there are two main types of mutations: single-base substitutions (GC to AT, GC to CG, and GC to TA), and single-base frameshifts (Huang *et al.*, 2003; Shockley *et al.*, 2013). The GC to TA transversions are also found as one of the most common base substitutions in the *sod1*Δ mutant of *S. cerevisiae* (Huang *et al.*, 2003). In *C. glabrata*, analysis of spontaneously arise mutations in the *sod1*Δ *sod2*Δ strain are mainly of the single-base substitution type (GC to TA and TA to CG) (Briones-Martin-Del-Campo *et al.*, 2014).

In a global analysis of genetic relationships, genes involved in the OSR and DNA replication were identified as the major guardians against spontaneous DNA damage. Their efficient repair involves DNA-damage checkpoint signaling and multiple DNA-repair pathways (Pan *et al.*, 2006). The base excision repair (BER) is the first line of defense against DNA damaged by oxidation (Dizdaroglu, 2005). BER is composed of 8-oxoguanine DNA glycosylase 1 (Ogg1), which remove the 8-oxoG lesions (Huang & Kolodner, 2005; Nash *et al.*, 1996). Recently, a data mining network study showed that Sod2 of *S. cerevisiae* associates with Ogg1 (Bonatto, 2007), and another report linked the SODs with DNA repair showing that the *sod1*Δ mutant down-regulate the *MEC1*-mediated checkpoint response (Carter *et al.*, 2005). This pathway participate in DNA damage checkpoint control, by arresting the cell cycle and inducing the transcription of genes that facilitate DNA repair (Elledge, 1996). Moreover, there is a direct association between Sod1 with Mec1 or Dun1 (Bonatto, 2007),allowing the transcription of genes involved in DNA synthesis and repair. The DNA damage agents (HU and MMS) activate the *MEC1*-dependent checkpoint response to oxidative damage (Branzei & Foiani, 2007). In addition to the *MEC1* pathway, BER has also been implicated in the response to MMS-induced damage (Xiao *et al.*, 1996).

Recently, an important finding shows that ROS induces Sod1 nuclear localization, which is crucial to protect against DNA damage. The ROS signaling is mediated by Mec1 and Dun1, where Dun1 interacts with Sod1. Furthermore, the nuclear Sod1 regulates the expression of OSR and DNA repair genes in yeast and in humans (Tsang *et al.*, 2014). Collectively, these findings demonstrate that SODs have a critical role not only in protecting DNA integrity but also in regulating the expression of the repair pathways.

Aging

In SP, cells obtain energy from mitochondrial respiration, and generation of $O_2^{\bullet-}$ as byproduct (Longo *et al.*, 1999). The accumulation of oxidative damage caused by $O_2^{\bullet-}$ is thought to be one the major contributor to aging (Longo *et al.*, 1999). All organisms are subject to a gradual deterioration of the physiological functions leading to the aging process. The deleterious effects of $O_2^{\bullet-}$ highlights the importance of the enzymes required for detoxification of this ROS. Thus, the SODs are central enzymes in aging. The yeast cells accumulate DNA damage during the aging process and the SODs play a critical role in preserving the genomic integrity (Muid *et al.*, 2014).

SODs mutations have been associated with a general pattern of premature aging in model organisms. In mice, the lack of MnSOD, causes dilated cardiomyopathy and neonatal death (Li *et al.*, 1995). Moreover, a 50% reduction in MnSOD activity increases oxidative DNA damage in nuclear and mitochondrial DNA, leading to aging (Van Remmen *et al.*, 2003). In contrast, mice lacking the Cu/Zn-SOD (*Sod1*−/−) showed no overt abnormalities during development, but showed a reduced lifespan and development liver tumors (Elchuri *et al.*, 2005). In *Drosophila melanogaster*, the Cu/Zn-SOD -null mutant shows a reduced adult life span, hypersensitivity to oxidative stress, loss of aconitase activity, and an increase in spontaneous mutation rates (Phillips *et al.*, 1989; Woodruff *et al.*, 2004). Conversly, yeast Cu/Zn-SOD overexpression extends the life span (Fabrizio *et al.*, 2003).

Yeast cells have been used as a model organism to study the role of the SODs in aging. Aging in *S. cerevisiae* is analyzed by their replicative life span (RLS) and their chronological life span (CLS). RLS is the total number of daughter cells that a single mother cell can generate. On the other hand, CLS is the longevity of a cell population that is not dividing (SP cells) but retains the ability to re-enter the cell cycle in the presence of nutrients (Kaeberlein *et al.*, 2007). The SODs are required for both RLS and CLS survival (Longo *et al.*, 1996; Unlu & Koc, 2007). *Sc*Sod2 is required under both low and normal oxygen conditions, whereas cytoplasmic *Sc*Sod1 is mainly required under normal aeration (Longo *et al.*, 1996). In addition, SODs overexpression in *S. cerevisiae* extends life span and delays the loss of aconitase activity (Fabrizio *et al.*, 2001; Fabrizio *et al.*, 2003). The aconitase (Aco1) is the only protein in the mitochondrial matrix that shows age-associated carbonylation and consequently the loss of its activity (Yan *et al.*, 1997). Furthermore, Aco1 is required for mitochondrial DNA (mtDNA) maintenance (Chen *et al.*, 2005). The activity of Aco1 is regulated by its redox reactive Fe-S center (Bulteau *et al.*, 2003). The disruption of Aco1 Fe-S center by ROS, indicate the presence of mitochondrial damage and this is signaled to the nucleus for reprogramming of gene expression leading to apoptosis.

In *C. glabrata*, *Cg*SOD2 is mainly required to CLS (Briones-Martin-Del-Campo *et al.*, 2014). Interestingly, there is also a decrease in CLS in SP when the synthesis of glutathione is compromised, indicating glutathione as a compensatory system to control ROS damage in the absence of SODs (Gutierrez-Escobedo *et al.*, 2013). In *S. pombe* the *sod1*Δ *gsh1*Δ double mutant (*GSH1* encode for the □-glutamyl-cysteine synthetase, catalyzing the first step in glutathione biosynthesis) is more sensitive to MD and lost viability upon entry into SP (Mutoh & Kitajima, 2007). In contrast, the *SOD2* role in aging has not been reported for *S. pombe* (Jeong *et al.*, 2001). In *C. neoformans var. gattii*, *SOD2* rather than *SOD1* was shown to be essential for survival during the stationary phase, especially in the presence of an increase of mitochondrial ROS (Narasipura *et al.*, 2005; Narasipura *et al.*, 2003). In filamentous fungi, there is evidence of the importance of Sods in aging. The *A. fumigatus sod1*Δ *sod2*Δ *sod3*Δ triple mutant shows a delay in conidial germination. Moreover, the viability of the conidia is affected during storage under aerobic conditions. This triple mutant lost all its viability after one year compared to only 3% of the parental strain under the same conditions (Lambou *et al.*, 2010). In *N. crassa*, the activities of the SODs, the catalases, and

the glutathione peroxidases are highly-correlated with conidial lifespan (Munkres *et al.*, 1984). During the senescence of the filamentous fungus *Podospora anserina*, the activity of *PaSOD2* decreases, whereas the activity of the cytoplasmic Cu/Zn-SOD1 (*PaSOD1*) increases (Borghouts *et al.*, 2001).

Virulence

Several studies suggest that the OSR of diverse pathogens is directly related to pathogenicity and survival against the respiratory burst in phagocytes. SODs contribute to the virulence of many bacteria and fungal pathogens by counteracting deleterious effects of ROS and evading killing by phagocytes (Miller & Britigan, 1997). Sod1 and Sod2 of *C. neoformans* var. *gattii*, *C. n* var. *neoformans*, and Sod1 and Sod5 of *C. albicans* are required for full virulence in a mouse model of infection (Giles *et al.*, 2005; Martchenko *et al.*, 2004; Narasipura *et al.*, 2005; Narasipura *et al.*, 2003). In *H. capsulatum*, *Hc*Sod3 protects cells from ROS-derived from phagocytes and deletion of its encoding gene reduces fungal virulence *in vivo* (Youseff *et al.*, 2012). The *sod1*Δ mutant of *C. neoformans* and *sod1*Δ *sod4*Δ and *sod5*Δ of *C. albicans* are significantly susceptible to killing by phagocytes (Frohner *et al.*, 2009; Hwang *et al.*, 2002; Narasipura *et al.*, 2003). In addition, the *sod1*Δ mutant of *C. neoformans* is defective in the production of several virulence factors, i.e. laccase, urease and phospholipase (Narasipura *et al.*, 2003). In contrast, *C. glabrata CTA1*, *SOD1* and *SOD2* are dispensable for colonization in a murine model of systemic infection (Briones-Martin-Del-Campo *et al.*, 2014); only the concomitant absence of *CgYAP1* and *CgSOD1* is detrimental to yeast survival in a primary mouse macrophage infection model (Roetzer *et al.*, 2011).

In *A. fumigatus*, SODs are recognized by infected human sera, serving mainly as antigens for IgA (Holdom *et al.*, 2000). Moreover, the triple *sod1*Δ *sod2*Δ *sod3*Δ mutant is sensitive to killing by alveolar macrophage in immunocompetent mice but show no defect in pathogenicity in murine aspergillosis models in immunocompromised animals (Lambou *et al.*, 2010).

Conclusions

It have been demonstrated that the role of SODs in cells is of critical importance. They are necessary to avoid the deleterious effect of superoxide and protect cells from oxidative damage. Because of this, SODs are involved in different parts of metabolism such as DNA integrity and repair, amino acid pathways, stress response, growth, metal regulation, protection of Fe-S cluster containing- enzymes, and microbial pathogenesis. The role of SODs correlates with their cellular localization. Thus, the intracellular SODs are involved in detoxifying superoxide, and the extracellular SODs are important to avoid exogenous oxidative stress. Moreover, the mitochondrial SODs prevent the superoxide accumulation and the consequent respiration-related aging. Although the SODs have been deeply

studied in model yeast like *S. cerevisiae*, more work is needed to characterize these enzymes in pathogenic yeasts and in particular in filamentous fungi.

References

Abrashev R, Dolashka P, Christova R, Stefanova L & Angelova M (2005) Role of antioxidant enzymes in survival of conidiospores of Aspergillus niger 26 under conditions of temperature stress. *J Appl Microbiol* **99**: 902-909.

Abrashev RI, Pashova SB, Stefanova LN, Vassilev SV, Dolashka-Angelova PA & Angelova MB (2008) Heat-shock-induced oxidative stress and antioxidant response in Aspergillus niger 26. *Can J Microbiol* **54**: 977-983.

Angelova MB, Pashova SB, Spasova BK, Vassilev SV & Slokoska LS (2005) Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. *Mycol Res* **109**: 150-158.

Barondeau DP, Kassmann CJ, Bruns CK, Tainer JA & Getzoff ED (2004) Nickel superoxide dismutase structure and mechanism. *Biochemistry* **43**: 8038-8047.

Bartnikas TB & Gitlin JD (2003) Mechanisms of biosynthesis of mammalian copper/zinc superoxide dismutase. *J Biol Chem* **278**: 33602-33608.

Bielski BH (1985) Fast kinetic studies of dioxygen-derived species and their metal complexes. *Philos Trans R Soc Lond B Biol Sci* **311**: 473-482.

Bilinski T, Krawiec Z, Liczmanski A & Litwinska J (1985) Is hydroxyl radical generated by the Fenton reaction in vivo? *Biochem Biophys Res Commun* **130**: 533-539.

Bonatto D (2007) A systems biology analysis of protein-protein interactions between yeast superoxide dismutases and DNA repair pathways. *Free Radic Biol Med* **43**: 557-567.

Bordo D, Djinovic K & Bolognesi M (1994) Conserved patterns in the Cu,Zn superoxide dismutase family. *J Mol Biol* **238**: 366-386.

Borghouts C, Werner A, Elthon T & Osiewacz HD (2001) Copper-modulated gene expression and senescence in the filamentous fungus Podospora anserina. *Mol Cell Biol* **21**: 390-399.

Borgstahl GE, Parge HE, Hickey MJ, Beyer WF, Jr., Hallewell RA & Tainer JA (1992) The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. *Cell* **71**: 107-118.

Branzei D & Foiani M (2007) Interplay of replication checkpoints and repair proteins at stalled replication forks. *DNA Repair (Amst)* **6**: 994-1003.

Briones-Martin-Del-Campo M, Orta-Zavalza E, Canas-Villamar I, Gutierrez-Escobedo G, Juarez-Cepeda J, Robledo-Marquez K, Arroyo-Helguera O, Castano I & De Las Penas A (2014) The superoxide dismutases of Candida glabrata protect against oxidative damage and are required for lysine biosynthesis, DNA integrity and chronological life survival. *Microbiology*.

Bulteau AL, Ikeda-Saito M & Szweda LI (2003) Redox-dependent modulation of aconitase activity in intact mitochondria. *Biochemistry* **42**: 14846-14855.

Carroll MC, Girouard JB, Ulloa JL, Subramaniam JR, Wong PC, Valentine JS & Culotta VC (2004) Mechanisms for activating Cu- and Zn-containing superoxide dismutase in the absence of the CCS Cu chaperone. *Proc Natl Acad Sci U S A* **101**: 5964-5969.

Carter CD, Kitchen LE, Au WC, Babic CM & Basrai MA (2005) Loss of SOD1 and LYS7 sensitizes Saccharomyces cerevisiae to hydroxyurea and DNA damage agents and downregulates MEC1 pathway effectors. *Mol Cell Biol* **25**: 10273-10285.

Casteilla L, Rigoulet M & Penicaud L (2001) Mitochondrial ROS metabolism: modulation by uncoupling proteins. *IUBMB Life* **52**: 181-188.

Cohen A, Nelson H & Nelson N (2000) The family of SMF metal ion transporters in yeast cells. *J Biol Chem* **275**: 33388-33394.

Costa V, Amorim MA, Reis E, Quintanilha A & Moradas-Ferreira P (1997) Mitochondrial superoxide dismutase is essential for ethanol tolerance of Saccharomyces cerevisiae in the post-diauxic phase. *Microbiology* **143 (Pt 5)**: 1649-1656.

Cox GM, Harrison TS, McDade HC, Taborda CP, Heinrich G, Casadevall A & Perfect JR (2003) Superoxide dismutase influences the virulence of Cryptococcus neoformans by affecting growth within macrophages. *Infect Immun* **71**: 173-180.

Cuellar-Cruz M, Briones-Martin-del-Campo M, Canas-Villamar I, Montalvo-Arredondo J, Riego-Ruiz L, Castano I & De Las Penas A (2008) High resistance to oxidative stress in the fungal pathogen *Candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. *Eukaryot Cell* **7**: 814-825.

Cuéllar-Cruz M, Castaño I, Arroyo-Helguera O & De Las Peñas A (2009) Oxidative stress response to menadione and cumene hydroperoxide in the opportunistic fungal pathogen *Candida glabrata*. *Mem Inst Oswaldo Cruz, Rio de Janeiro* **104**: 649-654.

Culotta VC (2000) Superoxide dismutase, oxidative stress, and cell metabolism. *Curr Top Cell Regul* **36**: 117-132.

Cyrne L, Martins L, Fernandes L & Marinho HS (2003) Regulation of antioxidant enzymes gene expression in the yeast Saccharomyces cerevisiae during stationary phase. *Free Radic Biol Med* **34**: 385-393.

Chang EC & Kosman DJ (1990) O2-dependent methionine auxotrophy in Cu,Zn superoxide dismutase-deficient mutants of Saccharomyces cerevisiae. *J Bacteriol* **172**: 1840-1845.

Chang M, Bellaoui M, Boone C & Brown GW (2002) A genome-wide screen for methyl methanesulfonate-sensitive mutants reveals genes required for S phase progression in the presence of DNA damage. *Proc Natl Acad Sci U S A* **99**: 16934-16939.

Chary P, Dillon D, Schroeder AL & Natvig DO (1994) Superoxide dismutase (sod-1) null mutants of Neurospora crassa: oxidative stress sensitivity, spontaneous mutation rate and response to mutagens. *Genetics* **137**: 723-730.

Chen XJ, Wang X, Kaufman BA & Butow RA (2005) Aconitase couples metabolic regulation to mitochondrial DNA maintenance. *Science* **307**: 714-717.

Diez B, Schleissner C, Moreno MA, Rodriguez M, Collados A & Barredo JL (1998) The manganese superoxide dismutase from the penicillin producer Penicillium chrysogenum. *Curr Genet* **33**: 387-394.

Dizdaroglu M (2005) Base-excision repair of oxidative DNA damage by DNA glycosylases. *Mutat Res* **591**: 45-59.

Dziadkowiec D, Krasowska A, Liebner A & Sigler K (2007) Protective role of mitochondrial superoxide dismutase against high osmolarity, heat and metalloid stress in saccharomyces cerevisiae. *Folia Microbiol (Praha)* **52**: 120-126.

Eitinger T (2004) In vivo production of active nickel superoxide dismutase from Prochlorococcus marinus MIT9313 is dependent on its cognate peptidase. *J Bacteriol* **186**: 7821-7825.

Elchuri S, Oberley TD, Qi W, Eisenstein RS, Jackson Roberts L, Van Remmen H, Epstein CJ & Huang TT (2005) CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* **24**: 367-380.

Elledge SJ (1996) Cell cycle checkpoints: preventing an identity crisis. *Science* **274**: 1664-1672.

Emri T, Pocsi I & Szentirmai A (1999) Analysis of the oxidative stress response of Penicillium chrysogenum to menadione. *Free Radic Res* **30**: 125-132.

Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiol Rev* **24**: 469-486.

Fabrizio P, Pozza F, Pletcher SD, Gendron CM & Longo VD (2001) Regulation of longevity and stress resistance by Sch9 in yeast. *Science* **292**: 288-290.

Fabrizio P, Liou LL, Moy VN, Diaspro A, Valentine JS, Gralla EB & Longo VD (2003) SOD2 functions downstream of Sch9 to extend longevity in yeast. *Genetics* **163**: 35-46.

Fanning S, Xu W, Solis N, Woolford CA, Filler SG & Mitchell AP (2012) Divergent targets of Candida albicans biofilm regulator Bcr1 in vitro and in vivo. *Eukaryot Cell* **11**: 896-904.

Flattery-O'Brien JA, Grant CM & Dawes IW (1997) Stationary-phase regulation of the Saccharomyces cerevisiae SOD2 gene is dependent on additive effects of HAP2/3/4/5- and STRE-binding elements. *Mol Microbiol* **23**: 303-312.

Flint DH, Tuminello JF & Emptage MH (1993) The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J Biol Chem* **268**: 22369-22376.

Fradin C, De Groot P, MacCallum D, Schaller M, Klis F, Odds FC & Hube B (2005) Granulocytes govern the transcriptional response, morphology and proliferation of Candida albicans in human blood. *Mol Microbiol* **56**: 397-415.

Frealle E, Noel C, Viscogliosi E, Camus D, Dei-Cas E & Delhaes L (2005) Manganese superoxide dismutase in pathogenic fungi: an issue with pathophysiological and phylogenetic involvements. *FEMS Immunol Med Microbiol* **45**: 411-422.

Frealle E, Noel C, Nolard N, Symoens F, Felipe MS, Dei-Cas E, Camus D, Viscogliosi E & Delhaes L (2006) Manganese superoxide dismutase based phylogeny of pathogenic fungi. *Mol Phylogenet Evol* **41**: 28-39.

Fridovich I (1995) Superoxide radical and superoxide dismutases. *Annu Rev Biochem* **64**: 97-112.

Frohner IE, Bourgeois C, Yatsyk K, Majer O & Kuchler K (2009) Candida albicans cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. *Mol Microbiol* **71**: 240-252.

Galiazzo F & Labbe-Bois R (1993) Regulation of Cu,Zn- and Mn-superoxide dismutase transcription in Saccharomyces cerevisiae. *FEBS Lett* **315**: 197-200.

Getzoff ED, Tainer JA, Weiner PK, Kollman PA, Richardson JS & Richardson DC

(1983) Electrostatic recognition between superoxide and copper, zinc superoxide dismutase. *Nature* **306**: 287-290.

Getzoff ED, Cabelli DE, Fisher CL, Parge HE, Viezzoli MS, Banci L & Hallewell RA (1992) Faster superoxide dismutase mutants designed by enhancing electrostatic guidance. *Nature* **358**: 347-351.

Giles SS, Batinic-Haberle I, Perfect JR & Cox GM (2005) Cryptococcus neoformans mitochondrial superoxide dismutase: an essential link between antioxidant function and high-temperature growth. *Eukaryot Cell* **4**: 46-54.

Ginsberg MD, Feliciello A, Jones JK, Avvedimento EV & Gottesman ME (2003) PKA-dependent binding of mRNA to the mitochondrial AKAP121 protein. *J Mol Biol* **327**: 885-897.

Gleason JE, Li CX, Odeh HM & Culotta VC (2014) Species-specific activation of Cu/Zn SOD by its CCS copper chaperone in the pathogenic yeast Candida albicans. *J Biol Inorg Chem* **19**: 595-603.

Gleason JE, Galaleldeen A, Peterson RL, Taylor AB, Holloway SP, Waninger-Saroni J, Cormack BP, Cabelli DE, Hart PJ & Culotta VC (2014) Candida albicans SOD5 represents the prototype of an unprecedented class of Cu-only superoxide dismutases required for pathogen defense. *Proc Natl Acad Sci U S A* **111**: 5866-5871.

Gonzalez-Parraga P, Hernandez JA & Arguelles JC (2003) Role of antioxidant enzymatic defences against oxidative stress H(2)O(2) and the acquisition of oxidative tolerance in Candida albicans. *Yeast* **20**: 1161-1169.

Grace SC (1990) Phylogenetic distribution of superoxide dismutase supports an endosymbiotic origin for chloroplasts and mitochondria. *Life Sci* **47**: 1875-1886.

Gralla EB & Valentine JS (1991) Null mutants of *Saccharomyces cerevisiae* Cu,Zn superoxide dismutase: characterization and spontaneous mutation rates. *J Bacteriol* **173**: 5918-5920.

Gralla EB & Kosman DJ (1992) Molecular genetics of superoxide dismutases in yeasts and related fungi. *Adv Genet* **30**: 251-319.

Gross DP, Burgard CA, Reddehase S, Leitch JM, Culotta VC & Hell K (2011) Mitochondrial Ccs1 contains a structural disulfide bond crucial for the import of this unconventional substrate by the disulfide relay system. *Mol Biol Cell* **22**: 3758-3767.

Gunasekaran U, Yang R & Gunasekaran M (1998) Regulation of superoxide dismutase synthesis in Candida albicans. *Mycopathologia* **141**: 59-63.

Gutierrez-Escobedo G, Orta-Zavalza E, Castano I & De Las Penas A (2013) Role of glutathione in the oxidative stress response in the fungal pathogen *Candida glabrata*. *Curr Genet* **59**: 91-106.

Hamilton AJ & Holdom MD (1997) Biochemical comparison of the Cu,Zn superoxide dismutases of Cryptococcus neoformans var. neoformans and Cryptococcus neoformans var. gattii. *Infect Immun* **65**: 488-494.

Hamilton AJ, Holdom MD & Jeavons L (1996) Expression of the Cu,Zn superoxide dismutase of Aspergillus fumigatus as determined by immunochemistry and immunoelectron microscopy. *FEMS Immunol Med Microbiol* **14**: 95-102.

Hampton MB, Kettle AJ & Winterbourn CC (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**: 3007-3017.

Henle ES, Roots R, Holley WR & Chatterjee A (1995) DNA strand breakage is correlated with unaltered base release after gamma irradiation. *Radiat Res* **143**: 144-150.

Herker E, Jungwirth H, Lehmann KA, Maldener C, Frohlich KU, Wissing S, Buttner S, Fehr M, Sigrist S & Madeo F (2004) Chronological aging leads to apoptosis in yeast. *J Cell Biol* **164**: 501-507.

Holbrook ED, Edwards JA, Youseff BH & Rappleye CA (2011) Definition of the extracellular proteome of pathogenic-phase Histoplasma capsulatum. *J Proteome Res* **10**: 1929-1943.

Holdom MD, Hay RJ & Hamilton AJ (1996) The Cu,Zn superoxide dismutases of Aspergillus flavus, Aspergillus niger, Aspergillus nidulans, and Aspergillus terreus: purification and biochemical comparison with the Aspergillus fumigatus Cu,Zn superoxide dismutase. *Infect Immun* **64**: 3326-3332.

Holdom MD, Lechenne B, Hay RJ, Hamilton AJ & Monod M (2000) Production and characterization of recombinant Aspergillus fumigatus Cu,Zn superoxide dismutase and its recognition by immune human sera. *J Clin Microbiol* **38**: 558-562.

Huang ME & Kolodner RD (2005) A biological network in Saccharomyces cerevisiae prevents the deleterious effects of endogenous oxidative DNA damage. *Mol Cell* **17**: 709-720.

Huang ME, Rio AG, Nicolas A & Kolodner RD (2003) A genomewide screen in Saccharomyces cerevisiae for genes that suppress the accumulation of mutations. *Proc Natl Acad Sci U S A* **100**: 11529-11534.

Hwang CS, Baek YU, Yim HS & Kang SO (2003) Protective roles of mitochondrial manganese-containing superoxide dismutase against various stresses in Candida albicans. *Yeast* **20**: 929-941.

Hwang CS, Rhie GE, Oh JH, Huh WK, Yim HS & Kang SO (2002) 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. *Microbiology* **148**: 3705-3713.

Imlay JA & Fridovich I (1991) Superoxide production by respiring membranes of Escherichia coli. *Free Radic Res Commun* **12-13 Pt 1**: 59-66.

Jensen LT, Sanchez RJ, Srinivasan C, Valentine JS & Culotta VC (2004) Mutations in Saccharomyces cerevisiae iron-sulfur cluster assembly genes and oxidative stress relevant to Cu,Zn superoxide dismutase. *J Biol Chem* **279**: 29938-29943.

Jeong JH, Kwon ES & Roe JH (2001) Characterization of the manganese-containing superoxide dismutase and its gene regulation in stress response of Schizosaccharomyces pombe. *Biochem Biophys Res Commun* **283**: 908-914.

Jung HI, Lee YY, Lim HW, Ahn KS, Park EH & Lim CJ (2002) Regulation of the manganese-containing superoxide dismutase gene from fission yeast. *Mol Cells* **14**: 300-304.

Kaeberlein M, Burtner CR & Kennedy BK (2007) Recent developments in yeast aging. *PLoS Genet* **3**: e84.

Keyer K & Imlay JA (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci U S A* **93**: 13635-13640.

Keyer K, Gort AS & Imlay JA (1995) Superoxide and the production of oxidative

DNA damage. *J Bacteriol* **177**: 6782-6790.

Kloppel C, Suzuki Y, Kojer K, Petrungaro C, Longen S, Fiedler S, Keller S & Riemer J (2011) Mia40-dependent oxidation of cysteines in domain I of Ccs1 controls its distribution between mitochondria and the cytosol. *Mol Biol Cell* **22**: 3749-3757.

Koziol S, Zagulski M, Bilinski T & Bartosz G (2005) Antioxidants protect the yeast Saccharomyces cerevisiae against hypertonic stress. *Free Radic Res* **39**: 365-371.

Kwon ES, Jeong JH & Roe JH (2006) Inactivation of homocitrate synthase causes lysine auxotrophy in copper/zinc-containing superoxide dismutase-deficient yeast *Schizosaccharomyces pombe*. *J Biol Chem* **281**: 1345-1351.

Laliberte J, Whitson LJ, Beaudoin J, Holloway SP, Hart PJ & Labbe S (2004) The Schizosaccharomyces pombe Pccs protein functions in both copper trafficking and metal detoxification pathways. *J Biol Chem* **279**: 28744-28755.

Lamarre C, LeMay JD, Deslauriers N & Bourbonnais Y (2001) Candida albicans expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase. *J Biol Chem* **276**: 43784-43791.

Lamb AL, Wernimont AK, Pufahl RA, Culotta VC, O'Halloran TV & Rosenzweig AC (1999) Crystal structure of the copper chaperone for superoxide dismutase. *Nat Struct Biol* **6**: 724-729.

Lambou K, Lamarre C, Beau R, Dufour N & Latge JP (2010) Functional analysis of the superoxide dismutase family in Aspergillus fumigatus. *Mol Microbiol* **75**: 910-923.

Lapinskas PJ, Cunningham KW, Liu XF, Fink GR & Culotta VC (1995) Mutations in PMR1 suppress oxidative damage in yeast cells lacking superoxide dismutase. *Mol Cell Biol* **15**: 1382-1388.

Lee J, Kwon ES, Kim DW, Cha J & Roe JH (2002) Regulation and the role of Cu,Zn-containing superoxide dismutase in cell cycle progression of Schizosaccharomyces pombe. *Biochem Biophys Res Commun* **297**: 854-862.

Lee J, Godon C, Lagniel G, Spector D, Garin J, Labarre J & Toledano MB (1999) Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *J Biol Chem* **274**: 16040-16046.

Lee JH, Choi IY, Kil IS, Kim SY, Yang ES & Park JW (2001) Protective role of superoxide dismutases against ionizing radiation in yeast. *Biochim Biophys Acta* **1526**: 191-198.

Li Y, Huang TT, Carlson EJ*, et al.* (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* **11**: 376-381.

Lin SJ & Culotta VC (1995) The ATX1 gene of Saccharomyces cerevisiae encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. *Proc Natl Acad Sci U S A* **92**: 3784-3788.

Lin SJ & Culotta VC (1996) Suppression of oxidative damage by Saccharomyces cerevisiae ATX2, which encodes a manganese-trafficking protein that localizes to Golgi-like vesicles. *Mol Cell Biol* **16**: 6303-6312.

Liochev SI & Fridovich I (1999) Superoxide and iron: partners in crime. *IUBMB Life* **48**: 157-161.

Liu X, Zhang X & Zhang Z (2010) Cu,Zn-superoxide dismutase is required for cell wall structure and for tolerance to cell wall-perturbing agents in Saccharomyces cerevisiae. *FEBS Lett* **584**: 1245-1250.

Liu XF & Culotta VC (1994) The requirement for yeast superoxide dismutase is bypassed through mutations in BSD2, a novel metal homeostasis gene. *Mol Cell Biol* **14**: 7037-7045.

Longo VD, Gralla EB & Valentine JS (1996) Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. Mitochondrial production of toxic oxygen species in vivo. *J Biol Chem* **271**: 12275-12280.

Longo VD, Liou LL, Valentine JS & Gralla EB (1999) Mitochondrial superoxide decreases yeast survival in stationary phase. *Arch Biochem Biophys* **365**: 131-142.

Luk E, Yang M, Jensen LT, Bourbonnais Y & Culotta VC (2005) Manganese activation of superoxide dismutase 2 in the mitochondria of Saccharomyces cerevisiae. *J Biol Chem* **280**: 22715-22720.

Lynch M & Kuramitsu H (2000) Expression and role of superoxide dismutases (SOD) in pathogenic bacteria. *Microbes Infect* **2**: 1245-1255.

Manfredini V, Roehrs R, Peralba MC, Henriques JA, Saffi J, Ramos AL & Benfato MS (2004) Glutathione peroxidase induction protects Saccharomyces cerevisiae sod1deltasod2delta double mutants against oxidative damage. *Braz J Med Biol Res* **37**: 159-165.

Mansour MK & Levitz SM (2002) Interactions of fungi with phagocytes. *Curr Opin Microbiol* **5**: 359-365.

Martchenko M, Alarco AM, Harcus D & Whiteway M (2004) Superoxide dismutases in Candida albicans: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene. *Mol Biol Cell* **15**: 456-467.

Matsuyama A, Arai R, Yashiroda Y*, et al.* (2006) ORFeome cloning and global analysis of protein localization in the fission yeast Schizosaccharomyces pombe. *Nat Biotechnol* **24**: 841-847.

McCord JM & Fridovich I (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* **244**: 6049-6055.

Meneghini R (1997) Iron homeostasis, oxidative stress, and DNA damage. *Free Radic Biol Med* **23**: 783-792.

Miller RA & Britigan BE (1997) Role of oxidants in microbial pathophysiology. *Clin Microbiol Rev* **10**: 1-18.

Miramon P, Dunker C, Windecker H, Bohovych IM, Brown AJ, Kurzai O & Hube B (2012) Cellular responses of Candida albicans to phagocytosis and the extracellular activities of neutrophils are critical to counteract carbohydrate starvation, oxidative and nitrosative stress. *PLoS One* **7**: e52850.

Muid KA, Karakaya HC & Koc A (2014) Absence of superoxide dismutase activity causes nuclear DNA fragmentation during the aging process. *Biochem Biophys Res Commun* **444**: 260-263.

Munkres KD, Rana RS & Goldstein E (1984) Genetically determined conidial longevity is positively correlated with superoxide dismutase, catalase, glutathione peroxidase, cytochrome c peroxidase, and ascorbate free radical reductase activities in Neurospora crassa. *Mech Ageing Dev* **24**: 83-100.

Mutoh N & Kitajima S (2007) Accelerated chronological aging of a mutant fission yeast deficient in both glutathione and superoxide dismutase having cu and zn as cofactors and its enhancement by sir2 deficiency. *Biosci Biotechnol Biochem* **71**: 2841-2844.

Narasipura SD, Chaturvedi V & Chaturvedi S (2005) Characterization of Cryptococcus neoformans variety gattii SOD2 reveals distinct roles of the two superoxide dismutases in fungal biology and virulence. *Mol Microbiol* **55**: 1782-1800.

Narasipura SD, Ault JG, Behr MJ, Chaturvedi V & Chaturvedi S (2003) Characterization of Cu,Zn superoxide dismutase (SOD1) gene knock-out mutant of Cryptococcus neoformans var. gattii: role in biology and virulence. *Mol Microbiol* **47**: 1681-1694.

Nash HM, Bruner SD, Scharer OD, Kawate T, Addona TA, Spooner E, Lane WS & Verdine GL (1996) Cloning of a yeast 8-oxoguanine DNA glycosylase reveals the existence of a base-excision DNA-repair protein superfamily. *Curr Biol* **6**: 968-980.

Nedeva TS, Petrova VY, Zamfirova DR, Stephanova EV & Kujumdzieva AV (2004) Cu/Zn superoxide dismutase in yeast mitochondria - a general phenomenon. *FEMS Microbiol Lett* **230**: 19-25.

Neeley WL & Essigmann JM (2006) Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products. *Chem Res Toxicol* **19**: 491-505.

Nikolaou E, Agrafioti I, Stumpf M, Quinn J, Stansfield I & Brown AJ (2009) Phylogenetic diversity of stress signalling pathways in fungi. *BMC Evol Biol* **9**: 44.

O'Brien KM, Dirmeier R, Engle M & Poyton RO (2004) Mitochondrial protein oxidation in yeast mutants lacking manganese-(MnSOD) or copper- and zinc-containing superoxide dismutase (CuZnSOD): evidence that MnSOD and CuZnSOD have both unique and overlapping functions in protecting mitochondrial proteins from oxidative damage. *J Biol Chem* **279**: 51817-51827.

Pan X, Ye P, Yuan DS, Wang X, Bader JS & Boeke JD (2006) A DNA integrity network in the yeast Saccharomyces cerevisiae. *Cell* **124**: 1069-1081.

Parker MW & Blake CC (1988) Iron- and manganese-containing superoxide dismutases can be distinguished by analysis of their primary structures. *FEBS Lett* **229**: 377-382.

Pereira MD, Eleutherio EC & Panek AD (2001) Acquisition of tolerance against oxidative damage in Saccharomyces cerevisiae. *BMC Microbiol* **1**: 11.

Phillips JP, Campbell SD, Michaud D, Charbonneau M & Hilliker AJ (1989) Null mutation of copper/zinc superoxide dismutase in Drosophila confers hypersensitivity to paraquat and reduced longevity. *Proc Natl Acad Sci U S A* **86**: 2761-2765.

Pinkham JL, Wang Z & Alsina J (1997) Heme regulates SOD2 transcription by activation and repression in Saccharomyces cerevisiae. *Curr Genet* **31**: 281-291.

Portnoy ME, Liu XF & Culotta VC (2000) Saccharomyces cerevisiae expresses three functionally distinct homologues of the nramp family of metal transporters. *Mol Cell Biol* **20**: 7893-7902.

Portnoy ME, Schmidt PJ, Rogers RS & Culotta VC (2001) Metal transporters that

contribute copper to metallochaperones in Saccharomyces cerevisiae. *Mol Genet Genomics* **265**: 873-882.

Rae TD, Schmidt PJ, Pufahl RA, Culotta VC & O'Halloran TV (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* **284**: 805-808.

Reddehase S, Grumbt B, Neupert W & Hell K (2009) The disulfide relay system of mitochondria is required for the biogenesis of mitochondrial Ccs1 and Sod1. *J Mol Biol* **385**: 331-338.

Reddi AR & Culotta VC (2013) SOD1 integrates signals from oxygen and glucose to repress respiration. *Cell* **152**: 224-235.

Rhie GE, Hwang CS, Brady MJ, Kim ST, Kim YR, Huh WK, Baek YU, Lee BH, Lee JS & Kang SO (1999) Manganese-containing superoxide dismutase and its gene from Candida albicans. *Biochim Biophys Acta* **1426**: 409-419.

Roetzer A, Klopf E, Gratz N, Marcet-Houben M, Hiller E, Rupp S, Gabaldon T, Kovarik P & Schuller C (2011) Regulation of *Candida glabrata* oxidative stress resistance is adapted to host environment. *FEBS Lett* **585**: 319-327.

Rohm M, Lindemann E, Hiller E*, et al.* (2013) A family of secreted pathogenesis-related proteins in Candida albicans. *Mol Microbiol* **87**: 132-151.

Schmidt PJ, Kunst C & Culotta VC (2000) Copper activation of superoxide dismutase 1 (SOD1) in vivo. Role for protein-protein interactions with the copper chaperone for SOD1. *J Biol Chem* **275**: 33771-33776.

Sehati S, Clement MH, Martins J, Xu L, Longo VD, Valentine JS & Gralla EB (2011) Metabolic alterations in yeast lacking copper-zinc superoxide dismutase. *Free Radic Biol Med* **50**: 1591-1598.

Shockley AH, Doo DW, Rodriguez GP & Crouse GF (2013) Oxidative damage and mutagenesis in Saccharomyces cerevisiae: genetic studies of pathways affecting replication fidelity of 8-oxoguanine. *Genetics* **195**: 359-367.

Slekar KH, Kosman DJ & Culotta VC (1996) The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection. *J Biol Chem* **271**: 28831-28836.

Smith MW & Doolittle RF (1992) A comparison of evolutionary rates of the two major kinds of superoxide dismutase. *J Mol Evol* **34**: 175-184.

Srinivasan C, Liba A, Imlay JA, Valentine JS & Gralla EB (2000) Yeast lacking superoxide dismutase(s) show elevated levels of "free iron" as measured by whole cell electron paramagnetic resonance. *J Biol Chem* **275**: 29187-29192.

Stoica BA, Rusu M, Petreus T & Nechifor M (2011) Manganese SOD mimics are effective against heat stress in a mutant fission yeast deficient in mitochondrial superoxide dismutase. *Biol Trace Elem Res* **144**: 1344-1350.

Strain J, Lorenz CR, Bode J, Garland S, Smolen GA, Ta DT, Vickery LE & Culotta VC (1998) Suppressors of superoxide dismutase (SOD1) deficiency in Saccharomyces cerevisiae. Identification of proteins predicted to mediate iron-sulfur cluster assembly. *J Biol Chem* **273**: 31138-31144.

Sturtz LA, Diekert K, Jensen LT, Lill R & Culotta VC (2001) A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. *J Biol Chem* **276**: 38084-38089.

Tainer JA, Getzoff ED, Richardson JS & Richardson DC (1983) Structure and

mechanism of copper, zinc superoxide dismutase. *Nature* **306**: 284-287.
Tainer JA, Getzoff ED, Beem KM, Richardson JS & Richardson DC (1982) Determination and analysis of the 2 A-structure of copper, zinc superoxide dismutase. *J Mol Biol* **160**: 181-217.
Takahashi H, Suzuki T, Shirai A, Matsuyama A, Dohmae N & Yoshida M (2011) Mitochondrial localization of fission yeast manganese superoxide dismutase is required for its lysine acetylation and for cellular stress resistance and respiratory growth. *Biochem Biophys Res Commun* **406**: 42-46.
Tarhan C, Pekmez M, Karaer S, Arda N & Sarikaya AT (2007) The effect of superoxide dismutase deficiency on zinc toxicity in Schizosaccharomyces pombe. *J Basic Microbiol* **47**: 506-512.
Tsang CK, Liu Y, Thomas J, Zhang Y & Zheng XF (2014) Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. *Nat Commun* **5**: 3446.
Unlu ES & Koc A (2007) Effects of deleting mitochondrial antioxidant genes on life span. *Ann N Y Acad Sci* **1100**: 505-509.
van Loon AP, Pesold-Hurt B & Schatz G (1986) A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. *Proc Natl Acad Sci U S A* **83**: 3820-3824.
Van Remmen H, Ikeno Y, Hamilton M*, et al.* (2003) Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol Genomics* **16**: 29-37.
Viriyakosol S, Singhania A, Fierer J, Goldberg J, Kirkland TN & Woelk CH (2013) Gene expression in human fungal pathogen Coccidioides immitis changes as arthroconidia differentiate into spherules and mature. *BMC Microbiol* **13**: 121.
Wallace MA, Liou LL, Martins J, Clement MH, Bailey S, Longo VD, Valentine JS & Gralla EB (2004) Superoxide inhibits 4Fe-4S cluster enzymes involved in amino acid biosynthesis. Cross-compartment protection by CuZn-superoxide dismutase. *J Biol Chem* **279**: 32055-32062.
Wilkinson SR, Prathalingam SR, Taylor MC, Ahmed A, Horn D & Kelly JM (2006) Functional characterisation of the iron superoxide dismutase gene repertoire in Trypanosoma brucei. *Free Radic Biol Med* **40**: 198-209.
Wintjens R, Noel C, May AC, Gerbod D, Dufernez F, Capron M, Viscogliosi E & Rooman M (2004) Specificity and phenetic relationships of iron- and manganese-containing superoxide dismutases on the basis of structure and sequence comparisons. *J Biol Chem* **279**: 9248-9254.
Woodruff RC, Phillips JP & Hilliker AJ (2004) Increased spontaneous DNA damage in Cu/Zn superoxide dismutase (SOD1) deficient Drosophila. *Genome* **47**: 1029-1035.
Xiao W, Chow BL & Rathgeber L (1996) The repair of DNA methylation damage in *Saccharomyces cerevisiae*. *Curr Genet* **30**: 461-468.
Yan LJ, Levine RL & Sohal RS (1997) Oxidative damage during aging targets mitochondrial aconitase. *Proc Natl Acad Sci U S A* **94**: 11168-11172.
Youn HD, Kim EJ, Roe JH, Hah YC & Kang SO (1996) A novel nickel-containing superoxide dismutase from Streptomyces spp. *Biochem J* **318 (Pt 3)**: 889-896.
Youseff BH, Holbrook ED, Smolnycki KA & Rappleye CA (2012) Extracellular superoxide dismutase protects Histoplasma yeast cells from host-derived

oxidative stress. *PLoS Pathog* **8**: e1002713.
Zabriskie TM & Jackson MD (2000) Lysine biosynthesis and metabolism in fungi. *Nat Prod Rep* **17**: 85-97.
Znaidi S, Barker KS, Weber S, Alarco AM, Liu TT, Boucher G, Rogers PD & Raymond M (2009) Identification of the Candida albicans Cap1p regulon. *Eukaryot Cell* **8**: 806-820.

Figures

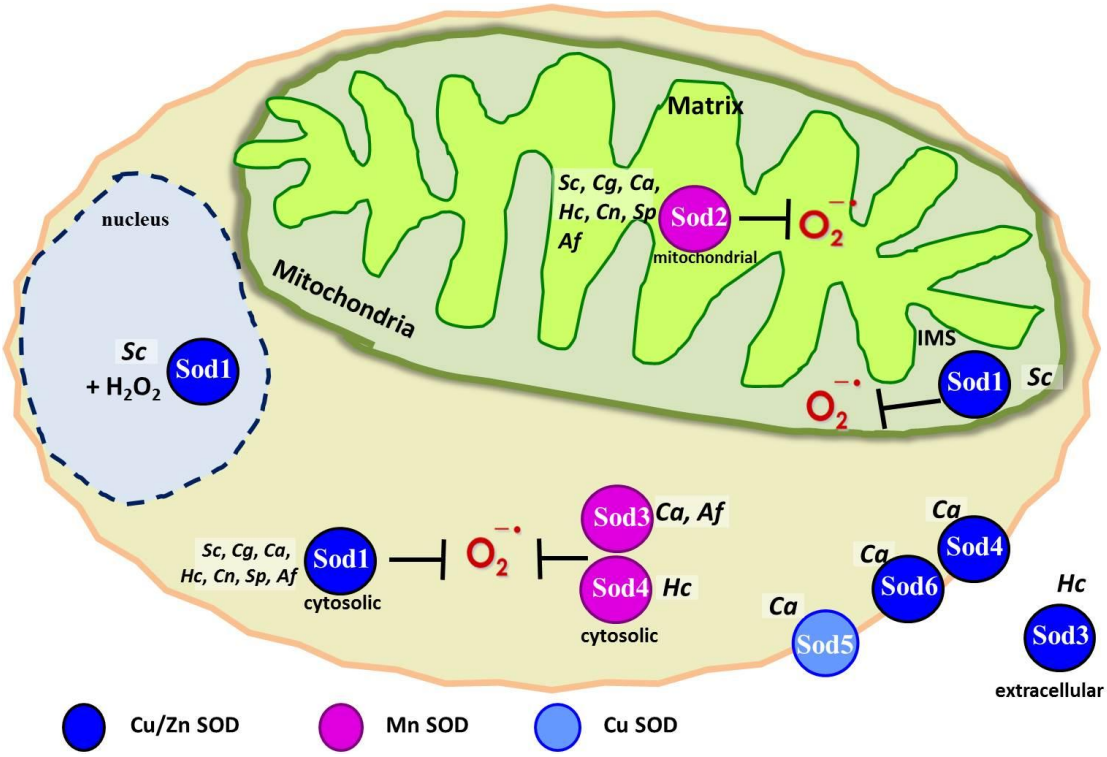


Fig. 1 SODs localization in fungi. Cellular localization and family type of the most studied SODs enzymes in fungi. Mn-superoxide dismutases (pink circle), Cu/Zn superoxide dismutases (dark blue circle), and Cu superoxide dismutases (light blue). Species abbreviations used are: *Sc* (*Saccharomyces cerevisiae*), *Cg* (*Candida glabrata*), *Ca* (*Candida albicans*), *Cn* (*Cryptococcus neoformans*), *Sp* (*Schizosaccharomyces pombe*), *Hc* (*Histoplasma capsulatum*), *Af* (*Aspergillus fumigatus*).

Table 1. SOD playing roles in fungi

Fungus / Stress	MD	PQ	H_2O_2	Ethanol	Salt	Heat	Virulence	MQ/ PMN	Aging
S. cerevisiae	**Sod1 Sod2**	**Sod2**	**Sod2**	**Sod1 Sod2**	**Sod2**	**Sod2**	**NA**	**NA**	**Sod1 Sod2**
S. pombe	**Sod1**		**Sod1**	**Sod2**	**Sod2**	**Sod2**	**NA**	**NA**	**Sod1 + Gsh1**
C. albicans	**Sod1 Sod2**	**Sod1 Sod2**	**Sod5**	**Sod2**	**Sod2**	**Sod2**	**Sod1 Sod5**	**Sod4 Sod5**	
C. glabrata	**Sod1 Sod2**	**ND**		**Sod2**	**ND**	**ND**	**No**	**Sod1 + Yap1**	**Sod2**
C. neoformans* var. *gattii	**Sod1 Sod2**	**Sod1 Sod2**		**Sod2**	**Sod1 Sod2**	**Sod2**	**Sod1 Sod2**	**Sod1**	**Sod2**
H. capsulatum		**Sod1**					**Sod3**	**Sod3**	**ND**

Abbreviations: ND = not determined; NA= not applicable; MD = menadione; PQ = paraquat; H_2O_2 = hydrogen peroxide; MQ = macrophages; PMN = polymorphonuclear leukocytes; Gsh1= γ-glutamyl-cysteine synthetase; Yap1= OSR transcription factor.

2. Resultados

2.1 Capitulo 1

Briones-Martin-del-Campo M, Orta-Zavalza E, Canas-Villamar I, Gutierrez-Escobedo G, Juarez-Cepeda J, Robledo-Márquez, K, Arroyo-Helguera O, Castaño I, De Las Peñas A. **The superoxide dismutases of *Candida glabrata* protect against oxidative damage and are required for lysine biosynthesis, DNA integrity and chronological life survival.** *Microbiology* mic.0.000006; published ahead of print December 5, 2014, doi:10.1099/mic.0.000006

Abstract

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

2.2 Capitulo 2

Briones-Martin-del-Campo M**,** Orta-Zavalza E, Juarez-Cepeda J, Gutierrez-Escobedo G, Canas-Villamar I, Castano I, De Las Penas A. **The oxidative stress response of the opportunistic fungal pathogen *Candida glabrata.*** *Rev Iberoam Micol.* 2014; 31(1):67–71.

Rev Iberoam Micol. 2014;31(1):67–71

Revista Iberoamericana de Micología

www.elsevier.es/reviberoammicol

Mycologic Forum

The oxidative stress response of the opportunistic fungal pathogen *Candida glabrata*

Marcela Briones-Martin-Del-Campo, Emmanuel Orta-Zavalza, Jacqueline Juarez-Cepeda, Guadalupe Gutierrez-Escobedo, Israel Cañas-Villamar, Irene Castaño, Alejandro De Las Peñas*

División de Biología Molecular, IPICYT, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, San Luis Potosí, México

ARTICLE INFO

Article history:
Received 26 August 2013
Accepted 27 September 2013
Available online 19 November 2013

Keywords:
Candida glabrata
Yap1
Cta1
Skn7
Glutathione
Reactive oxygen species

ABSTRACT

Organisms have evolved different strategies to respond to oxidative stress generated as a by-product of aerobic respiration and thus maintain the redox homeostasis within the cell. In particular, fungal pathogens are exposed to reactive oxygen species (ROS) when they interact with the phagocytic cells of the host which are the first line of defense against fungal infections. These pathogens have co-opted the enzymatic (catalases, superoxide dismutases (SODs), and peroxidases) and non-enzymatic (glutathione) mechanisms used to maintain the redox homeostasis within the cell, to resist oxidative stress and ensure survival within the host. Several virulence factors have been related to the response to oxidative stress in pathogenic fungi. The opportunistic fungal pathogen *Candida glabrata* (*C. glabrata*) is the second most common cause of candidiasis after *Candida albicans* (*C. albicans*). *C. glabrata* has a well defined oxidative stress response (OSR), which include both enzymatic and non-enzymatic mechanisms. *C. glabrata* OSR is controlled by the well-conserved transcription factors Yap1, Skn7, Msn2 and Msn4. In this review, we describe the OSR of *C. glabrata*, what is known about its core elements, its regulation and how *C. glabrata* interacts with the host.

This manuscript is part of the series of works presented at the "V International Workshop: Molecular genetic approaches to the study of human pathogenic fungi" (Oaxaca, Mexico, 2012).

Respuesta al estrés oxidante en el hongo patógeno oportunista *Candida glabrata*

Palabras clave:
Candida glabrata
Yap1
Cta1
Skn7
Glutatión
Especies reactivas del oxígeno

RESUMEN

Los microorganismos han establecido diferentes estrategias para controlar el estrés oxidante generado durante la respiración aeróbica y, por consiguiente, mantener la homeostasia redox en la célula. En particular, los hongos patógenos se exponen a especies reactivas del oxígeno cuando interactúan con las células fagocíticas del huésped que son la primera línea de defensa contra estos agentes infecciosos. Estos patógenos han reclutado sistemas enzimáticos (catalasas, superóxido dismutasas y peroxidasas) y no enzimáticos (glutatión) que normalmente utilizan para mantener la homeostasis redox en la célula, para resistir frente al estrés oxidante y garantizar la supervivencia dentro del huésped. Varios factores de virulencia se han relacionado con la respuesta al estrés oxidante de los hongos patógenos. El hongo patógeno oportunista *Candida glabrata* (*C. glabrata*) es la segunda causa más frecuente de candidiasis después de *Candida albicans* (*C. albicans*). *C. glabrata* tiene una respuesta bien definida al estrés oxidante, que incluye sistemas enzimáticos y no enzimáticos y está regulada por los factores de transcripción Yap1, Skn7, Msn2 y Msn4. En esta revisión, describimos los elementos de la respuesta de *C. glabrata* a dicho estrés, cómo se regula y cómo *C. glabrata* interacciona con el huésped.

Este artículo forma parte de una serie de estudios presentados en el «V International Workshop: Molecular genetic approaches to the study of human pathogenic fungi» (Oaxaca, México, 2012).

* Corresponding author.
E-mail address: cano@ipicyt.edu.mx (A. De Las Peñas).

1130-1406/$ – see front matter
http://dx.doi.org/10.1016/j.riam.2013.09.012

Yeast cells growing in an aerobic environment are exposed to reactive oxygen species (ROS) such as the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). ROS are formed as by-products, during normal aerobic metabolism, and could damage all biomolecules and cause cell death.[14] However, in order to maintain the redox homeostasis in the cell, a variety of enzymatic (catalases, SODs, and peroxidases) and non-enzymatic (glutathione) defense mechanisms are induced. This response is tightly controlled and is known as oxidative stress response (OSR).

Phagocytes are the first line of host defense against fungal infections. Upon interaction with the pathogen, phagocytes rapidly produce ROS through the NADPH oxidase complex.[1] Deficiencies in the NADPH oxidase result in increased susceptibility to fungal and bacterial infections underlying the importance of the oxidative killing of microbes. These data show how the pathogens antioxidant systems are necessary to survive the oxidative stress generated by host phagocytes during infection.[22] Until recently, it was not well-known how *Candida glabrata* responds to host cell phagocytosis and how it can survive and persist inside the phagolysosome.

C. glabrata is a haploid yeast found as a commensal in healthy individuals, but causes serious infections in immune compromised humans. In the past three decades, *C. glabrata* has become the second most common cause of candidiasis after *Candida albicans* (*C. albicans*). *C. glabrata* infections are difficult to treat and often result in high mortality in immune compromised hospitalized patients.[2] In the past 10 years, the molecular mechanisms enabling *C. glabrata* to become a successful human pathogen have been described. *C. glabrata* OSR appears to play a central role in the survival inside macrophages: *C. glabrata* neutralizes the ROS generated by the macrophage[38] by inducing antioxidant defenses,[28,29] inhibits the maturation of the phagolysosome[32] and ultimately replicates inside phagosomes.[15] In this review, we will focus on the OSR of *C. glabrata*.

Enzymatic defenses

Catalases

In aerobic organisms, catalases are ubiquitous enzymes that decompose H_2O_2 to water and oxygen. They function as scavengers of H_2O_2 to maintain the redox balance in the cell. Given that H_2O_2 has been found to be a signaling molecule, catalases are also important for growth regulation and development.[25] *C. glabrata* has one catalase CgCta1[5] (Table 1), a mono-functional 57-kDa heme-containing protein, classified as a small subunit catalase and is 85% similar to the *Saccharomyces cerevisiae* peroxisomal catalase, ScCta1. Although CgCta1 does not have a canonical C-terminal peroxisomal targeting signal (PTS1),[5] it has been shown to localize in the cytosol and accumulates in peroxisomes during respiration and inside phagocytic cells.[28] Furthermore, the expression of Cg*CTA1* is induced in the presence of oxidative stress and in carbon source deprivation.[29] Interestingly, the Cg*CTA1* locus does not maintain genic order when compared to other sequenced hemiascomycete yeasts.[23] In particular, the upstream region of *CTA1* is approximately 4.5 kb, much longer than the average 454 bp intergenic regions of *C. glabrata*.[36]

The in vitro resistance of *C. glabrata* to H_2O_2 is very high, compared to *S. cerevisiae* or *C. albicans*[5] and this in vitro resistance is mediated by the CgCta1; however, in a murine model of systemic infection the *cta1*△ mutant was not affected in the colonization phenotype of target organs in vivo.[5] This suggested that other antioxidant molecules could be compensating for the absence of CgCta1. Analysis of the double mutants lacking glutathione or thioredoxin suggested that theses antioxidant molecules are not responsible for compensating in vivo the absence of CgCta1.[10] To date, the role of CgCta1 as a virulence factor has not been established as in other pathogenic fungi.[8]

Superoxide dismutases

SODs are metalloenzymes that catalyze the dismutation of $O_2^{\bullet-}$ into H_2O_2 and oxygen. These enzymes protect cells against oxidative damage and scavenge $O_2^{\bullet-}$ radicals generated during aerobic metabolism. Based on their metal cofactor, SODs are classified in three families: copper/zinc (Cu,ZnSOD), iron or manganese SODs (FeSOD and MnSOD) and nickel SODs (NiSOD).[7] Most eukaryotes contain two SODs, a MnSOD localized in the mitochondrial matrix[37] and a highly abundant Cu,ZnSOD present in cytosol and mitochondrial inter-membrane space.[26] *C. glabrata* has two SOD genes, Cg*SOD1* (Cu,ZnSOD) and Cg*SOD2* (MnSOD) (Table 1). Interestingly, it has been shown that *Cryptococcus neoformans*, *C. albicans* and *Histoplasma capsulatum* SODs are required for survival against macrophages[13,24,40]; however, the survival rate of *C. glabrata sod1*△ mutant in macrophages was not diminished.[29]

In *S. cerevisiae*, *Schizosaccharomyces pombe* and *C. albicans*, *SOD1* expression is induced by oxidative stress,[6,18] and regulated by Yap1, a key transcriptional regulator of the OSR.[16,17,42] In contrast, *C. glabrata* Cg*SOD1* and Cg*SOD2* are constitutively expressed even in the presence of oxidative stress and independent of CgYap1.[29] Cg*SOD1* and Cg*SOD2* expression are highly induced under glucose starvation. Interestingly, the survival rate of *C. glabrata sod1*△ mutant in macrophages was diminished only in combination with a mutation in CgYap1.[29]

Glutathione and thioredoxin

Redox homeostasis is necessary to support important cellular processes. The oxidation status of thiols is maintained and rapidly

Table 1
Oxidative stress response genes in *Candida glabrata*.

Gene name	Systematic name	Function	Null mutant phenotype			
			Oxidative stress sensitivity	Phagocytes survival?	Important to virulence?	Ref.
CTA1	CAGL0K10868g	Catalase	H_2O_2	Yes	No	5
SOD1	CAGL0C04741g	Superoxide dismutase	MD	Yes	ND	31
SOD2	CAGL0E04356g	Superoxide dismutase	ND	ND	ND	31
TRR1	CAGL0A02530g	Thioredoxin reductase	No	ND	ND	12
GSH1	CAGL0L03630g	γ Glutamyl-cysteine synthetase	H_2O_2, MD, Cd	ND	ND	12,42
GSH2	CAGL0F00825g	Glutathione synthetase	H_2O_2, MD, Cd	ND	ND	12
YAP1	CAGL0H04631g	bZip transcription factor	H_2O_2, ONOO–	Yes	No	5,4,31
SKN7	CAGL0F09097g	Transcription factor	H_2O_2, t-BuOOH	Yes	Yes	5,34
MSN2	CAGL0F05995g	Cys_2-His_2 zinc finger transcription factor	H_2O_2	ND	No	5,29
MSN4	CAGL0M13189g	Cys_2-His_2 zinc finger transcription factor	H_2O_2	ND	No	5,29

Abbreviations: ND = not determined; H_2O_2 = hydrogen peroxide; t-BuOOH = tert-butyl hydroperoxide; ONOO– = peroxynitrite; MD = menadione; Cd = cadmium; bZIP = basic leucine zipper transcription factor.

restored by the action of two redox-balancing systems: glutathione (GSH) and thioredoxin pathways.[12] In yeast, the GSH system is constituted by GSH, glutaredoxins and a glutathione reductase. Similarly, the cytoplasmic thioredoxin pathway comprises two thioredoxins and a thioredoxin reductase.[35] *C. glabrata* has the majority of the components of both systems (Table 1); however, their role in the OSR of *C. glabrata* has recently been described.[10,39]

GSH is an essential tripeptide composed of glycine, cysteine and glutamate synthesized by the sequential action of Gsh1 and Gsh2.[21] Through its thiol group, GSH reacts directly with reactive species or acts as cofactor with specific enzymes (glutaredoxins, glutathione peroxidases or glutathione transferases) to detoxify ROS or xenobiotics. In *C. glabrata*, *GSH1* is essential[39]; however, a suppressor mutation in *CgPRO2* was isolated in the absence of *GSH1*. The *pro2-4* suppressor mutation could be acting through the synthesis of residual amounts of GSH.[10] Additionally, *C. glabrata* cells lacking glutathione are sensitive to oxidative stress and have a reduced late chronological life span.[10] Surprisingly, the *S. cerevisiae* specific GSH transporter (Sc*OPT1*) is not present in *C. glabrata*; however, there is evidence suggesting external uptake of GSH by an unknown transporter.[10] The differences between *S. cerevisiae* and *C. glabrata* in the GSH pathway and the absence of the GSH specific transporter suggest that GSH may play an important role during pathogenesis.

The thioredoxin system is a pivotal player in the redox homeostasis since some components affect the oxidative stress resistance in many microorganisms. In *C. glabrata*, there is evidence that suggest that the cytoplasmic thioredoxin system plays a minor role in H_2O_2 adaptation; however, the inability to disrupt simultaneously the three systems (GSH, thioredoxin and catalase) in *C. glabrata* suggests that these pathways could be complementing each other in order to respond to oxidative stress.[10]

Metallothioneins, phytochelatins and pigments

Some metals are essential micronutrients for physiological processes. Copper, zinc and other non-essential heavy metal ions, such as cadmium, lead, and mercury, are highly reactive and can produce oxidative stress. To diminish the toxic effects of heavy metals, some yeast synthesize phytochelatins (PCs), which are enzymatically synthesized cysteine-rich peptides derived from GSH and metallothioneins (MT), oligomeric thiolated molecules that act as chelators.[11] In the *C. glabrata* genome, two MT isoforms have been identified: MT-I (CAGL0D01265g) and MT-II (CAGL0H04257g).[19] *C. glabrata* appears to chelate cadmium with phytochelatin-like molecules[20]; however, the phytochelatin synthase coding gene has not been identified.

Pigments are important factors in pathogenic fungi for survival in their host. Production of the dark pigment melanin has been linked to pathogenicity. Recently it was shown that *C. glabrata* produces a pigment that protects against H_2O_2 and the attack by human neutrophils. This pigment is a by-product of the Ehrlich pathway of tryptophan degradation and its production is mainly driven by the aromatic aminotransferase I (Aro8).[3]

Transcriptional regulation by reactive oxygen species

CgYap1

A number of well-conserved transcription factors have been identified that control the OSR in *C. glabrata*.[5,28,29] Yap1 is a bZip transcription factor that contains cysteine rich domains in its N- and C-terminal portions. The transcription factor Yap1 of *C. glabrata* controls the OSR and accumulates transiently in the nucleus during phagocytosis.[28,29] In a genome wide analysis in *C. glabrata*, one set of genes (*CTA1*, *TRR1/2*, *TSA1/2*, *TRX2*, *GPX2* and *CCP1*) were dependent on the presence of both, Skn7 and Yap1, and defined the core of the OSR. A second Yap1-regulated group included genes encoding proteins with aldo-ketoreductase and oxidoreductase activities (*ADH6*, *GRE2*, *SCS7* and *OYE2*).[29] Interestingly, the loss of CgYap1 had no impact on virulence in both primary mouse macrophages or in a murine model of systemic infection.[4,29] It is possible that either the genes controlled by CgYap1 are dispensable to survive within the host or there are redundant mechanisms that compensate the lack of the genes controlled by this transcription factor.

CgSkn7

Skn7 is an oxidative and cell wall stress-responsive transcription factor highly conserved among fungi. It has been shown that in *C. glabrata* Skn7 controls the expression of a set of genes including *TRX2*, *TRR1*, *TSA1* and *CTA1* in response to the presence of H_2O_2 and is required for the adaptation response to oxidative stress.[5,30,34] Skn7 has been shown to be important for virulence in a murine model of systemic infection[31]; however, Skn7 was dispensable for prolonged survival in macrophages.[29]

CgMsn2 and CgMsn4

The Cys_2-His_2 zinc finger transcription factors Msn2 and Msn4 mediate the general stress response and functions in parallel with Yap1 and Skn7 to mediate the OSR in *C. glabrata*.[5,27] However, the function of Msn2 and Msn4-like proteins has diverged significantly among fungi. In a *Drosophila melanogaster* infection model, it was shown that Msn2 does not have a major impact on virulence.[27] Genes functionally connected to stress response, such as *HSP12*, *HSP42*, *DDR48*, *GPH1*, *GDB, TPS1*, *TPS2* or *PGM2* displayed a CgMsn2 and Msn4-associated upregulation.[27,41]

Concluding remarks

In the past 10 years, some of the *C. glabrata* virulence factors that contribute to pathogenesis have been identified, and in particular specific efforts have been made to study the interplay between *C. glabrata* and the host. As a successful fungal pathogen, *C. glabrata* quickly detects and responds to metabolic changes and to oxidative stress through the induction of protective enzymes against oxidative stress (catalase and SODs) and non-enzymatic defense systems (GSH) (Fig. 1). The transcription factors, Msn2, Msn4, Skn7 and Yap1, play a central role in the regulation of OSR. The fact that these transcriptional factors are dispensable for virulence (except for Skn7) suggests that *C. glabrata* survival depends on redundant pathways that can compensate each other (Fig. 1). It is possible that the ability of *C. glabrata* to suppress the production of ROS ensures its survival during phagocytosis.[33] Interestingly, although the structure of the MAP kinase pathways is relatively conserved among fungi, the role of MAPK pathways in *C. glabrata* remains largely unexplored despite the fact that these pathways are important for virulence.[9] The identification and characterization of the proteins involved in the detection of the oxidative signal will be essential to understand how *C. glabrata* evades killing by the phagocytic cells. Our knowledge about the OSR of *C. glabrata* is increasing rapidly and, in the next few years, future research will reveal major new insights into the regulatory networks and signal transduction pathways that regulate and coordinate the stress regulons in *C. glabrata*.

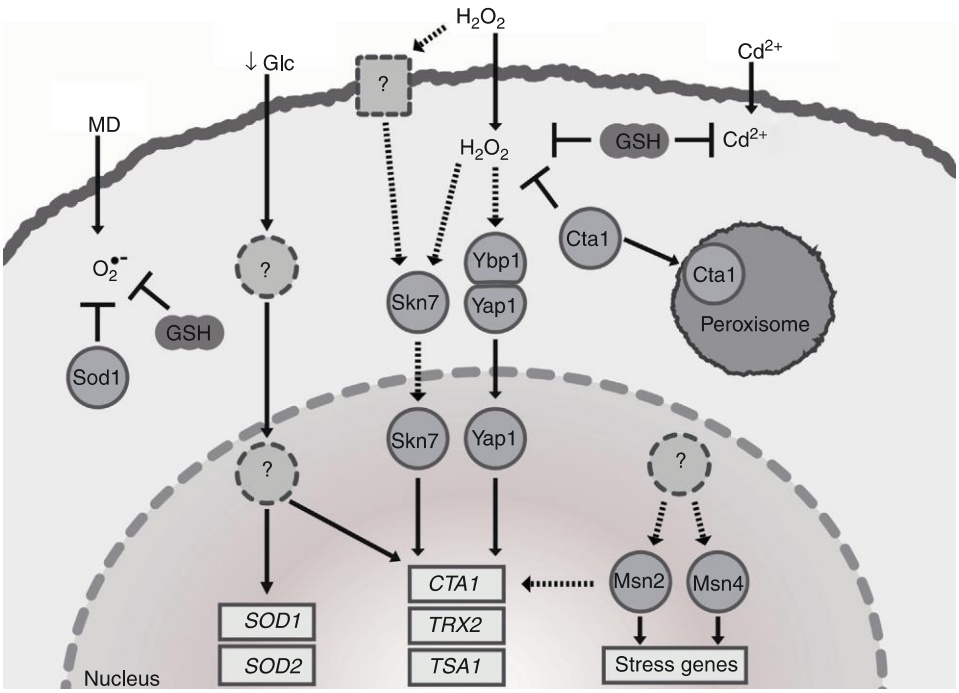


Fig. 1. Pathways of the OSR in *Candida glabrata*. *C. glabrata* responds with antioxidant defenses against external sources of oxidative stress such as menadione (MD), hydrogen peroxide (H_2O_2) and cadmium (Cd^{2+}). The peroxide-induced signal transduction pathway of *C. glabrata* is still unknown, but data suggest that H_2O_2 activates the transcription factors Yap1, Skn7 and Msn4. Yap1 response to H_2O_2 requires Ybp1. Yap1 and Skn7 activate transcription of the catalase (*CTA1*), thioredoxin (*TRX2*), and thioredoxin peroxidase (*TSA1*). The catalase detoxifies H_2O_2 in cytoplasm and peroxisomes. To neutralize the superoxide (O_2^-), *C. glabrata* induces the superoxide dismutase (Sod1) and the synthesis of glutathione (GSH). Sod1 converts O_2^- to H_2O_2. GSH participates in maintaining the redox balance. The transcription of *CTA1*, *SOD1* and *SOD2* is induced by glucose starvation (↓Glc). *SOD1* and *SOD2* do not respond to H_2O_2. Msn2 and Msn4 are involved in the activation of the general stress response. The discontinuous arrows and dashed-line circles represent not-well established pathways.

Conflict of interest

The authors declare that they have no conflict of interests.

Acknowledgements

This work was funded by a Consejo Nacional de Ciencia y Tecnología (CONACYT) fellowship to M.B.M.C. (209276), E.O.Z. (233455), J.J.C. (48549), G.G.E. (48580), and I.C.V. (224300). This work was funded by a CONACYT grant No. CB-2010-01-153929 to A.D.L.P. and grant No. CB-2010-01-151517 to I.C.N.

References

1. Babior BM. NADPH oxidase. Curr Opin Immunol. 2004;16:42–7.
2. Baddley JW, Patel M, Bhavnani SM, Moser SA, Andes DR. Association of fluconazole pharmacodynamics with mortality in patients with candidemia. Antimicrob Agents Chemother. 2008;52:3022–8.
3. Brunke S, Seider K, Almeida RS, Heyken A, Fleck CB, Brock M, et al. *Candida glabrata* tryptophan-based pigment production via the Ehrlich pathway. Mol Microbiol. 2010;76:25–47.
4. Chen KH, Miyazaki T, Tsai HF, Bennett JE. The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in *Candida glabrata*. Gene. 2007;386:63–72.
5. Cuellar-Cruz M, Briones-Martin-del-Campo M, Canas-Villamar I, Montalvo-Arredondo J, Riego-Ruiz L, Castano I, et al. High resistance to oxidative stress in the fungal pathogen *Candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p Skn7p, Msn2p, and Msn4p. Eukaryot Cell. 2008;7:814–25.
6. Cyrne L, Martins L, Fernandes L, Marinho HS. Regulation of antioxidant enzymes gene expression in the yeast *Saccharomyces cerevisiae* during stationary phase. Free Radic Biol Med. 2003;34:385–93.
7. Fridovich I. Superoxide radical superoxide dismutases. Annu Rev Biochem. 1995;64:97–112.
8. Giles SS, Stajich JE, Nichols C, Gerrald QD, Alspaugh JA, Dietrich F, et al. The *Cryptococcus neoformans* catalase gene family and its role in antioxidant defense. Eukaryot Cell. 2006;5:1447–59.
9. Gregori C, Schuller C, Roetzer A, Schwarzmuller T, Ammerer G, Kuchler K. The high-osmolarity glycerol response pathway in the human fungal pathogen *Candida glabrata* strain ATCC 2001 lacks a signaling branch that operates in baker's yeast. Eukaryot Cell. 2007;6:1635–45.
10. Gutierrez-Escobedo G, Orta-Zavalza E, Castano I, De Las Penas A. Role of glutathione in the oxidative stress response in the fungal pathogen *Candida glabrata*. Curr Genet. 2013;59:91–106.
11. Hamer DH, Thiele DJ, Lemontt JE. Function and autoregulation of yeast copperthionein. Science. 1985;228:685–90.
12. Holmgren A, Johansson C, Berndt C, Lonn ME, Hudemann C, Lillig CH. Thiol redox control via thioredoxin and glutaredoxin systems. Biochem Soc Trans. 2005;33:1375–7.
13. Hwang CS, Rhie GE, Oh JH, Huh WK, Yim HS, Kang SO. 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. Microbiology. 2002;148:3705–13.
14. Imlay JA. Pathways of oxidative damage. Annu Rev Microbiol. 2003;57:395–418.
15. Kaur R, Ma B, Cormack BP. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. Proc Natl Acad Sci USA. 2007;104:7628–33.
16. Lee J, Godon C, Lagniel G, Spector D, Garin J, Labarre J, et al. Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. J Biol Chem. 1999;274:16040–6.
17. Lee YY, Jung HI, Park EH, Sa JH, Lim CJ. Regulation of *Schizosaccharomyces pombe* gene encoding copper/zinc superoxide dismutase. Mol Cells. 2002;14:43–9.
18. Martchenko M, Alarco AM, Harcus D, Whiteway M. Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene. Mol Biol Cell. 2004;15:456–67.
19. Mehra RK, Garey JR, Butt TR, Gray WR, Winge DR. *Candida glabrata* metallothioneins. Cloning and sequence of the genes and characterization of proteins. J Biol Chem. 1989;264:19747–53.
20. Mehra RK, Mulchandani P, Hunter TC. Role of CdS quantum crystallites in cadmium resistance in *Candida glabrata*. Biochem Biophys Res Commun. 1994;200:1193–200.
21. Meister A. Glutathione, metabolism and function via the gamma-glutamyl cycle. Life Sci. 1974;15:177–90.
22. Missall TA, Lodge JK, McEwen JE. Mechanisms of resistance to oxidative and nitrosative stress: implications for fungal survival in mammalian hosts. Eukaryot Cell. 2004;3:835–46.
23. Nakagawa Y, Mizuguchi I. The nucleotide sequence determination of catalases of three medically important yeasts using newly designed degenerated primers. Nippon Ishinkin Gakkai Zasshi. 2005;46:35–42.

24. Narasipura SD, Ault JG, Behr MJ, Chaturvedi V, Chaturvedi S. Characterization of Cu,Zn superoxide dismutase (SOD1) gene knock-out mutant of *Cryptococcus neoformans* var *gattii*: role in biology and virulence. Mol Microbiol. 2003;47:1681–94.
25. Navarro RE, Stringer MA, Hansberg W, Timberlake WE, Aguirre J. catA, a new *Aspergillus nidulans* gene encoding a developmentally regulated catalase. Curr Genet. 1996;29:352–9.
26. Okado-Matsumoto A, Fridovich I. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. J Biol Chem. 2001;276:38388–93.
27. Roetzer A, Gregori C, Jennings AM, Quintin J, Ferrandon D, Butler G, et al. *Candida glabrata* environmental stress response involves *Saccharomyces cerevisiae* MSN2/4 orthologous transcription factors. Mol Microbiol. 2008;69:603–20.
28. Roetzer A, Gratz N, Kovarik P, Schuller C. Autophagy supports *Candida glabrata* survival during phagocytosis. Cell Microbiol. 2010;12:199–216.
29. Roetzer A, Klopf E, Gratz N, Marcet-Houben M, Hiller E, Rupp S, et al. Regulation of *Candida glabrata* oxidative stress resistance is adapted to host environment. FEBS Lett. 2011;585:319–27.
30. Ruprich-Robert G, Chapeland-Leclerc F, Boisnard S, Florent M, Bories G, Papon N. Contributions of the response regulators Ssk1p and Skn7p in the pseudohyphal development, stress adaptation, and drug sensitivity of the opportunistic yeast *Candida lusitaniae*. Eukaryot Cell. 2008;7:1071–4.
31. Saijo T, Miyazaki T, Izumikawa K, Mihara T, Takazono T, Kosai K, et al. Skn7 is involved in oxidative stress response and virulence of *Candida glabrata*. Mycopathologia. 2010;169:81–90.
32. Seider K, Brunke S, Schild L, Jablonowski N, Wilson D, Majer O, et al. The facultative intracellular pathogen *Candida glabrata* subverts macrophage cytokine production and phagolysosome maturation. J Immunol. 2011;187:3072–86.
33. Seider K, Heyken A, Luttich A, Miramon P, Hube B. Interaction of pathogenic yeasts with phagocytes: survival, persistence and escape. Curr Opin Microbiol. 2010;13:392–400.
34. Singh P, Chauhan N, Ghosh A, Dixon F, Calderone R. SKN7 of *Candida albicans*: mutant construction and phenotype analysis. Infect Immun. 2004;72:2390–4.
35. Toledano MB, Kumar C, Le Moan N, Spector D, Tacnet F. The system biology of thiol redox system in *Escherichia coli* and yeast: differential functions in oxidative stress, iron metabolism and DNA synthesis. FEBS Lett. 2007;581:3598–607.
36. Venkataram S, Fay JC. Is transcription factor binding site turnover a sufficient explanation for cis-regulatory sequence divergence? Genome Biol Evol. 2010;2:851–8.
37. Weisiger RA, Fridovich I. Mitochondrial superoxide simutase. Site of synthesis and intramitochondrial localization. J Biol Chem. 1973;248:4793–6.
38. Wellington M, Dolan K, Krysan DJ. Live *Candida albicans* suppresses production of reactive oxygen species in phagocytes. Infect Immun. 2009;77:405–13.
39. Yadav AK, Desai PR, Rai MN, Kaur R, Ganesan K, Bachhawat AK. Glutathione biosynthesis in the yeast pathogens *Candida glabrata* and *Candida albicans*: essential in *C. glabrata*, and essential for virulence in *C. albicans*. Microbiology. 2011;157:484–95.
40. Youseff BH, Holbrook ED, Smolnycki KA, Rappleye CA. Extracellular superoxide dismutase protects *Histoplasma* yeast cells from host-derived oxidative stress. PLoS Pathog. 2012;8:e1002713.
41. Zahringer H, Thevelein JM, Nwaka S. Induction of neutral trehalase Nth1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: variations of PKA effect during stress and growth. Mol Microbiol. 2000;35:397–406.
42. Znaidi S, Barker KS, Weber S, Alarco AM, Liu TT, Boucher G, et al. Identification of the *Candida albicans* Cap1p regulon. Eukaryot Cell. 2009;8:806–20.

2.3 Capitulo 3

Gallegos-Garcia V, Pan SJ, Juarez-Cepeda J, Ramirez-Zavaleta CY, Martin-del-Campo MB, Martinez-Jimenez V, Castano I, Cormack B, De Las Penas A. **A novel downstream regulatory element cooperates with the silencing machinery to repress *EPA1* expression in *Candida glabrata*.** *Genetics.* 2012; 190(4):1285-97.

Este documento fue publicado en la modalidad de acceso abierto en http://www.genetics.org/content/190/4/1285.long.

A Novel Downstream Regulatory Element Cooperates with the Silencing Machinery to Repress *EPA1* Expression in *Candida glabrata*

Verónica Gallegos-García,*[,1,2] Shih-Jung Pan,[†,1] Jacqueline Juárez-Cepeda,* Candy Y. Ramírez-Zavaleta,* Marcela Briones Martin-del-Campo,* Verónica Martínez-Jiménez,* Irene Castaño,* Brendan Cormack,[†] and Alejandro De Las Peñas*[,3]

*IPICYT, División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, San Luis Potosí, 78216, México and [†]Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT *Candida glabrata*, an opportunistic fungal pathogen, adheres to mammalian epithelial cells; adherence is mediated primarily by the Epa1 adhesin. *EPA1* is a member of a large gene family of ~23 paralogues, which encode putative adhesins. In this study, we address how *EPA1* transcription is regulated. Our data show that *EPA1* expression is subject to two distinct negative regulatory mechanisms. *EPA1* transcription is repressed by subtelomeric silencing: the Sir complex (Sir2–Sir4), Rap1, Rif1, yKu70, and yKu80 are required for full repression. Activation of *EPA1* occurs immediately after dilution of stationary phase (SP) cells into fresh media; however, transcription is rapidly repressed again, limiting expression to lag phase, just as the cells exit stationary phase. This repression following lag phase requires a *cis*-acting regulatory negative element (NE) located in the *EPA1* 3′-intergenic region and is independent of telomere proximity. Bioinformatic analysis shows that there are 10 copies of the NE-like sequence in the *C. glabrata* genome associated with other *EPA* genes as well as non-*EPA* genes.

CANDIDA glabrata, an opportunistic fungal pathogen normally present in the mucosal flora, can cause severe disseminated infections. *C. glabrata* is the second most common agent of candidiasis, accounting for 15–20% of *Candida* bloodstream infections worldwide (Pfaller and Diekema 2010). Some traits of *C. glabrata* that allow it to cause disease have been described (Kaur *et al.* 2005; Roetzer *et al.* 2011). These include resistance to oxidative stress (Cuellar-Cruz *et al.* 2008, 2009) and adherence to epithelial cells (Cormack *et al.* 1999; Castaño *et al.* 2005).

Adherence to host cells has been proposed to be an important initial step for virulence. Additionally, the ability to adhere to abiotic substrates and the adherence between microbial cells are essential attributes for biofilm formation in many pathogens. Adherence in pathogenic fungi has been shown to be mediated primarily by glycosylphosphatidylinositol-anchored cell wall proteins (GPI-CWPs) which are found broadly in different fungal species including *Saccharomyces cerevisiae*, *C. glabrata*, and *C. albicans* (Castaño *et al.* 2006). In *S. cerevisiae*, the *FLO* family of genes (*FLO1*, *FLO5*, and *FLO9–FLO11*) encode a group of (GPI-CWPs) that are required for flocculation, pseudohyphal growth, and biofilm formation on abiotic substrates (Kobayashi *et al.* 1998; Guo *et al.* 2000). *C. albicans* encodes ~104 putative GPI-CWPs including the Als family, the Hwp family, Hyr1, and Eap1 (De Groot *et al.* 2003a,b; Li and Palecek 2003), many of which are thought to mediate adhesion to host epithelial and endothelial cells as well as to extracellular matrix proteins (Hoyer 2001; Li and Palecek 2003; Sheppard *et al.* 2004; Klotz *et al.* 2004; Hoyer *et al.* 2008). *C. glabrata* can adhere to epithelial cells and also to inert surfaces. *EPA1* encodes the major epithelial adhesin in the BG2 strain, binding to *N*-acetyl lactosamine-containing glycoconjugates (Cormack *et al.* 1999; Zupancic *et al.* 2008). *EPA1* belongs to a large gene family (*EPA* family) of ~23 paralogues, of which *EPA6* and *EPA7* have also been shown to mediate

doi: 10.1534/genetics.111.138099
Manuscript received September 28, 2011; accepted for publication December 25, 2011
[1]These authors contributed equally to this work.
[2]Present address: Facultad de Enfermería, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, 78240, México.
[3]Corresponding author: Camino a la Presa San José 2055, División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, San Luis Potosí, 78216, México. E-mail: cano@ipicyt.edu.mx

adherence to epithelial cells *in vitro* (De Las Peñas *et al.* 2003; Castaño *et al.* 2005; De Groot *et al.* 2008).

Proper regulation of the expression of the *EPA* adhesin genes is thought to be of importance for survival and proliferation in the host environment. One layer of *EPA* gene transcriptional regulation is related to the fact that most *EPA* genes are encoded in subtelomeric loci, where they are subject to chromatin-based silencing mediated by the Sir complex (Sir2, Sir3, and Sir4), yKu70, yKu80, Rif1, and Rap1 (De Las Peñas *et al.* 2003; Castaño *et al.* 2005). Deletion of genes encoding the silencing factors results in the cells becoming hyperadherent, due to overexpression of some *EPA* genes, including *EPA6* and *EPA7* (Castaño *et al.* 2005). Other subtelomeric *EPAs* are not expressed even in *sir* mutant backgrounds, indicating additional gene-specific regulation for individual *EPA* genes (De Las Peñas *et al.* 2003; Castaño *et al.* 2005; Domergue *et al.* 2005).

In this article, we focus on the detailed regulation of *EPA1* transcription. *EPA1* resides 20.7 kb upstream from the right telomere in chromosome E and forms a cluster with two other *EPA* genes (*EPA1–EPA2–EPA3* telomere, Figure 1A) (De Las Peñas *et al.* 2003). Our data show that *EPA1* expression is tightly controlled negatively and positively. *EPA1* transcription is repressed by the Sir complex (Sir2, Sir3, and Sir4) and by Rap1, Rif1, yKu70, and yKu80. Transcription of *EPA1* is induced immediately after dilution of stationary phase (SP) cells into fresh media and concomitantly, the cells become adherent. Interestingly, *EPA1* expression is limited to lag phase, and is tightly repressed in long-term log phase (LP) cultures as well as in SP. We show that a *cis*-acting regulatory negative element (NE) localized at the intergenic region between *EPA1* and *EPA2*, 300 bp downstream of *EPA1* stop codon (TAA), plays a major role in transcriptional repression of *EPA1*.

Materials and Methods

Strains

All strains used in the study are described in Table 1.

Plasmids

All plasmids used in this study are described in Table 2.

Primers

All primers used for cloning are summarized in Table 3.

Media and growth conditions

All cell cultures were grown for 48 hr at 30°. SP cells are cells grown for 48 hr. LP cells are dividing cells. Lag phase is considered when SP cells are diluted into fresh media and cells are preparing for cell division. Yeast media were prepared as described (Sherman *et al.* 1986), and 2% agar was added to plates. YPD media contains yeast extract 10 g/liter, peptone 20 g/liter, supplemented with 2% glucose. When needed, YPD plates were supplemented with 400 μg/ml of hygromycin (A. G. Scientific). Synthetic complete media (SC) contains YNB without amino acids and nitrogen source (1.7 g/liter), NH_2SO_4 (5 g/liter), supplemented with 0.6% of casamino acids and 2% glucose and, when needed, supplemented with 50 mg/liter uracil and 0.9 g/liter 5-fluoroorotic acid (5-FOA, Toronto Research Chemicals) for 5-FOA plates. Bacterial media were prepared as described (Ausubel *et al.* 2001), and 1.5% agar was used for plates. Luria-Bertani (LB) media contained yeast extract at 5 g/liter, bactopeptone at 10 g/liter, and NaCl at 10 g/liter. When needed, LB plates were supplemented with 100 μg/ml of carbenicillin (Cb100, Invitrogen). Phosphate buffer saline (PBS) was 8 g/liter NaCl, 0.2 g/liter KCl, 1.65 g/liter $Na_2HPO_4 \cdot 7H_2O$, and 0.2 g/liter KH_2PO_4.

Yeast transformation

Yeast transformation was performed using the lithium acetate protocol as described previously (Castaño *et al.* 2003).

Construction of deletion strains

To construct deletion strains in this study, we used the one-step gene replacement procedure. Briefly, we cloned two fragments (the promoter region and the 3′-UTR, flanking the gene to be deleted) at each side of the hygromycin resistance cassette in the integrative *URA3* plasmid, pAP599 (see Table 1). The plasmid was digested with restriction enzymes that cut within the two cloned fragments generating homologous ends. After inactivation of the enzymes, *C. glabrata* was transformed with the digestion mix and transformants were selected on YPD −hygromycin or SC −Ura plates. PCR analysis was done to confirm the structure of the deletion. The absence of each deleted gene was also verified by the inability to PCR amplify an internal fragment of the gene. Strains constructed in this way are described in Table 1.

S1 nuclease protection assay

BG14 cells were grown for 48 hr at 30°. RNA was extracted as previously described (De Las Peñas *et al.* 2003). The *ACT1* and the *EPA1* probes (Table 3) were end labeled using [γ-^{32}P]-ATP with T4 polynucleotide kinase. A total of 30 μg of RNA was hybridized with each end-labeled probe at 55° overnight. The mix was digested at room temperature with 150 units of S1 nuclease (Invitrogen) for 30 min. The samples were then extracted with phenol, precipitated, and resuspended in 17 μl of 1× loading buffer. A total of 5μl of each sample was separated by electrophoresis on a 10% acrylamide gel and the signal detected using a phosphorimager.

EPA1 promoter::URA3 plate assay

Cells were grown for 48 hr at 30° in YPD and the cultures were adjusted to 0.5 OD_{600nm}. Ten-fold serial dilutions were spotted on YPD, SC −Ura, and SC +5-FOA plates and incubated at 30° for 48 hr. Ura^+ cells die on SC +5-FOA plates. Only cells with the *URA3* gene transcriptionally repressed can grow on SC +5-FOA.

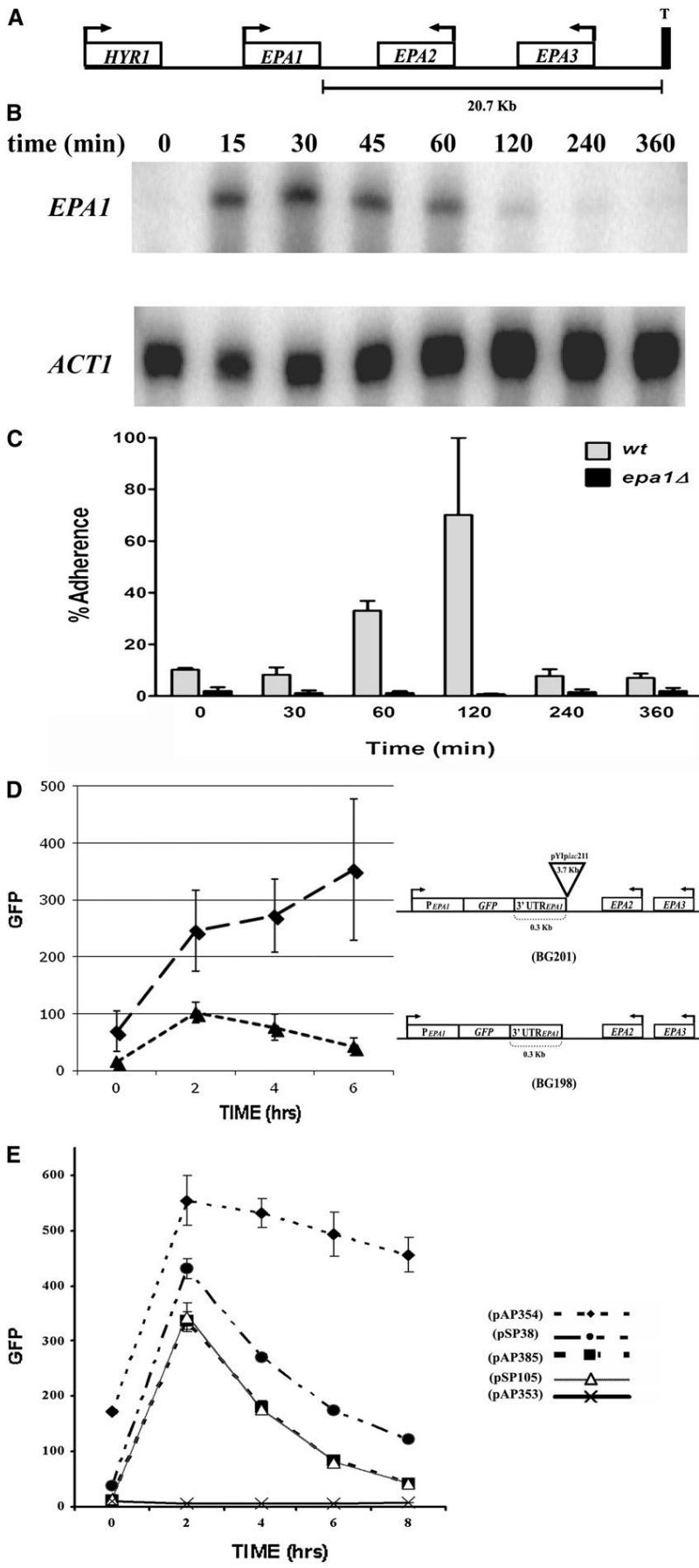


Figure 1 Regulation of the expression of *EPA1*. (A) Schematic representation of the *EPA1* genomic locus. (B) *EPA1* transcript levels measured by S1 nuclease protection. BG14 (WT) cells were grown for 48 hr and 30° in YPD media. Cells were diluted into fresh media and samples were taken at different time points (see *Materials and Methods*). Time 0 is undiluted stationary phase (SP) cells. (C) Adherence of *C. glabrata* cells to HeLa cells. *C. glabrata* wild-type strain BG14 (WT) and strain BG64 (*epa1*Δ) were grown for 48 hr at 30° in YPD media. Cells were diluted into fresh media and samples were taken at different time points. Cells were adjusted to OD_{600} of 1.0 in HBSS supplemented with 5 mM $CaCl_2$. Cell suspensions were diluted serially in sterile water and appropriate dilutions were made and plated on YPD plates to determine input colony forming units (CFU) (see *Materials and Methods*). Each experiment was made in triplicate. (D) *EPA1* promoter activity measured by FACS. Strains BG198 (P_{EPA1}::GFP) and BG201 (P_{EPA1}::GFP::pYI*plac*211) were grown for 48 hr at 30° in YPD media. Cells were diluted into fresh media and samples were taken every 2 hr. Yeast cells were washed and resuspended in 1 ml PBS and fluorescence was assessed by FACS analysis using a BD FACSCalibur flow cytometer (see *Materials and Methods*). *EPA1* promoter GFP fusion is at the chromosomal *EPA1* locus. GFP was used as reporter of the activity of the *EPA1* promoter. (E) *EPA1* promoter activity measured by FACS as in D, but cells were grown in SC −Ura media and all constructs are borne in plasmids. Strain BG14 (WT) carrying plasmids pAP353 (promoterless control, GFP::3′UTR$_{HIS3}$), pAP354 (P_{EPA1}::GFP::3′UTR$_{HIS3}$), pAP385 (P_{EPA1}::GFP::3′UTR$_{EPA1}$NE$_{(3.1Kb)}$), pSP38 (P_{EPA1}::GFP::3′UTR$_{HIS3}$::NE$_{(200bp)}$), and pSP105 (NE$_{200bp}$::P_{EPA1}::GFP::3′UTR$_{EPA1}$).

Table 1 Strains used in this study

Strain	Parent	Genotype	Reference
Escherichia coli strain			
DH10B		F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*Δ*M15* Δ*lacX74* *deoR recA1 endA1 araD139* Δ(*ara,leu*)7697*galU* *galK* λ$^-$ *rpsL nupG*	Calvin and Hanawalt (1988)
Candida glabrata strains			
BG2		Clinical isolate (strain B)	Fidel *et al.* (1996)
BG14	BG2	*ura3*Δ::Tn*903* G418^R	Cormack and Falkow (1999)
BG64	BG14	*epa1*Δ *ura3*Δ::Tn*903* G418^R	Cormack *et al.* (1999)
BG198	BG14	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*GFP*. *GFP* under the control of the *EPA1* promoter (Figure 1C)	De Las Peñas *et al.* (2003)
BG201	BG14	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*GFP* pYIp*lac*211 integrated 300bp from TAA of *EPA1*. *GFP* under the control of the *EPA1* promoter (Figure 1C)	This work
BG509	BG14	*ura3*Δ::Tn*903* G418^R *rif1*Δ::*hph* Hyg^R	Castaño *et al.* (2005)
BG592	BG14	*ura3*Δ::Tn*903* G418^R *rap1-21*	De Las Peñas *et al.* (2003)
BG646	BG14	*ura3*Δ::Tn*903* G418^R Tn*7* at intergenic region between *EPA1* and *EPA2*	De Las Peñas *et al.* (2003)
BG676	BG14	*ura3*Δ::Tn*903* G418^R *sir3*Δ::*hph* Hyg^R	De Las Peñas *et al.* (2003)
BG1048	BG14	*ura3*Δ::Tn*903* G418^R *sir2*Δ::*hph* Hyg^R	Domergue *et al.* (2005)
BG1050	BG14	*ura3*Δ::Tn*903* G418^R *sir4*Δ::*hph* Hyg^R	Rosas-Hernandez *et al.* (2008)
BG1080	BG14	*ura3*Δ::Tn*903* G418^R *hdf1*Δ::*hph* Hyg^R	Rosas-Hernandez *et al.* (2008)
BG1081	BG14	*ura3*Δ::Tn*903* G418^R *hdf2*Δ::*hph* Hyg^R	Rosas-Hernandez *et al.* (2008)
BG1124	BG1212	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3*. *EPA1* replaced by *URA3*. *URA3* under the control of the *EPA1* promoter	This work
BG1132	BG14	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat*. *EPA1* replaced by *URA3* and NE (negative element) replaced by the bacterial *cat* gene, chloramphenicol acetyl transferase from pACYC184 *URA3* under the control of the *EPA1* promoter	This work
*sir2*Δ			
CGM172	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *sir2*Δ::*hph* Hyg^R	This work
CGM174	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *sir2*Δ::*hph* Hyg^R	This work
*sir3*Δ			
CGM283	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *sir3*Δ::*hph* Hyg^R	This work
CGM285	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *sir3*Δ::*hph* Hyg^R	This work
*sir4*Δ			
CGM188	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *sir4*Δ::*hph* Hyg^R	This work
CGM190	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *sir4*Δ::*hph* Hyg^R	This work
*hdf1*Δ			
CGM198	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *hdf1*Δ::*hph* Hyg^R	This work
CGM184	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *hdf1*Δ::*hph* Hyg^R	This work
*hdf2*Δ			
CGM187	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *hdf2*Δ::*hph* Hyg^R	This work
CGM185	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *hdf2*Δ::*hph* Hyg^R	This work
*rif1*Δ			
CGM210	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *rif1*Δ::*hph* Hyg^R	This work
CGM212	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *rif1*Δ::*hph* Hyg^R	This work
*hst1*Δ			
CGM213	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *hst1*Δ::*hph* Hyg^R	This work
CGM214	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *hst1*Δ::*hph* Hyg^R	This work
*hst2*Δ			

(continued)

Table 1, *continued*

Strain	Parent	Genotype	Reference
CGM208	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *hst2*Δ::*hph* Hyg^R	This work
CGM217	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *hst2*Δ::*hph* Hyg^R	This work
rap1-21			
CGM349	BG592	*ura3*Δ::Tn*903* G418^R *rap1-21 epa1*Δ::*URA3*	This work
CGM374	BG592	*ura3*Δ::Tn*903* G418^R *rap1-21 epa1*Δ::*URA3* neΔ::*cat*	This work

In vitro adherence assays

Cervical carcinoma cells (HeLa) were cultured in 24-well plates in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 80 units ml^{-1} of penicillin G, 50 μg ml^{-1} of streptomycin sulfate, and 7% calf serum (HyClone Laboratories). The cells were maintained at 37° in 5% CO_2 until confluent growth. Each well was washed once with sterile water and PBS. Cells were fixed with 4% of paraformaldehyde. Plates were stored at 4° containing 1 ml of PBS supplemented with Pen/Strep (penicillin 100 units ml^{-1} and streptomycin 100 μg ml^{-1}). For adherence assay, *C. glabrata* cells were grown for 48 hr at 30° and, where indicated, diluted into fresh media. SP and LP cultures were adjusted to an OD_{600} of 1.0 with Hanks balanced salt solution (HBSS) supplemented with 5 mM $CaCl_2$. A total of 100 μl of cells was added to a 24-well plate containing fixed HeLa cells in 900 μl of PBS without antibiotics. The plates were incubated at room temperature for 1 hr. Nonadherent yeast cells were washed three times with HBSS with $CaCl_2$ and once with PBS. Adherent cells were recovered from the epithelial cells by scraping off the plate in 500 μl of PBS containing 0.1% Triton and 0.5% SDS. The cells were plated and viable accounts recorded.

FACS analysis of GFP expression

Strains were grown for 48 hr at 30° in SC −Ura media. Cells were diluted into fresh media to induce *EPA1* expression. *GFP* was used as reporter gene to measure the activity of the *EPA1* promoter. Yeast cells were washed with PBS and resuspended in 1 ml PBS and fluorescence was assessed by FACS analysis using a BD FACSCalibur flow cytometer with Cell Quest Pro software.

Results

EPA1 is induced in lag phase

C. glabrata strain BG14 SP cells do not adhere to cultured epithelial cells. However, upon dilution and growth in fresh media, BG14 cells become adherent. This *in vitro* adherence is mediated by the Epa1 adhesin. *epa1*Δ mutant cells are virtually nonadherent in any growth condition (Cormack *et al.* 1999). To begin to dissect the transcriptional regulation of *EPA1*, we measured transcript levels of *EPA1* in SP cells and in cells following dilution into fresh media. Little or no *EPA1* transcript is observed in *C. glabrata* SP cells, but dilution into fresh media results in significant *EPA1* transcription, which reaches maximal levels at 30 min following dilution (Figure 1B). However, 120 min after dilution, the *EPA1* transcript level has fallen significantly, and at 240 min postdilution, it returns to background level (Figure 1B). The cells become adherent at 60 min with maximal adherence at 120 min postdilution, but as cells grow in prolonged LP, they become nonadherent, since new *EPA1* transcription is repressed and preexisting Epa1 protein is diluted upon cell division (Figure 1C). These data indicate that *EPA1* expression is tightly regulated and is expressed neither in SP nor in long-term LP cultures, but rather is limited to lag phase, as cells exit SP.

EPA1 expression is controlled by positive and negative regulation

To analyze the regulation of *EPA1*, we replaced the *EPA1* ORF in its chromosomal location with the reporter gene GFP (BG198, P_{EPA1}::GFP). GFP fluorescence is measured in cells in SP and upon dilution into fresh media by fluorescence activated cell sorting (FACS). The expression profile of P_{EPA1}::GFP parallels that of *EPA1* transcript shown in Figure 1B. In SP cells, the P_{EPA1}::GFP reporter shows background levels of fluorescence; expression is highly induced immediately after dilution into fresh media (Figure 1D). We noticed that correct regulation of *EPA1* was lost in a mutant strain carrying an insertion of 3.797 kb (pYip*lac*211 plasmid, strain BG201) 300 bp downstream of the P_{EPA1}::GFP reporter stop codon: higher basal levels of expression and dilution of SP cells into fresh medium resulted in strong GFP induction; however, GFP reporter activity remained induced as cells divided, remaining elevated through LP and even in SP (Figure 1D). These data strongly suggest that normally, *EPA1* expression is repressed in LP cells and that in the insertion mutant strain, such repression is relieved (Figure 1D). The lack of repression of the reporter gene in the insertional mutant (BG201) could in principle be caused by disruption of a *trans*-acting repressor gene encoded downstream of *EPA1*. However, sequence analysis of the 3.1-kb intergenic region shows no evidence of any ORF sequences within the 3′-intergenic region; moreover, complementation experiments in which the entire 3.1-kb intergenic region was cloned on a plasmid and introduced into BG201 did not suppress the high GFP-expression phenotype (data not shown).

Next, we tested whether the transcriptional regulation of the *EPA1* locus can be reconstituted on a plasmid. We cloned in a *URA3* CEN-ARS plasmid (pGRB2.0) the P_{EPA1}::GFP

Table 2 Plasmids used in this study

Plasmid	Relevant genotype	Reference
pCYC184	Cloning vector Cm^R Tc^R	Chang and Cohen (1978)
pYIp*lac*211	Cloning vector, integrative vector *URA3* Ap^R	Gietz and Sugino (1988)
pMB11	Cloning vector, *sacB* counterselection Cm^R *ori* p15A	Lab collection
pGRB2.0	Cloning vector, pRS406 *URA3 C.g.CEN ARS* Ap^R	De Las Peñas *et al.* (2003)
pRS306	Cloning vector, integrative vector *URA3* Ap^R	Sikorski and Hieter (1989)
pAP599	Cloning vector for construction of knockout mutants with two *FRT* direct repeats flanking the hygromycin cassette to remove the selection marker for construction of multiple mutants in *C. glabrata*. [*FRT*-P_{PGK}::*hph*::3′UTR_{HIS3}-*FRT*] *URA3* Hyg^R Ap^R	Domergue *et al.* (2005)
pAP640	Cloning vector, pRS306 cut with *Nde*I/*Nae*I blunt ended with Klenow and religated. *ura3*Δ Ap^R	This work
pAP353	A 0.738-kb *Eco*RI/*Sal*I PCR fragment carrying *GFP* (no *Bst*XI site) and a 0.397-kb *Xho*I/*Kpn*I PCR fragment carrying *HIS3* 3′*UTR* were cloned into pGRB2.0. For *GFP* promoter fusions. *URA3* Ap^R. (Figure 1D)	This work
pAP354	A 2.5-kb *Bst*XI PCR fragment (primers 666/667) carrying the promoter region of *EPA1* was cloned into pAP353. *URA3* Ap^R P_{EPA1}::*GFP*::3′UTR_{HIS3} (Figure 1D)	This work
pAP385	A 3.1-kb *Xho*I PCR fragment (primers 723/724) carrying the 3′UTR_{EPA1} fragment cloned at *Xho*I site of pAP354. *URA3* Ap^R*EPA1*$_{Promoter}$::*GFP*::3′$UTR_{EPA1\text{-}3.1Kb}$ (Figure 1D)	This work
pAP407	Similar to pAP354 but 3′-UTR from *EPA1*.*URA3* Ap^R P_{EPA1}::*GFP*::3′$UTR_{EPA1\text{-}0.300Kb}$	Domergue *et al.* (2005)
pSP7	A 0.100-kb *Xho*I PCR fragment (primers 780/795) carrying the 3′ intergenic region of *EPA1*cloned into pAP407 digested with *Xho*I. *URA3* Ap^R (+400 bp; Figure 2, line 4)	This work
pSP19	A 0.50-kb *Xho*I PCR fragment (primers 780/841) carrying the 3′ *EPA1* (NE mapped) was cloned into pAP407 digested with *Xho*I. *URA3* Ap^R (+350 bp; Figure 2, line 5)	This work
pSP20	A 0.150-kb *Xho*I PCR fragment (primers 780/842) carrying the 3′ intergenic region of *EPA1*cloned into pAP407 digested with *Xho*I. *URA3* Ap^R (+450 bp; Figure 2, line 3)	This work
pSP21	A 0.200-kb *Xho*I PCR fragment (primers 780/843) carrying the 3′ intergenic region of *EPA1*cloned into pAP407 digested with *Xho*I. *URA3* Ap^R (+500 bp; Figure 2, line 1)	This work
pSP26	A 0.200-kb *Xho*I/*Sal*I PCR fragment (primers 776/839) carrying the 3′ UTR of *EPA1*cloned into pAP407 digested with *Xho*I/*Sal*I. *URA3* Ap^R (+200 bp; Figure 2, line 2)	This work
pSP31	A 0.250-kb *Xho*I PCR fragment (primers 843/913) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−50 bp; Figure 2, line 7)	This work
pSP32	A 0.200-kb *Xho*I PCR fragment (primers 780/843) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−100 bp; Figure 2, line 8)	This work
pSP33	A 0.150-kb *Xho*I PCR fragment (primers 914/843) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−150 bp; Figure 2, line 9)	This work
pSP34	A 0.100-kb *Xho*I PCR fragment (primers 915/843) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−200 bp; Figure 2, line 10)	This work
pSP35	A 0.50-kb *Xho*I PCR fragment (primers 916/843) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−250 bp; Figure 2, line 11)	This work
pSP38	Similar to pAP354 with the NE cloned immediately after the *HIS3* 3′-UTR. *URA3* Ap^R P_{EPA1}::*GFP*::3′UTR_{HIS3}::NE^+.	This work
pSP105	A 0.300-kb fragment (primers 1135/1136) carrying the NE cloned 5′ the *EPA1* promoter in pSP26. *URA3* Ap^R (Figure 1D)	This work

Table 3 Oligonucleotides used in this study

Primer	Sequence	Sites
666	TCGTTAAGCCATTGTGTTGGATCACTTTCAACACCAAATG	*Bst*XI
667	AAAATCATCCATTGTGTTGGGTTAATTGCAAAGACTAAAT	*Bst*XI
723	TGGTACTTCTCGAGTCCCACCAGTTGG	*Xho*I
724	CATAATAGTGATGAACATAGGGACCTAAAACCAGAAAAT	*Xho*I
776	CATAGGGGTCGACAACCAGAAAATATAATAAC	*Sal*I
777	AAAGTTCTCGAGTCTGGGAGAATAGAAAAGGCA	*Xho*I
795	ATTGACCTCTCGAGGAAGTTTAATTCGAGATT	*Xho*I
796	ACCTGAAACTCGAGAATAGTCCGTTACCTACC	*Xho*I
780	CTCCCAGACTCGAGAACTTTTGAGCAGGGACCA	*Xho*I
839	TAATATCTCTCGAGTCAAGTGTGACCAGGAAT	*Xho*I
841	GTGATTTGCTCGAGCTTTCTCTTGCTTTTGAA	*Xho*I
842	ATGATCATCTCGAGTTAGAATAATAAGTTGTT	*Xho*I
843	CCTTGCACCTCGAGCGTATAAACTCTCATATT	*Xho*I
913	TTCAGGGTCTCGAGTTTACATACGAAGCCTAA	*Xho*I
914	AAAGTTAGCTCGAGTCACGAAAATCCAGAAGA	*Xho*I
915	TAAACTTCCTCGAGAGGTCAATTGTCAAAAAA	*Xho*I
916	TATTCTAACTCGAGATGATCATATGAACATAC	*Xho*I
1135	CTCCCAGAGGATCCAACTTTTGAGCAGGGACCA	*Bam*HI
1136	CCTTGCACGGATCCCGTATAAACTCTCATATT	*Bam*HI
ACT1	CTCAAAATAGCGTGTGGCAAAGAGAAACCGGCGTAAATTGGA ACAACGTGGGTAACACCGTCACCAGAGTCCTTTTG	Probe for S1
EPA1	GCCAGTTCTAGGGTAATTGGGATCTAAATATGCTGCATCCCAA CATGGGTACGAACCCTTCTTCCGAAAATCTATCC	Probe for S1

fusion followed by the 3.1-kb *EPA1–EPA2* intergenic region (P_{EPA1}::GFP::3.1-kb intergenic region, pAP385). When this plasmid was transformed into *C. glabrata*, the GFP expression profile mirrored that observed from the chromosomal *EPA1* locus (BG198): activation of the *EPA1* promoter upon dilution into fresh media, followed by immediate repression (compare Figure 1, D and E, BG198 *vs.* pAP385). This suggests that the *EPA1* regulatory regions in pAP385 contain the *cis*-acting elements required for the positive as well as negative regulation in response to the growth state of cells, and that both regulatory mechanisms can function independently of the chromosomal location. When the 3.1-kb intergenic region was replaced with the *HIS3* 3′-UTR (P_{EPA1}::GFP::3′UTR_{HIS3}, pAP354), the resulting GFP expression profile mimics that derived from the insertional mutation in the chromosome (BG201): higher basal levels of expression, normal induction of *EPA1* transcription upon dilution, and loss of LP transcriptional repression (compare Figure 1, D and E, BG201 *vs.* pAP354). These results further support the important role for the 3.1-kb intergenic region in the transcriptional repression of *EPA1* locus, and suggest the presence in this 3′ region of a *cis*-acting element (NE) responsible for the repression of the expression of *EPA1*.

Mapping of the NE in the intergenic region between EPA1 and EPA2

To map the NE present in the intergenic region between *EPA1* and *EPA2*, we constructed a series of deletions in the 3.1-kb intergenic region carried on pAP385 (P_{EPA1}::GFP::3.1-kb intergenic region), transformed the resulting plasmids into strain BG14 and screened for loss of repression by monitoring GFP expression by FACS. As described above, when GFP is controlled by *EPA1* regulatory regions on the chromosome or in a plasmid, the GFP activity reaches the maximum at 2 hr after diluting SP *C. glabrata* cells into fresh media, then dropping to background levels by 6 hr postdilution. In Figure 2, the extent of *EPA1* transcriptional repression is indicated by the ratio (repression index, RI) of GFP reporter activity at 2 hr *vs.* 8 hr postdilution. First, we mapped the 3′-UTR of *EPA1* from +1 to +200 bp. This construct (pSP26, P_{EPA1}::GFP::+200 bp) is induced but has a RI of only 1.66 (compare with parental plasmid carrying the 3.1-kb intergenic region, RI of 8.01) (Figure 2, line 12). A construct with only 500 bp of the 3′-UTR, however, is fully induced and repressed, with a RI of 8.06 (Figure 2, line 1) at the same level as the parental plasmid carrying the 3.1-kb intergenic region (pAP385; Figure 2, line 12) or the P_{EPA1}::GFP in the chromosome (BG198) (Figure 1D). These data suggest that the NE may reside in the region between 200 to 500 bp downstream of *EPA1* ORF. Within this defined region, we further generated constructs containing deletions in 50-bp increments from either the 5′ or 3′ (Figure 2, line 3–11). These deletion series showed that the NE is contained in this +500-bp region. The first 50-bp deletion (from +450 to +500, relative to the stop codon) completely eliminate repression (RI of 2.79; Figure 2, line 3). Further 50-bp deletions from +300 to +450 had no additional impact (Figure 2, lines 3–6). The 5′ to 3′ deletion series revealed that the NE begins at +300 bp since deletion of +200 to +300 had no impact on the RI (Figure 2, lines 7 and 8), but deletion of +300 to +350 completely eliminated repression (RI of 1.96; Figure 2, line 9). These experiments indicate that the minimal region (NE), which confers the LP-specific repression of *EPA1*, is 200 bp long and is localized between +300 and +500 bp downstream of *EPA1* ORF, indicating a complex *cis*-acting element.

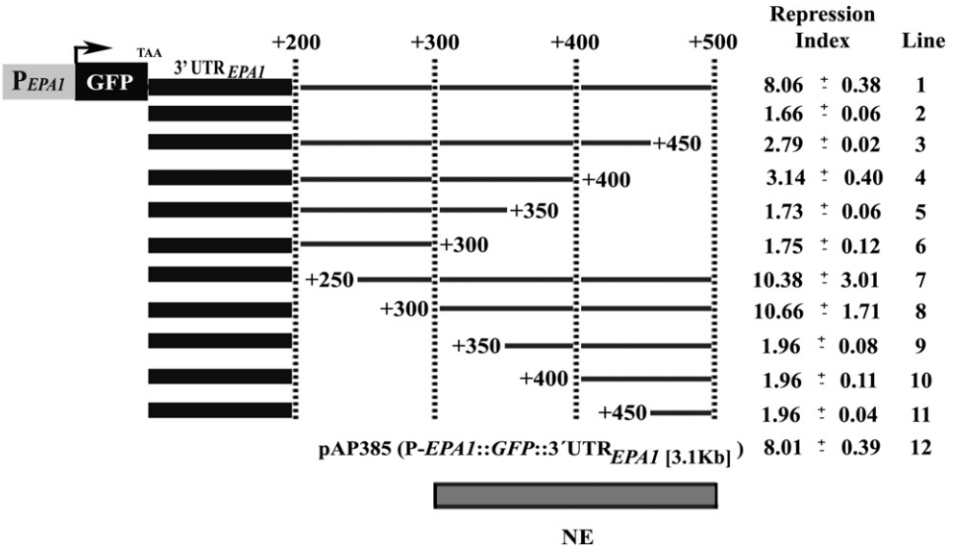


Figure 2 Mapping of the negative element (NE). The activity of the NE was assayed by measuring EPA1 promoter activity by FACS analysis of the GFP reporter fused to the EPA1 promoter. BG14 (WT) strain carrying a collection of plasmids containing serially deleted fragments (59 to 39 and 39 to 59) of the intergenic region between EPA1 and EPA2, were grown in SC 2 Ura media and assayed as described in Figure 1D legend. Fold of repression is the maximal expression of EPA1 at 2 hr divided by the expression at 8 hr. Line 1 is pSP21, line 2 is pSP26, line 3 is pSP20, line 4 is pSP7, line 5 is pSP19, line 6 is pAP407, line 7 is pSP31, line 8 is pSP32, line 9 is pSP33, line 10 is pSP34, line 11 is pSP35, and line 12 is pAP385 (Table 2). Experiments were done in triplicate and SDs are shown.

Characterization of the NE

We asked whether function of the NE depends on native polyadenylation sequences. To do this, we replaced the 200 bp EPA1 39-UTR sequence immediately after the stop codon of GFP with the 200 bp from the 39-UTR of C. glabrata HIS3. Following the HIS3 39-UTR, we placed the 300-bp NE region (+200 to +500, pSP38). In this construct, pSP38, EPA1 promoter is induced upon dilution into fresh media, followed by immediate repression; however, repression of this construct does not follow that of the EPA1 promoter in pSP385 (P_{EPA1}::GFP::3.1-kb intergenic region) or pSP105 (see below). This result indicates that the EPA1 polyadenylation sequences contribute partially to the overall negative regulation of EPA1 (Figure 1E). The next question we asked was whether the LP-specific repression mediated via the NE depends on its location relative to the ORF. To test this, we placed the 300-bp negative element region (from +200 to +500 bp downstream of EPA1 ORF) immediately in front of the EPA1 promoter fused to GFP (NE::P_{EPA1}::GFP::39UTR$_{EPA1}$, pSP105). The GFP expression profile is nearly identical to the parental construct (Figure 1E, compare pSP105 vs. pAP385) in which the EPA1 regulatory elements are in the original order. This shows that the regulation exerted by the NE can be location independent. These experiments confirm that the NE itself is a cis-acting regulatory element mediating the LP-specific repression. While the proximity of the NE to the 39-UTR raised the possibility that the NE functions to regulate transcript stability, the fact that the NE can function upstream of the ORF suggests rather that it acts at the level of transcription initiation.

The effect of the NE on transcription of the EPA1 locus has a substantial impact on adherence, as would be expected. We compared adherence of a wild-type strain BG14 with strain BG646. Both strains contain the functional EPA1 ORF at the normal chromosomal locus, except that the latter carries a Tn7 insertion localized at 300 bp downstream of EPA1 ORF which separates the EPA1 ORF from the NE by 3.4 kb. As expected, the parental strain BG14 in SP does not express EPA1 and showed little adherence to HeLa cells (Figure 3) but SP cells of the mutant strain BG646 were hyperadherent (Figure 3). Consistent with this experiment, RT–PCR analysis of strain BG646 showed an increased transcript level of EPA1 (data not shown). This experiment indicates that the regulation mediated through the NE influences the adhesion phenotype of C. glabrata cells.

EPA1 is controlled by the Sir complex and by yKu70, yKu80, Rap1, and Rif1

We have previously shown that the EPA1-3 loci (Figure 1A) is under the control of subtelomeric silencing. In those

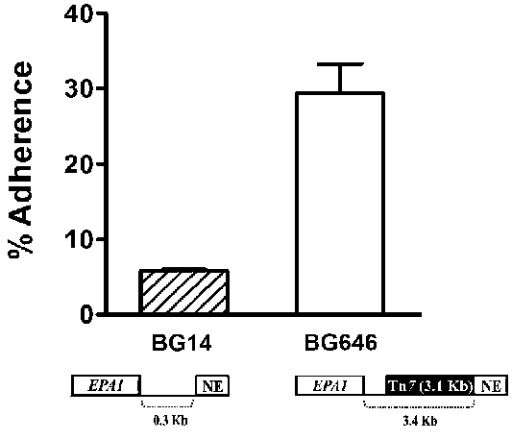


Figure 3 NE effect on adherence. C. glabrata wild-type strain BG14 (WT) (hatched bar) and strain BG646 containing a Tn7 insertion between EPA1 and the NE (open bar) were grown for 48 hr at 30° in YPD media. Cells were diluted to OD_{600} of 1.0 in HBSS supplemented with 5 mM $CaCl_2$. The adherence assays were done as described in the Figure 1C legend. Adherent cells were recovered from the epithelial cells and were plated for viable accounts. See Materials and Methods.

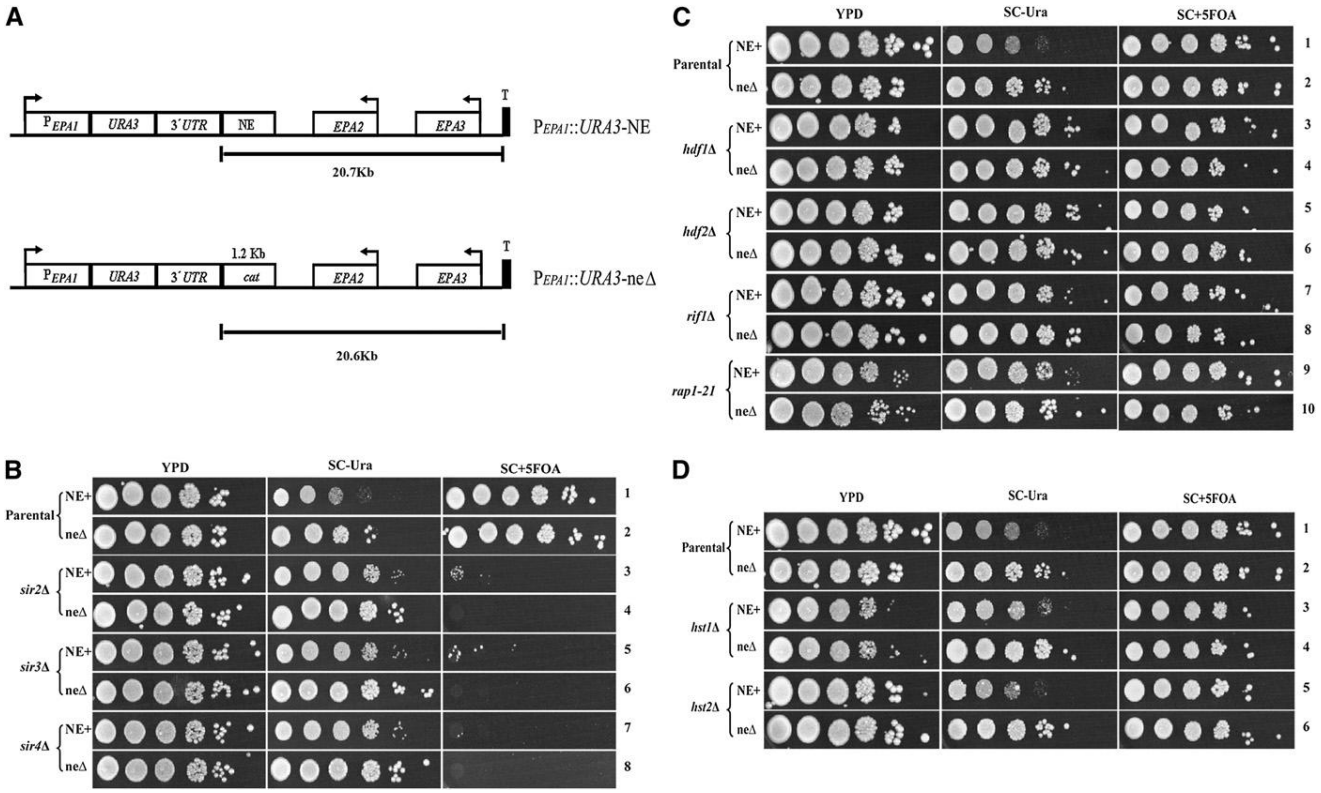


Figure 4 Silencing effect on the expression of *EPA1*. Schematic representation of the reporter strains. (A) *EPA1* was replaced by the *URA3* gene and the NE was replaced by the bacterial *cat* gene and recombined in the chromosome. *URA3* reports the activity of the *EPA1* promoter. (B) The parental strains NE$^+$ and neΔ and the strains carrying null mutations in *SIR2–SIR4* (*sir2*Δ–*sir4*Δ), (C) *HDF1* (yKu70), *HDF2* (yKu80), *RIF1*, and *rap1-21* (*hdf1*Δ, *hdf2*Δ, and *rap1-21*), and (D) *HST1* and *HST2* (*hst1*Δ and *hst2*Δ) were grown for 48 hr in YPD. Strains were diluted to OD_{600nm} 0.5 with distilled water and 10-fold serial dilutions were spotted onto YPD, SC −Ura and SC +5-FOA plates. Plates were incubated at 30°. Ura$^+$ cells die on SC +5-FOA plates. Only cells with the *URA3* gene transcriptionally repressed can grow on SC +5-FOA. See *Materials and Methods*.

experiments, a *URA3* reporter gene inserted at 20.7 kb from the telomere (300 bp from the TAA of *EPA1*), was largely not silenced, suggesting that the telomere position effect (TPE) ends before the *EPA1* locus (De Las Peñas *et al.* 2003; Rosas-Hernandez *et al.* 2008). However, in contrast to those data, mutations in *SIR2–SIR4* in fact increase the expression of *EPA1* (Castaño *et al.* 2005; Domergue *et al.* 2005). We therefore revisited whether the *EPA1* locus itself is controlled by silencing, by assessing the role of the Sir complex, yKu70, yKu80, Rap1, and Rif1 as well as the sirtuins Hst1 and Hst2. To monitor silencing of the *EPA1* locus we constructed a sensitive silencing reporter strain in which the *EPA1* ORF in its chromosomal location was replaced with the *URA3* ORF. Separately, we replaced a 1.0-kb fragment (carrying the NE) from the *EPA1/EPA2* intergenic region with a 1.2-kb fragment containing the *cat* gene (chloramphenicol acetyl transferase gene) from the bacterial plasmid pACYC184 to maintain the same distance from the telomeric repeats (Figure 4A). This pair of constructs permits assessment of silencing of the *EPA1* locus as well as the impact of the NE on that silencing.

The two strains (P_{EPA1}::*URA3*::NE$^+$, BG1124, and P_{EPA1}::*URA3*::neΔ, BG1132) were grown in YPD media and spotted onto YPD (for viable counts), SC −Ura (to assess activity of the *EPA1* promoter) and 5-FOA plates (to assess silencing of the *EPA1*::*URA3* locus, since the synthesis of Ura3 is toxic to the cell in the presence of 5-FOA). The NE$^+$ strain (P_{EPA1}::*URA3*::NE$^+$) grew poorly on SC −Ura medium (Figure 4B, line 1), whereas the neΔ strain (P_{EPA1}::*URA3*::neΔ) grew better but not to the same extent as the viable count on YPD (Figure 4B, line 2). This is consistent with the repressor role of the NE on the expression of *EPA1*. Both parental strains (P_{EPA1}::*URA3*::NE$^+$ and P_{EPA1}::*URA3*::neΔ) grew on 5-FOA plates at almost the same extent as the viable count on YPD, suggesting that *EPA1* is subject to silencing independent of the NE (Figure 4B, lines 1 and 2). Consistent with this, even the neΔ strain (P_{EPA1}::*URA3*::neΔ) did not grow as well on the SC −Ura plates as on the YPD or 5-FOA plates (Figure 4B, line 2), suggesting NE-independent repression of *EPA1*. These results suggest two levels of negative regulation of *EPA1*. One depends on the NE; in addition, a NE-independent repression is indicated by the growth of both strains (NE$^+$ and neΔ) on 5-FOA plates and by the reduced growth of the neΔ strain (P_{EPA1}::*URA3*::neΔ) on SC −Ura (Figure 4B, line 2).

To assess the impact of known silencing genes on repression of the *EPA1* locus, we deleted the *SIR2–SIR4*, *HDF1*

Table 4 Role of silencing proteins on NE function independently of telomere

Strain	Genotype	Plasmid	Repression index
BG14	WT	pSP21	16.26 ± 2.00
BG509	*rif1*Δ	pSP21	15.06 ± 1.83
BG592	*rap1-21*	pSP21	13.04 ± 1.87
BG676	*sir3*Δ	pSP21	16.71 ± 2.10
BG1048	*sir2*Δ	pSP21	14.62 ± 1.78
BG1050	*sir4*Δ	pSP21	15.78 ± 0.78
BG1080	*hdf1*Δ	pSP21	8.74 ± 1.54
BG1081	*hdf2*Δ	pSP21	8.66 ± 2.10
BG14	WT	pSP26	2.54 ± 0.40

(yKu70), *HDF2* (yKu80), *RIF1*, *HST1*, and *HST2* genes in the strains carrying the P_{EPA1}::*URA3*::NE and P_{EPA1}::*URA3*::neΔ constructs on the chromosome. In addition, in both strains, we replaced the essential *RAP1* gene with the silencing defective *rap1-21* allele. Strains were grown and spotted onto YPD, SC −Ura and 5-FOA plates. *sir2*Δ–*sir4*Δ mutant strains did not grow on 5-FOA plates, indicating that silencing is completely relieved independently of the presence of the NE (Figure 4B, lines 3–8). Consistent with this, growth of the *sir2*Δ–*sir4*Δ mutant strains on SC −Ura plates was better than the corresponding parental strains. Notably, growth of *sir2*Δ, *sir3*Δ, or *sir4*Δ NE^+ strains on SC −Ura plates was consistently worse than the corresponding *sir2*Δ, *sir3*Δ, or *sir4*Δ neΔ strains (Figure 4B, lines 3, 5, and 7 and lines 4, 6, and 8 on SC −Ura plates). This suggests that the function of the NE is independent of the Sir proteins.

We analyzed the effect of yKu70, yKu80, Rif1, and Rap1 on the expression of *EPA1*. Loss of *HDF1* (yKu70), *HDF2* (yKu80), *RIF1*, and *RAP1* in the presence (NE^+) or absence (neΔ) of the NE did not substantially affect silencing of *EPA1* since the vast majority of *hdf1*Δ, *hdf2*Δ, *rif1*Δ, or *rap1-21* mutant strains grow on 5-FOA plates (Figure 4C, compare lines 1 and 2 with lines 3–10). The *hdf1*Δ, *hdf2*Δ, *rif1*Δ, and *rap1-21* NE^+ strains grew better than the parental NE^+ strain on SC −Ura plates (Figure 4C, compare line 1 with lines 3, 5, 7, and 9), suggesting a derepression of *EPA1* transcription in these backgrounds, even though the effect on silencing was minimal. Notably, for the *hdf1*Δ and *hdf2*Δ mutants, growth of the NE^+ and neΔ strains on SC −Ura plates were the same, suggesting that *HDF1* (yKu70) and *HDF2* (yKu80) are required for function of the NE (Figure 4C, lines 3–6). The sirtuins Hst1 and Hst2 do not participate in the regulation of *EPA1* since *hst1*Δ and *hst2*Δ strains in the presence (NE^+) or absence (neΔ) of the NE behave the same as their corresponding parental strains (Figure 4D).

To assess the role of Sir2–Sir4, yKu70, yKu80, Rap1, and Rif1, on NE function independently of the telomere, we assayed the repression index of P_{EPA1}::GFP::+500 bp (pSP21; Figure 2, line 1) in each mutant background. Consistent with no effect of Sir2–Sir4, Rif1, and Rap1 on NE function, the RI in these backgrounds was no different than wild-type strain BG14. By contrast, for yKu70 and yKu80, the RI was decreased to 8.74 and 8.66, respectively, consistent with a role for yKu70 and yKu80 in NE-mediated repression (Table 4).

The NE is present 10 times in the C. glabrata genome

Finally, we asked whether additional copies of the NE were present in the *C. glabrata* genome and associated to other *EPA*s. We carried out a Blast search with the 200-bp sequence of the NE and found that the last 60 bp are present 10 times in the *C. glabrata* genome. This sequence is associated both with *EPA* genes as well as other genes (Figure 5).

Discussion

Adherence to specific cell tissues is important for pathogens. Their capacity to adhere to cells, tissues, abiotic surfaces, as well as their ability to form biofilms is often tied to expression of families of cell surface proteins whose transcription is tightly controlled. In *C. glabrata*, the *EPA* genes encode GPI-anchored cell wall proteins of which *EPA1*, *EPA6*, and *EPA7* have been shown to mediate adherence to epithelial cells *in vitro* (Cormack *et al.* 1999; De Las Peñas *et al.* 2003; Castaño *et al.* 2005; Domergue *et al.* 2005). Interestingly, many of these *EPA* genes are subject to chromatin-based subtelomeric silencing. This epigenetic regulation of adhesins is advantageous, since pathogens need not commit all cells in the population to express a particular adhesin, allowing a balance between adherence, colonization, and dissemination (De Las Peñas *et al.* 2003; Halme *et al.* 2004; Domergue *et al.* 2005).

A 3′ cis-acting element (NE) negatively regulates the expression of EPA1

In this study, we showed that *EPA1* expression is negatively regulated by two independent mechanisms: subtelomeric silencing and a telomere-independent, novel mode of negative regulation, dependent on a *cis*-acting NE contained in a 200-bp fragment required for full activity, located 300 bp downstream from the stop codon of *EPA1* in the intergenic region between *EPA1* and *EPA2* (Figure 2). The NE can still repress transcription independent of the 3′-UTR used and if placed upstream of the *EPA1* promoter (Figure 1E), underscoring that the NE is a transcriptional, rather than a post-transcriptional regulatory element. Furthermore, the *EPA1* 3′-UTR has a partial contribution on the expression of *EPA1*.

To our knowledge, *cis*-acting elements located outside promoters, or 3′ of the ORF affecting the expression of promoters have not previously been reported in yeast for Pol II promoters, though similar *cis*-acting elements have been described in yeast for Pol III transcribed genes and in other organisms, some localized inside introns (Errede *et al.* 1987; Martin *et al.* 2001; Stark *et al.* 2001; Calderwood *et al.* 2003; Delaloy *et al.* 2008). In yeast, activators or repressors generally function when their corresponding *cis*-acting elements are located at distances no greater than 700 bp (Guarente and Hoar 1984; Struhl 1984; Keegan *et al.* 1986) upstream of the start site of transcription. Notably, *cis*-acting elements

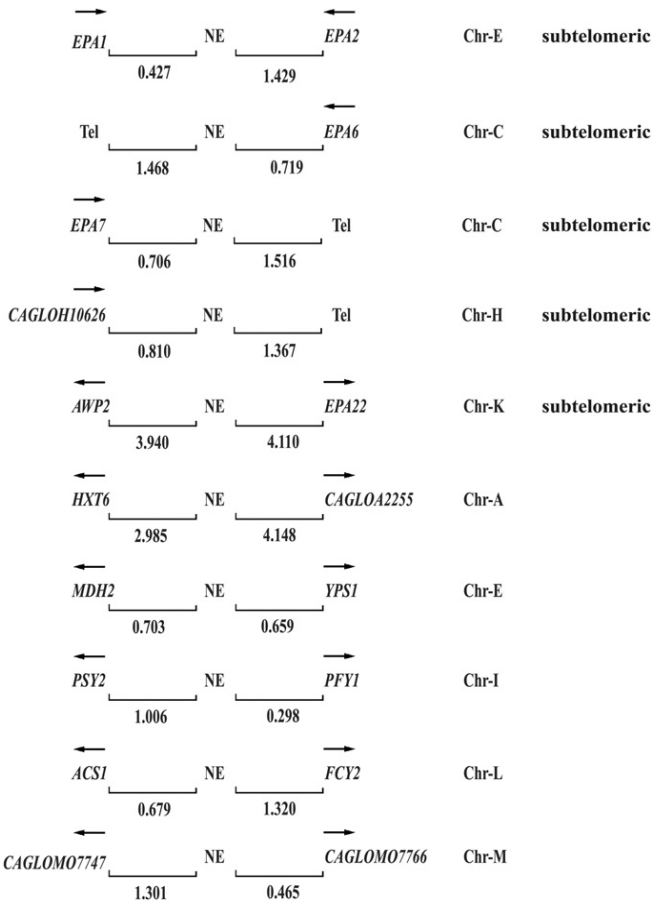


Figure 5 Blast analysis of the NE in the *C. glabrata* genome. The last 60 bp of the NE is associated with other *EPA*s and non-*EPA* genes. *EPA1*, *EPA2*, *EPA6*, *EPA7*, *AWP2*, and *CAGLOH10626* (15) encode cell wall proteins, all are subtelomeric and the NE associated is localized at their 3′ ends, except for *AWP2* and *EPA22*, which is localized between these two divergently transcribed genes. The NE associated to non-*EPA* genes is located at the 5′ regions (promoters) of these divergently expressed genes. *HXT6* (high-affinity glucose transporter of the major facilitator superfamily), *MDH2* (cytoplasmic malate dehydrogenase), *YPS1* (GPI-anchored aspartyl protease), *PSY2* (putative subunit of an evolutionarily conserved protein phosphatase complex containing the catalytic subunit Pph3p and the regulatory subunit Psy4p), *PFY1* (profilin, binds actin involved in cytoskeleton organization), *ACS1* (acetyl-coA synthetase isoform), *FCY2* (purine-cytosine permease), and CAGLOA2255, CAGL0M07747, and CAGL0M07766 are of unknown function (*Saccharomyces* Genome Database http://www.yeastgenome.org/ and *C. glabrata* Genome Database http://www.genolevures.org/cagl.html). Arrows indicate direction of transcription; numbers show the distance in kilobases between the negative element (NE) and the genes or the telomere (Tel); and Chr-(letter) denotes chromosome notation.

(UAS or operator) heterologously positioned at distances >700 bp on the 3′ end of a gene, can activate/repress if the reporter gene and the *cis*-element are localized near a telomere (de Bruin *et al.* 2001; Zaman *et al.* 2002). One explanation is that yeast telomeres can fold back and form a higher order structure or loop, allowing the *cis*-acting element with a tethered *trans*-acting factor to interact with the promoter and in this way activate or repress transcription of that gene. A similar mechanism could occur between the NE and some element in the *EPA1* promoter, in which looping of the DNA would establish an interaction leading to repression of *EPA1*. In support of this hypothesis, the NE does not function to repress transcription of five other promoters (*HHT2*, *MET3*, *PGK1*, *PDC1*, and *EGD1*) when carried on a CEN ARS plasmid (data not shown).

Interestingly, there have been no reports in other *Candida* species of transcriptional regulation dependent on a *cis*-element 3′ to the ORF. However, in an example with clear parallels to *EPA1* regulation, *C. albicans*, *ALS1* and *ALS7*, which, like *EPA1*, encode cell surface adhesions, are also induced in lag phase (Green *et al.* 2005). Whether this regulation depends on a 3′ element has not, to our knowledge, been tested.

Repression of EPA1 expression by the NE depends on HDF1 (yKu70) and HDF2 (yKu80) but is independent of the telomere

EPA1 repression after induction analyzed in the context of the chromosome, can be recapitulated in a plasmid (BG198 *vs.* pAP385, Figure 1, D and E), indicating that all regulatory elements for proper regulation of *EPA1* are contained in the plasmid (pAP385) and that NE-mediated repression of *EPA1* transcription is telomere independent. This telomere-independent regulation of *EPA1* expression must coordinate with telomere-dependent silencing.

Surprisingly, our genetic data show that the NE-mediated repression depends on *HDF1* (yKu70) and *HDF2* (yKu80) (Figure 4C, lines 3–6). yKu70 and yKu80 are essential to repair double strand breaks by nonhomologous end joining (Rosas-Hernandez *et al.* 2008), prevent native chromosome ends from degradation and fusion, and initiate silencing by recruiting Sir4 to the telomere (Tham and Zakian 2002). Interestingly, *C. glabrata* yKu70/yKu80 are not required for subtelomeric silencing at the *EPA1* telomere (chromosome E Right) (Rosas-Hernandez *et al.* 2008). How might yKu70 and yKu80 repress transcription of *EPA1* through the NE? Given that it has been shown that the Ku complex also associates with subtelomeric regions (Martin *et al.* 1999) and can nucleate silencing when tethered (Tham and Zakian 2002; Rusche *et al.* 2003), a possible model is that Ku associates directly with the NE ultimately leading to repression. We suggest that the repression mechanism might result from Ku-mediated interactions between the NE and elements in the *EPA1* promoter that form a transcriptionally repressed chromatin loop.

EPA1 expression is silenced

We have shown previously that *EPA1* expression is regulated by the silencing machinery (Castaño *et al.* 2005; Domergue *et al.* 2005). However, the analysis of *URA3* reporter genes inserted in the *EPA1–EPA3* region suggested that subtelomeric silencing ends in the intergenic region between *EPA1* and *EPA2* (De Las Peñas *et al.* 2003). We revisited this in the current study, and our genetic experiments confirm that Sir2–Sir4 silence the expression of *EPA1*. We suggest that differences in the reporter constructs used affect the

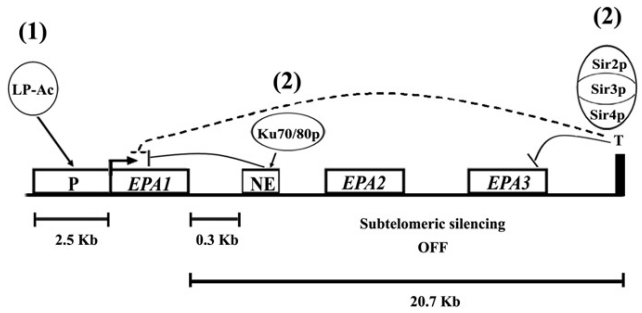


Figure 6 Model of *EPA1* regulation. In stationary phase (SP), *EPA1* is not expressed and cells are nonadherent. Upon dilution into fresh media, (1) a log-phase specific transcriptional activator (LP-Ac) induces expression of *EPA1* and cells become adherent. This transcriptional activation is counteracted in log phase (LP) by the concerted action of silencing and the NE (2). The Sir complex silences the expression of *EPA1* and yKu70/yKu80 repress *EPA1* expression through the NE. These two regulatory mechanisms assure that *EPA1* is not expressed even in the presence of the LP-Ac, which is active throughout LP.

silencing assay. The *URA3* reporter silencing experiments were done with two different promoters—the *EPA1* promoter fused to the *URA3* gene (shown in this article) and the *URA3* gene driven by its own promoter in the previous analysis (De Las Peñas *et al.* 2003; Castaño *et al.* 2005; Rosas-Hernandez *et al.* 2008). We suggest that the *EPA1–URA3* construct more faithfully reported silencing than the heterologous *URA3* gene either because the *EPA1* promoter is weaker and more sensitive to silencing or potentially because the *URA3* reporter inherently can be an imperfect measure of silencing (Stillman *et al.* 2011).

Our data suggest, therefore, that there are two distinct mechanisms that maintain the expression of *EPA1* tightly controlled: negative regulation by silencing, which depends on the Sir complex and telomere proximity, and a telomere-independent, Sir-independent repression by a NE at the 3′ end of the gene that depends on yKu70 and yKu80. These mechanisms operate independently of one another but in conjunction tightly control expression of *EPA1*. In our current model of *EPA1* transcriptional regulation, upon dilution into fresh media, a log-phase–specific transcriptional activator (LP-Ac, Figure 6) induces *EPA1* expression (Figure 1B). After one cell division, both silencing (Sir complex dependent) and the NE with yKu70/80 cooperate to repress transcription. These two regulatory mechanisms counteract the putative LP-Ac that is present and active throughout LP, keeping the expression of *EPA1* repressed. This mode of regulation keeps the expression of *EPA1* tightly repressed, but poised to be transiently induced in the appropriate environment. We do not know the identity of the activator; however, the transcription factors Flo8 and Mss1 are candidates since both transcription factors are normally required for the expression of *EPA6* under inducing environmental conditions (Mundy and Cormack 2009).

It is worth pointing out that traditional gene expression analyses in yeast do not usually use cognate 3′-UTR/3′-intergenic regions or assess their potential impact on regulation. Our data suggest that it might be worth doing so.

Acknowledgments

We thank B. Ma for careful reading of the manuscript. This work was funded by a Consejo Nacional de Ciencia y Tecnología (CONACYT) fellowship to V.G.-G. (143037), J.J.-C. (48549), M.B.-M.-C. (209276) and V.M.-J. (205619). This work was funded by CONACYT grant no. CB-2005-48279 to A.D.L.P and by National Institutes of Health grant 5R01AI046223 to B.C. We will always remember Meg.

Literature Cited

Ausubel, F., R. Brent, R. E. Kingston, D. Moore, J. G. Seidman *et al.*, 2001 *Current Protocols in Molecular Biology.* John Wiley & Sons, New York.

Calderwood, M. S., L. Gannoun-Zaki, T. E. Wellems, and K. W. Deitsch, 2003 Plasmodium falciparum var genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. J. Biol. Chem. 278: 34125–34132.

Calvin, N. M., and P. C. Hanawalt, 1988 High-efficiency transformation of bacterial cells by electroporation. J. Bacteriol. 170: 2796–2801.

Castaño, I., R. Kaur, S. Pan, R. Cregg, A. De Las Peñas *et al.*, 2003 Tn7-based genome-wide random insertional mutagenesis of Candida glabrata. Genome Res. 13: 905–915.

Castaño, I., S. J. Pan, M. Zupancic, C. Hennequin, B. Dujon *et al.*, 2005 Telomere length control and transcriptional regulation of subtelomeric adhesins in Candida glabrata. Mol. Microbiol. 55: 1246–1258.

Castaño, I., A. De Las Peñas, and B. Cormack, 2006 Function and regulation of adhesin gene families in *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida glabrata*, p. 684 in *Molecular Principles of Fungal Pathogenesis*, edited by J. Heitman, J. E. Edwards, S. G. Filler, and A. P. Mitchell. ASM Press, New York.

Chang, A. C., and S. N. Cohen, 1978 Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134: 1141–1156.

Cormack, B. P., and S. Falkow, 1999 Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen Candida glabrata. Genetics 151: 979–987.

Cormack, B. P., N. Ghori, and S. Falkow, 1999 An adhesin of the yeast pathogen Candida glabrata mediating adherence to human epithelial cells. Science 285: 578–582.

Cuellar-Cruz, M., M. Briones-Martin-del-Campo, I. Canas-Villamar, J. Montalvo-Arredondo, L. Riego-Ruiz *et al.*, 2008 High resistance to oxidative stress in the fungal pathogen Candida glabrata is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. Eukaryot. Cell 7: 814–825.

Cuellar-Cruz, M., I. Castaño, O. Arroyo-Helguera, and A. De Las Peñas, 2009 Oxidative stress response to menadione and cumene hydroperoxide in the opportunistic fungal pathogen Candida glabrata. Mem. Inst. Oswaldo Cruz 104: 649–654.

de Bruin, D., Z. Zaman, R. A. Liberatore, and M. Ptashne, 2001 Telomere looping permits gene activation by a downstream UAS in yeast. Nature 409: 109–113.

De Groot, P. W. J., K. J. Hellingwerf, and F. M. Klis, 2003a Genome-wide identification of fungal GPI proteins. Yeast 20: 781–796.

De Groot, P. W. J., H. L. Dekker, A. D. De Boer, K. J. Hellingwerf, C. G. De Koster *et al.*, 2003b Identification of cell wall proteins

of the fungal pathogen Candida albicans and other fungi using mass-spectrometric and genome-wide computational approaches. Yeast 20: S57–S57.

De Groot, P. W. J., E. A. Kraneveld, Q. Y. Yin, H. L. Dekker, U. Gross *et al.*, 2008 The cell wall of the human pathogen Candida glabrata: differential incorporation of novel adhesin-like wall proteins. Eukaryot. Cell 7: 1951–1964.

De Las Peñas, A., S. J. Pan, I. Castaño, J. Alder, R. Cregg *et al.*, 2003 Virulence-related surface glycoproteins in the yeast pathogen Candida glabrata are encoded in subtelomeric clusters and subject to RAP1- and SIR-dependent transcriptional silencing. Genes Dev. 17: 2245–2258.

Delaloy, C., E. Elvira-Matelot, M. Clemessy, X. O. Zhou, M. Imbert-Teboul *et al.*, 2008 Deletion of WNK1 First Intron Results in Misregulation of Both Isoforms in Renal and Extrarenal Tissues. Hypertension 52: 1149–1154.

Domergue, R., I. Castaño, A. De Las Peñas, M. Zupancic, V. Lockatell *et al.*, 2005 Nicotinic acid limitation regulates silencing of Candida adhesins during UTI. Science 308: 866–870.

Errede, B., M. Company, and C. A. Hutchison 3rd. 1987 Ty1 sequence with enhancer and mating-type-dependent regulatory activities. Mol. Cell. Biol. 7: 258–265.

Fidel, P. L. Jr. J. L. Cutright, L. Tait, and J. D. Sobel, 1996 A murine model of Candida glabrata vaginitis. J. Infect. Dis. 173: 425–431.

Gietz, R. D., and A. Sugino, 1988 New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74: 527–534.

Green, C. B., X. Zhao, K. M. Yeater, and L. L. Hoyer, 2005 Construction and real-time RT-PCR validation of Candida albicans PALS-GFP reporter strains and their use in flow cytometry analysis of ALS gene expression in budding and filamenting cells. Microbiology 151: 1051–1060.

Guarente, L., and E. Hoar, 1984 Upstream activation sites of the CYC1 gene of Saccharomyces cerevisiae are active when inverted but not when placed downstream of the "TATA box". Proc. Natl. Acad. Sci. USA 81: 7860–7864.

Guo, B., C. A. Styles, Q. H. Feng, and G. R. Fink, 2000 A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating. Proc. Natl. Acad. Sci. USA 97: 12158–12163.

Halme, A., S. Bumgarner, C. Styles, and G. R. Fink, 2004 Genetic and epigenetic regulation of the FLO gene family generates cell-surface variation in yeast. Cell 116: 405–415.

Hoyer, L. L., 2001 The ALS gene family of Candida albicans. Trends Microbiol. 9: 176–180.

Hoyer, L. L., C. B. Green, S. H. Oh, and X. Zhao, 2008 Discovering the secrets of the Candida albicans agglutinin-like sequence (ALS) gene family—a sticky pursuit. Med. Mycol. 46: 1–15.

Kaur, R., R. Domergue, M. L. Zupancic, and B. P. Cormack, 2005 A yeast by any other name: Candida glabrata and its interaction with the host. Curr. Opin. Microbiol. 8: 378–384.

Keegan, L., G. Gill, and M. Ptashne, 1986 Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science 231: 699–704.

Klotz, S. A., N. K. Gaur, D. F. Lake, V. Chan, J. Rauceo *et al.*, 2004 Degenerate peptide recognition by Candida albicans adhesins Als5p and Als1p. Infect. Immun. 72: 2029–2034.

Kobayashi, O., N. Hayashi, R. Kuroki, and H. Sone, 1998 Region of Flo1 proteins responsible for sugar recognition. J. Bacteriol. 180: 6503–6510.

Li, F., and S. P. Palecek, 2003 EAP1, a Candida albicans gene involved in binding human epithelial cells. Eukaryot. Cell 2: 1266–1273.

Martin, M. P., V. L. Gerlach, and D. A. Brow, 2001 A novel upstream RNA polymerase III promoter element becomes essential when the chromatin structure of the yeast U6 RNA gene is altered. Mol. Cell. Biol. 21: 6429–6439.

Martin, S. G., T. Laroche, N. Suka, M. Grunstein, and S. M. Gasser, 1999 Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. Cell 97: 621–633.

Mundy, R. D., and B. Cormack, 2009 Expression of Candida glabrata adhesins after exposure to chemical preservatives. J. Infect. Dis. 199: 1891–1898.

Pfaller, M. A., and D. J. Diekema, 2010 Epidemiology of invasive mycoses in North America. Crit. Rev. Microbiol. 36: 1–53.

Roetzer, A., T. Gabaldon, and C. Schuller, 2011 From Saccharomyces cerevisiae to Candida glabrata in a few easy steps: important adaptations for an opportunistic pathogen. FEMS Microbiol. Lett. 314: 1–9.

Rosas-Hernandez, L. L., A. Juarez-Reyes, O. E. Arroyo-Helguera, A. De Las Peñas, S. J. Pan *et al.*, 2008 yKu70/yKu80 and Rif1 regulate silencing differentially at telomeres in Candida glabrata. Eukaryot. Cell 7: 2168–2178.

Rusche, L. N., A. L. Kirchmaier, and J. Rine, 2003 The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu. Rev. Biochem. 72: 481–516.

Sheppard, D. C., M. R. Yeaman, W. H. Welch, Q. T. Phan, Y. Fu *et al.*, 2004 Functional and structural diversity in the Als protein family of Candida albicans. J. Biol. Chem. 279: 30480–30489.

Sherman, F., G. R. Fink, and J. B. Hicks, 1986 *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19–27.

Stark, K., D. L. Kirk, and R. Schmitt, 2001 Two enhancers and one silencer located in the introns of regA control somatic cell differentiation in Volvox carteri. Genes Dev. 15: 1449–1460.

Stillman, B., M. P. Rossmann, W. J. Luo, O. Tsaponina, and A. Chabes, 2011 A common telomeric gene silencing assay is affected by nucleotide metabolism. Mol. Cell 42: 127–136.

Struhl, K., 1984 Genetic properties and chromatin structure of the yeast gal regulatory element: an enhancer-like sequence. Proc. Natl. Acad. Sci. USA 81: 7865–7869.

Tham, W. H., and V. A. Zakian, 2002 Transcriptional silencing at Saccharomyces telomeres: implications for other organisms. Oncogene 21: 512–521.

Zaman, Z., C. Heid, and M. Ptashne, 2002 Telomere looping permits repression "at a distance" in yeast. Curr. Biol. 12: 930–933.

Zupancic, M. L., M. Frieman, D. Smith, R. A. Alvarez, R. D. Cummings *et al.*, 2008 Glycan microarray analysis of Candida glabrata adhesin ligand specificity. Mol. Microbiol. 68: 547–559.

Communicating editor: F. Winston

2.4 Capitulo 4

Orta-Zavalza E, Briones-Martin-del-Campo Castano I, De Las Penas A. **Catalase Activity Assay in *Candida glabrata*.** *Bio-protocol*. 2014; 4(6).

Este documento fue publicado en la modalidad de acceso abierto en http://www.bio-protocol.org/e1072.

Catalase Activity Assay in *Candida glabrata*

Emmanuel Orta-Zavalza, Marcela Briones-Martin-del-Campo1, Irene Castano and Alejandro De Las Penas*

Molecular Biology Department, IPICYT A. C., San Luis Potosi, Mexico
*For correspondence: cano@ipicyt.edu.mx

[Abstract] Commensal and pathogenic fungi are exposed to hydrogen peroxide (H_2O_2) produced by macrophages of the host. Pathogenic fungi counteract the harmful effects of H_2O_2 with the enzyme catalase (EC 1.11.1.6), which decomposes two molecules of H_2O_2 to two molecules of H_2O and O_2. Contribution of antioxidant systems on fungal virulence is actively studied. Measurement of catalase activity can contribute to the elucidation of the factors that influence the regulation of this pivotal enzyme. Here we describe a simple spectrophotometric method in which the activity of catalase is measured in total yeast extracts. Decomposition of H_2O_2 by the yeast extract is followed by the decrease in absorbance at 240 nm. The difference in absorbance through time (ΔA_{240}) is inferred as the measure of catalase activity.

Materials and Reagents

1. Yeast strains
   Note: BG14 was used as the C. glabrata parental strain. The hst1Δ and the cta1Δ null mutants were used as a positive and the negative controls, respectively.
2. Catalase from bovine liver (Sigma-Aldrich, catalog number: C9322)
3. Bradford reagent (Sigma-Aldrich, catalog number: B6916)
4. Bovine serum albumin - fraction V (Sigma-Aldrich, catalog number: 85040C)
5. Zirconia/silica beads (0.5 mm diameter) (Bio Spec Products, catalog number: 11079105z)
6. Sterile water
7. Ice
8. One tablet of protease inhibitors cOmplete ULTRA Mini *EASYpack* is used in 10 ml of phosphate buffer (Roche Diagnostics, catalog number: 05 892 970 001)
9. H_2O_2 (Sigma-Aldrich, catalog number: 349887)
10. Catalase lyophilized powder (Sigma-Aldrich, catalog number: C9322)
11. YPD broth (see Recipes)
12. 50 mM Phosphate buffer (PB) (pH 7.0) (see Recipes)
13. 30 mM H_2O_2 (see Recipes)
14. Catalase solution (see Recipes)

Equipment

1. Orbital incubator shaker
2. Microfuge tubes
3. 50 ml conical tubes
4. Corning 96 well clear flat bottom (Corning, catalog number: 3595)
5. Standard 10 mm light path quartz cuvette with PTFE cover
6. UV/Vis Spectrophotometer (Shimadzu, model: UV-1700, catalog number: 206-55401-92)
7. Microplate spectrophotometer system (Benchmark Plus Microplate reader) (Bio-Rad Laboratories, catalog number: 170-6931)
8. Centrifuge (Beckman Coulter, model: Allegra® 25R, catalog number: 369464)
9. Microfuge
10. Stopwatch
11. Parafilm

Procedure

A. Preparation of total soluble extracts
   1. Yeast strains are grown overnight in 5 ml of Yeast extract-Peptone-Dextrose broth or selective media at 30 °C.
   2. Dilute overnight cultures in 50 ml of fresh medium in order that after seven duplications, the yeast cultures reach an OD_{600} = 0.5 at 30 °C.
   3. Centrifuge the cells for 5 min at 2,600 *x g*. Discard supernatant. Temperature of centrifuge is not relevant.
   4. Wash the cells with 25 ml of sterile water and discard supernatant.
   5. Resuspend the cells in 0.5 ml PB with protease inhibitors and transfer to a microfuge tube. Keep the samples on ice.
   6. Add 50 µl of zirconia/silica beads to each sample.
   7. Disrupt the cells by vortexing at maximum speed for 1 min and place on ice for another minute. Repeat 20 times.
   8. Centrifuge the lysate at 25,000 *x g* for 30 min at 4 °C to remove cell debris and zirconia/silica beads
   9. Transfer supernatant to a clean microfuge tube. At this point, lysates are ready for quantification of total protein and measurement of catalase activity. Alternatively, samples can be stored at -20 °C.

B. Bradford assay for protein quantitation
   1. Fill the wells of a microplate with 250 µl of Bradford regent.

2. Prepare a standard curve of absorbance versus nanograms of protein using fresh BSA standards (100, 200, 400, 600, 800 ng/µl). Use PB as solvent.
3. Dilute the lysates 1:20 or 1:50 with sterile water.
4. Load 5 µl of the standards and diluted lysates to the Bradford reagent. Incubate room temperature for 5 min.
5. Measure the absorbance at OD_{595} in a microplate spectrophotometer.
6. Determine the amount of protein of the samples from the standard curve. Consider the dilution factor.

C. Catalase activity assay

1. Set up the spectrophotometer by first turning on the instrument and then the UV light. Set up a kinetics program to record every 30 s at a wavelength of 240 nm for 2 min.
2. Calibrate the spectrophotometer using 3 ml of PB in a 3-ml quartz cuvette as a blank.
3. Dilute the lysate samples 1:50 with PB.
4. In a quartz cuvette, mix 1 ml PB with 1 ml of the diluted sample. To begin the assay, add 1 ml of the H_2O_2 solution (H_2O_2 to a final concentration of 10 mM). The initial absorbance must be between 0.550 and 0.520. If necessary, add H_2O_2 to increase the absorbance and Phosphate Buffer to decrease the absorbance.
5. Mix the content by inversion and Immediately place the cuvettes into the spectrophotometer. Follow the decrease in absorbance at OD_{240} with a stopwatch for 2 min.
6. A catalase solution must be used as a control. Pippete 2.9 ml of PB in the cuvette, add 1 ml 30 mM H_2O_2 and 100 µl of the catalase solution (~10 units). Record the initial and final absorbance in a one-minute period. Use 2 ml of PB and 1 ml of 30 mM H_2O_2 as blank.
7. Calculate the catalase activity using the following formula (Cuellar-Cruz *et al.*, 2009)

$$U/mg = \frac{(A_0 - A_{60}) \times V_t}{\varepsilon_{240} \times d \times V_s \times C_t \times 0.001}$$

Where

$(A_0 - A_{60})$ is the difference between the initial and final absorbance.

V_t is the total volume of the reaction (3 ml).

ε_{240} is the molar extinction coefficient for H_2O_2 at OD_{240} (34.9 mol/cm).

d is the optical length path of cuvette (1 cm).

V_s is the volume of the sample in ml.

C_t is the protein concentration of the sample in mg/ml.

8. Example of catalase activities of extracts from *C. glabrata* strains in exponential phase of growth:

Strain	Act [U/mg]
BG14	4.4057
	4.0087
	1.0869
hst1Δ	9.5256
	0.2598
	6.0690
cta1Δ	0.0000

Note: Catalase activity of the BG14 strain is higher in stationary phase ≈ 10 U/mg. However catalase activity of a cta1Δ is always < 2 U/mg or undetectable.

Notes

1. For catalase, the dependence of the H_2O_2 decomposition on temperature is small, so measurements can be carried out between 0 and 37 °C, however 20 °C is recommended.
2. For the catalse assay, each test cuvette will need to be run one at a time, so do not prepare the next test cuvette until the run with the preceding cuvette is complete.
3. Low concentrations of H_2O_2 are used to avoid bubbling.
4. Mixing of the samples can be facilitated by the use of parafilm.

Recipes

1. Yeast extract-Peptone-Dextrose broth (1 L)
   Dissolve 10 g yeast extract and 20 g peptone in 950 ml of distilled water
   Autoclave (121 °C, 15 lb/in^2 for 15 min)
   Add 50 ml 40% (w/v) dextrose (2% final; sterilized separately by autoclaving or filtering)
2. 50 mM Phosphate Buffer (PB) (pH 7.0) (1 L)
   Dissolve 2.724 g KH_2PO_4 in 400 ml of distilled water
   Dissolve 5.34 g Na_2HPO_4 in 600 ml of distilled water
   Mix solutions [proportion (1:1.5)]
   pH to 7.0 with 1 M KOH
   To obtain cell lysates, prepare 10 ml of PB and add one tablet of cOmplete protease inhibitors.
3. 30 mM H_2O_2 (100 ml)
   Dilute 0.26 ml of 35% H_2O_2 with PB to 100 ml. Prepare fresh to each activity assay. The solution can be at room temperature during the experiment.

4. Catalase solution
   Dissolve 10 mg of catalase lyophilized powder in 1 ml of cold PB
   Immediately before use, dilute 5 µl of catalase solution to 1 ml cold PB to obtain a solution with ~100 U/ml. Stored at -20 °C for 6 months.

Acknowledgments

This protocol is based on the methodology reported by Aebi (1984), and by Weydert and Cullen (2010). Our adapted method was first published in Cuellar-Cruz *et al.* (2009). This work was funded by a CONACYT grant no. CB-2010-153929 to A.D.L.P. Finally, we thank Guadalupe Gutierrez-Escobedo for technical assistance.

References

1. Aebi, H. (1984). Catalase *in vitro*. *Methods Enzymol* 105: 121-126.
2. Cuellar-Cruz, M., Castano, I., Arroyo-Helguera, O. and De Las Penas, A. (2009). Oxidative stress response to menadione and cumene hydroperoxide in the opportunistic fungal pathogen *Candida glabrata*. *Mem Inst Oswaldo Cruz* 104(4): 649-654.
3. Weydert, C. J. and Cullen, J. J. (2010). Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nat Protoc* 5(1): 51-66.

3. Resultados adicionales

Expresión de Sod2 a los siete días de fase estacionaria.
Para conocer la localización intracelular de Sod2 durante la fase estacionaria (FE) tardía de *C. glabrata*, construimos fusiones traduccionales del gen *SOD2* con la proteína fluorescente GFP. La cepa conteniendo esta fusión, se creció en medio rico (YPD) durante 7 días a 30°C. Se tiñeron con DAPI para revelar el núcleo (estructuras redondas de color azul). Como mencionamos anteriormente, Sod2-GFP a los 2 días de FE se localiza en la mitocondria, la cual se observa como puntos alrededor del núcleo, tinción con DAPI y Mitotracker). A los siete días, Sod2-GFP fluorece en estructuras alargadas que siguen la periferia de la célula (FIGURA 1). Este dato confirma que Sod2-GFP está en la mitocondria durante una FE tardía.

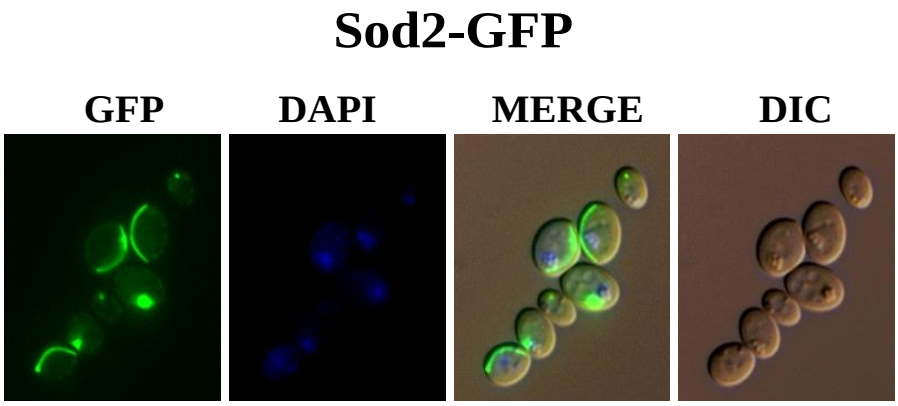


FIGURA 1. Localización celular de Sod2 en fase estacionaria de 7dias. Células en FE de 7 días de la cepa parental que expresa Sod2-GFP se cultivaron en medio YPD y se tiñeron con DAPI (núcleo). Se muestra imagen representativa.

Actividad de SOD en las mutantes de SOD de *C. glabrata*
Para determinar el aporte enzimático de Sod1 y Sod2 a la actividad total de SOD en *C. glabrata*, decidimos cuantificar la actividad de SOD durante la fase logarítmica (FL) y estacionaria de la cepa parental y las mutantes *sod1*Δ, *sod2*Δ y *sod1*Δ *sod2*Δ de *C. glabrata* utilizando el método colorimétrico WST-1 (FIGURA 2). Los valores de las mutantes en SOD se normalizaron con respecto a su cepa parental *y* se compararon con las actividades de SOD de las mutantes sencillas en SOD de *S. cerevisiae*. Encontramos que las actividades de SOD de las mutantes *sod1*Δ, *sod2*Δ y *sod1*Δ *sod2*Δ de *C. glabrata* fueron similares en las dos fases de crecimiento (comparar Fig. A y B). La actividad de SOD en la mutante *sod1*Δ de *C. glabrata* alrededor del 10% y la actividad de la mutante *sod2*Δ fue aprox. Del 60 % en comparación con la cepa parental (FIGURA 2). La mutante *sod1*Δ *sod2*Δ no mostro actividad de SOD. Con estos datos confirmamos que *C. glabrata* contiene dos SOD y que Sod1 la que aporta mayor actividad de SOD a la célula.

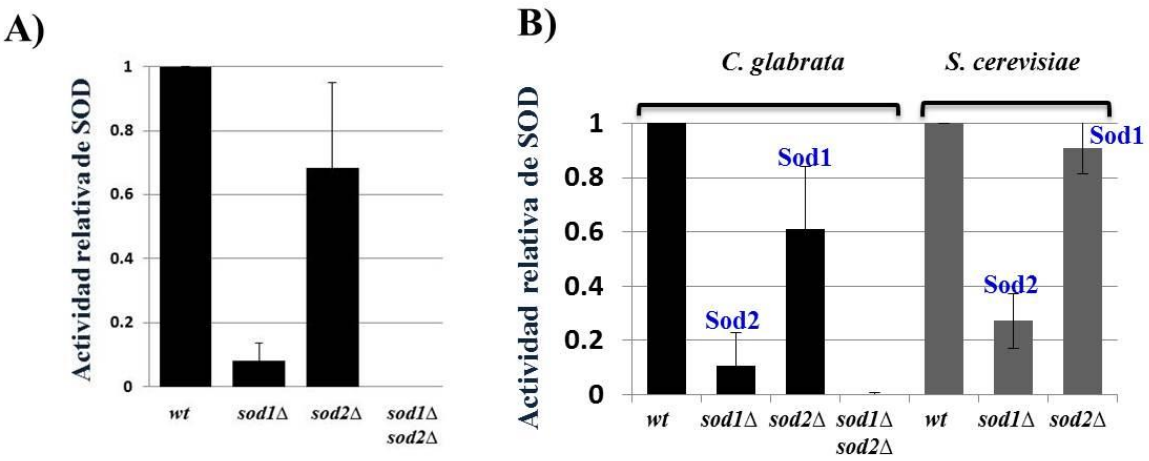


FIGURA 2. Actividad de SOD en la cepa silvestre y las mutantes *sod1*Δ, *sod2*Δ y *sod1*Δ *sod2*Δ de *C. glabrata* y *sod1*Δ y *sod2*Δ de *S. cerevisiae.* Las cepas BG14 (wt), CGM787 (*sod1*Δ), CGM656 (*sod2*Δ) y CGM937 (*sod1*Δ *sod2*Δ) de *C. glabrata* y BY4749 (parental), L108 (*sod1*Δ) y L109 (*sod2*Δ) de *S. cerevisiae* se cultivaron en YPD a 30˚C. Se hicieron extractos proteicos de estas cepas y su actividad total de SOD se cuantificó con el método colorimétrico WST-1. Los valores se normalizaron con respecto a las cepas silvestres de *C. glabrata* o *S. cerevisiae*. Los experimentos se realizaron 3 veces y las barras de dispersión indican SD. A) Fase logarítmica de siete días. B) FE dos días.

Sod1 de *C. glabrata* es necesaria para resistir el estrés oxidante crónico generado por menadiona y H_2O_2

Para determinar la función de las SOD de *C. glabrata* en la respuesta a estrés oxidante durante un tratamiento crónico, evaluamos el crecimiento celular de la cepa silvestre y las mutantes *sod1*Δ, *sod2*Δ y *sod1*Δ*sod2*Δ en presencia de menadiona (MD) y H_2O_2 Los experimentos se hicieron con células de FE y FL. Encontramos que las células en FE y FL se comportan de forma similar en los tratamientos crónicos. Durante el estrés crónico con MD, encontramos que la cepa parental fue resistente a 0.08mM y la mutante *sod2*Δ fue sensible a esta concentración. En cambio la mutante *sod1*Δ y la doble mutante *sod1*Δ *sod2*Δ fueron sensibles a 0.06mM de MD (FIGURA 3). Estos datos indican que Sod1 es necesaria para resistir el estrés oxidante generado por MD.

En presencia de H_2O_2, observamos que la cepa parental resistió 20 mM de H_2O_2 y la mutante *sod1Δ* solo a 5 mM. La mutante *sod2Δ* perdió viabilidad a H_2O_2 15 mM y no creció a 20 mM (FIGURA 3). La cepa *sod1Δ sod2Δ* no creció a ninguna de las concentraciones de H_2O_2 probadas. Estos resultados sugieren que, principalmente Sod1 y en menor medida Sod2, están involucradas en la resistencia a menadiona y H_2O_2 durante un tratamiento crónico.

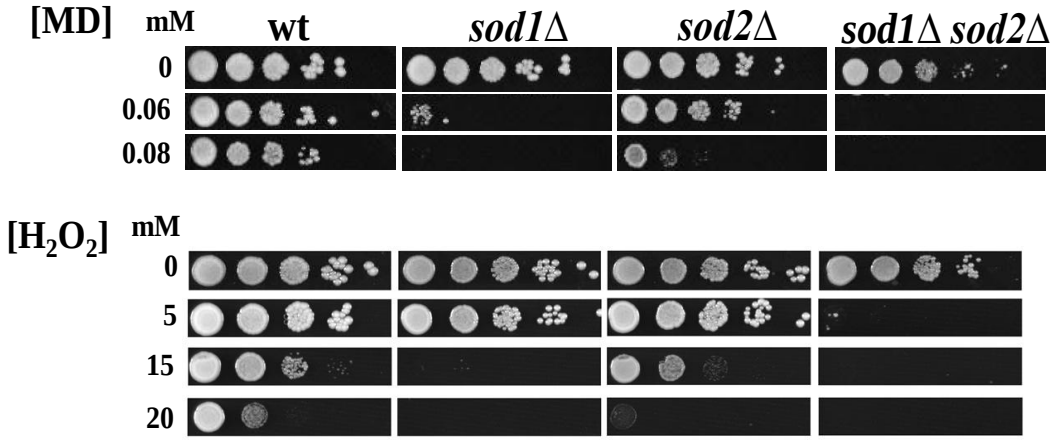


FIGURA 3. Resistencia a MD y H_2O_2 de la cepa parental y las mutantes *sod1*Δ, *sod2*Δ y *sod1*Δ *sod2*Δ de *C. glabrata.* Las cepas BG14 (wt), CGM787 (*sod1*Δ), CGM656 (*sod2*Δ) y CGM937 (*sod1*Δ *sod2*Δ) se cultivaron por 48 h en YPD a 30˚C (FE) y se diluyeron a una OD_{600nm} de 0.5 con el medio usado del mismo cultivo. Se hicieron diluciones seriadas y se gotearon en cajas de YPD conteniendo MD 0.06 y 0.08mM; y H_2O_2 .5, 10, 15 y 20 mM. Las cajas se incubaron a 30°C durante 48 h.

El aumento a dos copias de *SOD1* y *SOD2* no tienen efecto en la resistencia a menadiona

Para conocer si la carga genética de las SODs es importante en la respuesta a estrés oxidante en *C. glabrata*, analizamos la resistencia a MD de la cepa parental con una copia extra de SODs en forma episomal. Encontramos que la cepa parental conteniendo una copia extra de cualquiera de las SODs tiene la misma resistencia a MD que la cepa parental con una sola copia (FIGURA 4). Resultados similares se encontraron en el tratamiento agudo (datos no mostrados). Estos datos sugieren que posiblemente una sola copia de *SOD1* o *SOD2* no sea suficiente para aumentar la resistencia a MD o que simplemente el aumento en la carga genética de las SOD no favorece la resistencia a MD.

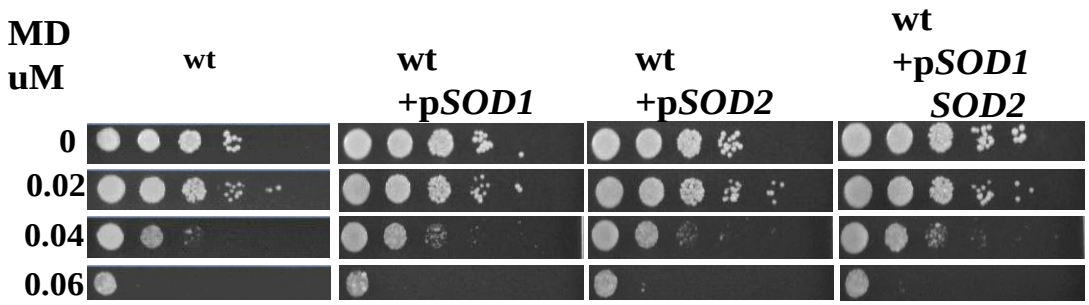


FIGURA 4. Resistencia a MD de la cepa parental con una o dos copias de los genes SOD de *C. glabrata.* Las cepas CGM1719 (wt), CGM1734 (wt + p*SOD1*), CGM1736 (*wt + pSOD2*) y CGM1749 (*wt + pSOD1, pSOD2*) se cultivaron por 48 h

en YPD a 30˚C (FE) y se diluyeron a una OD_{600nm} de 0.5 con el medio usado del mismo cultivo. Se hicieron diluciones seriadas y se gotearon en cajas de YPD conteniendo MD 0.02, 0.04 y 0.06mM. Las cajas se incubaron a 30°C durante 48 h.

Análisis de crecimiento en ausencia de lisina de las cepas parental, *lys4*Δ y *sod1*Δ complementadas con los genes *LYS4, LYS12, LYS21* y *LYS20* de *C. glabrata*

Previamente observamos que la doble mutante *sod1*Δ *sod2*Δ es auxótrofa a lisina, esto sugiere que las SODS de *C. glabrata* están involucradas en el mantenimiento de la vía de biosíntesis de lisina, la vía α-aminoadipato (AAA). Estudios anteriores han demostrado que la auxotrofía a lisina de la mutante *sod1*Δ de *S. cerevisiae* es debido al daño oxidante causado por el superóxido. Se ha propuesto que la homoaconitasa (Lys4), segunda enzima de la vía-AAA, es un blanco para el superóxido (Wallace *et al.*, 2004). Basados en estas observaciones, determinamos si la sobreproducción de Lys4 podía suprimir la auxotrofia a lisina de la doble mutante *sod1*Δ *sod2*Δ. Clonamos el gen *LYS4* bajo el promotor fuerte de *PGK1* en el vector de expresión pGRB2.2 y el plásmido resultante se introdujo en la mutante sencilla *sod1*Δ y en la doble mutante *sod1*Δ *sod2*Δ. Encontramos que la sobreexpresión de *LYS4* no suprimió la auxotrofía a lisina ni tampoco el lento crecimiento de *sod1*Δ ya sea bajo un promotor fuerte o bajo su propio promotor (FIGURA 5). Como control, demostramos que los plásmidos que expresan a Lys4 si complementan a la mutante *lys4*Δ y no tienen efecto aparente en la cepa parental (construcciones de la figura X: wt/p*LYS4 UTR'*s, wt/pP_{PGK1}*LYS4 UTR'*s, *lys4*Δ*/pLYS4 UTR's, lys4*Δ*/pP*$_{PGK1}$*LYS4 UTR's*).

Además, también demostramos que la sobreexpresión de los genes *LYS12, LYS21* y *LYS20* tampoco suprime la auxotrofia a lisina de la doble mutante *sod1*Δ *sod2*Δ, ni el lento crecimiento de la mutante sencilla *sod1*Δ (FIGURA 6).

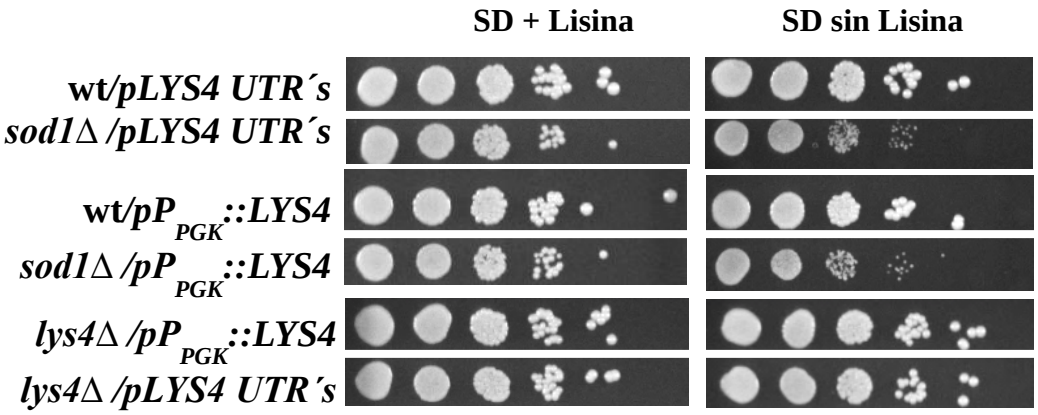


FIGURA 5. Crecimiento en presencia y ausencia de lisina de cepas complementadas con *LYS4* de *C. glabrata*. Las cepas parental, *sod1*Δ o *lys4*Δ complementadas con un plásmido que contenía Lys4 bajo su propio promotor o bajo un promotor fuerte [wt/p*LYS4* UTR´s (CGM1646), *sod1*Δ/p*LYS4* UTR´s

(CGM1753), wt/pP_{PGK}::*LYS4* (CGM1644), *sod1*Δ/pP_{PGK}::*LYS4* (CGM1751), *lys4*Δ/pP_{PGK}::*LYS4* (CGM1688), *lys4*Δ/p*LYS4* UTR´s (CGM1690)] fueron cultivadas en medio SD suplementado con Lys durante 48 h a 30 ºC. Cultivos en FE se lavaron y se resuspendieron en agua destilada. Se hicieron diluciones seriadas y se gotearon en cajas de medio SD con o sin 30 mg / l de Lisina. Las placas se incubaron durante 48 h a 30 º C.

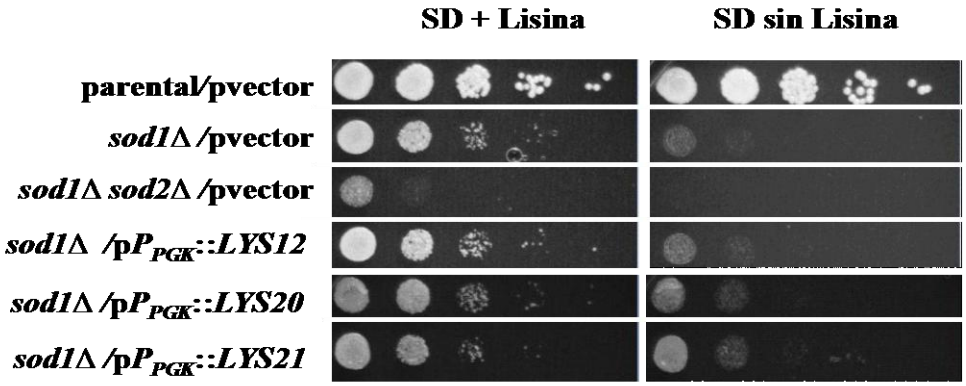


FIGURA 6. Crecimiento en presencia y ausencia de lisina de cepas sobre-expresando *LYS12, LYS20* o *LYS21* de *C. glabrata*. Las cepas parental (CGM1717), la mutante sencilla *sod1*Δ (CGM1721), la doble mutante *sod1*Δ *sod2*Δ (CGM1725) y la mutante *sod1*Δ sobre-expresando *LYS12* (CGM1885)*, LYS20* (CGM1887) o *LYS21* (CGM1889) fueron cultivadas en medio SD suplementado con Lys durante 48 h a 30 ºC. Cultivos en FE se lavaron y se resuspendieron en agua destilada. Se hicieron diluciones seriadas y se gotearon en cajas de medio SD con o sin 30 mg /l de Lisina. Las placas se incubaron durante 48 h a 30 º C.

Análisis de crecimiento en ausencia de lisina de las mutantes *lys4*Δ*, lys5*Δ, y *lys9*Δ de *C. glabrata***

Como parte de la búsqueda del gen o los genes en la vía-AAA responsables de la auxotrofia a lisina de la doble mutante *sod1*Δ *sod2*Δ, analizamos si las mutantes *lys4*Δ*, lys5*Δ*, lys9*Δ*, lys12*Δ y *lys14*Δ eran auxótrofas a lisina. Encontramos que *lys4*Δ*, lys5*Δ*,* y *lys9*Δ son auxótrofas a lisina, en cambio la mutante *lys12*Δ es de lento crecimiento en ausencia de lisina y la mutante *lys14*Δ no es auxótrofa a lisina (FIGURA 7).

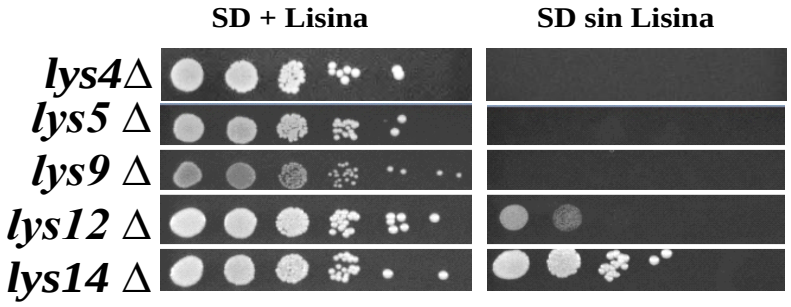


FIGURA 7. Auxotrofia a lisina de algunas mutantes en los genes biosintéticos de lisina. Las cepas *lys4*Δ (CGM1663), *lys5*Δ (CGM1740), *lys9*Δ (CGM1741), *lys12*Δ (CGM1742) y *lys14*Δ (CGM1743) fueron cultivadas en medio SD suplementado con Lys durante 48 h a 30 ºC. Cultivos en FE se lavaron y se resuspendieron en agua destilada. Se hicieron diluciones seriadas y se gotearon en cajas de medio SD con o sin 30 mg / l de Lisina. Las placas se incubaron durante 48 h a 30 º C.

Crecimiento de la doble mutante *sod1*Δ *sod2*Δ y la mutante sencilla *lys5*Δ de *C. glabrata* en presencia de diferentes concentraciones de lisina

Como hemos demostrado la doble mutante *sod1*Δ *sod2*Δ es auxótrofa a lisina. Para conocer la mínima concentración de lisina que necesita esta mutante para crecer igual que la parental, decidimos realizar el siguiente experimento: cultivos saturados de la cepa parental, la mutante *lys5*Δ y la doble mutante *sod1*Δ *sod2*Δ se cultivaron en medio mínimo con distintas concentraciones de lisina. Se realizaron curvas de crecimiento usando el equipo Bioscreen. Encontramos que la cepa parental tuvo crecimiento similar en las distintas concentraciones de lisina probadas (dato no mostrado). La mutante auxótrofa a lisina *lys5*Δ recupero su crecimiento a partir de la concentración mínima de lisina probada (30mg/L) y continuo mejorando su tiempo de duplicación con las concentraciones mayores (FIGURA 8, inciso A) hasta crecer similar a la cepa parental. En cambio, la doble mutante *sod1*Δ *sod2*Δ recupero su crecimiento y tuvo tiempos de duplicación similares con todas las concentraciones de lisina probadas (FIGURA 8, inciso B). Estos datos muestran que aunque las dos cepas *sod1*Δ *sod2*Δ y *lys5*Δ son auxótrofas a lisina la recuperación de su crecimiento al añadir lisina es distinta. El tiempo de duplicación de la mutante *lys5*Δ depende de la concentración de lisina añadida, en cambio en la doble mutante *sod1*Δ *sod2*Δ es independiente.

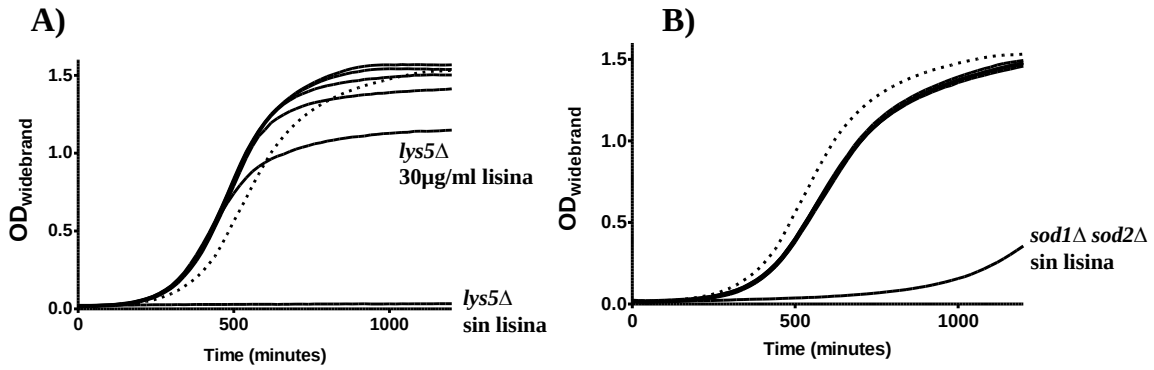


FIGURA 8. Auxotrofia a lisina de las mutantes *lys5*Δ y *sod1*Δ *sod*2Δ de *C. glabrata*. Las cepas BG14 (wt), CGM1740 (*lys5*Δ) (A) y CGM1725 (*sod1*Δ *sod2*Δ) (B) se cultivaron por 48 h en medio SD suplementado con Lys a 30˚C. Se diluyeron a una OD_{600nm} de 0.1 en medio SD sin lisina o con 30, 50, 60, 80 y 120 mg / l de lisina. La línea punteada en ambas graficas es la cepa BG14 (wt) usada como control de crecimiento en medio sin lisina. Las células se lavaron y diluyeron a una densidad de $2x10^6$ células/mL. El crecimiento se evaluó en el equipo Bioscreen C MBR durante 24h a 30°C y agitación constante.

Tiempo de vida cronológica de *S. cerevisiae*, *C. glabrata* y las mutantes en SOD

Diversos estudios de *C. glabrata* demuestran que esta levadura oportunista es más resistente a distintos tipos de estrés que *S. cerevisiae* (Cuellar-Cruz *et al.*, 2008; Cuéllar-Cruz *et al.*, 2009). Estas características hacen de *C. glabrata* una levadura resistente al ambiente hostil. De acuerdo con estos hallazgos decidimos evaluar la viabilidad de *C. glabrata* durante una FE tardía y compararla con la de *S. cerevisiae.* Para esto, cultivos saturados de las dos cepas de levadura fueron crecidas en medio SD a 30°C durante 12 días consecutivos. A partir del día cuatro se tomaron muestras que se diluyeron en medio YPD para analizar su viabilidad en el equipo Bioscreen (ver material y métodos). Se toma el día dos como el 100% de viabilidad. Al quinto día, *C. glabrata* mostro el doble de viabilidad que *S. cerevisiae* (66% vs aprox. 30%). Para el doceavo día *S. cerevisiae* había perdido el 90% de su viabilidad, en cambio *C. glabrata* mostraba alrededor del 40%. Estos datos indican que *C. glabrata* tiene una vida cronológica más larga que *S. cerevisiae* en las condiciones probadas (FIGURA 9).

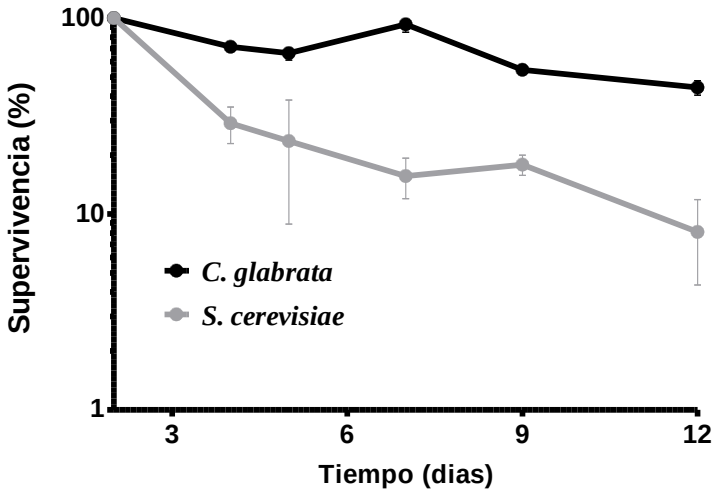


FIGURA 9. Vida cronológica de *C. glabrata* y *S. cerevisiae.* Cultivos saturados de cepas de de *C. glabrata* (BG14) y *S. cerevisiae* (BY4742) se cultivaron en medio SD durante 72 horas a 30 ° C. El día 2 representa el 100% de viabilidad. Las muestras se tomaron en los días 2, 4, 5, 7, 9 y 12 y se diluyeron en medios YPD fresco. La viabilidad se obtuvo con el método de cuenta viable. Las barras de error indican la desviación estándar de cuatro réplicas biológicas. Ver Materiales y Métodos.

Para determinar si las SODs tienen un papel en la vida cronológica de *C. glabrata*, analizamos la viabilidad de las mutantes en SOD durante la FE tardía por el método de cuenta viable. Brevemente, hicimos diluciones seriadas de la muestra tomada a diferentes días de la FE, plateamos en medio YPD y contamos las CFUs. Se toma el día tres como el 100% de viabilidad. Después del séptimo día, la doble mutante *sod1*Δ *sod2*Δ de *C. glabrata* mostro perdida de viabilidad. En cambio la cepa parental y las mutantes sencillas *sod1*Δ y *sod2*Δ se mantuvieron con viables durante los doce días de FE (FIGURA 10). Estos datos indican que la viabilidad de *C. glabrata* en FE tardía en ausencia de aminoácidos depende de la función concomitante de las SODs.

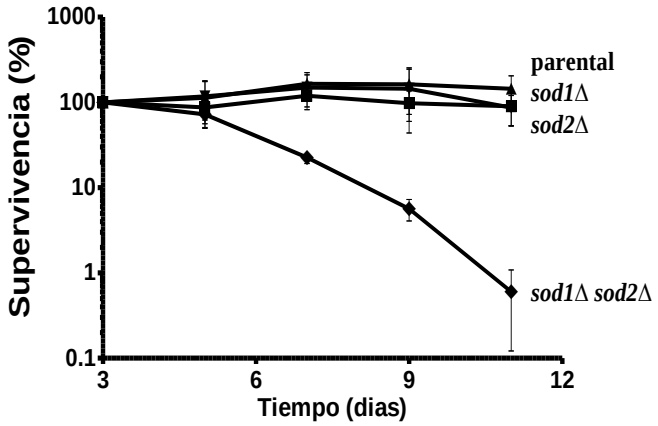


FIGURA 10. Vida cronológica de las mutantes *sod1*Δ, *sod2*Δ y *sod1*Δ *sod2*Δ de *C. glabrata* con el método de cuenta viable. Cultivos saturados de las cepas BG2 (wt), CGM1146 (*sod1*Δ), CGM1131 (*sod2*Δ) y CGM1133 (*sod1*Δ *sod2*Δ) se cultivaron en medio SD en ausencia de aminoácidos durante 72 horas a 30 ° C. El día 3 representa el 100% de viabilidad. Las muestras se tomaron en los días 3, 5, 7, 9, y 11 y se diluyeron en medio YPD fresco. La viabilidad se obtuvo con el método de cuenta viable. Las barras de error indican la desviación estándar de cuatro réplicas biológicas. Ver Materiales y Métodos.

Niveles de $O_2^{\bullet-}$ detectado en células de fase estacionaria al 2, 4 y 7 días

En este trabajo hemos demostrado que las mutantes en SOD tienen un mayor daño oxidante que la cepa parental. Esto se debe a que el $O_2^{\bullet-}$ endógeno producido en la mitocondria no se elimina adecuadamente. Para demostrar la acumulación de $O_2^{\bullet-}$, cuantificamos sus niveles intracelulares por citometria de flujo y flurometria usando la sonda selectiva dihidroetidium (DHE) (Benov & Fridovich, 1998). Cultivos en FE de la cepa parental y las mutantes en SOD fueron cultivadas en medio SD conteniendo lisina. Se tomaron muestras a los 2, 4 y 7 días de FE y se tiñeron con DHE. A los dos días, encontramos que la fluorescencia emitida por la sonda DHE fue similar en la cepa parental y en la mutante sencilla *sod2*Δ. Y aunque las diferencias no son estadísticamente significativas, las mutantes *sod1*Δ y *sod1*Δ *sod2*Δ muestran la tendencia de tener menor fluorescencia con respecto a la cepa parental (FIGURA 11, A). En cambio, a los 4 días de FE la fluorescencia de las mutantes *sod2*Δ y *sod1*Δ *sod2*Δ es mayor que en la cepa parental y la mutante *sod1*Δ (FIGURA 11, B). Cuando la FE llega a los 7 días, observamos que las mutantes en SOD tienden a tener mayor fluorescencia que la cepa parental (FIGURA 12). Estos datos sugieren que *SOD1* y *SOD2* de *C. glabrata* regulan de forma diferente y en momentos distintos los niveles de $O_2^{\bullet-}$ durante la FE.

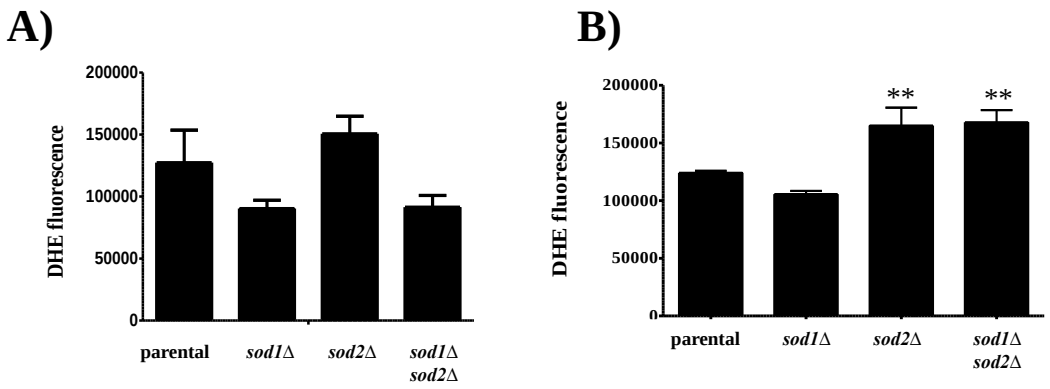


FIGURA 11. Niveles de $O_2^{\bullet-}$ en las mutantes de SOD a los 2 y 4 días de FE. Se detectaron los niveles de

fluorescencia usando la sonda DHE (400µM) en el fluórometro. Cultivos saturados de la cepa parental Ura$^-$ (CGM1719), y en las mutantes *sod1*Δ (CGM1721), *sod2*Δ (CGM1723), y *sod1*Δ *sod2*Δ (CGM1725) se crecieron en medio SD con 120ug/ml de lisina durante A) 2 y B) 4 días en FE. Los datos representan la media ± desviación estándar de cultivos por triplicado. Resultados similares se encontraron usando otras concentraciones de DHE (100 y 200µM).**, P < 0.01. Ver Materiales y Métodos.

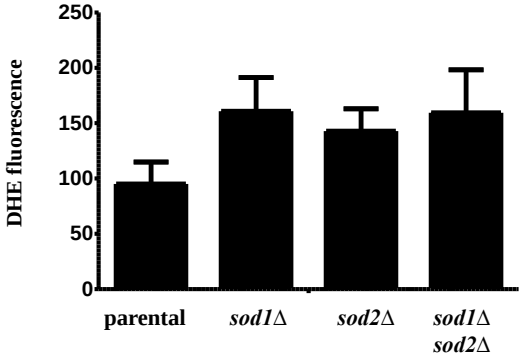


FIGURA 12. Niveles de $O_2^{\bullet-}$ en las mutantes de SOD a los 7 días de FE. Los niveles de fluorescencia detectados por dihidroetidio (DHE, 50µM) en el citómetro de flujo fueron medidos en la cepa parental Ura$^-$ (CGM1719), y en las mutantes *sod1*Δ (CGM1721), *sod2*Δ (CGM1723), y *sod1*Δ *sod2*Δ (CGM1725) después de 7 días en fase estacionaria. Resultados similares se encontraron usando otras concentraciones de DHE (25 y 150 µM). Los datos representan la media ± desviación estándar de cultivos por cuadruplicado. Ver Materiales y Métodos.

Expresión de *SOD1* y *SOD2* de *C. glabrata* durante el tratamiento con menadiona y en presencia de una fuente de carbono no fermentable.

a. Inducción de SODs en fase estacionaria

Las SOD son enzimas clave en la respuesta a estrés oxidante en diferentes organismos. Su regulación transcripcional es esencial para llevar a cabo una respuesta rápida y adecuada contra especies reactivas de oxigeno en especial el superóxido. En *C. glabrata*, esta descrito que *SOD1* y *SOD2* se transcriben constitutivamente y no están controlados por Yap1 o Skn7, factores de transcripción importantes para la respuesta a estrés oxidante (Roetzer *et al.*, 2011). En este trabajo, decidimos analizar la participación de otros dos factores de transcripción que tiene también un papel en la respuesta a estrés oxidante en *C. glabrata* (Cuellar-Cruz *et al.*, 2008). Estos factores transcripcionales llamados Msn2 y Msn4 son conocidos por formar un heterodimero que responde a señales de estrés general en *S. cerevisiae*.

Para investigar la regulación transcripcional de los genes *SOD1* y *SOD2* de *C. glabrata* y la participación de factores de transcripción, construimos fusiones transcripcionales con GFP: (P_{SOD1}::GFP::3´UTR$_{SOD1}$ y P_{SOD2}::GFP::3´UTR$_{SOD2}$). Cada fusión se transformó en la cepa parental, la doble mutante *sod1*Δ *sod2*Δ*,* las mutantes dobles en los factores de transcripción de estrés *yap1*Δ *skn7*Δ*, msn2*Δ *msn4*Δ y en la cuádruple mutante *yap1*Δ *skn7*Δ *msn2*Δ *msn4*Δ. Cultivos saturados de estas cepas se crecieron hasta FE en medio CAA y se cuantifico la fluorescencia de GFP en un citómetro de flujo. La fluorescencia se normalizo con respecto a la cepa parental. Encontramos que la doble mutante *sod1*Δ *sod2*Δ tuvo más fluorescencia relativa que la cepa parental. Como demostramos anteriormente, esta doble mutante tiene un nivel más alto de superóxido que la cepa parental (FIGURA 13). Por lo tanto, podemos inferir que el aumento de la fluorescencia se debe a que el exceso de superóxido induce la expresión episomal de las fusiones transcripcionales de SODs con GFP. Por otra parte, la mutante doble *yap1*Δ *skn7*Δ tuvo fluorescencia similar a la cepa parental. Este dato confirma lo descrito en la literatura sobre que Yap1 y Skn7 no regulan a las SODs en *C. glabrata* (Roetzer *et al.*, 2011). Sorprendentemente, encontramos que las cepas *msn2*Δ*msn4*Δ y *yap1*Δ *skn7*Δ *msn2*Δ *msn4*Δ que contienen las fusiones de *SOD1* y *SOD2* mostraron menor fluorescencia que la cepa parental (FIGURA 13). Este dato sugiere que posiblemente los factores de transcripción Msn2 y Msn4 estén involucrados en la regulación de la expresión de las SODs durante FE. Para apoyar esta hipótesis, cuantificamos la actividad de SOD total en cultivos de FE de la mutante cuádruple *yap1*Δ *skn7*Δ *msn2*Δ *msn4*Δ*.* Encontramos que esta mutante muestra menor actividad de SOD que la cepa parental (FIGURA 14), lo cual indica que estos factores de transcripción si regulan la expresión basal de las SODs en FE.

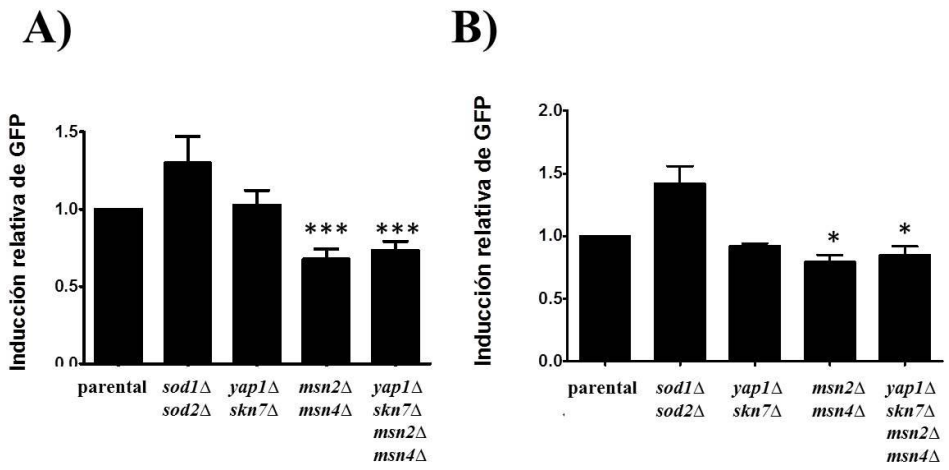


FIGURA 13. Expresión basal de *SOD1* y *SOD2* en fase estacionaria. Cultivos saturados de las cepas parental Ura+ (CGM1080), *sod1*Δ *sod2*Δ (CGM1088), *yap1*Δ *skn7*Δ (CGM1256), *msn2*Δ *msn4*Δ (CGM1258) y *yap1*Δ *skn7*Δ *msn2*Δ *msn4*Δ (CGM1084) complementadas con el plásmido pMB66 (P_{SOD1}::GFP::3´UTR$_{SOD1}$) en el inciso (A); y de las cepas B) parental Ura+ (CGM1082), *sod1*Δ *sod2*Δ (CGM1090), *yap1*Δ *skn7*Δ (CGM1276), *msn2*Δ *msn4*Δ (CGM1278), y *yap1*Δ *skn7*Δ *msn2*Δ *msn4*Δ (CGM1086) complementadas con el plásmido pMB68 (P_{SOD2}::GFP::3´UTR$_{SOD2}$) en el inciso (B) fueron crecidas en medio SC durante 48 horas a 30 °C. Se cuantifico la fluorescencia de GFP por célula en el citómetro de flujo Beckman coulter. Se normalizo a 1.0 con respecto a la parental. Las barras de error indican desviación estándar. ***, P<0.0001; prueba ANOVA una vía, post-prueba Dunnett *vs*. cepa parental. Ver Materiales y Métodos.

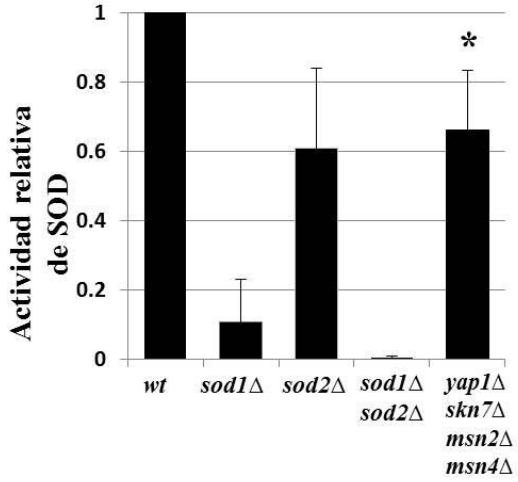


FIGURA 14. Actividad de SOD en la cepa silvestre y las mutantes *sod1*Δ, *sod2*Δ, *sod1*Δ *sod2*Δ y *yap1*Δ *skn7*Δ *msn2*Δ *msn4*Δ de *C. glabrata.* Cultivos saturados de la cepa parental Ura + (CGM1), y las mutantes *sod1*Δ (CGM787), *sod2*Δ (CGM656), *sod1*Δ *sod2*Δ (CGM937), *yap1*Δ *skn7*Δ *msn2*Δ *msn4*Δ (CGM527)

crecieron en YPD a 30˚C. Se hicieron extractos proteicos de estas cepas y su actividad total de SOD se cuantificó con el método colorimétrico WST-1. Los valores se normalizaron con respecto a la cepa parental. Las barras de error indican desviación estándar. *, P<0.05; prueba ANOVA una vía, post-prueba Dunnett *vs*. cepa parental. Ver Materiales y Métodos.

b. Inducción de SODs en glucosa y glicerol

El cambio de glucosa a glicerol o etanol induce la respiración mitocondrial y la expresión de genes de defensa antioxidante para contrarrestar el incremento de las especies reactivas de oxígeno generadas en la mitocondria. Recientemente se demostró la inducción de *SOD1* de *C. glabrata* durante la privación de glucosa. Además, en este trabajo demostramos que Sod2 se requiere para un óptimo crecimiento en glicerol y etanol. Lo que sugiere que las SODs están relacionadas con la fuente de carbono que utiliza *C. glabrata*.

Para conocer la expresión de las SODs en presencia de glucosa o glicerol y si ésta depende de los factores de transcripción Yap1, Skn7, Msn2 y/o Msn4 medimos la fluorescencia de las fusiones transcripcionales con GFP (P_{SOD1}::GFP::3´UTR$_{SOD1}$ y P_{SOD2}::GFP::3´UTR$_{SOD2}$). Cultivos saturados en presencia de glucosa de la cepa parental y las mutantes dobles, triples y cuádruples de los factores de transcripción Yap1, Skn7, Msn2 y/o Msn4 conteniendo las fusiones P_{SOD1}::GFP o P_{SOD2}::GFP fueron diluidos en medio fresco con glucosa o glicerol. Después de una hora, se cuantifico la fluorescencia de GFP en el citómetro de flujo. Encontramos que las SODs de *C. glabrata* se indujeron en medio fresco con glucosa o glicerol en comparación con FE (FIGURA 15) y aún en ausencia de los factores de transcripción Yap1, Skn7, Msn2 y/o Msn4. Estos resultados sugieren que las SODs responden al cambio a medio fresco y/o a la fuente de carbono, pero lo hacen independientemente de los factores de transcripción de respuesta a estrés.

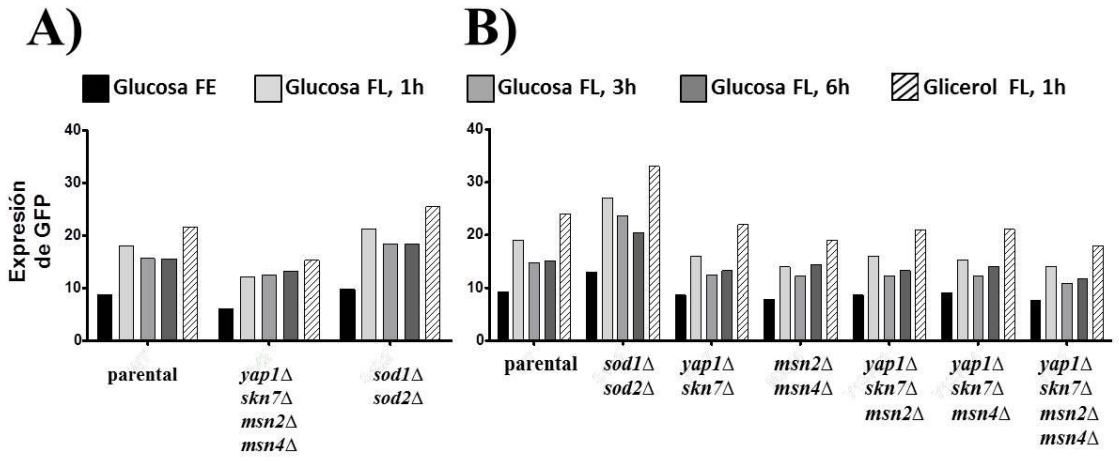


FIGURA 15. Inducción de las SODs en FL. Cultivos saturados de las cepas A) parental Ura+ (CGM1080), *yap1*Δ *skn7*Δ *msn2*Δ *msn4*Δ (CGM1084) y *sod1*Δ *sod2*Δ (CGM1088) complementadas con el plásmido pMB66 (P_{SOD1}::GFP::3´UTR_{SOD1}) y de las cepas B) parental Ura+ (CGM1082), *sod1*Δ *sod2*Δ (CGM1090), *yap1*Δ *skn7*Δ (CGM1276), *msn2*Δ *msn4*Δ (CGM1278), *yap1*Δ *skn7*Δ *msn2*Δ (CGM1280), *yap1*Δ *skn7*Δ *msn4*Δ (CGM1282) y *yap1*Δ *skn7*Δ *msn2*Δ *msn4*Δ (CGM1086) complementadas con el plásmido pMB68 (P_{SOD2}::GFP::3´UTR_{SOD2}) fueron crecidos en medio SC y diluidos a OD_{600nm} de 0.1 en medio fresco con glucosa o glicerol. Se cuantifico la fluorescencia de GFP por célula emitida a 1, 3 y 6h en FL con glucosa y 1h en FL con glicerol. Se usó el citómetro de flujo Beckman coulter. Ver Materiales y Métodos.

Sod1 tiene un papel en la recombinación no homologa (NHEJ) de *C. glabrata*

En resultados anteriores demostramos que las SODs de *C. glabrata* tienen un papel importante en la estabilidad del ADN, ya que protegen al genoma de mutaciones causadas por daño oxidante. Existe la posibilidad de que las SODs y los mecanismos de reparación del ADN estén interconectados. Nosotros hemos demostrado que las mutantes en SOD de *C. glabrata* son sensibles a las genotoxinas HU y MMS. Estos agentes activan los puntos de control del ciclo celular en respuesta al daño oxidante, lo que provoca arresto celular y activación de la maquinaria de reparación del ADN.

Para continuar la investigación, decidimos determinar la habilidad de las mutantes en SODs para reparar cortes de ADN de doble cadena. Las mutantes se transformaron con plásmidos linearizados. Dentro de la célula es necesario que este ADN episomal se recircularice para poder ser replicado y obtener transformantes estables que puedan crecer. La vía de reparación de cortes de doble cadena de ADN es la recombinación no homologa (NHEJ, por sus siglas en ingles). Por lo tanto, el número de transformantes obtenidas en cada cepa con el plásmido linealizado se normalizó para el número de colonias obtenidas con el plásmido sin cortar. De esta forma se midió la eficiencia de unión de extremos no homólogos. Sorprendentemente encontramos que las mutantes *sod1*Δ y *sod1*Δ *sod2*Δ tienden a ser más eficientes en la reparación de plásmidos linealizados que la cepa parental (FIGURA 16). Esto sugiere que Sod1 interviene de forma negativa en la actividad de la vía NHEJ.

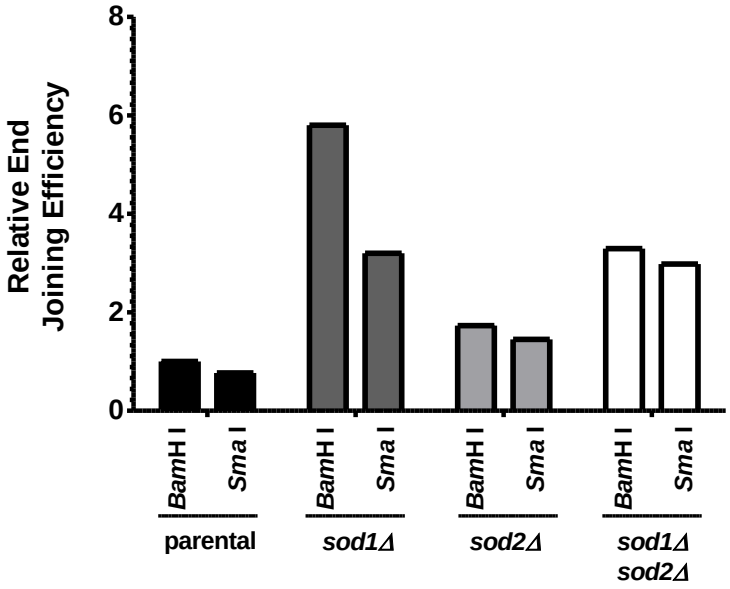


FIGURA 16. Papel de las SODs de *C. glabrata* en la recombinación no homologa (NHEJ). Determinación de la eficiencia de la vía de unión de extremos no homólogos *in vivo* utilizando un plásmido *CEN.URA3* (pGRB2.0) linealizado ya sea con *Sma* I (extremos romos) o *Bam*H I (extremos cohesivos). Las cepas usadas fueron: parental (CGM1), *sod1*Δ (CGM787), *sod2*Δ (CGM656), *sod1*Δ *sod2*Δ (CGM937). La unión de extremos se calculo como el número de transformantes obtenidas para cada cepa con el plásmido linealizado dividido entre el número de colonias obtenidas con el plásmido sin cortar. El valor de la cepa parental con *Bam* HI se normalizó a 1.

4. Discusión

Los patógenos pueden evadir la respuesta oxidante de las células fagociticas gracias a los mecanismos enzimáticos y no enzimáticos con los que cuenta para eliminar las especies reactivas de oxigeno (ERO). De esta forma los patógenos pueden sobrevivir y persistir en el huésped. Recientemente se ha estudiado la respuesta a estrés oxidante de la levadura patógena oportunista *C. glabrata*. Este hongo ha emergido como un importante patógeno en pacientes inmunocomprometidos. Causa infecciones en mucosas e infecciones invasivas diseminadas y en E.U.A. es el segundo lugar en infecciones sistémicas nosocomiales causadas por hongos (solo después de *C. albicans*) (Pfaller *et al.*, 2000). En los últimos años, se han descrito algunos factores de virulencia para *C. glabrata* como la adherencia a células epiteliales (Cormack *et al.*, 1999; De Las Penas *et al.*, 2003; Domergue *et al.*, 2005), formación de biofilms (Iraqui *et al.*, 2005), alta resistencia a fluconazol (Hitchcock *et al.*, 1993; Komshian *et al.*, 1989; Willocks *et al.*, 1991) y una gran resistencia a estrés oxidante (Cuellar-Cruz *et al.*, 2008; Cuéllar-Cruz *et al.*, 2009). Además, se ha visto que *C. glabrata* puede sobrevivir al ataque oxidante de los macrófagos (Kaur *et al.*, 2007; Seider *et al.*, 2011). Los mecanismos que permiten a *C. glabrata* adaptarse durante la infección crónica y persistir en el huésped inmunocomprometido aun no están descritos.

Para conocer la respuesta a estrés oxidante de *C. glabrata*, hemos investigado la resistencia de este hongo a diferentes oxidantes. Demostramos que *C. glabrata* resiste concentraciones más altas de H_2O_2 que *C. albicans* y que *S. cerevisiae* (Cuellar-Cruz *et al.*, 2008). Los factores de transcripción (FT) que controlan esta resistencia son Yap1, Skn7, Msn2 y Msn4 y esta mediada por la única catalasa, Cta1, que tiene *C. glabrata*. También hemos encontrado que *C. glabrata* no es tan resistente a menadiona (MD) como *C. albicans*, debido posiblemente a que *C. albicans* tiene 6 genes que codifican para SODs. El daño que produce la MD se debe al aumento de $O_2^{\bullet-}$ en la célula (Bolton *et al.*, 2000). Este $O_2^{\bullet-}$ puede ser eliminado por SODs. De forma interesante, las cepas de laboratorio de *S. cerevisiae* son menos resistente a MD que *C. glabrata* y *C. albicans* y en cambio aislados clínicos de *S. cerevisiae* son más resistentes a MD y a H_2O_2 (Cuéllar-Cruz *et al.*, 2009). Este último dato sugiere que para poder ser un patógeno exitoso se requiere de una adecuada respuesta a estrés oxidante. Para continuar el estudio de la respuesta a estrés oxidante de *C. glabrata*, decidimos caracterizar funcionalmente a las SODs.

En este trabajo presentamos evidencias de que la enzima citosolica *SOD1* es más importante que la mitocondrial *SOD2* ante una exposición aguda a menadiona (MD) y en fase logarítmica (FL) y ante la exposición crónica a MD y H_2O_2. En cambio *SOD2* es necesaria ante una exposición a oxidantes en fase estacionaria (FE) y para crecer en fuentes de carbono no fermentables. La ausencia de las dos SODs en *C. glabrata* provoca auxotrofía a lisina y una alta tasa de mutación espontánea. Además, reportamos que *C. glabrata* es más longeva que *S. cerevisiae* en FE y *SOD2* de *C. glabrata* participa en detener el envejecimiento de la levadura.

Auxotrofia a lisina de la doble mutante *sod1*Δ *sod2*Δ

En este trabajo demostramos que la doble mutante en SODs de *C. glabrata* es auxótrofa a lisina. Esto quiere decir que Sod1 y Sod2 son importantes para la biosíntesis de lisina. Lo que relaciona el estrés oxidante generado por $O_2^{\bullet-}$ con el metabolismo de aminoácidos. En otros organismos se ha demostrado que las mutantes en SOD son auxótrofas de aminoácidos. La mutante *sodA sodB* de *E. coli* es auxótrofa a aminoácidos de cadena ramificada (Kuo *et al.*, 1987) y las mutantes *sod1*Δ de *S. cerevisiae* y *S. pombe* son auxótrofas a lisina bajo condiciones aeróbicas (Kwon *et al.*, 2006). Se ha reportado que estas auxotrofias se deben a la inactivación por $O_2^{\bullet-}$ de diferentes dehidratasas involucradas en la biosíntesis de esos aminoácidos. Las dehidratasas son enzimas que contienen grupos [4Fe-4S] los cuales son oxidados por el $O_2^{\bullet-}$, provocando la liberación de una molécula de hierro (D'Autréaux & Toledano, 2007). En *S. cerevisiae,* se sugiere que la auxotrofía a lisina de la mutante *sod1*Δ resulta por la inactivación de la homoaconitasa (Lys4) y en *S. pombe* por la homocitrato sintasa (Kwon *et al.*, 2006; Wallace *et al.*, 2004). Estas enzimas participan en la biosíntesis de lisina (Xu *et al.*, 2006). Lys4 está localizada en la matriz mitocondrial y se predice que tiene el dominio [4Fe-4S](Irvin & Bhattacharjee, 1998; Xu *et al.*, 2006). Por otro lado Lys20/Lys21 son las homocitrato sintasa de *S. cerevisiae*, pero no tienen grupo [4Fe-4S]. Se ha demostrado que la ausencia de *SOD1* en *S. cerevisiae* provoca la inactivación de Lys4 solo en presencia de oxígeno (Wallace *et al.*, 2004) concluyendo que la inactivación de Lys4 por $O_2^{\bullet-}$ es la causa de la auxotrofía en *S.c.sod1*Δ. Esto tendría sentido, si no fuera por el hecho de que Sod1 se localiza en el espacio intermembranal de la mitocondria (EIM) y Lys4 en la matriz mitocondrial. Entonces, ¿cómo es que Sod1 puede proteger a Lys4 del ataque por $O_2^{\bullet-}$ si están en compartimentos diferentes? Se ha sugerido que los niveles altos de $O_2^{\bullet-}$ en el EIM en la mutante *sod1*Δ, difunden atreves de la membrana interna y oxidan a Lys4 en la matriz mitocondrial. También se sugirió que el $O_2^{\bullet-}$ del EIM en *sod1*Δ reacciona con el NO endógeno para formar HOONO el cual difunde a la matriz e inactiva a Lys4 (Liochev & Fridovich, 2005).

Nuestros datos muestran que no hay auxotrofias a lisina en las mutantes sencillas *sod1*Δ y *sod2*Δ, pero si en la doble mutante *sod1*Δ *sod2*Δ. Estos resultados indican que el papel en la protección de la vía de biosíntesis de lisina de Sod1 y Sod2 de *C. glabrata* es redundante. Esto quiere decir que se requiere al menos la presencia de una de ellas para proteger la vía de biosíntesis. Los datos presentados en este trabajo no alcanzan a elucidar la causa de la auxotrofía a lisina de la doble mutante *sod1*Δ *sod2*Δ de *C. glabrata*. Observamos que al sobreexpresamos los genes *LYS4*, *LYS12*, *LYS20*, *LYS21* en la doble mutante *sod1*Δ *sod2*Δ no se suprimía la auxotrofia a lisina lo que nos indica que un gen diferente o más de un gen dentro de la via de biosíntesis de lisina son los responsables de la auxotrofia. Falta probar la actividad de la enzima Lys4 en la mutante *sod1*Δ *sod2*Δ. Para conocer si esta enzima está involucrada en la auxotrofía a lisina de la mutante *sod1*Δ *sod2*Δ de *C. glabrata*, podríamos medir la actividad de Lys4 en la doble mutante *sod1*Δ *sod2*Δ de *C. glabrata* y comparar con la cepa silvestre. Si la actividad fuera menor en la mutante *sod1*Δ *sod2*Δ, supondríamos que esta enzima está siendo inactivada por $O_2^{\bullet-}$ y que esto puede ser la causa de la auxotrofía. Para saber si el $O_2^{\bullet-}$ es el causante directo de la

auxotrofía a lisina podríamos crecer a la mutante *sod1*Δ *sod2*Δ en ausencia de lisina pero ahora en condiciones de anaerobiosis y observar si se borra el fenotipo descrito. Esta reportado que la auxotrofía a lisina de la mutante *sod1*Δ de *S. cerevisiae* es dependiente de oxigeno (Gralla & Valentine, 1991; Wallace *et al.*, 2004). Si la auxotrofía a lisina es a causa de la inactivación de proteína que contiene el grupo [4Fe-4S], el hierro libre podría ser mayor en la doble mutante *sod1*Δ *sod2*Δ. Esto sugeriría que el $O_2^{\bullet-}$ esta oxidando a grupos [4Fe-4S] y liberando hierro.

Aumento en el daño oxidante al ADN en la doble mutante *sod1*Δ *sod2*Δ

El ADN es uno de los blancos más importantes de las EROs, y mantener su integridad es vital para la célula. Si las lesiones en el ADN no se reparan, se ocasionan mutaciones que contribuyen al envejecimiento celular como sustitución de bases hasta rearreglos cromosomales (Imlay, 2003).

En este trabajo reportamos que la doble mutante *sod1*Δ *sod2*Δ de *C. glabrata* tiene una tasa de mutación espontánea mayor que las mutantes sencillas y la cepa parental. Este resultado indica que la ausencia de ambas SODs provoca daño al ADN, lo que sugiere un papel protector. El papel de las SODs en el mantenimiento de la estabilidad del ADN está bien documentado en los microorganismos. La primera evidencia de que el $O_2^{\bullet-}$ estaba implicado en el daño al ADN, fue en la mutante en SOD de *E. coli* la cual es hipermutagénica (Farr *et al.*, 1986). Después, se observo altas tasas de mutación en las mutantes de SOD de *S. cerevisiae* (Gralla & Valentine, 1991; Huang *et al.*, 2003), *Neurospora crassa* (Sod1) (Chary *et al.*, 1994), *Drosophila melanogaster* (Sod1) (Woodruff *et al.*, 2004), entre otros. Pero, ¿Cuál es el papel de las SOD en la estabilidad del ADN? Existen varias hipótesis. La más antigua es que las SOD protegen al ADN del daño ocasionado por el radical hidroxilo (•OH) y el hierro libre, los cuales se generan a través del $O_2^{\bullet-}$. El •OH puede adicionar dobles enlaces a las bases o extraer un átomo de H al grupo metilo de la timina (Cooke *et al.*, 2003). El $O_2^{\bullet-}$ puede aumentar el hierro libre en la célula al oxidar los grupos [4Fe-4S] de las proteínas y este hierro catalizar lesiones en el ADN. Si la cantidad de hierro libre aumenta, el daño oxidante al ADN se acelera (Keyer *et al.*, 1995). Otra hipótesis, es que las SODs actúan como detectores del $O_2^{\bullet-}$ bajo condiciones de un desbalance redox, activando y controlando los mecanismos de reparación del ADN. Datos recientes sobre interacciones proteicas de las SOD de *S. cerevisiae*, sugieren que posiblemente Sod1 participe en el circuito de detección del daño oxidante al ADN y actué en cooperación para el control del ciclo celular. En cambio, Sod2 parece interaccionar con las vías de reparación del daño al ADN: unión de extremos no homólogos (NHEJ) y reparación por escisión de nucleótidos (NER) (Bonatto, 2007). Datos prelimianres en el laboratorio muestran que Sod1 podría tener un papel en la recombinación no homologa (NHEJ) de *C. glabrata* pero es necesario continuar con los experimentos para confirmar posibles fenotipos.

Nuestro modelo actual sobre el papel que desempeñan las SODs de ***C. glabrata*** en la protección al ADN es que al no estar presentes estas enzimas la cantidad de $O_2^{\bullet-}$ en la levadura aumenta y ataca a los grupos [4Fe-4S] y libera hierro. El $O_2^{\bullet-}$, H_2O_2 y el hierro libre generan •OH el cual oxida al ADN ocasionando mutaciones. Es posible que las vías de reparación del ADN estén en descontrol o inactivas,

debido a la ausencia de las SOD. Todo esto conlleva a la muerte celular o a la generación de mutantes supresoras. Cabe señalar que el experimento de tasa de mutación se hizo con células de FE en donde sabemos que tanto Sod1 como Sod2 son esenciales para eliminar las ERO. ¿Es posible que la tasa de mutación para las mutantes en SOD cambie si lo hacemos con células de FL? Hay que considerar que las células en FE se distinguen por salir del ciclo celular. Por lo tanto el daño oxidante se acumula debido a que las ERO no se puede diluir en células hijas. Este daño solo puede ser prevenido o reparado. Es posible que no observemos diferencias importantes entre las mutantes y la silvestre en la tasa de mutación espontanea en FL debido a que el daño oxidante se hubiese diluido en las hijas. Si esto fuese cierto, mientras más larga sea la FE, mayor el daño al DNA (Finkel & Holbrook, 2000). Aunque no tenemos ese experimento si tenemos el dato de que la cantidad de $O_2^{\bullet -}$ en la levadura al día 7 (FE tardía) es alta en las mutantes de SOD. Para elucidar la vía por la cual las SOD protegen al ADN del daño oxidante podríamos evaluar si el hierro libre causa lesiones al ADN, para esto podemos añadir quelantes de hierro (deferipron o 2,2 dipiridilo) y observar si la genotoxicidad disminuye.Para saber si alguno de los sistemas de reparación del ADN están involucrados, podemos introducir de forma episomal en la mutante *sod1*Δ *sod2*Δ genes involucrados en los principales sistemas de reparación y valorar si la tasa de mutación disminuye. Incluso podemos seleccionar en la mutante *sod1*Δ *sod2*Δ transformada con una librería genómica de *C. glabrata* (construida en el laboratorio), supresoras que revirtieran el fenotipo de la doble mutante. Es posible que pudiéramos encontrar genes de los sistemas de reparación o metabolismo del hierro.

5. Perspectivas

Los resultados obtenidos durante mi doctorado nos permitieron conocer el papel de las superóxido dismutasas de *C. glabrata* durante la respuesta a estrés oxidante. Además, encontramos que estas SODs están involucradas en otros aspectos del metabolismo general de la levadura. Sod2 tiene un papel primordial durante la fase estacionaria de *C. glabrata*, impidiendo el daño oxidante debido a la respiración celular y evitando el envejecimiento de la levadura. Por otro lado, Sod1 ayuda al buen funcionamiento de la vía de biosíntesis de lisina. Nuestros datos sugieren que Sod1 evita el daño oxidante a una o varias de las enzimas de esta vía. Esto debido a que probablemente contienen grupos Fe-S en su sitio activo y que son blancos del superóxido. Además, tenemos evidencia de que las SODs protegen al ADN del daño oxidante.

Aunque hemos obtenido avances importantes sobre el estudio de las SODs en *C. glabrata*, quedan varias preguntas aún sin resolver. Por ejemplo, sería interesante poder identificar el blanco que es protegido por las SODs y que debido a su oxidación, las células de *C. glabrata* se vuelven auxótrofas a lisina. Nosotros consideramos que es muy probable que no solo una enzima de la vía de biosíntesis de lisina este siendo dañada. Para poder descubrir la causa exacta de la auxotrofia a lisina de la mutante en SODs de *C. glabrata* llevaremos a cabo los siguientes experimentos: 1) medir la actividad de homoaconitasa en la doble mutante *sod1*Δ *sod2*Δ de *C. glabrata* para clarificar si Lys4 (homoaconitasa) es o no es la enzima oxidada. 2) Podríamos utilizar la librería de *C. glabrata* con la que contamos en el laboratorio para identificar genes que supriman la auxotrofia a lisina. 3) Cuantificar los niveles de intermediarios en la vía de biosíntesis de lisina para reconocer el punto en el cual se detiene la producción de lisina en la doble mutante *sod1*Δ *sod2*Δ de *C. glabrata*.

Otro tema muy interesante que sigue inconcluso es el papel de las SODs en la protección al ADN. Está demostrado que el superóxido daña los grupos Fe-S de algunas enzimas como por ejemplo la aconitasa y homoaconitasa. Este daño oxidante provoca la inactivación de las enzimas. Las SODs tienen un papel clave en la protección de las enzimas con grupos Fe-S. Recientemente, se ha observado que proteínas involucradas en las vías de reparación al ADN tienen grupos Fe-S en su sitio activo. Por otra parte, las mutantes en SODs de *C. glabrata*, *S. cerevisiae* y otros hongos son sensibles a genotóxicos y muestran daño al ADN (tasa de mutación alta). Entonces, ¿a qué se debe el aumento de tasa de mutación espontanea en las mutantes de SOD de *C. glabrata*? La explicación más estudiada es que la ausencia de SODs en la célula provoca un aumento de especies reactivas de oxigeno (ERO), las cuales dañan directamente el ADN. Pero nosotros pensamos en otra posibilidad que aún no hemos probado: ¿qué tal si las ERO en la célula dañan a las enzimas que contienen grupos Fe-S y que están involucradas en las vías de reparación al ADN? Si esto sucediera, se podría explicar el daño al ADN debido a que no es posible repararlo porque las vías están inactivas. Para probar esta hipótesis es necesario tener evidencias de varios hechos: 1) identificar enzimas de las vías de reparación de *C. glabrata* que contienen grupos Fe-S. 2) Si este tipo de enzimas existen, entonces podríamos

evaluar si están activas o no en la doble mutante *sod1*Δ *sod2*Δ de *C. glabrata*. 3) Correlacionar el tipo de mutaciones que se generan en esta mutante con la vía de reparación que podría estar dañada.

Sorpresivamente, hemos encontrado en este trabajo que las SODs no tiene un papel en la virulencia de *C. glabrata*. Esto no concuerda con lo que se sabe en otros hongos oportunistas patógenos como *C. albicans* y *C. neoformans*. Una posibilidad es que las mutantes de SOD en *C. glabrata* colonicen los órganos de ratón al mismo nivel que la cepa parental debido a su efecto mutagénico. Hemos pensado que si la doble mutante *sod1*Δ *sod2*Δ es capaz de mutar con mayor frecuencia que la parental, entonces es posible que estas mutaciones permitan la adecuada colonización en el ratón. Por tanto sería conveniente evaluar el estado de estas mutantes en el contexto *in vivo*. Podríamos evaluar si las mutantes de SODs de *C. glabrata* generan mutaciones estando dentro del ratón o en presencia de macrófagos. Así podríamos recuperar las células en contacto con ratones o macrófagos y evaluar la integridad del ADN. Si encontráramos mutaciones podríamos también identificar los genes en los cuales sucedieron los cambios genéticos y probar si estos genes están involucrados en la colonización de *C. glabrata* o si ayudan a las mutantes de SOD a sobrevivir a estas condiciones.

6. Material y métodos

Cepas de *E. coli*
Las cepas utilizadas en este trabajo están descritas en la Tabla 1.

Plásmidos
Todos los plásmidos usados en este trabajo están descritos en la Tabla 2.

Oligonucleótidos
Los oligonucleótidos usados en este trabajo están descritos en la Tabla 3.

Tabla 1. Cepas usadas en este trabajo

Cepa	Parental	Genotipo y/o descripción	Referencia o fuente
Cepas de *E. coli*			
DH10B		F *mcrA* (*mrr-hsdRMS-mcrBC*) 80d*lacZ M15 lacX74 deoR recA1 endA1 araD139* (*ara*,*leu*)*7697 galU galK rpsL nupG*	(Calvin & Hanawalt, 1988)
Cepas de *S. cerevisiae*			
BY4742		*MAT*α *his3*Δ*1*, *leu2*Δ, *lys2*Δ*, ura3*Δ	(Brachmann *et al.*, 1998)
L108	BY4742	*MAT*α *his3*Δ*1*, *leu2*Δ, *lys2*Δ*, ura3*Δ, *sod1*Δ::*KanMX* Kan^R	Colección del laboratorio de Rine
L109	BY4742	*MAT*α *his3*Δ*1*, *leu2*Δ, *lys2*Δ*, ura3*Δ, *sod1*Δ::*KanMX* Kan^R	Colección del laboratorio de Rine
Cepas de *C. glabrata*			
BG2		Aislado clínico (cepa B)	(Fidel *et al.*, 1996)
BG14	BG2	*ura3*Δ*::*Tn*903* G418^R*;* parental Ura$^-$ strain used in this study.	(Cormack *et al.*, 1999)
CGM139	BG14	*URA3*	(De Las Penas *et al.*, 2003)
CGM527		*ura3*Δ*::*Tn*903* G418^R *yap1*Δ *skn7*Δ *msn4*Δ *msn2*Δ*::hph* Hyg^R	(Cuellar-Cruz *et al.*, 2008)
CGM656	BG14	*ura3*Δ*::*Tn*903* G418^R; *sod2*Δ*::NatMX4* Nat^R Ura$^-$ (PCR de fusión, oligonucleótidos #228/#229)	Este trabajo
CGM787	BG14	*ura3*Δ*::*Tn*903* G418^R; *sod1*Δ*::NatMX4* Nat^R Ura$^-$ (PCR de fusión, oligonucleótidos #232/#233)	Este trabajo
CGM927	CGM656	*ura3*Δ*::*Tn*903* G418^R; *sod2*Δ Nat^S Ura$^-$	Este trabajo
CGM937	CGM927	*ura3*Δ*::*Tn*903* G418^R; *sod2*Δ *sod1*Δ*::NatMX4* Nat^R Ura$^-$ (PCR de fusión, oligonucleótidos #232/#233)	Este trabajo
CGM1080	BG14	*ura3*Δ*::*Tn*903* G418^R pMB66 Ura$^+$	Este trabajo
CGM1082	BG14	*ura3*Δ*::*Tn*903* G418^R pMB68 Ura$^+$	Este trabajo

CGM1084	CGM527	*ura3*Δ*::*Tn*903* G418^R *yap1*Δ *skn7*Δ *msn4*Δ *msn2*Δ*::hph* Hyg^R pMB66 Ura$^+$	Este trabajo
CGM1086	CGM527	*ura3*Δ*::*Tn*903* G418^R *yap1*Δ *skn7*Δ *msn4*Δ *msn2*Δ*::hph* Hyg^R pMB68 Ura$^+$	Este trabajo
CGM1088	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB66 Ura$^+$	Este trabajo
CGM1090	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB68 Ura$^+$	Este trabajo
CGM1131	CGM656	*URA3 sod2*Δ Nat^R Ura$^+$	Este trabajo
CGM1133	CGM937	*URA3 sod2*Δ *sod1*Δ Nat^R Ura$^+$	Este trabajo
CGM1146	CGM787	*URA3 sod1*Δ Nat^R Ura$^+$	Este trabajo
CGM1256	CGM835	*ura3*Δ*::*Tn*903* G418^R *yap1*Δ *skn7*Δ Hyg^R pMB66 Ura$^+$	Este trabajo
CGM1258	CGM385	*ura3*Δ*::*Tn*903* G418^R *msn4*Δ *msn2*Δ Hyg^R pMB66 Ura$^+$	Este trabajo
CGM1276	CGM835	*ura3*Δ*::*Tn*903* G418^R *yap1*Δ *skn7*Δ Hyg^R pMB68 Ura$^+$	Este trabajo
CGM1278	CGM385	*ura3*Δ*::*Tn*903* G418^R *msn4*Δ *msn2*Δ Hyg^R pMB68 Ura$^+$	Este trabajo
CGM1280	CGM837	*ura3*Δ*::*Tn*903* G418^R *yap1*Δ *skn7*Δ *msn2*Δ Hyg^R pMB68 Ura$^+$	Este trabajo
CGM1282	CGM477	*ura3*Δ*::*Tn*903* G418^R *yap1*Δ *skn7*Δ *msn4*Δ Hyg^R pMB68 Ura$^+$	Este trabajo
CGM1637	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB131 Ura$^+$	Este trabajo
CGM1639	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB101 Ura$^+$	Este trabajo
CGM1640	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB127 Ura$^+$	Este trabajo
CGM1642	CGM787	*ura3*Δ*::*Tn*903* G418^R *sod1*Δ*::NatMX4* Nat^R pMB101 Ura$^+$	Este trabajo
CGM1644	BG14	*ura3*Δ*::*Tn*903* G418^R pMB131 Ura$^+$	Este trabajo
CGM1646	BG14	*ura3*Δ*::*Tn*903* G418^R pMB142 Ura$^+$	Este trabajo
CGM1648	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB101 Ura$^+$	Este trabajo
CGM1650	CGM937	*ura3*Δ*::*Tn*903* G418^R; *sod2*Δ *sod1*Δ Nat^S Ura$^-$	Este trabajo
CGM1651	CGM656	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ*::NatMX4* Nat^R pMB144 Ura$^+$	Este trabajo
CGM1653	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB144 Ura$^+$	Este trabajo
CGM1663	BG14	*ura3*Δ*::*Tn*903* G418^R; *lys4*Δ*::NatMX4* Nat^R Ura$^-$ (PCR de fusión, oligonucleótidos #970/#972)	Este trabajo
CGM1670	BG14	*ura3*Δ*::Tn903 G418^R SOD1-GFP::hph* Hyg^R Ura$^-$ (pMB146/*Bst*E II-*Sac* I)	Este trabajo
CGM1672	BG14	*ura3*Δ*::Tn903 G418R SOD2-GFP::hph* Hyg^R Ura$^-$ (pMB122/*Blp* I)	Este trabajo
CGM1688	CGM1663	*ura3*Δ*::*Tn*903* G418^R; *lys4*Δ*::NatMX4* Nat^R pMB131 Ura$^+$	Este trabajo

CGM1690	CGM1663	*ura3*Δ*::*Tn*903* G418^R; *lys4*Δ*::NatMX4* Nat^R pMB142 Ura$^+$	Este trabajo
CGM1701	CGM1670	*ura3*Δ*::Tn903 G418^R SOD1-GFP* Hyg^S Ura$^-$	Este trabajo
CGM1703	CGM1672	*ura3*Δ*::Tn903 G418^R SOD2-GFP* Hyg^S Ura$^-$	Este trabajo
CGM1705	CGM1650	*ura3*Δ*::*Tn*903* G418^R; *sod2*Δ *sod1*Δ Nat^S pMB131 Ura$^+$	Este trabajo
CGM1707	CGM1650	*ura3*Δ*::*Tn*903* G418^R; *sod2*Δ *sod1*Δ Nat^S pMB142 Ura$^+$	Este trabajo
CGM1717	BG14	*ura3*Δ*::*Tn*903* G418^R pGRB2.0 Ura$^+$	Este trabajo
CGM1719	BG14	*ura3*Δ*::*Tn*903* G418^R pGRB2.0 Ura$^+$	Este trabajo
CGM1721	CGM787	*ura3*Δ*::*Tn*903* G418^R *sod1*Δ*::NatMX4* Nat^R pGRB2.0 Ura$^+$	Este trabajo
CGM1723	CGM656	*ura3*Δ*::*Tn*903* G418^R *sod1*Δ*::NatMX4* Nat^R pGRB2.0 Ura$^+$	Este trabajo
CGM1725	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pGRB2.0 Ura$^+$	Este trabajo
CGM1734	BG14	*ura3*Δ*::*Tn*903* G418^R pMB101 Ura$^+$	Este trabajo
CGM1736	BG14	*ura3*Δ*::*Tn*903* G418^R pMB144 Ura$^+$	Este trabajo
CGM1738	CGM1707	*ura3*Δ*::*Tn*903* G418^R; *sod2*Δ *sod1*Δ pMB142, pMB135 Ura$^+$ Nat^R	Este trabajo
CGM1740	BG14	*lys5*Δ Hyg^R Ura$^+$	(Castano *et al.*, 2003)
CGM1741	BG14	*lys9*Δ Hyg^R Ura$^+$	(Castano *et al.*, 2003)
CGM1742	BG14	*lys12*Δ Hyg^R Ura$^+$	(Castano *et al.*, 2003)
CGM1743	BG14	*lys14*Δ Hyg^R Ura$^+$	(Castano *et al.*, 2003)
CGM1749	BG14	*ura3*Δ*::*Tn*903* G418^R pMB127 Ura$^+$	Este trabajo
CGM1751	CGM787	*ura3*Δ*::*Tn*903* G418^R; *sod1*Δ*::NatMX4* Nat^R pMB131 Ura$^+$	Este trabajo
CGM1753	CGM787	*ura3*Δ*::*Tn*903* G418^R; *sod1*Δ*::NatMX4* Nat^R pMB142 Ura$^+$	Este trabajo
CGM1795	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB150 Ura$^+$	Este trabajo
CGM1797	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB152 Ura$^+$	Este trabajo
CGM1799	CGM937	*ura3*Δ*::*Tn*903* G418^R *sod2*Δ *sod1*Δ *::NatMX4* Nat^R pMB154 Ura$^+$	Este trabajo
CGM1816	CGM937	*ura3*Δ*::*Tn*903* G418^R; *sod1*Δ*::NatMX4*; *SOD2-GFP::hph* Hyg^R Nat^R Ura$^-$, (pMB122/*Blp* I)	Este trabajo
CGM1817	CGM937	*ura3*Δ*::*Tn*903* G418^R; *sod2*Δ; *SOD1-GFP::hph* Hyg^R Ura$^-$ (pMB146/*Bst*E II)	Este trabajo
CGM1885	CGM787	*ura3*Δ*::*Tn*903* G418^R; *sod1*Δ*::NatMX4* Nat^R pMB150 Ura$^+$	Este trabajo
CGM1887	CGM787	*ura3*Δ*::*Tn*903* G418^R; *sod1*Δ*::NatMX4* Nat^R pMB152 Ura$^+$	Este trabajo

CGM1889	CGM787	*ura3*Δ*::*Tn*903* G418^R; *sod1*Δ*::NatMX4* Nat^R pMB154 Ura$^+$	Este trabajo

Tabla 2. Plásmidos usados en este trabajo

Plásmido	Genotipo y/o descripción	Referencia o fuente
Plasmidos para clonación y construcción		
pMB11	Vector de clonación, *sacB* contra selección, Cm^R *ori* p15A	Colección de laboratorio
pGRB2.0	Vector de clonación, pRS406 *URA3 C.g.CEN ARS* Ap^R	(De Las Penas *et al.*, 2003)
pGRB2.2	Vector de clonación derivado de pGRB2.0, para fusiones con el promotor *PGK1 URA3 C.g.CEN ARS* Ap^R	(Domergue *et al.*, 2005)
pOZ16	Vector para marcar con *GFP*. Contiene un fragmento de 0.715 kb que codifica para *GFP* seguido de un fragment de 0.289 kb de la secuencia no codificante Cg*CTA1* 3' UTR y un cassette *hph*. Los cassette Cg*CTA1* 3' UTR y *hph* están flanqueados por dos repetidos directos FRT para remover el marcador de selección. [*GFP*-FRT-3'UTR$_{CgCTA1}$-P$_{ScPGK1}$::*hph*::3'UTR$_{ScHIS3}$-FRT] *URA3* Hyg^R Ap^R	Colección de laboratorio
pMZ21	Contiene a *FLP1* dirigido por el promotor *EPA1* de *C. glabrata*. La Flp recombinasa expresada de este plásmido puede reconocer los sitios *FRT* y reciclar el cassette *hph*. *URA3, Cg CEN ARS,* Ap^R	Colección del laboratorio de Cormack
pBC34.1	pUC19::Cg*URA3*; fragmento 2.2-kb *Pst* I; Ap^r	(Cormack *et al.*, 1999)
Plásmidos para complementación		
pMB101	Vector de complementación de *SOD1.* Un fragmento de 1.469 kb *Spe* I/*Sac* I conteniendo el ORF de *SOD*1 y sus secuencias no codificantes 5'y 3' (oligonucleótidos #783/#1086) clonado en pGRB2.0. *URA3*, *Cg CEN ARS*, Ap^R	Este trabajo
pMB144	Vector de complementación de *SOD2.* Un fragmento de 2.225 kb *Kpn* I/*Bam* HI conteniendo el ORF de *SOD*2 y sus secuencias no codificantes 5' y 3' (oligonucleótidos #786/#1106) clonado en pGRB2.0. *URA3*, *Cg CEN ARS*, Ap^R	Este trabajo
pMB127	Vector de complementación de *SOD1* y *SOD2.* Un fragmento de 2.225 kb *Kpn* I/*Bam* HI conteniendo el ORF de *SOD*2 y sus secuencias no codificantes 5' y 3' (oligonucleótidos #786/#1106) clonado en pMB101. *URA3*, *Cg CEN ARS*, Ap^R	Este trabajo
pMB131	Vector de complementación de *LYS4.* Un fragmento de 2.070 kb *Bam* HI/*Sal* I conteniendo el ORF de *LYS4* bajo el promotor de *PGK1* y el terminador 3´UTR$_{ScHIS3}$ (oligonucleótidos #1121/#1122) clonado en pGRB2.2. *URA3*, *Cg CEN ARS*, Ap^R	Este trabajo
pMB142	Vector de complementación de *LYS4.* Un fragmento de 3.3 kb *Bam* HI/*Sac* I conteniendo el ORF de *LYS4* y sus secuencias	Este trabajo

	no codificantes 5'y 3' (oligonucleótidos #1119/#1120) clonado en pGRB2.0. *URA3*, *Cg CEN ARS*, Ap^R	
pMB150	Vector de complementación de *LYS12.* Un fragmento de 1.119 kb *Xho* I/*Spe* I conteniendo el ORF de *LYS12* bajo el promotor de *PGK1* y el terminador 3´UTR_{ScHIS3} (oligonucleótidos #1131/#1134) clonado en pGRB2.2. *URA3*, *Cg CEN ARS*, Ap^R	Este trabajo
pMB152	Vector de complementación de *LYS20.* Un fragmento de 1.296 kb *Xho* I/*Spe* I conteniendo el ORF de *LYS20* bajo el promotor de *PGK1* y el terminador 3´UTR_{ScHIS3} (oligonucleótidos #1143/#1146) clonado en pGRB2.2. *URA3*, *Cg CEN ARS*, Ap^R	Este trabajo
pMB154	Vector de complementación de *LYS21.* Un fragmento de 1.314 kb *Xho* I/*Spe* I conteniendo el ORF de *LYS21* bajo el promotor de *PGK1* y el terminador 3´UTR_{ScHIS3} (oligonucleótidos #1155/#1158) clonado en pGRB2.2. *URA3*, *Cg CEN ARS*, Ap^R	Este trabajo
Para etiquetar con *GFP*		
pMB122	Vector con la fusión traduccional *SOD2-GFP*. Un fragmento de 1.07 kb *Bam*H I/*Spe* I conteniendo el ORF de *SOD2* sin codón de paro (oligonucleótidos #961/#962) y un fragmento de 0.993 kb *Xho* I/*Kpn* I de la secuencia no codificante *SOD2* 3' (oligonucleótidos #1100/#518) clonado en pOZ16. El fragmento del ORF de *SOD2* fue puesto en fase con el epitope *GFP*. [*SOD2*::*GFP*-FRT-3'UTR_{CgCTA1}-P_{ScPGK1}::*hph*::3'UTR_{ScHIS3}-FRT-3'UTR_{SOD2}] *URA3* Hyg^R Ap^R	Este trabajo
pMB146	Vector con la fusión traduccional *SOD1-GFP*. Un fragmento de 0.706 kb *Bam*H I/*Spe* I conteniendo el ORF de *SOD1* sin codón de paro (oligonucleótidos #958/#959) y un fragmento de 0.677 kb *Cla* I/*Pvu* II de la secuencia no codificante *SOD1* 3' (oligonucleótidos #1101/#1084) clonado en pOZ16. El fragmento del ORF de *SOD1* fue puesto en fase con el epitope *GFP*. [*SOD1*::*GFP*-FRT-3'UTR_{CgCTA1}-P_{ScPGK1}::*hph*::3'UTR_{ScHIS3}-FRT-3'UTR_{SOD1}] *URA3* Hyg^R Ap^R	Este trabajo
pMB66	Vector con la fusión transcripcional *SOD1-GFP*. Un fragmento de 1.7 kb *Kpn* I/*Sac* I conteniendo el ORF de *GFP* y las secuencias no codificantes 5' (oligonucleótidos #781/#780) y 3' (oligonucleótidos #782/#783) de *SOD1* clonado en pAP353. *URA3*, *Cg CEN ARS*, Ap^R	Este trabajo
pMB68	Vector con la fusión transcripcional *SOD2-GFP*. Un fragmento de 2.2 kb *Kpn* I/*Sac* I conteniendo el ORF de *GFP* y las secuencias no codificantes 5' (oligonucleótidos #786/#784) y 3' (oligonucleótidos #787/#785) de *SOD2* clonado en pAP353. *URA3*, *Cg CEN ARS*, Ap^R	Este trabajo

Tabla 3. Oligonucleótidos usados en este trabajo

Primer	Secuencia	Descripción o sitio de restricción (s)
224	CACGGCGCGCCTAGCAGCGGCGTTTCCTTGTAATGTATACCTCTG	-
226	GTCAGCGGCCGCATCCCTGCGCTAATTTTTTCTTCAACTCTCTC	-
228	GCAATATGCCAACCCTTG	-
229	GATTTATGTGGTTTGTAGTATCGAAA	-
230	GTCAGCGGCCGCATCCCTGCCTACCACCAGCTGCCAGC	-
231	CACGGCGCGCCTAGCAGCGGTTTCTATTGTAGTAGTCTTTTTATTTGG	-
232	TTGGATGGCGTTATGGAGTGC	-
233	GTCCTTGCCGAGCTCGAA	-
518	CTGGTTCAGCTTTCCGACG	-
780	GCGTGTCGACTATTGTAGTAGTCTTTTTATTTGG	Sal I
781	GCCGGTACCGCAGTGTGAGCAAAC	Kpn I
782	TTGGGATCCACCAGCTGCCAGC	BamH I, Xho I
783	CCT**GAGCTC**TAGGGGTGGTGAAAAGG	Sac I
784	CGTGTCGACGTTTCCTTGTAATGTATACCTC	Sal I
785	CCTGAGCTCGATTGTAGTTGGAACAAGG	Sac I
786	GCC**GGTACC**GTTGGCCAGGAATAAG	Kpn I
787	CTCGGATCCATTAGCTAATTTTTTCTTCAACTCTCTC	BamH I
961	TAT**ACTAGT**CTGGCCAAACTTGCTTAG	Spe I
962	GTA**GGATCC**GTTTAGTGACTTTGGATGTTTCG	BamH I
970	CACTCTGAGGAAGACACTGC	-
972	GAGCATATCCGTGTATGTGG	-
975	GCTCGTCTCTCTGATTTGG	-
976	CTTTGAACCCATTCTACCC	-
958	TAT**ACTAGT**CTACCCACCCAAGAATG	Spe I
959	GTA**GGATCC**GGTTGGTTAGGCCGATGACACC	BamH I
1084	CGG**GGTACC**AATGGCAGAGCTTGG	Kpn I
1086	TAA**ACTAGT**GTGAGCAAACCCG	Spe I
1100	ATT**CTCGAG**CCAC**CAGCTG**CCAGCG	Xho I and Pvu II
1101	CTC**CTCGAG**ATTAGCTAATTTTTTCTTCAACTCTCTC	Xho I
1106	ATT**GGATCC**GATTGTAGTTGGAACAAGG	BamH I
1119	GCGGGATCCAGTTTTGCACAGAAAGG	BamH I
1120	CTTGAGCTCAATTCGGCTTTCAACC	Sac I
1121	GCAGGATCCATGGTGGTACTGCGTAGGTC	BamH I
1122	GTAGTCGACTCACAATTGAGACTTGACCC	Sal I
1131	TAA**ACTAGT**ATGCTAAGAGGAAGTGCTACTAG	Spe I
1134	ATC**CTCGAG**TTATATCTTGGACAAGATGTCG	Xho I
1143	TAA**ACTAGT**ATGACCGCAAATAATAATCC	Spe I
1146	ATC**CTCGAG**TTAATCAACTCTTGCTTTCTTTTG	Xho I
1155	TAA**ACTAGT**ATGGATCAATACCAACAAGTAAC	Spe I
1158	ATC**CTCGAG**TTATTGGGATAGCTTAGATTTCTTC	Xho I

Medios de cultivo

El medios de cultivo para bacteria utilizado fue Luria-Bertani (LB) el cual contiene extracto de levadura 5g/L y triptona 10g/L, NaCl 10g/L. Para medio sólido añadir agar 15g/L. Cuando fuese necesario se suplemento con carbenicilina 100µg/ml o cloranfenicol 20µg/ml, y/o sacarosa 5%. Para recuperar las bacterias recien transformadas con ADN se utilizo el medio de cultivo SOC, el cual contiene extracto de levadura 5g/L, triptona 20g/L, NaCl 10mM, KCl 2.5mM, $MgSO_4$ 10mM y $MgCl_2$ 10mM. Después de esterilizarlo se suplemento con glucosa 0.4%.

Los medios de cultivo para levadura utilizados en este trabajo fueron: Medio YPD que contiene extracto de levadura 10 g/L, peptona 20 g/L y suplementado con glucosa 2%. Para los experimentos en fuentes de carbono no fermentables se sustituyo la glucosa por glicerol 2% o etanol 2%. Para medio solido se añadió agar 2% y se suplementaron con nourseotricina (Werner agentes biológicos) 100 mg/L (Nat100), o higromicina B (AG Científico) 460 mg/L (Hyg460), o penicilina (100 U)-estreptomicina (100 mg/L) (Gibco BRL). El medio completo sintético (SC) se compone de YNB sin sulfato de amonio, $(NH_4)_2SO_4$ 5g/L, suplementado con casaminoácidos 0,6% y glucosa 2%. De ser necesario se le añadió uracilo 25 mg/L, ácido 5-fluoroorótico (5-FOA, Toronto Research Chemicals) 1.1g/L para cajas de 5-FOA, o ácido 5-fluroantranilico (5-FAA, Sigma Aldrich) 0,5 g/L para cajas de 5-FAA. El medio SD está constituido por base de nitrógeno de levadura sin sulfato de amonio, $(NH_4)_2SO_4$ 5g/L, y se suplementó con glucosa 2% y, cuando fue necesario, con uracilo 25 mg/ L y/o lisina (30 o 120 mg/L, Sigma Aldrich).

Transformaciones

Todos los plásmidos construidos se introdujeron en *Escherichia coli* DH10 por electroporación {Ausubel, 2000 #1}. Las transformaciones de levadura con ADN de plásmido lineal o superenrollado se realizaron con el método de acetato de litio como se describe a continuación: las cepas a transformar con ADN se crecieron durante una noche en medio YPD o SC. Con estos cultivos se inoculó 50ml de medio fresco (YPD o SC) hasta llegar a una OD_{600nm} de 0.8. Se centrifugaron y se lavaron con agua grado mQ y acetato de litio 0.1M para obtener un botón de células competentes para transformación resuspendidas en 300µL de acetato de litio 0.1M. Además se preparo la mezcla de transformación la cual consta de: 240µL de polietilenglicol 50%, 36µL de acetato de litio 1M, 25µL de esperma de salmón de cadena sencilla 2mg/mL desnaturalizado con calor y el ADN a transformar (alrededor de 500µg). Se mezclaron 50µL de las células competentes y con la suspensión de ADN y se incubó a 30°C durante 45 min. Al término de la incubación se añadió 45µL de DMSO y se incubó nuevamente a 42°C, 15 min. La suspensión de transformación se centrifugo y el botón de células transformadas con el ADN se re suspendió en 600µL de agua mQ (para seleccionar Ura+) o en 1mL de YPD (para seleccionar resistencia a antibióticos). Para seleccionar las transformantes, sembrar en medio solido SC (transformantes Ura+) o en medio solido YPD Nat100 o Hyg460. Incubar a 30°C durante 2 días. Las transformantes obtenidas se estrían en medio solido de selección correspondiente para ser purificadas. Para diagnosticar que la transformación del ADN fue exitosa, se lleva a cabo una PCR de colonia o a partir de ADN extraído de cultivos de las

transformantes. Seleccionar dos clonas diferentes para cada transformación y guardarlas a-80°C en glicerol 10%.

Extracción de ADN

a) Purificación del ADN por extracción con fenol-cloroformo-alcohol isoamílico y precipitación con etanol

Para purificar ADN de digestiones, ligaciones o algún otra reacción de biología molecular. La mezcla de ADN se lleva a un volumen final de 100µL con agua mQ. Se le añaden 32µL de acetato de amonio 9M para una concentración final de 2.5M y 128µL de Fenol-Cloroformo-Isoamílico 24:24:1 (v/v). Centrifugar para separar fase acuosa y transferirla a un tubo con 1µL de glicógeno. Añadir 2.5 volúmenes de etanol 100% e incubar a -20°C por 30 minutos. Centrifugar y descartar el sobrenadante. Lavar la pastilla de ADN con 1ml de etanol 70% y centrifugar y descartar el sobrenadante. Secar la pastilla incubando a 50°C. Resuspender la pastilla en 50µL de Tris-HCl 10mM pH 8.

b) Extracción de ADN plasmídico

Los plásmidos de cultivos bacterianos se extrajeron y purificaron con el kit de Qiagen Mini Prep o con el kit Wizard ADN purification® (Promega, para aislar ADN plasmídico de volúmenes grandes). Para extraer ADN de geles de agarosa, se usó el kit Gel Extraction QIAquick®. Para purificar productos de PCR, se usó el kit PCR Purification QIAquick®.

c) Extracción de ADN genómico de levadura

Para extraer ADN genómico se cultivaron las levaduras durante una noche en medio YPD. Este cultivo se centrifugo, se descarto el sobrenadante y el botón de células se resuspendieron en 500µL de solución A la cual contiene Tris 0.05M, EDTA 0.01M, NaCl 0.15M, Triton 1% y SDS 1%. Después de mezclar, se adiciono 500µL de Fenol-Cloroformo-Isoamílico 24:24:1 (v/v), se agito durante 6 minutos y se incubó a 44°C por 30 minutos. Después se centrifugo la mezcla para separar la fase acuosa y adicionarle 50µL de la solución A sin SDS y 0.3µL de coctel de RNAsa. Esta mezcla se incubo a 44°C por 30 min. Para precipitar el ADN se adicionó 1mL de etanol 100% y se centrifugo. El botón de ADN se lavo con 1mL de etanol 70% y se resuspendió en 150µL de Tris-HCl 10mM pH 8.

Construcción de fusiones transcripcionales de SODs con GFP

Los plásmidos usados para las fusiones transcripcionales fueron construidos por amplificación de las secuencias promotoras (5´ UTR) y terminadoras (3´UTR) de los genes de SOD a partir del ADN genómico de la cepa BG14. Se amplifico el promotor de *SOD1* con los oligonucleótidos #781/#780, y de *SOD2* con #786/#784. Los oligonucleótidos para el 3´UTR de *SOD1* fueron #782/#783, y para *SOD2* #787/#785. Estos fragmentos fueron clonados en el pAP353 que contiene el gen reportero GFP para generar los plásmidos P_{SOD1}::*GFP*::3´UTR_{SOD1} (pMB66) y P_{SOD2}::*GFP*::3´UTR_{SOD2} (pMB68). Cada fusión se transformó en la cepa parental, la doble mutante *sod1*Δ *sod2*Δ y en las mutantes dobles, triples y cuádruples en los factores de transcripción de estrés Yap1, Skn7, Msn2 y Msn4.

Las transformantes fueron seleccionadas en medio solido de SC. Para cada transformación, dos clonas distintas se obtuvieron y guardaron a -80°C.

Ensayo de envejecimiento (CLS)

a) Material:

- Tubos de vidrio delgados para "tubos de envejecimiento" estarán 14 días en el roller a 30°C.
- Tubos gordos de vidrio con 2ml de agua para 1era dilución OD= 0.5.
- Tubos Ependorfs con 1ml de YPD, etiquetados.
- Placas de Bioscreen.
- YPD liquido, alícuotas de mínimo 30 ml. Una alícuota por día de muestreo (día 2, 3, 4, 5, 7, 9, 11, 14. Es decir 8 días de muestreo = 8 alícuotas cada una de 30 ml YPD). El volumen de las alícuotas depende del número de cepas que se analizaran.
- Agua MQ.
- Pipeta de repetición, puntas de 50 ml y de 1 ml (1 punta para cada día de muestreo).
- Puntas azules y amarillas o blancas.
- Celdas para OD.
- Vasos de precipitado estériles (1 vaso por día, se utilizarán para tomar el YPD y el agua en la pipeta de repetición).
- Laptop con Excel
- Espectrofotómetro
- Campana de extracción
- Vortex
- Balanza granataria
- NO usar centrifuga.

b) Procedimiento general:

Nota importante: seguir los pasos igual en todas las tomas de muestra, no variar nada. Mismo tipo de puntas, mismos volúmenes etc.

i. Poner un overnight de las cepas en medio mínimo (glucosa, YNB, sulfato de amonio y si es necesario uracilo).
ii. Inocular la SP de las cepas en tubos de vidrio delgado con 5ml de medio mínimo.
iii. Colocar los tubos de SP en el roller a 30°C. Ahí se quedaran aprox. 14 días. La incubadora debe estar húmeda: colocar un traste con agua.
iv. Tomar muestras cada 2 días más o menos. De preferencia tomar muestra diariamente los primeros 5 días porque es importante tener las primeras viabilidades de las cepas ya que serán el punto de referencia para los cálculos: 2, 3, 4, 5, 7, 9, 12, 14 días.

v. Análisis de datos de Bioscreen en los software: Excel y Graph Pad Prism.

Nota importante: Antes de comenzar la toma de muestra cada día, procurar no sacar los "tubos de envejecimiento" del roller hasta no tener listo los tubos ependorfs con 1ml YPD y los tubos gordos de vidrio con 2ml de agua. (Para evitar cold shock).

c) Protocolo:
   i. Alicuotar 2ml de agua en los tubos de vidrio gordos.
   ii. Alicuotar 1ml de YPD en los tubos Ependorf etiquetados. Usar una alicuota de 50ml de YPD, pipeta de repetición y punta de 50ml.
   iii. Pesar los "tubos de envejecimiento" (Peso inicial)
   iv. Medir las OD a tubos de envejecimiento (suponemos que los triplicados tienen OD parecida). Este paso solo se hará en el 1er día de muestreo.
   v. Supongamos que la OD de los tubos es de: 20. Entonces tomar 50ul de cada "tubo de envejecimiento" y colocarlo en el tubo de vidrio gordo que contiene 2ml de agua para generar una dilucion a 0.5.
   vi. Pesar los "tubos de envejecimiento" (Peso final)
   vii. Regresar los tubos de envejecimiento al roller.
   viii. Medir las ODs a la dilución 0.5 tomando 1ml de los 2ml de la dilución (hecha en el paso 4).
   ix. Registrar las OD en Excel (estarán alrededor de 0.5)
   x. Toma aprox. 20ul y los colocarlos en los ependorfs que tienen 1ml de YPD (2da dilución = 0.01 de OD).
   xi. De estos ependorfs tomar 300ul para colocarlos en los pozos de la caja de Bioscreen, por duplicado.
   xii. Programar el Bioscreen a 30°C, 420-480nm wideband, 20min/lectura, shaking máximo.

Para la toma de muestras de los siguientes días hay que ajustar el volumen de agua que se evaporó en la incubación en el roller: es decir, mantener el volumen de los tubos de envejecimeinto constante durante todo el experimento. Entonces: Realizar pasos 1, 2 y 3 del protocolo. El paso 4 ya no se hace. Calcular cuánta agua hay que añadir, antes de continuar con el paso 5:
Volumen de agua a añadir = (Peso final de la toma de muestra anterior - peso inicial de la toma de muestra de hoy).

Ejemplo: volumen de agua a añadir al tubo #17 en la toma de muestra día 3 = (Peso final del día 2 es 5.2 g - peso inicial del día 3 que es 5.05 g) = 0.15 g = 150 uL de agua a añadir.
Despues, adicionar el volumen de agua a todos los tubos usando pipeta de repetición. Y por último, continuar con los pasos del 5 al 12 del protocolo.

Expresión de GFP por análisis de FACS

Cultivos saturados de las cepas en medio SC crecieron hasta FE o se diluyeron en medio fresco (inicio de FL). GFP se utilizó como gen reportero para medir la actividad de los promotores de *SOD1* y *SOD2*. Las células de levadura se lavaron con PBS y se resuspendieron en 1 ml de PBS y la fluorescencia se evaluó por FACS análisis utilizando un flujo Beckman Coulter citómetro. La fluorescencia indicada es la salida directa del canal FL2 (detección de fluorescencia verde) sin compensación. En algunos experimentos la fluorescencia se normalizo con respecto a la cepa parental. Se analizaron un total de 10.000 células por cada muestra.

Extracción de proteínas

Las cepas fueron crecidas a 30°C in medio SC hasta FE. Las proteínas se aislaron por homogeneización de las células con perlas de vidrio en 0,3 ml de tampón de lisis (1 × PBS suplementado con 1 × inhibidores de la proteasa generales [Sigma Fast ®]). Las células se rompieron por agitación a 4 ° C durante 1 min y se colocaron en hielo durante otro minuto (repetido 15 veces) y los lisados se centrifugaron a 25 200 x g. durante 15 min a 4 ° C para eliminar los desechos celulares y perlas de vidrio. El sobrenadante se utilizó para medir la actividad de SOD y su contenido proteico usando el ensayo de Bradford (Fermentas ®) (54). La albúmina sérica bovina de Sigma se utilizó como estándar.

Cuantificación de la actividad total de SOD

La actividad total de SOD se cuantifico en extractos proteicos de cultivos en FE o en FL y fue determinada espectrofotométricamente con el método colorimétrico de WST-1 (Peskin & Winterbourn, 2000) (SOD assay, for the measurement of superoxide dismutase (SOD) inhibition activity, cat. no. kt-019, Kamiya B Biomedical company; Superoxide dismutase activity kit catalog # 900-157 www.assaydesigns.com) que se describe a continuación. El superóxido reduce el WST-1 y forma un compuesto de color llamado formazan que se detecta a 450nm en un lector de placas y se cuantifica. La presencia de SOD en la reacción elimina el superóxido y por lo tanto inhibe la producción de formazan. Se determino la IC50 (50% de la actividad de inhibición de la SOD) para cada muestra y se calcula la actividad de SOD. Una unidad es definida como la cantidad de SOD requerida para inhibir el 50% de la taza de formación del formazan.

Preparación de Soluciones Stock: La solución de WST-1 10mM contiene 0.03257g de WST-1 en 5ml de agua mQ. Se aliquotó y guardo a -20°C en obscuridad. La enzima Xantina Oxidasa (XO) 255mU/ml se obtuvo al diluir 25µL de XO en 1ml de Tris 50mM, se mezclo el reactivo antes usarse para evitar separación de fases. Esta solución se preparó al momento de usarla y se mantuvo en hielo. Se preparo una solución de SOD (Sigma) a 6ng/µL para hacer la curva de estándares. Para preparar 100ml del buffer de ensayo 10x: se mezclaron 5 ml de Tris 1M pH 8.5, 0.039g DTPA (diethylenetriamine-pentaacetic acid) y 0.0136g hipoxantina. Se filtro y guardo en la obscuridad a 4°C (vigencia 3meses). La solución de catalasa 2mg/ml contiene 0.01g de catalasa (Sigma) en 5ml de PBS. Se alicuotó y guardo

4°C. Preparar mezcla de reacción: Buffer de ensayo 1x, WST-1 50uM y catalasa 0.01mg/ml y suplementar al momento con XO 4.5mU/ml (1mU/pozo).
Preparación de los controles de reacción: Se prepararon tres diferentes controles: C1, C2 y C3. El control **C1** contiene: 200µL de la mezcla de reacción con XO y 10µL de PBS. El control **C3** contiene: 200µL de la mezcla de reacción sin XO y 10µL de PBS. El control **C2** contiene: 200µL de la mezcla de reacción sin XO y 10µL de extracto proteico. Cada muestra tiene su propio C2.
Preparación de los estándares de SOD: Se prepararon soluciones 0.075, 0.15, 0.3, 0.6 y 1.2ng/µL de SOD, usando el stock de 6 ng/µL, a un volumen final de 100 µL. Se tomaron 8µL de cada solución y se colocaron en la placa de pozos, para obtener los estándares: 0, 0.6, 1.2, 2.4, 4.8 y 9.6ng de SOD.
Preparación de las muestras: Se diluyeron los extractos proteicos de la cepa parental y las mutantes en SOD para obtener 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4 y 0.8µg/µL por muestra. Se tomaron 10µL de estas diluciones y se colocaron en la placa de pozos para obtener el rango de 0.1, 0.2, 0.4, 0.8, 1, 2, 4 y 8µg/pozo por muestra.
Procedimiento general: Se colocaron por duplicado los extractos proteicos de las muestras y los estándares de SOD en la placa de pozos. Se comenzó la reacción al añadir 200µL de la mezcla de reacción suplementada con XO a todos los pozos excepto a los controles C2 y C3 (controles sin XO). Se mezclo el contenido de los pozos y se observo la aparición de un color amarillo (formazan). Se incubo la placa de pozos en el lector de placas a 25°C, se hicieron lecturas de Abs a 450nm cada 2 min durante 20 min.

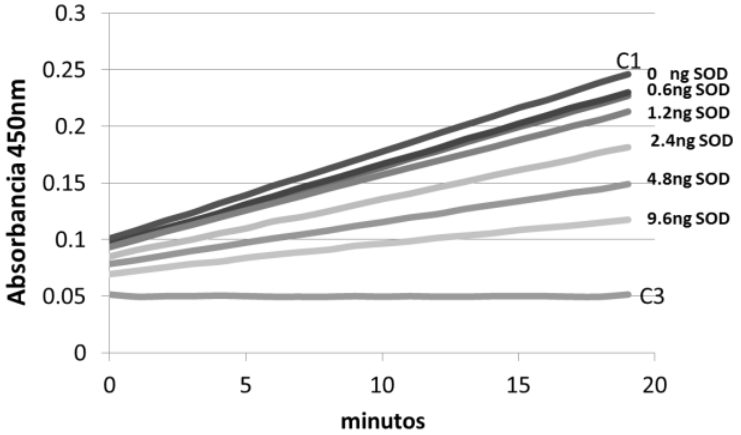


FIGURA 17. Cambio en la absorbancia a 450 nm *vs* el tiempo para los estándares de SOD

Análisis de datos: graficamos la Abs de los estándares de SOD *vs* tiempo (min). La curva de los estándares de SOD se utiliza como control positivo para verificar que el ensayo está funcionando, no para calcular las unidades de SOD en las muestras. Determinamos el % de inhibición de la reacción en las muestras al aplicar la formula:
Taza de inhibición, % = {[(Abs C1 - Abs C3) - (Abs muestra - Abs C2)] / (Abs C1 - Abs C3)} * 100

Graficamos la taza de inhibición, % *vs* Log (µg de muestra en c/pozo), calculamos la ecuación de regresión lineal y obtenemos los µg de muestra cuando la taza de inhibición es 50% (IC50). Una unidad de SOD se define como la cantidad de enzima que inhibe en un 50% la tasa de formación de formazan. Así, la actividad de SOD (U/mg de proteína) en las muestras es igual a {1**/** [anti Log (µg de muestra al 50% inhibición)]}*1000.

Zimogramas de actividad para SOD

Extractos proteicos de cultivos en FE se usaron para determinar la actividad de SOD. Entre 30 y 50 mg de proteína total se separaron en geles de poliacrilamida al 12% en condiciones no desnaturalizantes. Los geles se corrieron a 30 mA durante 3 h en una celda Miniprotean II (Bio-Rad). La actividad de SOD se reveló al teñir el gel con nitroazul de tetrazolio (NBT) y riboflavina (Beauchamp & Fridovich, 1971). Brevemente, después de la electroforesis, el gel se enjuagó con agua destilada y se incubó inmerso en una solución NBT (0.6mg/ml) durante 15 min en agitación constante y obscuridad. Se traslado a otra solución conteniendo 90 µL de TEMED y 200 µL de riboflavina 1mg/ml (conc. final de 10µg/ml, 4°C y proteger de la luz) en un volumen total de 20mL en agua destilada. Se incubó durante 15 min en agitación y obscuridad. Por último, el gel se expuso a la luz en agitación constante hasta la aparición de bandas descoloridas (actividad de SOD in situ) sobre un fondo azul.

Soluciones para preparar y correr geles de poliacrilamida no desnaturalizantes:
Solución 30% acrilamida / 0.8% bisacrilamida contiene una mezcla de 30g de acrilamida y 0.8g de bisacrilamida en agua destilada a un volumen total de 100ml. Filtrar (poro de 0.45µm) y guardar a 4°C en la obscuridad. Precaución el monomero de acrilamida es neurotóxico.
El Tris 2M a pH 8.5 contiene 36.33g de trizma base disuelto en 80ml de agua destilada. Ajustar pH a 8.5 con HCL 6N y aforar a 150ml. Guardar a 4°C.
Persulfato de amonio 10%.
El buffer de corrida 5x contiene 15g de trisma Base y 72g de glicina aun volumen total de 1L de agua mQ. Dura un mes a 4°C.

Cuantificación de superóxido

Las cepas se crecieron en medio SD suplementado con 120mg / l de lisina durante 48 h a 30 ºC. Los niveles de superóxido se cuantificaron con la sonda dihidroetidio (DHE) por dos protocolos diferentes: micro-espectro-fluorometria y citometria de flujo (FACS). Brevemente, se recolectaron 2×10^7 células por centrifugación y el botón se lavó una vez con PBS. Se resuspendieron en una solución de DHE, 200µM para fluorometria y 50µM para FACS, se incubaron durante 10 min en la oscuridad. Se lavaron con PBS y se registró la fluorescencia (DHE: λex = 485 nm, λem = 595 nm) y absorbancia. Los datos de fluorescencia obtenidos por fluorometria se normalizaron usando los valores de los controles sin teñir. Los resultados se expresan en unidades de fluorescencia relativa por absorbancia (RFU/Abs). Los datos de fluorescencia evaluados por FACS se analizaron usando el citómetro de flujo FACSCALIBUR BD. La fluorescencia de DHE indicada es la

salida directa del canal FL3 (detección de fluorescencia roja) sin compensación. Se analizaron un total de 10.000 células por cada muestra.

Microscopia de fluorescencia

Las cepas se crecieron en medio YPD hasta FE o FL a 30°C. El botón de 1ml de células se lavó una vez con PBS y se resuspendieron en 0,5 ml de PBS. Las suspensiones celulares se incubaron durante 10 min con 40 ng/ml de DAPI (4 ', 6'-diamino-2-fenilindol), DAPI 500ng/ml (para visualizar núcleos) o con 40 nM de MitoTracker (para visualizar mitocondrias). Las células se lavaron y analizaron directamente por microscopia de fluorescencia empleando un microscopio Axio Imager.M2 (Carl Zeiss, Inc.) y usando un objetivo de 100X. Las imágenes fueron analizados con el software de procesamiento de imágenes AxioVision v 4.8.2.0.

Ensayo de unión de extremos no homólogos vivo (NHEJ)

Fue hecho como se describe previamente (Rosas-Hernandez *et al.*, 2008). Brevemente, el plásmido pGRB2.0 (*CEN* ARS *URA3*) fue linealizado con *Bam*H I o *Sma* I para generar extremos cohesivos o romos, respectivamente. Este ADN lineal y el plásmido sin cortar se transformaron en la cepa parental (CGM1), y en las mutantes *sod1*Δ (CGM787), *sod2*Δ (CGM656), y *sod1*Δ *sod2*Δ (CGM937). Diluciones seriadas fueron sembradas en medio SC e incubadas a 30°C durante 2 días. Las transformantes se contaron y la eficiencia relativa de unión de extremos no homólogos se calculo como sigue. El número de transformantes obtenidas con plásmido linealizado se dividió entre el numero de transformantes obtenidas con el plásmido superenrollado. La eficiencia de la cepa parental (del plásmido digerido con Bam HI) se normalizo a 1.0.

7. Referencias

Abrashev, R., Dolashka, P., Christova, R., Stefanova, L. & Angelova, M. (2005). Role of antioxidant enzymes in survival of conidiospores of Aspergillus niger 26 under conditions of temperature stress. *J Appl Microbiol* **99**, 902-909.

Abrashev, R. I., Pashova, S. B., Stefanova, L. N., Vassilev, S. V., Dolashka-Angelova, P. A. & Angelova, M. B. (2008). Heat-shock-induced oxidative stress and antioxidant response in Aspergillus niger 26. *Can J Microbiol* **54**, 977-983.

Angelova, M. B., Pashova, S. B., Spasova, B. K., Vassilev, S. V. & Slokoska, L. S. (2005). Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. *Mycol Res* **109**, 150-158.

Barondeau, D. P., Kassmann, C. J., Bruns, C. K., Tainer, J. A. & Getzoff, E. D. (2004). Nickel superoxide dismutase structure and mechanism. *Biochemistry* **43**, 8038-8047.

Bartnikas, T. B. & Gitlin, J. D. (2003). Mechanisms of biosynthesis of mammalian copper/zinc superoxide dismutase. *J Biol Chem* **278**, 33602-33608.

Beauchamp, C. & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* **44**, 276-287.

Benov, L. & Fridovich, I. (1998). Superoxide dependence of the toxicity of short chain sugars. *J Biol Chem* **273**, 25741-25744.

Bielski, B. H. (1985). Fast kinetic studies of dioxygen-derived species and their metal complexes. *Philos Trans R Soc Lond B Biol Sci* **311**, 473-482.

Bilinski, T., Krawiec, Z., Liczmanski, A. & Litwinska, J. (1985). Is hydroxyl radical generated by the Fenton reaction in vivo? *Biochem Biophys Res Commun* **130**, 533-539.

Bolton, J. L., Trush, M. A., Penning, T. M., Dryhurst, G. & Monks, T. J. (2000). Role of quinones in toxicology. *Chem Res Toxicol* **13**, 135-160.

Bonatto, D. (2007). A systems biology analysis of protein-protein interactions between yeast superoxide dismutases and DNA repair pathways. *Free Radic Biol Med* **43**, 557-567.

Bordo, D., Djinovic, K. & Bolognesi, M. (1994). Conserved patterns in the Cu,Zn superoxide dismutase family. *J Mol Biol* **238**, 366-386.

Borghouts, C., Werner, A., Elthon, T. & Osiewacz, H. D. (2001). Copper-modulated gene expression and senescence in the filamentous fungus Podospora anserina. *Mol Cell Biol* **21**, 390-399.

Borgstahl, G. E., Parge, H. E., Hickey, M. J., Beyer, W. F., Jr., Hallewell, R. A. & Tainer, J. A. (1992). The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. *Cell* **71**, 107-118.

Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P. & Boeke, J. D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**, 115-132.

Branzei, D. & Foiani, M. (2007). Interplay of replication checkpoints and repair proteins at stalled replication forks. *DNA Repair (Amst)* **6**, 994-1003.

Briones-Martin-Del-Campo, M., Orta-Zavalza, E., Canas-Villamar, I., Gutierrez-Escobedo, G., Juarez-Cepeda, J., Robledo-Marquez, K., Arroyo-Helguera, O.,

Castano, I. & De Las Penas, A. (2014). The superoxide dismutases of Candida glabrata protect against oxidative damage and are required for lysine biosynthesis, DNA integrity and chronological life survival. *Microbiology*.

Bulteau, A. L., Ikeda-Saito, M. & Szweda, L. I. (2003). Redox-dependent modulation of aconitase activity in intact mitochondria. *Biochemistry* **42**, 14846-14855.

Calvin, N. M. & Hanawalt, P. C. (1988). High-efficiency transformation of bacterial cells by electroporation. *J Bacteriol* **170**, 2796-2801.

Carroll, M. C., Girouard, J. B., Ulloa, J. L., Subramaniam, J. R., Wong, P. C., Valentine, J. S. & Culotta, V. C. (2004). Mechanisms for activating Cu- and Zn-containing superoxide dismutase in the absence of the CCS Cu chaperone. *Proc Natl Acad Sci U S A* **101**, 5964-5969.

Carter, C. D., Kitchen, L. E., Au, W. C., Babic, C. M. & Basrai, M. A. (2005). Loss of SOD1 and LYS7 sensitizes Saccharomyces cerevisiae to hydroxyurea and DNA damage agents and downregulates MEC1 pathway effectors. *Mol Cell Biol* **25**, 10273-10285.

Castano, I., Kaur, R., Pan, S., Cregg, R., Penas Ade, L., Guo, N., Biery, M. C., Craig, N. L. & Cormack, B. P. (2003). Tn7-based genome-wide random insertional mutagenesis of *Candida glabrata*. *Genome Res* **13**, 905-915.

Casteilla, L., Rigoulet, M. & Penicaud, L. (2001). Mitochondrial ROS metabolism: modulation by uncoupling proteins. *IUBMB Life* **52**, 181-188.

Cohen, A., Nelson, H. & Nelson, N. (2000). The family of SMF metal ion transporters in yeast cells. *J Biol Chem* **275**, 33388-33394.

Cooke, M. S., Evans, M. D., Dizdaroglu, M. & Lunec, J. (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J* **17**, 1195-1214.

Cormack, B. P., Ghori, N. & Falkow, S. (1999). An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. *Science* **285**, 578-582.

Costa, V., Amorim, M. A., Reis, E., Quintanilha, A. & Moradas-Ferreira, P. (1997). Mitochondrial superoxide dismutase is essential for ethanol tolerance of Saccharomyces cerevisiae in the post-diauxic phase. *Microbiology* **143 (Pt 5)**, 1649-1656.

Cox, G. M., Harrison, T. S., McDade, H. C., Taborda, C. P., Heinrich, G., Casadevall, A. & Perfect, J. R. (2003). Superoxide dismutase influences the virulence of Cryptococcus neoformans by affecting growth within macrophages. *Infect Immun* **71**, 173-180.

Cuellar-Cruz, M., Briones-Martin-del-Campo, M., Canas-Villamar, I., Montalvo-Arredondo, J., Riego-Ruiz, L., Castano, I. & De Las Penas, A. (2008). High resistance to oxidative stress in the fungal pathogen *Candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. *Eukaryot Cell* **7**, 814-825.

Cuéllar-Cruz, M., Castaño, I., Arroyo-Helguera, O. & De Las Peñas, A. (2009). Oxidative stress response to menadione and cumene hydroperoxide in the opportunistic fungal pathogen *Candida glabrata*. *Mem Inst Oswaldo Cruz, Rio de Janeiro* **104**, 649-654.

Culotta, V. C. (2000). Superoxide dismutase, oxidative stress, and cell metabolism. *Curr Top Cell Regul* **36**, 117-132.

Cyrne, L., Martins, L., Fernandes, L. & Marinho, H. S. (2003). Regulation of antioxidant enzymes gene expression in the yeast Saccharomyces cerevisiae during stationary phase. *Free Radic Biol Med* **34**, 385-393.

Chang, E. C. & Kosman, D. J. (1990). O2-dependent methionine auxotrophy in Cu,Zn superoxide dismutase-deficient mutants of Saccharomyces cerevisiae. *J Bacteriol* **172**, 1840-1845.

Chang, M., Bellaoui, M., Boone, C. & Brown, G. W. (2002). A genome-wide screen for methyl methanesulfonate-sensitive mutants reveals genes required for S phase progression in the presence of DNA damage. *Proc Natl Acad Sci U S A* **99**, 16934-16939.

Chary, P., Dillon, D., Schroeder, A. L. & Natvig, D. O. (1994). Superoxide dismutase (sod-1) null mutants of Neurospora crassa: oxidative stress sensitivity, spontaneous mutation rate and response to mutagens. *Genetics* **137**, 723-730.

Chen, X. J., Wang, X., Kaufman, B. A. & Butow, R. A. (2005). Aconitase couples metabolic regulation to mitochondrial DNA maintenance. *Science* **307**, 714-717.

D'Autréaux, B. & Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *nature reviews* **8**, 813-824.

De Las Penas, A., Pan, S. J., Castano, I., Alder, J., Cregg, R. & Cormack, B. P. (2003). Virulence-related surface glycoproteins in the yeast pathogen *Candida glabrata* are encoded in subtelomeric clusters and subject to RAP1- and SIR-dependent transcriptional silencing. *Genes Dev* **17**, 2245-2258.

Diez, B., Schleissner, C., Moreno, M. A., Rodriguez, M., Collados, A. & Barredo, J. L. (1998). The manganese superoxide dismutase from the penicillin producer Penicillium chrysogenum. *Curr Genet* **33**, 387-394.

Dizdaroglu, M. (2005). Base-excision repair of oxidative DNA damage by DNA glycosylases. *Mutat Res* **591**, 45-59.

Domergue, R., Castano, I., De Las Penas, A., Zupancic, M., Lockatell, V., Hebel, J. R., Johnson, D. & Cormack, B. P. (2005). Nicotinic acid limitation regulates silencing of Candida adhesins during UTI. *Science* **308**, 866-870.

Dziadkowiec, D., Krasowska, A., Liebner, A. & Sigler, K. (2007). Protective role of mitochondrial superoxide dismutase against high osmolarity, heat and metalloid stress in saccharomyces cerevisiae. *Folia Microbiol (Praha)* **52**, 120-126.

Eitinger, T. (2004). In vivo production of active nickel superoxide dismutase from Prochlorococcus marinus MIT9313 is dependent on its cognate peptidase. *J Bacteriol* **186**, 7821-7825.

Elchuri, S., Oberley, T. D., Qi, W., Eisenstein, R. S., Jackson Roberts, L., Van Remmen, H., Epstein, C. J. & Huang, T. T. (2005). CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* **24**, 367-380.

Elledge, S. J. (1996). Cell cycle checkpoints: preventing an identity crisis. *Science* **274**, 1664-1672.

Emri, T., Pocsi, I. & Szentirmai, A. (1999). Analysis of the oxidative stress response of Penicillium chrysogenum to menadione. *Free Radic Res* **30**, 125-132.

Fabrizio, P., Pozza, F., Pletcher, S. D., Gendron, C. M. & Longo, V. D. (2001). Regulation of longevity and stress resistance by Sch9 in yeast. *Science* **292**, 288-290.

Fabrizio, P., Liou, L. L., Moy, V. N., Diaspro, A., Valentine, J. S., Gralla, E. B. & Longo, V. D. (2003). SOD2 functions downstream of Sch9 to extend longevity in yeast. *Genetics* **163**, 35-46.

Fanning, S., Xu, W., Solis, N., Woolford, C. A., Filler, S. G. & Mitchell, A. P. (2012). Divergent targets of Candida albicans biofilm regulator Bcr1 in vitro and in vivo. *Eukaryot Cell* **11**, 896-904.

Farr, S. B., D'Ari, R. & Touati, D. (1986). Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. *Proc Natl Acad Sci U S A* **83**, 8268-8272.

Fidel, P. L., Jr., Cutright, J. L., Tait, L. & Sobel, J. D. (1996). A murine model of *Candida glabrata* vaginitis. *J Infect Dis* **173**, 425-431.

Finkel, T. & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.

Flattery-O'Brien, J. A., Grant, C. M. & Dawes, I. W. (1997). Stationary-phase regulation of the Saccharomyces cerevisiae SOD2 gene is dependent on additive effects of HAP2/3/4/5- and STRE-binding elements. *Mol Microbiol* **23**, 303-312.

Flint, D. H., Tuminello, J. F. & Emptage, M. H. (1993). The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J Biol Chem* **268**, 22369-22376.

Fradin, C., De Groot, P., MacCallum, D., Schaller, M., Klis, F., Odds, F. C. & Hube, B. (2005). Granulocytes govern the transcriptional response, morphology and proliferation of Candida albicans in human blood. *Mol Microbiol* **56**, 397-415.

Frealle, E., Noel, C., Viscogliosi, E., Camus, D., Dei-Cas, E. & Delhaes, L. (2005). Manganese superoxide dismutase in pathogenic fungi: an issue with pathophysiological and phylogenetic involvements. *FEMS Immunol Med Microbiol* **45**, 411-422.

Frealle, E., Noel, C., Nolard, N., Symoens, F., Felipe, M. S., Dei-Cas, E., Camus, D., Viscogliosi, E. & Delhaes, L. (2006). Manganese superoxide dismutase based phylogeny of pathogenic fungi. *Mol Phylogenet Evol* **41**, 28-39.

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annu Rev Biochem* **64**, 97-112.

Frohner, I. E., Bourgeois, C., Yatsyk, K., Majer, O. & Kuchler, K. (2009). Candida albicans cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. *Mol Microbiol* **71**, 240-252.

Galiazzo, F. & Labbe-Bois, R. (1993). Regulation of Cu,Zn- and Mn-superoxide dismutase transcription in Saccharomyces cerevisiae. *FEBS Lett* **315**, 197-200.

Getzoff, E. D., Tainer, J. A., Weiner, P. K., Kollman, P. A., Richardson, J. S. & Richardson, D. C. (1983). Electrostatic recognition between superoxide and copper, zinc superoxide dismutase. *Nature* **306**, 287-290.

Getzoff, E. D., Cabelli, D. E., Fisher, C. L., Parge, H. E., Viezzoli, M. S., Banci, L. & Hallewell, R. A. (1992). Faster superoxide dismutase mutants designed by enhancing electrostatic guidance. *Nature* **358**, 347-351.

Giles, S. S., Batinic-Haberle, I., Perfect, J. R. & Cox, G. M. (2005). Cryptococcus neoformans mitochondrial superoxide dismutase: an essential link between antioxidant function and high-temperature growth. *Eukaryot Cell* **4**, 46-54.

Ginsberg, M. D., Feliciello, A., Jones, J. K., Avvedimento, E. V. & Gottesman, M. E. (2003). PKA-dependent binding of mRNA to the mitochondrial AKAP121 protein. *J Mol Biol* **327**, 885-897.

Gleason, J. E., Li, C. X., Odeh, H. M. & Culotta, V. C. (2014a). Species-specific activation of Cu/Zn SOD by its CCS copper chaperone in the pathogenic yeast Candida albicans. *J Biol Inorg Chem* **19**, 595-603.

Gleason, J. E., Galaleldeen, A., Peterson, R. L., Taylor, A. B., Holloway, S. P., Waninger-Saroni, J., Cormack, B. P., Cabelli, D. E., Hart, P. J. & other authors (2014b). Candida albicans SOD5 represents the prototype of an unprecedented class of Cu-only superoxide dismutases required for pathogen defense. *Proc Natl Acad Sci U S A* **111**, 5866-5871.

Gonzalez-Parraga, P., Hernandez, J. A. & Arguelles, J. C. (2003). Role of antioxidant enzymatic defences against oxidative stress H(2)O(2) and the acquisition of oxidative tolerance in Candida albicans. *Yeast* **20**, 1161-1169.

Grace, S. C. (1990). Phylogenetic distribution of superoxide dismutase supports an endosymbiotic origin for chloroplasts and mitochondria. *Life Sci* **47**, 1875-1886.

Gralla, E. B. & Valentine, J. S. (1991). Null mutants of *Saccharomyces cerevisiae* Cu,Zn superoxide dismutase: characterization and spontaneous mutation rates. *J Bacteriol* **173**, 5918-5920.

Gralla, E. B. & Kosman, D. J. (1992). Molecular genetics of superoxide dismutases in yeasts and related fungi. *Adv Genet* **30**, 251-319.

Gross, D. P., Burgard, C. A., Reddehase, S., Leitch, J. M., Culotta, V. C. & Hell, K. (2011). Mitochondrial Ccs1 contains a structural disulfide bond crucial for the import of this unconventional substrate by the disulfide relay system. *Mol Biol Cell* **22**, 3758-3767.

Gunasekaran, U., Yang, R. & Gunasekaran, M. (1998). Regulation of superoxide dismutase synthesis in Candida albicans. *Mycopathologia* **141**, 59-63.

Gutierrez-Escobedo, G., Orta-Zavalza, E., Castano, I. & De Las Penas, A. (2013). Role of glutathione in the oxidative stress response in the fungal pathogen *Candida glabrata*. *Curr Genet* **59**, 91-106.

Hamilton, A. J. & Holdom, M. D. (1997). Biochemical comparison of the Cu,Zn superoxide dismutases of Cryptococcus neoformans var. neoformans and Cryptococcus neoformans var. gattii. *Infect Immun* **65**, 488-494.

Hamilton, A. J., Holdom, M. D. & Jeavons, L. (1996). Expression of the Cu,Zn superoxide dismutase of Aspergillus fumigatus as determined by immunochemistry and immunoelectron microscopy. *FEMS Immunol Med Microbiol* **14**, 95-102.

Hampton, M. B., Kettle, A. J. & Winterbourn, C. C. (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**, 3007-3017.

Henle, E. S., Roots, R., Holley, W. R. & Chatterjee, A. (1995). DNA strand breakage is correlated with unaltered base release after gamma irradiation. *Radiat Res* **143**, 144-150.

Herker, E., Jungwirth, H., Lehmann, K. A., Maldener, C., Frohlich, K. U., Wissing, S., Buttner, S., Fehr, M., Sigrist, S. & other authors (2004). Chronological aging leads to apoptosis in yeast. *J Cell Biol* **164**, 501-507.

Hitchcock, C. A., Pye, G. W., Troke, P. F., Johnson, E. M. & Warnock, D. W. (1993). Fluconazole resistance in Candida glabrata. *Antimicrob Agents Chemother* **37**, 1962-1965.

Holbrook, E. D., Edwards, J. A., Youseff, B. H. & Rappleye, C. A. (2011). Definition of the extracellular proteome of pathogenic-phase Histoplasma capsulatum. *J Proteome Res* **10**, 1929-1943.

Holdom, M. D., Lechenne, B., Hay, R. J., Hamilton, A. J. & Monod, M. (2000). Production and characterization of recombinant Aspergillus fumigatus Cu,Zn superoxide dismutase and its recognition by immune human sera. *J Clin Microbiol* **38**, 558-562.

Huang, M. E. & Kolodner, R. D. (2005). A biological network in Saccharomyces cerevisiae prevents the deleterious effects of endogenous oxidative DNA damage. *Mol Cell* **17**, 709-720.

Huang, M. E., Rio, A. G., Nicolas, A. & Kolodner, R. D. (2003). A genomewide screen in Saccharomyces cerevisiae for genes that suppress the accumulation of mutations. *Proc Natl Acad Sci U S A* **100**, 11529-11534.

Hwang, C. S., Baek, Y. U., Yim, H. S. & Kang, S. O. (2003). Protective roles of mitochondrial manganese-containing superoxide dismutase against various stresses in Candida albicans. *Yeast* **20**, 929-941.

Hwang, C. S., Rhie, G. E., Oh, J. H., Huh, W. K., Yim, H. S. & Kang, S. O. (2002). 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. *Microbiology* **148**, 3705-3713.

Imlay, J. A. (2003). Pathways of oxidative damage. *Annu Rev Microbiol* **57**, 395-418.

Imlay, J. A. & Fridovich, I. (1991). Superoxide production by respiring membranes of Escherichia coli. *Free Radic Res Commun* **12-13 Pt 1**, 59-66.

Iraqui, I., Garcia-Sanchez, S., Aubert, S., Dromer, F., Ghigo, J. M., d'Enfert, C. & Janbon, G. (2005). The Yak1p kinase controls expression of adhesins and biofilm formation in Candida glabrata in a Sir4p-dependent pathway. *Mol Microbiol* **55**, 1259-1271.

Irvin, S. D. & Bhattacharjee, J. K. (1998). A unique fungal lysine biosynthesis enzyme shares a common ancestor with tricarboxylic acid cycle and leucine biosynthetic enzymes found in diverse organisms. *J Mol Evol* **46**, 401-408.

Jensen, L. T., Sanchez, R. J., Srinivasan, C., Valentine, J. S. & Culotta, V. C. (2004). Mutations in Saccharomyces cerevisiae iron-sulfur cluster assembly genes and oxidative stress relevant to Cu,Zn superoxide dismutase. *J Biol Chem* **279**, 29938-29943.

Jeong, J. H., Kwon, E. S. & Roe, J. H. (2001). Characterization of the manganese-containing superoxide dismutase and its gene regulation in stress response of Schizosaccharomyces pombe. *Biochem Biophys Res Commun* **283**, 908-914.

Jung, H. I., Lee, Y. Y., Lim, H. W., Ahn, K. S., Park, E. H. & Lim, C. J. (2002). Regulation of the manganese-containing superoxide dismutase gene from fission yeast. *Mol Cells* **14**, 300-304.

Kaeberlein, M., Burtner, C. R. & Kennedy, B. K. (2007). Recent developments in yeast aging. *PLoS Genet* **3**, e84.

Kaur, R., Ma, B. & Cormack, B. P. (2007). A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. *Proc Natl Acad Sci U S A* **104**, 7628-7633.

Keyer, K. & Imlay, J. A. (1996). Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci U S A* **93**, 13635-13640.

Keyer, K., Gort, A. S. & Imlay, J. A. (1995). Superoxide and the production of oxidative DNA damage. *J Bacteriol* **177**, 6782-6790.

Kloppel, C., Suzuki, Y., Kojer, K., Petrungaro, C., Longen, S., Fiedler, S., Keller, S. & Riemer, J. (2011). Mia40-dependent oxidation of cysteines in domain I of Ccs1 controls its distribution between mitochondria and the cytosol. *Mol Biol Cell* **22**, 3749-3757.

Komshian, S. V., Uwaydah, A. K., Sobel, J. D. & Crane, L. R. (1989). Fungemia caused by Candida species and Torulopsis glabrata in the hospitalized patient: frequency, characteristics, and evaluation of factors influencing outcome. *Rev Infect Dis* **11**, 379-390.

Koziol, S., Zagulski, M., Bilinski, T. & Bartosz, G. (2005). Antioxidants protect the yeast Saccharomyces cerevisiae against hypertonic stress. *Free Radic Res* **39**, 365-371.

Kuo, C. F., Mashino, T. & Fridovich, I. (1987). alpha, beta-Dihydroxyisovalerate dehydratase. A superoxide-sensitive enzyme. *Journal of Biological Chemistry* **262**, 4724-4727.

Kwon, E. S., Jeong, J. H. & Roe, J. H. (2006). Inactivation of homocitrate synthase causes lysine auxotrophy in copper/zinc-containing superoxide dismutase-deficient yeast *Schizosaccharomyces pombe*. *J Biol Chem* **281**, 1345-1351.

Laliberte, J., Whitson, L. J., Beaudoin, J., Holloway, S. P., Hart, P. J. & Labbe, S. (2004). The Schizosaccharomyces pombe Pccs protein functions in both copper trafficking and metal detoxification pathways. *J Biol Chem* **279**, 28744-28755.

Lamarre, C., LeMay, J. D., Deslauriers, N. & Bourbonnais, Y. (2001). Candida albicans expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase. *J Biol Chem* **276**, 43784-43791.

Lamb, A. L., Wernimont, A. K., Pufahl, R. A., Culotta, V. C., O'Halloran, T. V. & Rosenzweig, A. C. (1999). Crystal structure of the copper chaperone for superoxide dismutase. *Nat Struct Biol* **6**, 724-729.

Lambou, K., Lamarre, C., Beau, R., Dufour, N. & Latge, J. P. (2010). Functional analysis of the superoxide dismutase family in Aspergillus fumigatus. *Mol Microbiol* **75**, 910-923.

Lapinskas, P. J., Cunningham, K. W., Liu, X. F., Fink, G. R. & Culotta, V. C. (1995). Mutations in PMR1 suppress oxidative damage in yeast cells lacking superoxide dismutase. *Mol Cell Biol* **15**, 1382-1388.

Lee, J., Kwon, E. S., Kim, D. W., Cha, J. & Roe, J. H. (2002). Regulation and the role of Cu,Zn-containing superoxide dismutase in cell cycle progression of Schizosaccharomyces pombe. *Biochem Biophys Res Commun* **297**, 854-862.

Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J. & Toledano, M. B. (1999). Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *J Biol Chem* **274**, 16040-16046.

Lee, J. H., Choi, I. Y., Kil, I. S., Kim, S. Y., Yang, E. S. & Park, J. W. (2001). Protective role of superoxide dismutases against ionizing radiation in yeast. *Biochim Biophys Acta* **1526**, 191-198.

Li, Y., Huang, T. T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C. & other authors (1995). Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* **11**, 376-381.

Lin, S. J. & Culotta, V. C. (1995). The ATX1 gene of Saccharomyces cerevisiae encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. *Proc Natl Acad Sci U S A* **92**, 3784-3788.

Lin, S. J. & Culotta, V. C. (1996). Suppression of oxidative damage by Saccharomyces cerevisiae ATX2, which encodes a manganese-trafficking protein that localizes to Golgi-like vesicles. *Mol Cell Biol* **16**, 6303-6312.

Liochev, S. I. & Fridovich, I. (1999). Superoxide and iron: partners in crime. *IUBMB Life* **48**, 157-161.

Liochev, S. I. & Fridovich, I. (2005). Cross-compartment protection by SOD1. *Free Radic Biol Med* **38**, 146-147.

Liu, X., Zhang, X. & Zhang, Z. (2010). Cu,Zn-superoxide dismutase is required for cell wall structure and for tolerance to cell wall-perturbing agents in Saccharomyces cerevisiae. *FEBS Lett* **584**, 1245-1250.

Liu, X. F. & Culotta, V. C. (1994). The requirement for yeast superoxide dismutase is bypassed through mutations in BSD2, a novel metal homeostasis gene. *Mol Cell Biol* **14**, 7037-7045.

Longo, V. D., Gralla, E. B. & Valentine, J. S. (1996). Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. Mitochondrial production of toxic oxygen species in vivo. *J Biol Chem* **271**, 12275-12280.

Longo, V. D., Liou, L. L., Valentine, J. S. & Gralla, E. B. (1999). Mitochondrial superoxide decreases yeast survival in stationary phase. *Arch Biochem Biophys* **365**, 131-142.

Luk, E., Yang, M., Jensen, L. T., Bourbonnais, Y. & Culotta, V. C. (2005). Manganese activation of superoxide dismutase 2 in the mitochondria of Saccharomyces cerevisiae. *J Biol Chem* **280**, 22715-22720.

Lynch, M. & Kuramitsu, H. (2000). Expression and role of superoxide dismutases (SOD) in pathogenic bacteria. *Microbes Infect* **2**, 1245-1255.

Manfredini, V., Roehrs, R., Peralba, M. C., Henriques, J. A., Saffi, J., Ramos, A. L. & Benfato, M. S. (2004). Glutathione peroxidase induction protects Saccharomyces cerevisiae sod1deltasod2delta double mutants against oxidative damage. *Braz J Med Biol Res* **37**, 159-165.

Mansour, M. K. & Levitz, S. M. (2002). Interactions of fungi with phagocytes. *Curr Opin Microbiol* **5**, 359-365.

Martchenko, M., Alarco, A. M., Harcus, D. & Whiteway, M. (2004). Superoxide dismutases in Candida albicans: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene. *Mol Biol Cell* **15**, 456-467.

Matsuyama, A., Arai, R., Yashiroda, Y., Shirai, A., Kamata, A., Sekido, S., Kobayashi, Y., Hashimoto, A., Hamamoto, M. & other authors (2006). ORFeome cloning and global analysis of protein localization in the fission yeast Schizosaccharomyces pombe. *Nat Biotechnol* **24**, 841-847.

McCord, J. M. & Fridovich, I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* **244**, 6049-6055.

Meneghini, R. (1997). Iron homeostasis, oxidative stress, and DNA damage. *Free Radic Biol Med* **23**, 783-792.

Miller, R. A. & Britigan, B. E. (1997). Role of oxidants in microbial pathophysiology. *Clin Microbiol Rev* **10**, 1-18.

Miramon, P., Dunker, C., Windecker, H., Bohovych, I. M., Brown, A. J., Kurzai, O. & Hube, B. (2012). Cellular responses of Candida albicans to phagocytosis and the extracellular activities of neutrophils are critical to counteract carbohydrate starvation, oxidative and nitrosative stress. *PLoS One* **7**, e52850.

Muid, K. A., Karakaya, H. C. & Koc, A. (2014). Absence of superoxide dismutase activity causes nuclear DNA fragmentation during the aging process. *Biochem Biophys Res Commun* **444**, 260-263.

Munkres, K. D., Rana, R. S. & Goldstein, E. (1984). Genetically determined conidial longevity is positively correlated with superoxide dismutase, catalase, glutathione peroxidase, cytochrome c peroxidase, and ascorbate free radical reductase activities in Neurospora crassa. *Mech Ageing Dev* **24**, 83-100.

Mutoh, N. & Kitajima, S. (2007). Accelerated chronological aging of a mutant fission yeast deficient in both glutathione and superoxide dismutase having cu and zn as cofactors and its enhancement by sir2 deficiency. *Biosci Biotechnol Biochem* **71**, 2841-2844.

Narasipura, S. D., Chaturvedi, V. & Chaturvedi, S. (2005). Characterization of Cryptococcus neoformans variety gattii SOD2 reveals distinct roles of the two superoxide dismutases in fungal biology and virulence. *Mol Microbiol* **55**, 1782-1800.

Narasipura, S. D., Ault, J. G., Behr, M. J., Chaturvedi, V. & Chaturvedi, S. (2003). Characterization of Cu,Zn superoxide dismutase (SOD1) gene knock-out mutant of Cryptococcus neoformans var. gattii: role in biology and virulence. *Mol Microbiol* **47**, 1681-1694.

Nash, H. M., Bruner, S. D., Scharer, O. D., Kawate, T., Addona, T. A., Spooner, E., Lane, W. S. & Verdine, G. L. (1996). Cloning of a yeast 8-oxoguanine DNA glycosylase reveals the existence of a base-excision DNA-repair protein superfamily. *Curr Biol* **6**, 968-980.

Nedeva, T. S., Petrova, V. Y., Zamfirova, D. R., Stephanova, E. V. & Kujumdzieva, A. V. (2004). Cu/Zn superoxide dismutase in yeast mitochondria - a general phenomenon. *FEMS Microbiol Lett* **230**, 19-25.

Neeley, W. L. & Essigmann, J. M. (2006). Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products. *Chem Res Toxicol* **19**, 491-505.

Nikolaou, E., Agrafioti, I., Stumpf, M., Quinn, J., Stansfield, I. & Brown, A. J. (2009). Phylogenetic diversity of stress signalling pathways in fungi. *BMC Evol Biol* **9**, 44.

O'Brien, K. M., Dirmeier, R., Engle, M. & Poyton, R. O. (2004). Mitochondrial protein oxidation in yeast mutants lacking manganese-(MnSOD) or copper- and zinc-containing superoxide dismutase (CuZnSOD): evidence that MnSOD and CuZnSOD have both unique and overlapping functions in protecting mitochondrial proteins from oxidative damage. *J Biol Chem* **279**, 51817-51827.

Pan, X., Ye, P., Yuan, D. S., Wang, X., Bader, J. S. & Boeke, J. D. (2006). A DNA integrity network in the yeast Saccharomyces cerevisiae. *Cell* **124**, 1069-1081.

Parker, M. W. & Blake, C. C. (1988). Iron- and manganese-containing superoxide dismutases can be distinguished by analysis of their primary structures. *FEBS Lett* **229**, 377-382.

Pereira, M. D., Eleutherio, E. C. & Panek, A. D. (2001). Acquisition of tolerance against oxidative damage in Saccharomyces cerevisiae. *BMC Microbiol* **1**, 11.

Peskin, A. V. & Winterbourn, C. C. (2000). A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). *Clin Chim Acta* **293**, 157-166.

Pfaller, M. A., Jones, R. N., Doern, G. V., Sader, H. S., Messer, S. A., Houston, A., Coffman, S. & Hollis, R. J. (2000). Bloodstream infections due to Candida species: SENTRY antimicrobial surveillance program in North America and Latin America, 1997-1998. *Antimicrob Agents Chemother* **44**, 747-751.

Phillips, J. P., Campbell, S. D., Michaud, D., Charbonneau, M. & Hilliker, A. J. (1989). Null mutation of copper/zinc superoxide dismutase in Drosophila confers hypersensitivity to paraquat and reduced longevity. *Proc Natl Acad Sci U S A* **86**, 2761-2765.

Pinkham, J. L., Wang, Z. & Alsina, J. (1997). Heme regulates SOD2 transcription by activation and repression in Saccharomyces cerevisiae. *Curr Genet* **31**, 281-291.

Portnoy, M. E., Liu, X. F. & Culotta, V. C. (2000). Saccharomyces cerevisiae expresses three functionally distinct homologues of the nramp family of metal transporters. *Mol Cell Biol* **20**, 7893-7902.

Portnoy, M. E., Schmidt, P. J., Rogers, R. S. & Culotta, V. C. (2001). Metal transporters that contribute copper to metallochaperones in Saccharomyces cerevisiae. *Mol Genet Genomics* **265**, 873-882.

Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C. & O'Halloran, T. V. (1999). Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* **284**, 805-808.

Reddehase, S., Grumbt, B., Neupert, W. & Hell, K. (2009). The disulfide relay system of mitochondria is required for the biogenesis of mitochondrial Ccs1 and Sod1. *J Mol Biol* **385**, 331-338.

Reddi, A. R. & Culotta, V. C. (2013). SOD1 integrates signals from oxygen and glucose to repress respiration. *Cell* **152**, 224-235.

Rhie, G. E., Hwang, C. S., Brady, M. J., Kim, S. T., Kim, Y. R., Huh, W. K., Baek, Y. U., Lee, B. H., Lee, J. S. & other authors (1999). Manganese-containing superoxide dismutase and its gene from Candida albicans. *Biochim Biophys Acta* **1426**, 409-419.

Roetzer, A., Klopf, E., Gratz, N., Marcet-Houben, M., Hiller, E., Rupp, S., Gabaldon, T., Kovarik, P. & Schuller, C. (2011). Regulation of *Candida glabrata* oxidative stress resistance is adapted to host environment. *FEBS Lett* **585**, 319-327.

Rohm, M., Lindemann, E., Hiller, E., Ermert, D., Lemuth, K., Trkulja, D., Sogukpinar, O., Brunner, H., Rupp, S. & other authors (2013). A family of secreted pathogenesis-related proteins in Candida albicans. *Mol Microbiol* **87**, 132-151.

Rosas-Hernandez, L. L., Juarez-Reyes, A., Arroyo-Helguera, O. E., De Las Penas, A., Pan, S. J., Cormack, B. P. & Castano, I. (2008). yKu70/yKu80 and Rif1 regulate silencing differentially at telomeres in Candida glabrata. *Eukaryot Cell* **7**, 2168-2178.

Schmidt, P. J., Kunst, C. & Culotta, V. C. (2000). Copper activation of superoxide dismutase 1 (SOD1) in vivo. Role for protein-protein interactions with the copper chaperone for SOD1. *J Biol Chem* **275**, 33771-33776.

Sehati, S., Clement, M. H., Martins, J., Xu, L., Longo, V. D., Valentine, J. S. & Gralla, E. B. (2011). Metabolic alterations in yeast lacking copper-zinc superoxide dismutase. *Free Radic Biol Med* **50**, 1591-1598.

Seider, K., Brunke, S., Schild, L., Jablonowski, N., Wilson, D., Majer, O., Barz, D., Haas, A., Kuchler, K. & other authors (2011). The facultative intracellular pathogen *Candida glabrata* subverts macrophage cytokine production and phagolysosome maturation. *J Immunol* **187**, 3072-3086.

Shockley, A. H., Doo, D. W., Rodriguez, G. P. & Crouse, G. F. (2013). Oxidative damage and mutagenesis in Saccharomyces cerevisiae: genetic studies of pathways affecting replication fidelity of 8-oxoguanine. *Genetics* **195**, 359-367.

Slekar, K. H., Kosman, D. J. & Culotta, V. C. (1996). The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection. *J Biol Chem* **271**, 28831-28836.

Smith, M. W. & Doolittle, R. F. (1992). A comparison of evolutionary rates of the two major kinds of superoxide dismutase. *J Mol Evol* **34**, 175-184.

Srinivasan, C., Liba, A., Imlay, J. A., Valentine, J. S. & Gralla, E. B. (2000). Yeast lacking superoxide dismutase(s) show elevated levels of "free iron" as measured by whole cell electron paramagnetic resonance. *J Biol Chem* **275**, 29187-29192.

Stoica, B. A., Rusu, M., Petreus, T. & Nechifor, M. (2011). Manganese SOD mimics are effective against heat stress in a mutant fission yeast deficient in mitochondrial superoxide dismutase. *Biol Trace Elem Res* **144**, 1344-1350.

Strain, J., Lorenz, C. R., Bode, J., Garland, S., Smolen, G. A., Ta, D. T., Vickery, L. E. & Culotta, V. C. (1998). Suppressors of superoxide dismutase (SOD1) deficiency in Saccharomyces cerevisiae. Identification of proteins predicted to mediate iron-sulfur cluster assembly. *J Biol Chem* **273**, 31138-31144.

Sturtz, L. A., Diekert, K., Jensen, L. T., Lill, R. & Culotta, V. C. (2001). A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. *J Biol Chem* **276**, 38084-38089.

Tainer, J. A., Getzoff, E. D., Richardson, J. S. & Richardson, D. C. (1983). Structure and mechanism of copper, zinc superoxide dismutase. *Nature* **306**, 284-287.

Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S. & Richardson, D. C. (1982). Determination and analysis of the 2 A-structure of copper, zinc superoxide dismutase. *J Mol Biol* **160**, 181-217.

Takahashi, H., Suzuki, T., Shirai, A., Matsuyama, A., Dohmae, N. & Yoshida, M. (2011). Mitochondrial localization of fission yeast manganese superoxide dismutase is required for its lysine acetylation and for cellular stress resistance and respiratory growth. *Biochem Biophys Res Commun* **406**, 42-46.

Tarhan, C., Pekmez, M., Karaer, S., Arda, N. & Sarikaya, A. T. (2007). The effect of superoxide dismutase deficiency on zinc toxicity in Schizosaccharomyces pombe. *J Basic Microbiol* **47**, 506-512.

Tsang, C. K., Liu, Y., Thomas, J., Zhang, Y. & Zheng, X. F. (2014). Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. *Nat Commun* **5**, 3446.

Unlu, E. S. & Koc, A. (2007). Effects of deleting mitochondrial antioxidant genes on life span. *Ann N Y Acad Sci* **1100**, 505-509.

van Loon, A. P., Pesold-Hurt, B. & Schatz, G. (1986). A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. *Proc Natl Acad Sci U S A* **83**, 3820-3824.

Van Remmen, H., Ikeno, Y., Hamilton, M., Pahlavani, M., Wolf, N., Thorpe, S. R., Alderson, N. L., Baynes, J. W., Epstein, C. J. & other authors (2003). Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol Genomics* **16**, 29-37.

Viriyakosol, S., Singhania, A., Fierer, J., Goldberg, J., Kirkland, T. N. & Woelk, C. H. (2013). Gene expression in human fungal pathogen Coccidioides immitis changes as arthroconidia differentiate into spherules and mature. *BMC Microbiol* **13**, 121.

Wallace, M. A., Liou, L. L., Martins, J., Clement, M. H., Bailey, S., Longo, V. D., Valentine, J. S. & Gralla, E. B. (2004). Superoxide inhibits 4Fe-4S cluster enzymes involved in amino acid biosynthesis. Cross-compartment protection by CuZn-superoxide dismutase. *J Biol Chem* **279**, 32055-32062.

Wilkinson, S. R., Prathalingam, S. R., Taylor, M. C., Ahmed, A., Horn, D. & Kelly, J. M. (2006). Functional characterisation of the iron superoxide dismutase gene repertoire in Trypanosoma brucei. *Free Radic Biol Med* **40**, 198-209.

Willocks, L., Leen, C. L., Brettle, R. P., Urquhart, D., Russell, T. B. & Milne, L. J. (1991). Fluconazole resistance in AIDS patients. *J Antimicrob Chemother* **28**, 937-939.

Wintjens, R., Noel, C., May, A. C., Gerbod, D., Dufernez, F., Capron, M., Viscogliosi, E. & Rooman, M. (2004). Specificity and phenetic relationships of iron- and manganese-containing superoxide dismutases on the basis of structure and sequence comparisons. *J Biol Chem* **279**, 9248-9254.

Woodruff, R. C., Phillips, J. P. & Hilliker, A. J. (2004). Increased spontaneous DNA damage in Cu/Zn superoxide dismutase (SOD1) deficient Drosophila. *Genome* **47**, 1029-1035.

Xiao, W., Chow, B. L. & Rathgeber, L. (1996). The repair of DNA methylation damage in *Saccharomyces cerevisiae*. *Curr Genet* **30**, 461-468.

Xu, H., Andi, B., Qian, J., West, A. H. & Cook, P. F. (2006). The alpha-aminoadipate pathway for lysine biosynthesis in fungi. *Cell Biochem Biophys* **46**, 43-64.

Yan, L. J., Levine, R. L. & Sohal, R. S. (1997). Oxidative damage during aging targets mitochondrial aconitase. *Proc Natl Acad Sci U S A* **94**, 11168-11172.

Youn, H. D., Kim, E. J., Roe, J. H., Hah, Y. C. & Kang, S. O. (1996). A novel nickel-containing superoxide dismutase from Streptomyces spp. *Biochem J* **318 (Pt 3)**, 889-896.

Youseff, B. H., Holbrook, E. D., Smolnycki, K. A. & Rappleye, C. A. (2012). Extracellular superoxide dismutase protects Histoplasma yeast cells from host-derived oxidative stress. *PLoS Pathog* **8**, e1002713.

Zabriskie, T. M. & Jackson, M. D. (2000). Lysine biosynthesis and metabolism in fungi. *Nat Prod Rep* **17**, 85-97.

Znaidi, S., Barker, K. S., Weber, S., Alarco, A. M., Liu, T. T., Boucher, G., Rogers, P. D. & Raymond, M. (2009). Identification of the Candida albicans Cap1p regulon. *Eukaryot Cell* **8**, 806-820.