Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Oxidative stress response in *Paracoccidioides brasiliensis*

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Genet. Mol. Res. 4 (2): 409-429 (2005)
Received January 18, 2005
Accepted May 5, 2005
Published June 30, 2005

ABSTRACT. Survival of pathogenic fungi inside human hosts depends on evasion from the host immune system and adaptation to the host environment. Among different insults that *Paracoccidioides brasiliensis* has to handle are reactive oxygen and nitrogen species produced by the human host cells, and by its own metabolism. Knowing how the parasite deals with reactive species is important to understand how it establishes infection and survives within humans. The initiative to describe the *P. brasiliensis* transcriptome fostered new approaches to study oxidative stress response in this organism. By examining genes related to oxidative stress response, one can evaluate the parasite's ability to face this condition and infer about possible ways to overcome this ability. We report the results of a search of the *P. brasiliensis* assembled expressed sequence tag database for homologous sequences involved in oxidative stress response. We described several genes coding proteins involved in antioxidant defense, for example, catalase and superoxide dismutase isoenzymes, peroxiredoxin, cytochrome c peroxidase, glutathione synthesis enzymes, thioredoxin, and the transcription factors Yap1

and Skn7. The transcriptome analysis of *P. brasiliensis* reveals a pathogen that has many resources to combat reactive species. Besides characterizing the antioxidant defense system in *P. brasiliensis*, we also compared the ways in which different fungi respond to oxidative damage, and we dentified the basic features of this response.

Key words: *Paracoccidioides brasiliensis*, Expressed sequence tags, Oxidative stress, Antioxidant, Free radicals, Transcription factors

INTRODUCTION

Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants (Sies, 1997). It is a general biological condition and a challenge to aerobic organisms. In multicellular organisms, including man, it takes part in physiology and pathology. The course and outcome of oxidative stress depend on properties of the reactive oxygen species (ROS) and the antioxidant defenses that are involved. The term ROS covers oxygen molecules in different redox and excitation states, as well as compounds of oxygen with hydrogen, chlorine and nitrogen (Sigler et al., 1999).

Primary ROS include the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$). These can produce additional secondary reactive oxygen metabolites, which are products of lipid peroxidation (hydroperoxides, alkoxyl and peroxyl radicals, epoxides, or aldehydes) and peroxynitrite ($ONOO^-$), a product of the reaction between superoxide with nitrogen monoxide (Beckman and Koppenol, 1996; Sigler et al., 1999). These reactive species can damage lipids, proteins, and nucleic acids, and can cause mutations in DNA, which might contribute to tumor formation. Aerobic life implies ROS production. Various metabolic processes produce ROS. The mitochondrial respiratory chain is an important source of ROS; peroxisomal metabolism is another contributor. Peroxisomes house some oxidative enzymes producing ROS, such as H_2O_2, which forms during β-oxidation of fatty acids (Van den Bosch et al., 1992). Formation of ROS in biological systems is important because these species can damage all biological molecules, but may also act as a second messenger in signal transduction (Allen and Tresini, 2000; Vivancos et al., 2004).

We searched for the antioxidants and oxidative stress response of the human pathogen *Paracoccidioides brasiliensis*, based on information provided by transcriptome analysis. We also compared the ways in which different fungi respond to oxidative damage and we identified the basic features of the response.

MATERIAL AND METHODS

We identified the supposed genes involved in oxidative stress by searching the *P. brasiliensis* expressed sequence tag (PbEAST) database and by using tools provided by the bioinformatics group of the *P. brasiliensis* functional genome project (Felipe et al., 2003). Analysis of the selected genes was made using common bioinformatics tools.

RESULTS AND DISCUSSION

Oxidative burst as part of the host response to *Paracoccidioides brasiliensis*

In many pathologies, oxidative stress can be an outcome of a loss of cell homeostasis or of a response against a pathogen. *Paracoccidioides brasiliensis* occurs as an intracellular parasite in the human host. The immune cells' oxidative burst, a reaction characterized by increased oxygen uptake and ROS production, challenges parasite viability (Hampton et al., 1998). On infection of the human host, *P. brasiliensis* has to interact with various effector cells (macrophages, polymorphonuclear leukocytes, monocytes). For successful colonization, it should resist their microbiostatic and microbicidal mechanisms. It is not known why some people eliminate *P. brasiliensis*, whereas others are unable to avoid the spread of this parasite to tissues. The microbicidal capability of macrophages relates to reactive oxygen and nitrogen intermediates production, with nitric oxide (NO) being one of the most potent cytotoxic agents (MacMicking et al., 1997). Patients with paracoccidioidomycosis (PCM) produce lower levels of IFN-γ and TNF-α, suggesting that these cytokines are important in two ways. First, generation of NO decreases when IFN-γ and TNF-α diminish (Souto et al., 2000). Gonzales et al. (2000) showed that cytokine-induced production of NO by macrophages inhibits the transformation from conidium to yeast, an important step for the stablishment of infection. NO may be protective, acting as an effector molecule of macrophage cytotoxicity and as a regulator of inflammatory responses. It probably has a secondary effect, causing immunosuppression and controlling spread of the fungus (Bocca et al., 1998, 1999). Nascimento et al. (2002) showed that NO is essential for resistance because inducible NO synthase-deficient mice are susceptible to *P. brasiliensis* infection, and persistent NO production correlates with susceptibility to *P. brasiliensis* infection.

Much of the NO toxicity is due to the peroxynitrite anion ($ONOO^-$). The reactivity of $ONOO^-$ is roughly the same as that of the hydroxyl radical and NO_2. Its toxicity arises from the ability to nitrate and hydroxylate the aromatic rings of amino acid residues directly, and to react with sulfhydryls, lipids, proteins, and DNA. Peroxynitrite anion can also affect cellular energy status by inactivating key mitochondrial enzymes, and it may trigger calcium release from the mitochondria. The peroxynitrite anion, and perhaps a few other reactive nitrogen species, but not NO, can nitrate tyrosine residues, potentially leading to protein dysfunction. These reactions can have devastating effects on cellular physiology and viability (Barzilai et al., 2002).

We have previously described *P. brasiliensis* genes coding proteins likely to interfere with NADPH oxidase activity and to convert reactive nitrogen species into less toxic forms (Felipe et al., 2005). Among the latest were copper and zinc-containing superoxide dismutase, peroxiredoxins, glucose-6-phosphate dehydrogenase, and flavohemoglobin. Peroxiredoxins may protect against NO because of their activity towards peroxynitrite (see below). We found one gene (PbAEST 2685) homologous to an NO reductase (NOR) gene from *Fusarium oxysporum*. The enzyme coded by this gene may protect *P. brasiliensis* against NO toxicity. The *P. brasiliensis* NOR (EC 1.7.99.7) homolog showed 48 and 43% identity to the *Aspergillus oryzae* and *Fusarium oxysporum* NOR genes, respectively. NOR participates in denitrification, a process discovered more than a century ago, and now known to occur not only in bacteria, but also in archaea and in the mitochondria of fungi. NORs are present in several pathogens that are not denitrifiers (Takaya and Shoun, 2000). Denitrifying activity is widely distributed among fungi

and yeast (Shoun et al., 1992; Usuda et al., 1995; Tsuruta et al., 1998; Zhang et al., 2001). Part of the denitrifying process in fungi is associated with the respiratory chain, coupled to the synthesis of ATP (Kobayashi et al., 1996).

Fusarium oxysporum is the first example of a denitrifying eukaryote. To detoxify NO, the fungus NOR converts NO to N_2O. *Fusarium oxysporum* produces two isoforms of P450, P450norA and P450norB, located in the mitochondria and cytosol, respectively (Nakahara and Shoun, 1996). These isoenzymes show no observable difference in their catalytic properties (Takaya et al., 2002). They are encoded by the same gene (CYP55A1) and are translated from different initiation codons (Takaya et al., 1996). Recent advances in genome projects revealed the presence of a *Neurospora crassa* cDNA sequence that is homologous to these P450nors (http:// www.genome.ou.edu/fungal.html).

Anjum et al. (2002) identified two loci within the *Neisseira meningitides* MC58 genome, which may take part in pathogenesis. The gene predictably encodes a 151-amino acid polypeptide (NorB) with similarity to cytochrome c from other organisms (Ambler et al., 1981). NorB keeps NO concentration low and takes part in evading the host immune system during infection. The fungal denitrifying set of enzymes include nitrate reductase, formate dehydrogenase, nitrite reductase, and NOR. The *N. meningitides* NOR belongs to the cytochrome P450 superfamily based on the primary and tertiary structures, and was thus designated P450nor (Takaya et al., 2002). The NOR enzyme in *P. brasiliensis* may have a role in helping the fungus to evade the host immune response during infection.

Association between reactive oxygen intermediates release and resistance to infection occurs in several infectious diseases. For instance, monocytes and macrophages kill the pathogenic fungus *C. albicans* by mechanisms dependent on O_2. The superoxide anion ($O_2^{\bullet-}$) is essential for the oxidative death of *C. albicans* by monocytes and macrophages. H_2O_2 and $ONOO^-$ also increase the death rates of *C. albicans* (Vazquez-Torres and Balish, 1997).

Calvi et al. (2003) evaluated the effect of IFN-γ on the activation state and the fungicidal capability of monocytes from PCM patients. These authors showed an association between higher levels of H_2O_2 and higher fungicidal effect in patients, which suggests that O_2 metabolites are involved in the killing of fungus by these cells. Recent experiments showed that incubating monocytes with catalase during challenge with *P. brasiliensi*s prevents fungicidal activity, indicating that H_2O_2 may kill this pathogen. This was expected, because H_2O_2 is one of the main molecules involved in microbicidal or tumoricidal effects of human monocytes (Murray and Cartelli, 1983; Moonis et al., 1992). On the other hand, IFN-γ-activated murine peritoneal macrophages kill *P. brasiliensis* yeast cells in an oxidative burst-independent manner (Brummer et al., 1988).

Besides host cell ROS and reactive nitrogen species production, changes in redox equilibrium due to endogenous ROS production could cause *P. brasiliensis* to die. The mitochondrial electron transport chain and the β-oxidation dehydrogenases in peroxisomes are potential sources of ROS in *P. brasiliensis*. We identified a gene homologous to a multifunctional β-oxidation gene (PbAEST 2420), which has an e-value of e^{-63} in relation to gene XP-330945.1 from *N. crassa*. A supposed urate oxidase gene is also present in the *P. brasiliensis* transcriptome database. Urate oxidase (EC 1.7.3.3, also called uricase) is an enzyme of the purine breakdown pathway located in peroxisomes (Goldman and Blobel, 1978). Uricase converts uric acid into hydrogen peroxide and allantoin, which after several additional steps results in ammonia. The *Neurospora* uricase is a tetrameric enzyme composed of four identical subunits, each

with a molecular mass of about 33,000 kDa (Wang and Marzluf, 1980). Nahm and Marzluf (1987) studying *N. crassa* uricase, showed that a single enzyme species occurs in this fungus and that, on induction, increased enzyme activity parallels with increase enzyme synthesis. In *Aspergillus nidulans*, the purine degradation pathway has been thoroughly characterized by genetic and physiological studies. The uricase gene in this organism (uaZ) encodes a 301-amino acid protein that shows high similarities with 10 other urate oxidases (Oestreicher and Scazzocchio, 1993). The comparison among these proteins shows the possible copper binding site (Wu et al., 1989), the signal for peroxisomal entry (Gould et al., 1990), and two motifs of conserved amino acids.

Aspergillus flavus uricase consists of four identical subunits with molecular mass of about 32,000 kDa (Conley and Priest, 1980). Legoux et al. (1992) described the cDNA and genomic sequences of *A. flavus uaZ* and its expression in *Escherichia coli*. The *uaZ* gene encodes an open reading frame of 302 amino acids and contains two short introns. They identified a consensus sequence (Val-Leu-Lys-Ser-Thr-Asn-Ser) in *A. flavus* uricase, described for other organisms as Val-Leu-Lys-Thr-Thr-Gln-Ser. In addition, they observed a second consensus sequence in all uricases sequenced so far, which is Ser-Pro-Ser-Val-Gln-lys/His-Asn-Thr-Leu-Tyr. In *A. flavus* this sequence changes to Ser-Ala-Ser-Val-Gln-Ala-Thr-Met-Tyr. The deduced amino acid sequence of the *P. brasiliensis* PbEAST 264 (contig 265) matches the amino terminal sequence of uricase, when aligned with the *A. flavus* uricase (Legoux et al., 1992). The two conserved motifs described in *A. niger* and *A. flavus* uricase were not found in the predicted *P. brasiliensis* urate oxidase. However, some conserved amino acid residues, identified as aligning *A. flavus* with 10 other urate oxidases from different organisms, are present in the PbEAST 264 predicted protein. The amino acids are Glu, Leu, Tyr, Asn, and Ser, which in *A. flavus* uricase correspond to amino acids 32, 39, 47, 52, and 53, respectively (Legoux et al., 1992). There was 78% amino acid identity between PbAEST 264 urate oxidase and the *A. flavus* enzyme. Although this result was obtained through alignment of a short-amino acid sequence, it represents what has been observed for this enzyme from various organisms. Characterizing *Pb* uricase gene and protein should help to open new perspectives for using this protein as a drug target in PCM treatment, particularly due to the fact that this protein is absent in humans.

The antioxidant defense of *Paracoccidioides brasiliensis*

Antioxidants can be enzymes or non-enzymatic scavenging substances of low molecular weight. We searched the PbAEST database to check for enzymatic antioxidants. There are many low-molecular weight antioxidants. Among them are lipophilic molecules, such as α-tocopherol (vitamin E), carotenoids (including vitamin A), polyphenols (including flavonoids), and hydrophilic antioxidants such as glutathione (GSH), thioredoxin and glutaredoxin. We focused on the biosynthetic pathway of GSH.

Glutathione synthesis and metabolism

GSH (γ-L-glutamyl-L-cysteinylglycine) is an abundant thiol tripeptide that takes part in the metabolism of xenobiotics and ROS, amino acid transport, protein and nucleic acid synthesis, and modulation of enzyme activity (Sigler et al., 1999). It protects against oxidative stress by

maintaining the cytosol of the cells more reduced. GSH may react with the hydroxyl radical directly, producing water (Sigler et al., 1999). Its synthesis occurs by the consecutive action of γ-glutamyl-cysteine synthetase and GSH synthetase. Through the action of GSH peroxidase (GPx), it is converted into the oxidized form (GSSG). The latter is reduced back to GSH by GSH reductase, using NADPH as an electron donor. The resulting $NADP^+$ is regenerated to NADPH through the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from the pentose phosphate pathway. Apart from GPx, all other enzymes of the GSH system exist in *P. brasiliensis* (Table 1).

Table 1. Genes involved in glutathione biosynthesis and NADPH regeneration in *Paracoccidioides brasiliensis*.

PbAEST	Annotation	Number of EST	Organism/accession number/e-value
1485	Gamma-glutamylcysteine synthetase	2	*Aspergillus nidulans*/5e-44
2805	Glutamate-cysteine ligase	14	*Magnaporthe grisea*/1e-28
5388	Gamma-glutamylcysteine synthetase	1	*M. grise*/3e-70
1446	Glutathione synthetase	2	*Neurospora crassa*/2e-45
1304	Glutathione reductase	2	*A. nidulans*/e-121
5023	Glucose-6-phosphate dehydrogenase	1	*N. crassa*/7e-41
553	6-phosphogluconate dehydrogenase	14	*Aspergillus oryzae*/0.0

PbAEST = *P. brasiliensis* assembled expressed sequence tag.

GSH is an essential antioxidant in *S. cerevisiae* and is required for protection against H_2O_2 (Grant et al., 1996, 1998). *Saccharomyces cerevisiae* mutants in the genes coding for GSH redutase and GSH synthetase are sensitive to H_2O_2. These mutants have a survival rate two times lower than that of the wild-type yeast, when treated with 4 mM H_2O_2 for an hour (Duncan et al., 1996; Grant et al., 1999).

Superoxide dismutases and catalase

Superoxide dismutase (SOD) is a primary antioxidant defense against ROS and was the first enzyme reported to use a free radical as a substrate. It dismutates the superoxide radical ($O_2^{\bullet -}$) into molecular oxygen and H_2O_2. SODs may have different co-factors in the active site, which distinguishes the four types of isoenzymes: CuZnSOD, MnSOD, FeMnSOD, and NiSOD (Halliwell and Gutteridge, 1999). Copper- and zinc-containing and Mn-containing enzymes are cytosolic and mitochondrial enzymes, respectively. The MnSODs are dimeric or tetrameric enzymes and have one Mn^{+3} atom in each subunit (Fridovich, 1998). In eukaryotic cells, the MnSOD is synthesized in the cytosol and then imported into the mitochondria. The CuZnSODs from eukaryotic cells are homodimers (Tainer et al., 1982). Human CuZnSOD is a homodimer formed by noncovalently linked subunits of 16-19 kDa (Yoo et al., 1999). We found four genes corresponding to two SOD isoezymes in the *P. brasiliensis* transcriptome (Table 2). Of these, one is a manganese-containing protein and another is a copper- and zinc-containing enzyme.

CuZnSOD is important for the growth of fungi at high temperatures. In *C. albicans*, for example, the activity of CuZnSOD increases 100 times when these organisms are incubated at the 37°C (Romandini et al., 1994). *Candida albicans* mutants in the CuZnSOD gene show

Table 2. Superoxide dismutase homologs in *Paracoccidioides brasiliensis*.

PbAEST	Annotation	Number of EST	Organism/accession number/e-value
2509	Superoxide dismutase [Cu-Zn]	19	*Aspergillus fumigatus*/NP_012638.1/1e-68
1649	Superoxide dismutase	22	*Emericella nidulans*/6e-87
2587	[Mn]	5	*Neurospora crassa*/8e-55
5824		1	*E. nidulans*/2e-50

PbAEST = *P. brasiliensis* assembled expressed sequence tag.

a delay of five days in hyphal growth when compared to wild-type cells. These mutants also are sensitive to menadione and H_2O_2 (Hwang et al., 2002). The expression of CuZnSOD increases three times in *Cryptococcus neoformans* grown at 37°C, when compared with growth at 25°C (Cox et al., 2003). In *C. albicans* and *C. neoformans*, CuZnSOD takes part in virulence. This was concluded because CuZnSOD mutants of these organisms are less virulent to BALB/c mice (Hwang et al., 2002). Exposure of *C. albicans* to blood causes increased expression of the genes coding ROS detoxification enzymes, including CuZnSOD (Sod1), a catalase (Cat1), a supposed thiol-specific antioxidant protein (IPF2431), and a thioredoxin reductase (Trr1) (Fradin et al., 2003). These data show that *C. albicans* respond to signals present in the blood, most probably leukocytes.

Catalases (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) are widespread enzymes in aerobic organisms. They catalyze the reaction $2\ H_2O_2 \rightarrow O_2 + H_2O$. Three unrelated catalase gene families can be distinguished: manganese catalase, which has been reported only in prokaryotes, catalase peroxidases, widely distributed in the prokaryotic kingdom and also found in lower eukaryotes, and a family that may be referred to as true catalases, corresponding to homotetramic, heme-containing enzymes, found ubiquitously in eukaryotes and in many prokaryotes (Schonbaum and Chance, 1976; Klotz et al., 1997). Only heme catalases have been described for higher organisms (Schonbaum and Chance, 1976). The existence of a series of pathologies related to catalases going wrong, which comprise, among other characteristics, increased susceptibility to thermal injury (Leff, 1993), high mutation rates (Halliwell and Aruoma, 1991), inflammation (Halliwell and Gutteridge, 1990), and accelerated aging (Taub et al., 1999), exemplifies the importance of these enzymes.

Moreira et al. (2004) isolated a complete cDNA encoding a peroxisomal catalase of *P. brasiliensis* (PbcatP). The PbcatP revealed canonical motifs of monofunctional typical catalases and the peroxisome-PTS-1-targeting signal. PbcatP expression was induced in cells treated with H_2O_2, and the authors speculated that the enzymatic activity was protective against endogenously produced oxygen radicals and exogenous H_2O_2. These authors observed that the protein and its transcript were regulated during *P. brasiliensis* development, increasing during the mycelium-to-yeast transition. We observed complete identity between the predicted open reading frame product of PbAEST 5460 (Table 3) and the monofunctional peroxisomal catalase (P) of *P. brasiliensis* described by Moreira et al. (2004). PbAEST 5460 alignment performed using BLAST programs (Altschul et al., 1997) showed an e-value of $2e^{-24}$ in relation to gene AF428076, corresponding to PbcatP. Amino acid sequence analysis of PbAEST 5460 (52 amino acids) showed a region in the predicted open reading frame that is the haem binding site (RLFSYPDTH), also present in PbcatP, as in all other heme catalases that have been studied.

Table 3. Catalase homologs in *Paracoccidioides brasiliensis*.

PbAEST	Annotation	Number of EST	Organism/accession number/e-value
621	Peroxisomal	10	*Ajellomyces capsulatus*/AF189369/0.0
5460	Catalase P	1	*A. capsulatus*/AF189369/5e-25
3142	Catalase isozyme A	1	*A. capsulatus*/AF189368/2e-61

PbAEST = *P. brasiliensis* assembled expressed sequence tag.

Some studies have described how important oxidative stress is in *P. brasiliensis* host defense (McEwen et al., 1984; Melloni-Bruneri et al., 1996). However, publications on *P. brasiliensis* catalases and SOD are scarce. Catalase is important in fungal pathogenicity and development. For example, catalase takes part in *C. albicans* survival from neutrophil attack and within the host. Disruption of the *C. albicans* catalase gene results in higher sensitivity to damage by neutrophils and to exogenous peroxide. The *C. albicans CAT1* gene, which encodes a protein with catalase activity, is involved in oxidant susceptibility; its deletion generates cells that are less virulent in the mouse model of acute systemic infection (Wysong et al., 1998). The mitogen-activated protein kinase (MAPK) signal transduction cascade may act in activating the expression of the catalase gene in *C. albicans* (Nakagawa et al., 1999).

Catalase plays an important role in the pathogenicity of *Hystoplasma capsulatum* (Johnson et al., 2002). There are three catalase genes in *H. capsulatum*, CATA, CATB (encoding the M antigen) (Zancope-Oliveira et al., 1999), and CATP. All three catalase enzymes are synthesized by yeast cells during exposure to the respiratory burst of neutrophils and macrophages (Johnson et al., 2002). *Aspergillus fumigatus* produces two mycelial catalases, one that is monofunctional and one that is bifunctional catalase-peroxidase (Hearn et al., 1992). Paris et al. (2003) examined the role of all of the conidial and mycelial catalases of *A. fumigatus* in the pathogenicity of the fungus and observed the expression of three active catalases, one in conidia and two in mycelium. The conidial catalase does not protect conidia against the oxidative burst of macrophages, but it protects against H_2O_2 *in vitro*. ROS produced by alveolar macrophages play an essential role in killing *A. fumigatus* conidia (Philippe et al., 2003). However, the mycelial catalases are needed to scavenge harmful peroxide *in vitro* and in the rat model of infection, but they provide only partial resistance to polymorphonuclear leukocytes. A lack of catalase activity could make *A. fumigatus* vulnerable to rapid killing by the H_2O_2-generating phagocytes, and thus, *A. fumigatus* CAT1 has been supposed to be a virulence factor (Calera et al., 1997; Hamilton and Holdom, 1999).

Aspergillus nidulans offers an excellent experimental model to study cell growth regulation and differentiation by activated oxygen species. *Aspergillus nidulans* CatA and CatB enzymes belong to the monofunctional catalase family large subunit. They have different localizations; CatA has its activity associated with spores (Navarro et al., 1996) and CatB immunolocalizes in the hypha cell wall and in the cytosol (Kawasaki and Aguirre, 2001). Navarro et al. (1996) showed the preferential accumulation of CatA in *A. nidulans* conidia. This might reflect increased production of H_2O_2 and other forms of activated oxygen during development. Other catalases have been associated with fungal development, such as *Bacillus subtillis* catalase 2, which accumulates in stationary phase cells and spores (Loewen and Switala, 1987), and *N. crassa* catalase 3, which accumulates in conidia, like CatA (Chary and Natvig, 1989). Thus,

developmental control of catalases is likely to be widespread in prokaryotes and eukaryotes. Catalase and other antioxidant enzymes are linked to conidial longevity (Munkres, 1990).

Jakubowski et al. (2000), examining oxidative stress during prolonged incubation of stationary cultures of *S. cerevisiae*, observed that the extent of this stress increased in antioxidant-deficient strains, lacking SOD dismutases and catalases. They also observed a decreased GSH content. In yeast, catalase is inducible by various types of stress. Transcription of the CTT1 gene, which codes yeast catalase, is regulated by the cAMP level (Kong et al., 1996), and the lack of cAMP induces the biosynthesis of catalase. A drop in cAMP content is observed after various types of insults, in particular oxidative and heat stress (Yermilov et al., 1995).

Díaz et al. (2001) observed an increase in Cat1 of *N. crassa* during asexual spore formation. This spore catalase showed an unusual resistance to inactivation by temperature and various denaturants, characteristics that are especially suitable for spore survival. Previous research has associated ROS in inducing and controlling asexual sporulation in *N. crassa* (Hansberg, 1996).

Peroxidases and the thioredoxin system

Cytochrome-c peroxidase (CCP) is another key enzyme for controlling H_2O_2 concentration. It decomposes H_2O_2 at the expense of cytochrome c (Erman and Vitello, 2002). This heme protein was discovered in *S. cerevisiae* in 1940 by Altscul, Abrams and Hogness (reviewed in Yonetani and Ray, 1965). It is coded by the yeast nuclear genome and is synthesized as a preprotein that is processed to produce the mature enzyme upon heme addition (Kaput et al., 1989). CCP is localized in the mitochondrial intermembrane space of *S. cerevisiae* grown aerobically, where it protects the organisms from damage caused by high concentrations of H_2O_2. While classic peroxidases oxidize various substrates, including aromatic amines, phenols and lignin, CCP shows low affinity for these substrates, and therefore is a specific peroxidase (Halliwell and Gutteridge, 1999; Erman and Vitello, 2002).

Since its discovery in yeast, CCP has been shown to be absent in mammals and to be present in bacteria of the genus *Pseudomonas* (Villalain et al., 1984; Ridout et al., 1995; Alves et al., 1999), *Paracoccus denitrificans* (Pettigrew, 1991; Hu et al., 1997), *Methylococcus capsulatus* (Zahn et al., 1997), *Rhodobacter capsulatus* (Hu et al., 1998), and *Nitrosomonas europaea* (Arciero and Hooper, 1994). The trematodes *Schistosoma mansoni* and *Fasciola hepatica* also possess CCP activity, which locates in the intermembrane space of mitochondria (Campos et al., 1995, 1999). The presence of CCP in parasites that lack catalase indicates CCP as a major H_2O_2-detoxification enzyme. A partially purified CCP from *F. hepatica* has the ability to inhibit 2-deoxyribose degradation *in vitro* by blocking hydroxyl radical production (Campos et al., 1999). In experiments using the yeast *S. cerevisiae*, CCP gene expression increases when the cells are treated with 1 mM peroxinitrite. Therefore, this enzyme may participate in the detoxification of peroxinitrate as well as H_2O_2 (Kwon et al., 2003). *Paracoccidioides brasiliensis* also has CCP. We found a singlet (PbAEST 3183) with similarity to *S. cerevisiae* CCP having an e-value of 3e-8 in relation to the gene NP-012992.1. The presence of this specific enzyme in *P. brasiliensis* indicates that this fungus has a conserved extra H_2O_2 detoxification mechanism in relation to the human host.

Peroxiredoxins [Prx; also called thioredoxin peroxidase or thiol-specific antioxidant, reduce hydrogen peroxide, peroxynitrite, and a wide range of organic alkyl hydroperoxides

(ROOH) to water and the corresponding alcohol (Bryk et al., 2000; Wood et al., 2003a). They exist in all organisms, have highly conserved primary sequences and are produced at high levels in cells. They differ from other peroxidases in that they have no redox co-factors, such as metals and prosthetic groups. After peroxide reduction, Prx are reduced through electron transfer from thiol-containing donor molecules, such as thioredoxin and GSH (Wood et al., 2003b). The Prx superfamily is divided into three groups: 1-Cys Prx, "typical" and "atypical" 2-Cys Prx, according to the presence of one or two conserved cysteine residues and according to the catalytic reaction. Typical 2-Cys Prx are obligate homodimers, use thioredoxin as an electron donor, and have two conserved cysteine residues. The cysteine residues are commonly positioned near amino acid residue 50 (Cys-50) and 170 (Cys-170). The preceding cysteine domains match to a Phe-Val-Cys-Pro sequence, and the posterior domain to Glu-Val-Cys-Pro. Atypical 2-Cys are functionally monomeric and use thioredoxin as an electron donor. 1-Cys Prx have a conserved peroxidatic cysteine, but the identity of its redox partner remains elusive (Wood et al., 2003a). 2-Cys regulates peroxide-mediated signaling cascades (Fujii and Ikeda, 2002).

In *S. cerevisiae*, a protein named Prxp1 is homologous to human 1-Cys Prx (peroxiredoxin 6) (Wood et al., 2003b). This protein is a mitochondrial 1-Cys Prx and uses as electron donor the mitochondrial thioredoxin. Prx-1p protects yeast cells against heat shock and exogenous H_2O_2 exposure and may be more efficient in removing peroxides produced endogenously. Prx-1p-null cells are sensitive to oxidant conditions (Pedrajas et al., 2000). Peroxiredoxin homologs have been identified in the *P. brasiliensis* transcriptome (Felipe et al., 2003) (Table 4). Analysis of Prx sequences (Table 4) revealed that PbAEST 190 corresponds to a 1-Cys-Prx homolog (Figure 1). As peroxiredoxin 6 in humans is a GPx (Wood et al, 2003b) and we have not found a GPx gene in *P. brasiliensis*, we supposed that PbAEST 190 code a Prx with GPx activity. A real time RT-PCR analysis showed that a Prx gene is highly expressed in *P. brasiliensis* yeast cells grown under three different conditions in different media (Marques et al., 2004).

Table 4. Peroxiredoxins and thioredoxin homologs in *Paracoccidioides brasiliensis*.

PbAEST	Annotation	Number of EST	Organism/accession number/e-value
1333	Member of a glutaredoxin subfamily in Sc together with GRX4 & GRX5	4	*Saccharomyces cerevisiae*/NC_001136/ 2e-42
190	Tsa1	7	*Ajellomyces capsulatus*/AAG31645.1/3e-96
1715	Ahp1p	8	*S. cerevisiae*/NP_013210.1/4e-24
1811	PMP20 peroxisomal	4	*Schizosaccharomyces pombe*/NP_587706.1/2e-12
1923	Thioredoxin	11	*Emericella nidulans*/P29429/1e-25
4207	Trx1p	1	*S. cerevisiae*/NP_013144.1/3.1e-08
5873	TrxB_Schpo thioredoxin reductase	1	*S. pombe*/Q92375/9e-23

PbAEST = *P. brasiliensis* assembled expressed sequence tag.

Thioredoxins are small proteins (~12 kDa), containing an active site with a redox-active disulfide. They function in electron transfer by the reversible oxidation of two vicinal SH-groups

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PbAEST190       ----------------------------------------MAEEQRAPLRLGSTAPNFKA
Prx1p           MFSRICSAQLKRTAWTLPKQAHLQSQTIKTFATAPILCKQFKQSDQPRLRINSDAPNFDA
Prx6            ----------------------------------------MPGGLLLGDVAPNFEA
                                                         * :.. ****.*
                                          Cp
PbAEST190       VTTKGEIDFHEFIGDKYVVLFSHPDDFTPTCTTELGAFAKLEPEFTARGVKLIGLSANTL
Prx1p           DTTVGKINFYDYLGDSWGVLFSHPADFTPVCTTEVSAFAKLKPEFDKRNVKLIGLSVEDV
Prx6            NTTVGRIRFHDFLGDSWGILFSHPRDFTPVCTTELGRAAKLAPEFAKRNVKLIALSIDSV
                 ** *.* *::::**.: :***** ****.****:.  *** ***  *.****.** : :

PbAEST190       KSHYDWINDINEVTG----SDLQFPIIADADRKISYMYDMIDYQDTTNVDEKGMAMTIRS
Prx1p           ESHEKWIQDIKEIAKV---KNVGFPIIGDTFRNVAFLYDMVDAEGFKNIND-GSLKTVRS
Prx6            EDHLAWSKDINAYNCEEPTEKLPFPIIDDRNRELAILLGMLDPAEK---DEKGMPVTARV
                :.*  * :**:        ..: **** *  *::: : .*:*       :: *   * *

PbAEST190       VFIIDPNKKIRLTISYPASTGRNAAEVLRVVDALQTTDKNGVNTPINWNVGDDVIIPP--
Prx1p           VFVIDPKKKIRLIFTYPSTVGRNTSEVLRVIDALQLTDKEGVVTPINWQPADDVIIPPSV
Prx6            VFVFGPDKKLKLSILYPATTGRNFDEILRVVISLQLTAEKRVATPVDWKDGDSVMVLPTI
                **::.*.**::* : **::.***  *:***: :** * :: * **::*: .*.*:: *
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Figure 1. Amino acid sequence partial alignment of two 1Cys-Prx versus PbAEST190 (Table 4). The peroxidase sequences shown are mitochondrial peroxiredoxin (1-Cys Prx) with thioredoxin peroxidase activity from *Saccharomyces cerevisiae* (NP_009489) and *H. sapiens* peroxiredoxin 6, which is also a 1-Cys Prx (BC035857). Cp indicates the peroxidatic cysteine, which is shaded. Alignment was performed using ClustalW.

to an -S-S- bridge. They are reduced by thioredoxin reductase and are involved in antioxidant defense, ribonucleotide reduction and reduction of peroxidases and transcription factors (Rahlfs et al., 2002). The thioredoxin system comprises thioredoxin reductase, thioredoxins and thioredoxin-dependent peroxidases. Thioredoxin and thioredoxin reductase are proteins that uphold the redox state of the cell. Thioredoxin and thioredoxin reductase homologs found in *P. brasiliensis* are shown in Table 4. Glutaredoxins belong to the thioredoxin superfamily and are small heat-stable disulfide oxidoreductases. A glutaredoxin homolog is expressed at higher levels in the pathogenic yeast phase than in the mycelial phase of *P. brasiliensis* (Marques et al., 2003).

Glutathione-S-transferase

Glutathione-S-transferase (GST) conjugates GSH to DNA and lipid hydroperoxides, as well as mutagens, carcinogens, and other toxic chemical substances (Ketterer and Meyer, 1989). The enzymes also present binding properties toward a wide range of endogenous and exogenous ligands, interaction with kinases and GSH-dependent peroxidase and isomerase activities (Morel et al., 2004). Two distinct supergene families encode proteins with GST activity; first, at least 16 genes encode proteins expressed in tissue cytosols, and second, at least six genes are expressed in membranes. Based primarily on protein sequence similarity, the soluble GSTs have been divided into eight families that are highly conserved between species: Alpha, Mu, Pi, Theta, Kappa, Sigma, Zeta, and Omega (Hayes and Pulford, 1995; Hayes and Strange, 2000; Strange et al., 2001). Our analysis led to the identification of a class Omega glutathione transferase (Board et al., 2000) homolog in *P. brasiliensis*, as well as a microsomal homolog of GST (Table 5). A third PbAEST (832) was included in our results, because it has a GST signature and appears to be a member of the GST structural family. It would be interesting to determine if the enzymatic characteristics of the *P. brasiliensis* Omega GST is different from those of other GSTs.

Table 5. Glutathione-S-transferase homologs in *Paracoccidioides brasiliensis*.

PbAEST	Annotation	Number of EST	Organism/accession number/e-value
832	Glutathione-S-transferase	8	*Schizosaccharomyces pombe*/ CAA22828.1/7e-26
5013	Related to microsomal glutathione-S-transferase 3	1	*Neurospora crassa*/CAC18210.1/2e-21
2296	Glutathione-S-transferase omega 1	6	*Glycine max*/*AAG34801.1*/8e-18

PbAEST = *P. brasiliensis* assembled expressed sequence tag.

Metal chelators and the iron redox cycle in *Paracoccidioides brasiliensis*

The Cu^{+2} and Fe^{+2} ions play an important role in ROS production. In general, organisms need these ions for transport, protection against oxidative stress, cell growth, and development. However, they can catalyze hydroxyl radical formation by the Fenton reaction: $Fe^{+2} + H_2O_2 + H^+ \rightarrow Fe^{+3} + H_2O + HO^{\bullet}$ (Gutteridge, 1983). Therefore, the levels of Fe^{+2} and Cu^{+2} in cells should be carefully controlled.

Mitochondria need micromolar concentrations of iron to support the heme and the iron-sulfur cluster biosynthetic pathways (Petrat et al., 2002). The occurrence of Friedreich ataxia, a severe neuro- and cardio-degenerative disease in humans, in which mitochondria are unable to handle iron properly, exemplifies the importance of iron homeostasis (Rotig et al., 1997; Koutnikova et al., 1997). Friedreich ataxia is caused by defects in a protein known as frataxin, a protein of yet unknown function. In *S. cerevisiae*, frataxin loss causes iron accumulation in mitochondria, hindering respiration and causing oxidative damage to mitochondrial and nuclear DNA by Fenton chemistry (Babcock et al., 1997; Foury and Cazzalini, 1997; Wilson and Roof, 1997; Karthikeyan et al., 2002). Recent data suggest that yeast frataxin controls the iron needed for the living body biosynthesis of iron-sulfur clusters (Muhlenhoff et al., 2002; Duby et al., 2002) and of heme (Lesuisse et al., 2003). New evidence supports the hypothesis that frataxin functions both as a chaperone for Fe^{2+} when mitochondrial iron is limiting, and as a storage compartment for Fe^{3+} when iron is in excess (Park et al., 2003). In *P. brasiliensis*, there is a gene coding for frataxin (PbAEST 858), this gene having an e-value of $3e^{-15}$ in relation to the frataxin homolog (Yfh1p; NP-010163.1) from *S. cerevisiae*. Studies on *P. brasiliensis* frataxin may help to clarify the function of this protein in humans (Table 6).

Metallochaperones deliver copper to specific cellular targets (Luk et al., 2003). Yeast Lys7p (human CCS) delivers copper to Cu-Zn Sod1p in the cytosol (Cullota et al., 1997), and Atx1p (human HAH1 or ATOX1) specifically escorts copper to the secretory pathway for incorporation into copper enzymes destined for the cell surface or extracellular milieu (Lin et al., 1997). Atx1p specifically delivers copper to the Ccc2p copper transporter in the Golgi. Also, when overproduced, Atx1p substitutes for Sod1 in preventing oxidative damage. It may be a scavenger of free radicals (Portnoy et al., 1999). Homolog to both, Lys7p and Atx1p, were identified in the *P. brasiliensis* database. Metalloproteins, low-molecular weight cysteine-rich proteins responsible for the control of zinc and copper ions, were not identified in our database. Neither was ferritin, an intracellular protein responsible for intracellular iron storage. Whether these proteins exist in *P. brasiliensis* remains to be discovered.

Table 6. Iron homeostasis and metal chelator homologs in *Paracoccidioides brasiliensis*.

PbAEST	Annotation	Number of EST	Organism/accession number/e-value
858	Yeast frataxin homologue	4	*Saccharomyces cerevisiae*/NP_010163.1/3e-15
2607	Atx1p	3	*S. cerevisiae*/NP_014140.1/1e-09
541	Lys7p	6	*S. cerevisiae*/NP_013752.1/5e-51

PbAEST = *P. brasiliensis* assembled expressed sequence tag.

Transcription regulatory genes

To deal with the toxic and eventually lethal effects of ROS, cells have developed anti-oxidant mechanisms to respond to such threats. These mechanisms in lower eukaryotes have so far eluded complete understanding. However, it is obvious that this cellular response occurs, at least partially, at the transcriptional level. The various signaling pathways used by fungal cells to control the expression of genes involved in the oxidative stress response have been reviewed recently (Moye-Rowley, 2003). The mechanisms regulating the fungal response to oxidative challenge can be broadly classified into two types: nuclear localization control and activity regulation by protein phosphorylation through MAPK cascades. One of the first examples of a transcription factor in which nuclear localization is regulated by oxidative stress is Yap1p protein from *S. cerevisiae* (Costa and Moradas-Ferreira, 2001). It contains the basic region leucine zipper (bZip). Nuclear localization and trafficking between the nucleus and cytoplasm is essential for Yap1p regulation. Yap1 binds to the specific DNA sequence termed YRE (Yap1 response element, 50-TTA(C/G)TAA-30) (Fernandes et al., 1997). Mutants lacking YAP1 are highly sensitive to H_2O_2 (Schnell et al., 1992; Kuge and Jones, 1994). Yap1p constitutes a direct oxidant sensor in *S. cerevisiae*. A YAP1 homolog was found in the *P. brasiliensis* transcriptome (Table 7). YAP1 homologs are also found in *Schizosaccharomyces pombe* and *Candida albicans*, where they are named PAP1 and CAP1. Pap1p is required for resistance of *S. pombe* to oxidative stress, and it is activated by hydrogen peroxide (Toone et al., 1998; Vivancos et al., 2004). It seems to control the response against oxidative stress through the StyI/Spc1 MAPK pathway. Activated MAPK Sty1 has Atf1 as its substrate and histidine as upstream sensors for ROS (Toone et al., 1998, 2001). *Saccharomyces cerevisiae*, on the other hand, does not express the analogous histidine kinases seen in *S. pombe*, consistent with the different requirements for oxidant sensing in these two yeasts (Moye-Rowley, 2003). Hog1p is a crucial participant in osmotic stress (as is Sty in *S. pombe*) but does not play a significant role in the oxidative stress response (Toone and Jones, 1998). As with Yap1p, Cap1p from *C. albicans* localizes in the nucleus in response to oxidative stress (Zhang et al., 2000). C*ap1*Δ mutants are hypersensitive to hydrogen peroxide and to various other oxidants in solid medium (Alarco and Raymond, 1999; Alonso-Monge et al., 2003).

Another factor linked to oxidative stress tolerance is Skn7 (Krems et al., 1996), which exists in the *P. brasiliensis* transcriptome (Table 7). Data from Raitt et al. (2000) have shown that Skn7p connects with the normal response to oxidative stress in *S. cerevisiae*. A protein homologous to Skn7p, Prr1, has been described in *S. pombe* and responds to oxidative stress similarly to Skn7p in *S. cerevisiae* (Ohmiya et al., 1999). Another Skn7p homolog (*Ca* KKN7, accession number orf 19.971) is found in *C. albicans*, although a functional analysis of this gene

Table 7. Transcription factor homologs in *Paracoccidioides brasiliensis*.

PbAEST	Annotation	Number of EST	Organism/accession number/e-value
1731	Yap1p	4	*Neurospora crassa*/CAB91681.2/4e-16
4548	Yap3p	1	*Saccharomyces cerevisiae*/NP_011854.1/8.1e-08
2533	Skn7p	2	*Schizosaccharomyces pombe*/sp|O14283|/5e-26
3937	Mac1-dependent regulator (Mdr1p)	1	*S. cerevisiae*/NP_011614.1|/3e-31
356	Hog1	2	*S. cerevisiae*/NP_592843.1/1.1e-44
1426	Hap3p (HAPC)	3	*Aspergillus oryzae*/BAA28356.1|/9e-65
3390	Putative transcriptional activator (Hap)	1	*Aspergillus nidulans*/AB052971/1e-29

PbAEST = *P. brasiliensis* assembled expressed sequence tag.

has not been reported (Moye-Rowley, 2003). The presence of both Yap1p and Skn7p is required for the H_2O_2-mediated activation of the thioredoxin-encoding TRX2 gene and the GPx-encoding GPX2 gene, demonstrating that Skn7p function is linked to Yap1p (Morgan et al., 1997; Tsuzi et al., 2004). Yap1p also activates transcription of the GSH synthetase gene, which codes for the enzyme catalyzing the rate-imiting step of GSH biosynthesis (Wu and Moye-Rowley, 1994).

Thorpe et al. (2004), screened the *S. cerevisiae* deletion mutant collection by using five different ROS; they identified genes and cellular functions needed to preserve cellular viability when ROS are present. These genes include glutathione peroxidase GPx3, TRX2, CCP1, pentose phosphate pathway enzymes (GND1 and RPE1) and the transcriptional factors YAP1 and SKN7. As expected, genes encoding the specific oxidative-stress transcription factors, YAP1 and SKN7, were found to be essential for viability during all forms of oxidative stress. The YAP1 deletion mutant was more sensitive than the SKN7 deletion mutant. Yap1 has a larger role than Skn7p during oxidative stress and responds more specifically to ROS than does Skn7p (Godon et al., 1998).

Another transcriptional regulator involved in the oxidative stress response in *S. cerevisiae*, Hap3, has a homolog in *P. brasiliensis* (Table 7). In *S. cerevisiae*, transcription of the peroxiredoxin gene, TSA2, is responsive to various reactive oxygen and nitrogen species. Transcription of TSA2 is under Hap1p, rox1p and Hap2/3/5p control (Wong et al., 2003). HAP2/3/4 and HAP1 are normally found to activate genes involved in respiratory functions. They are involved in mediating higher activity of the inducible polyubiquitin gene (UBI4) during respiratory growth, which may reflect the contribution of UBI4 expression to tolerance to oxidative stress (Watt and Piper, 1997).

Abnormally low levels of copper can be toxic. The toxicity of copper may arise, in part, from cellular damage caused by reactive oxygen intermediates. Homeostasis mechanisms exist in all cells to regulate the cellular concentration of copper ions, minimizing the deleterious effects of excess copper ions. In *S. cerevisiae*, copper ions regulate gene expression through two transcription activators, Ace1 and Mac1. Thus, the known genes regulated by Mac1 in *S. cerevisiae* are functionally important in copper homeostasis. The SOD1 gene is transcriptionally activated when the extracellular copper exceeds 1 μM, as a protective response to the cytotoxicity of copper (Gross et al., 2000). The *P. brasiliensis* transcriptome has an MAC1-dependent regulator (Table 7) which may be involved with copper homeostasis.

Msn2/4p binds to a general stress response element (consensus CCCCT) and directly mediates stress response element-dependent transcriptional activation induction under pleiotropic stress conditions (Martínez-Pastor et al., 1996). Msn2p is a transcription factor involved in regulating the *S. cerevisiae* catalase gene (CTT1) (Martínez-Pastor et al., 1996). Although we have not found Msn2 and Msn4 homologs in the *P. brasiliensis* transcriptome, and they have been reported not to exist in *C. albicans* (Enjalbert et al., 2003), we cannot exclude the possibility of their existence in the former organism, because our database covers only ~80% of this parasite's genome (Felipe et al., 2005). Future studies about the functional role of these transcription factors described in the *P. brasiliensis* transcriptome could provide insight into the intricate nature of the fungal response to various oxidants.

CONCLUDING REMARKS

Our main findings: 1) a review of the antioxidant defense system of *P. brasiliensis* showed the presence of a specific gene (CCP), a gene not previously described in pathogenic fungi (GST Omega homolog) and a gene coding a 1-Cys Prx homolog that probably codes a mitochondrial enzyme having GPx activity; 2) an NOR homolog in *P. brasiliensis* that mediates NO detoxification is an important mechanism of immune system evasion, and 3) the transcription factors involved in the response against oxidative stress are similar to *S. cerevisiae* factors, indicating that nuclear localization may be the main mechanism of transcriptional response control.

ACKNOWLEDGMENTS

Research supported by MCT/CNPq, CNPq, CAPES, FUB, and UFG. We are thankful to Hugo Costa Paes for English revision.

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