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*Review*

# Virulence insights from the *Paracoccidioides brasiliensis* transcriptome

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**ABSTRACT.** *Paracoccidioides brasiliensis*, the etiologic agent of paracoccidioidomycosis, is a dimorphic fungus, which is found as mycelia at 22-26°C and as yeasts at 37°C. A remarkable feature common to several pathogenic fungi is their ability to differentiate from mycelium to yeast morphologies, or vice-versa. Although *P. brasiliensis* is a recognized pathogen for humans, little is known about its virulence genes. In this sense, we performed a search for putative virulence genes in the *P. brasiliensis* transcriptome. BLAST comparative analyses were done among *P. brasilienses* assembled expressed sequence tags (PbAESTs) and the sequences deposited in GenBank. As a result, the putative virulence PbAESTs were grouped into five classes, metabolism-, cell wall-, detoxification-related, secreted factors, and other determinants. Among these, we have identified orthologs of the glyoxylate cycle enzymes, a metabolic pathway involved in the virulence of bacteria and fungi. Besides the previously described α- and β-glucan synthases, orthologs to chitin synthase and mannosyl transferases, also important in cell wall synthesis and stabilization, were identified. With respect to the enzymes involved in the intracellular survival of *P. brasiliensis*, orthologs to su-

 

peroxide dismutase, thiol peroxidase and an alternative oxidase were also found. Among the secreted factors, we were able to find phospholipase and urease orthologs in *P. brasiliensis* transcriptome. Collectively, our results suggest that this organism may possess a vast arsenal of putative virulence genes, allowing the survival in the different host environments.

**Key words:** *Paracoccidioides brasiliensis*, Dimorphism, Virulence, Transcriptome analysis, Pathogenicity, Host-pathogen interaction

## INTRODUCTION

The incidence of fungal systemic diseases in healthy and in immunocompromised individuals is showing a worldwide increasing pattern in the last years, converting fungal diseases into an important medical research field. Since the treatment of these systemic infections is still compromised by the high costs, drug side effects and the development of resistant fungal strains, the discovery of new treatment approaches is of prime relevance. In the last decade, genomic approaches have proved to be a landmark in the characterization of fungal virulence factors, becoming a starting point for the knowledge of fungal molecular pathogenesis. In order to obtain information concerning the *P. brasiliensis* mycelium and yeast transcriptomes, a laboratory network from the central region of Brasil, has undertaken an expressed sequence tag (EST) genome project entitled "Functional and Differential Genome of the Fungus *Paracoccidioides brasiliensis*" (http://www.biomol.unb.br/Pb), as described by Felipe et al. (2003). Single-pass 5' sequencing from non-normalized cDNA libraries of mycelium and yeast cells generated a total of 19,718 high-quality ESTs. Upon CAP3 assembly, 2,655 contigs and 3,367 singlets, which constitute the so-called 6,022 *P. brasiliensis* assembled EST (PbAEST) database were generated (Felipe et al., 2005).

The dimorphic fungus *P. brasiliensis* is the etiologic agent of paracoccidioidomycosis (PCM), an important human systemic mycosis endemic in Central and South America (Brummer et al., 1993). Since 85% of PCM cases occur in Brazil, this disease represents a major health problem, being classified as the first cause of deaths among systemic mycoses, and the eighth, among all infectious and parasitic diseases (Restrepo et al., 2001; Coutinho et al., 2002). Infection is probably acquired by inhalation of airborne propagules derived from the mycelial saprophytic form of *P. brasiliensis* (Restrepo et al., 2001). Once in the lungs, this fungus undergoes a dimorphic transition, converting to the yeast form, an essential step for the establishment of the pulmonary infection, which alternatively can be eradicated, contained in a granuloma or disseminate to the rest of the body (Franco et al., 1993). These different outcomes of the fungus-host interaction will depend mainly on the host immunological response and fungal virulence. Clinical and experimental data indicate that cell-mediated immune response is the main mechanism of defense against *P. brasiliensis* infection, whereas specific antibodies produced in large amounts do not confer protection (Calich and Kashino, 1998; Kashino et al., 2000; Bernard et al., 2001; Fornari et al., 2001; Marques Mello et al., 2002). The protective cell-mediated

immune response in PCM is characterized by the production of cytokines (TNF-α, IL-12 and IFN-γ), which are required for the activation of macrophages, the main defensive cell against *P. brasiliensis* (Cano et al., 1998; Souto et al., 2000; Arruda et al., 2002). In the absence of such cytokines, such as in susceptible hosts, macrophages serve as a protected environment in which fungus can undergo intracellular replication and disseminates from the lungs to other organs, as observed in histoplasmosis (Brummer et al., 1988a,b, 1989, 1990; Moscardi-Bacchi et al., 1994; Gonzalez et al., 2000; Woods, 2003).

## FUNGAL VIRULENCE ATTRIBUTES

In a review on virulence, the understanding of this complex concept is imperative. The early pathogen-centered view of virulence states that it is an intrinsic and invariable microbial characteristic. This concept has been redefined in order to incorporate the host immune factors (Casadevall and Pirofski, 1999, 2003). Experimental evidence has shown the importance of the host immune response in the outcome of host-pathogen interactions, i.e., acapsular strains of *Cryptococcus neoformans* have reduced virulence in immunocompetent mice, whereas in immunodeficient mice these strains cause menigoencephalitis, similar to that caused by capsulated strains (Salkowski and Balish, 1991; Chang and Kwon-Chung, 1994). In this context, virulence is considered a microbial attribute strongly associated to the host susceptibility.

Differently from pathogenic bacteria and viruses, environmental pathogenic fungi do not require infection to replicate. In addition, person to person transmission is relatively rare. Thus, how virulence, a trait mostly maintained by selective evolutionary pressure, arises and is sustained in fungi? This question has now begun to be addressed. Steenbergen et al. (2001, 2004) and Mylonakis et al. (2002) have shown that environmental fungal pathogens such as *C. neoformans*, *Blastomyces dermatitidis*, *Sporothrix schenckii*, and *Histoplasma capsulatum* may have evolved some of their virulence properties towards humans and animals due to environmental selective pressures imposed by amoeboid and nematode predators, such as *Acanthamoeba castellanii*, *Dictyostelium discoideum* and *Caenorhabditis elegans*. Considering the environmental habitat of *P. brasiliensis* and its close phylogenetic relationship with *H. capsulatum* and *B. dermatitidis* (Leclerc et al., 1994; Guarro et al., 1999), studies must be performed in order to assess the influence of such ameboid and nematode predators on the acquirement and maintenance of *P. brasiliensis* virulence.

A number of potential virulence factors and events are considered important for invasive fungi, including dimorphism, growth at elevated temperatures, adherence to host cells, cell wall components, enzyme production, i.e., proteinases, lipases, phospholipases, and others (Hogan et al., 1996; San-Blas et al., 2000; Van Burik and Magee, 2001). Only recently, the specific genetic and molecular mechanisms of these potential virulence factors have become a matter of investigation (Kwon-Chung, 1998; Odds et al., 2001; Yang, 2003). Genomic and transcriptome sequencing efforts, coupled to sophisticated molecular biological tools, have made it possible to find direct proven evidence of whether a given factor is required for fungal virulence. The standard protocol for molecular pathogenesis studies in fungi has been the use of site-directed mutagenesis (gene-disruption), followed by the study of these specific null mutants and the respective reconstituted strains in a relevant animal model. For a gene to be considered part of the virulence composite, the infection caused by the null mutant must be attenuated when compared to that caused by the wild-type and reconstituted strains. This approach is based on the

"Molecular Koch's postulates", originally described for bacteria (Falkow, 1988, 2004), which has been used for several fungal genes (Perfect and Cox, 2000; Odds et al., 2001; Navarro-Garcia et al., 2001). In spite of the great progress on the studies of *P. brasiliensis* pathobiology, the absence of efficient gene-disruption and transformation systems preclude the study of putative virulence genes based on the molecular Koch's postulates. However, the *P. brasiliensis* transcriptome project (Felipe et al., 2003) allowed us to search for orthologs of virulence genes found in other pathogenic fungi that cause systemic mycosis. Among the genes assigned by several studies as virulence genes, we were able to find 30 orthologs in our transcriptome database (Table 1). Their predicted amino acid sequences were determined by comparative analyses with fungus sequences deposited in GenBank or other specific sites (for *Coccidiodes immitis* and *Histoplasma capsulatum*). The e-value and similarity results from some of these organisms were determined (Table 1). As one would expect, these genes are well distributed among all fungi listed, showing a high similarity score. We noted that tsa1, a putative thiol-specific antioxidant protein, and ags1, an α-1,3-glucan synthase, are relatively rare, with orthologs found only in few pathogenic fungi and none in non-pathogenic ones.

The *P. brasiliensis* virulence orthologs were placed in groups, based either on their functional or structural characteristics, such as: metabolism-, cell wall-, detoxification-related genes, secreted factors and other determinants (Navarro-Garcia et al., 2001). In this review, we examined specific gene products that were assigned as virulence factors by a molecular genetic approach (molecular Koch's postulates).

## METABOLISM-RELATED GENES

*P. brasiliensis*, a facultative intracellular pathogen, is able to survive and replicate within the phagosome of nonactivated murine and human macrophages (Brummer et al., 1988a,b, 1989, 1990; Moscardi-Bacchi et al., 1994; Gonzalez et al., 2000). Thus, this fungus may have evolved mechanisms that counteract the metabolic constraints imposed by phagocytic cells. The phagosome is believed to be a poor source of complex carbon, such as carbohydrates. Instead, intracellular pathogens may find only two-carbon (C2) compounds for energy production such as acetate, a product of fatty-acid degradation, for energy production (Finlay and Falkow, 1997; Lorenz and Fink, 2002). In agreement with its ability to survive in such inhospitable habitat, we identified two *C. albicans* ortholog genes in the *P. brasiliensis* transcriptome, *ICL1* and *MLS1*, encoding isocitrate lyase and malate synthase, respectively, which activities are specific and limited to the glyoxylate cycle. This cycle bypasses the two decarboxylation steps of the tricarboxylic acid cycle, thus allowing C2 compounds to serve as carbon sources in gluconeogenesis (Lorenz and Fink, 2002).

It has been found that the glyoxylate cycle belongs to the virulence repertoire of both bacteria (*Mycobacterium tuberculosis)* and fungi (*C. albicans*). Upon phagocytosis, *M. tuberculosis* and *C. albicans* respond inducing the glyoxylate cycle, as shown by the upregulation of *ICL* and *MLS* genes, when compared to the growth of these microorganisms in acellular cultures (Graham et al., 1999; Lorenz and Fink, 2001; Schnappinger et al., 2003). Furthermore, *icl* was also upregulated in the lungs of mice chronically infected with *M. tuberculosis*, suggesting a role of the glyoxylate cycle in the ability of *M. tuberculosis* to cause disease (Timm et al., 2003). Experimental virulence studies with a null mutant strain for the *icl* gene in *M. tuberculosis* showed that the mutant had its survival capacity decreased in IFN-γ-activated, but not

**Table 1.** *Paracoccidioides brasiliensis* putative virulence genes.

| PbAEST | Gene | *Candida albicans* | | *Cryptococcus neoformans* | | *Aspergillus fumigatus* | | *Coccidioides immitis* | | *Histoplasma capsulatum* | | *Magnaporthe grisea* | | *Neurospora crassa* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Accession number | e-value | Accession number | e-value | Accession number | e-value | Contig[1] | e-value | Contig[2] | e-value | Accession number | e-value | Accession number | e-value |
| Metabolism genes | | | | | | | | | | | | | | | |
| 495 | *ade2* | AAC49742 | 2e-83 | AAC98316 | 2e-62 | | - | 1.12 | 6e-06 | 4.113 | 5.3e-112 | EAA55605 | 8e-56 | XP_330630 | 3e-52 |
| 2396 | *ura3* | CAC08811.1 | 2e-39 | - | - | | 2e-83 | 1.13 | 1e-04 | 0.27 | 3.8e-81 | - | - | - | - |
| 668 | *nmt* | AAA34351 | 8e-80 | AAA17547 | 1e-60 | | e-115 | 1.76 | 7e-06 | 43.4 | 6.4e-96 | EAA56874 | 2e-70 | EAA30036 | 9e-85 |
| 3750 | *fas2* | JC4086 | 7e-33 | - | - | | - | 1.69 | 1e-23 | 5.30 | 3.1e-38 | - | - | XP_327594 | 4e-41 |
| 1219 | *hem3* | CAA21999 | 1e-58 | - | - | | - | - | - | 3.33 | 3.5e-96 | EAA52168 | 5e-60 | XP_331268 | 1e-57 |
| 3819 | *tps1* | CAA69223 | 8e-47 | AAT40476 | 3e-52 | | - | 1.23 | 5e-27 | 0.106 | 2.3e-83 | AAN46744 | 9e-56 | XP_330365 | 6e-56 |
| 1688 | *icl1* | AAF34690 | e-104 | AAL56614 | 6e-98 | | e-137 | 1.27 | 3e-93 | 0.199 | 1.2e-119 | EAA52203 | e-129 | XP_323570 | e-126 |
| 829 | *mls1* | AAF34695 | 2e-88 | - | - | | e-117 | 1.183 | 1e-41 | 2.61 | 7.5e-176 | EAA47570 | e-113 | XP_322865 | e-119 |
| 1730 | *pabaA* | EAK90820 | 3e-05 | - | - | | 1e-12 | - | - | 3.19 | 1.6e-14 | EAA50296 | 7e-07 | EAA31658 | 2e-07 |
| Cell wall genes | | | | | | | | | | | | | | | |
| 4346 | *chs3* | P30573 | 7e-22 | EAL23166 | 9e-44 | | 1e-36 | 1.96 | 1e-29 | 3.18 | 2.1e-52 | BAA74449 | 3e-39 | EAA27095 | 1e-59 |
| 4968 | *gna1* | BAA36496 | 1e-13 | - | - | | - | 1.125 | 3e-04 | 8.39 | 1.3e-33 | EAA47591 | 5e-24 | XP_329092 | 3e-29 |
| 1063 | *mnt1* | CAA67930 | 9e-49 | - | - | | - | - | - | 1.15 | 1.5e-32 | EAA51170 | 3e-24 | XP_326396 | 3e-24 |
| 2980 | *pmt1* | AAC31119 | 5e-43 | - | - | | 2e-17 | 1.36 | 6e-12 | 26.5 | 3.4e-78 | EAA50668 | 5e-77 | XP_332024 | 1e-79 |
| 2375 | *phr1* | AAF73430 | 8e-36 | - | - | | 1e-70 | 1.136 | 4e-06 | 25.24 | 1.4e-57 | EAA55065 | 1e-56 | XP_327067 | 3e-53 |
| 1370 | *phr2* | AAB80716 | e-113 | - | - | | e-116 | 1.23 | 7e-23 | 2.111 | 1.2e-158 | EAA57400 | e-113 | XP_331301 | e-116 |
| 4988 | *ags1* | - | - | - | - | | 4e-71 | - | - | 0.129 | 2.1e-49 | - | - | - | - |
| Detoxification | | | | | | | | | | | | | | | |
| 621 | *cat1* | CAA07164 | 1e-172 | - | - | - | - | 1.84 | 2e-96 | 4.47 | 3.5e-138 | EAA56471 | 2e-81 | XP_324526 | e-139 |
| 1098 | *aox1* | AAF21993 | 8e-26 | AAM22475 | 2e-48 | AAL87459 | 3e-15 | 1.18 | 3e-08 | 4.95 | 30e-73 | AAG49588 | 7e-30 | AAC37481 | 7e-36 |
| 2509 | *sod1* | AAC12872 | 2e-56 | AAK01665 | 4e-51 | AAD42060 | 2e-59 | 1.116 | 1e-06 | 27.7 | 4.6e-51 | EAA47382 | 3e-50 | XP_329323 | 4e-57 |
| 190 | *tsa1* | - | - | AAP68994 | 3e-18 | - | - | 1.77 | 2e-09 | 0.205 | 8.6e-70 | - | - | - | - |

Continued on next page

**Table 1.** Continued.

| PbAEST | Gene | *Candida albicans* | | *Cryptococcus neoformans* | | *Aspergillus fumigatus* | | *Coccidioides immitis* | | *Histoplasma capsulatum* | | *Magnaporthe grisea* | | *Neurospora crassa* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Accession number | e-value | Accession number | e-value | Accession number | e-value | Contig[1] | e-value | Contig[2] | e-value | Accession number | e-value | Accession number | e-value |
| Other secreted factors and fungal determinants | | | | | | | | | | | | | | | |
| 3553 | *mdr1* | CAA76194 | 2e-27 | - | - | CAD29613 | 2e-19 | - | - | 8.22 | 9.8e-36 | EAA57091 | 3e-26 | XP_325932 | 8e-22 |
| 3306 | *plb1* | AAF08980 | 2e-38 | - | - | AAQ85123 | 7e-31 | 1.67 | 4e-06 | 54.1 | 1.8e-66 | EAA56932 | 1e-16 | CAE76554 | 2e-16 |
| 4267 | *top1* | EAK95233 | 6e-55 | AAC18442 | 5e-51 | - | - | 1.93 | 2e-28 | 4.21 | 8.5e-68 | EAA46696 | 4e-57 | EAA28797.1 | 2e-61 |
| 5012 | *vps34* | CAA70254 | 2e-29 | - | - | - | - | 1.113 | 3e-13 | 30.9 | 1.1e-103 | EAA47826 | 2e-57 | XP_324836 | 7e-59 |
| 2456 | *ure1* | - | - | AAC62257 | 6e-76 | - | - | 1.96 | 4e-09 | 4.67 | 6.8e-81 | EAA55673 | 7e-92 | XP_330563 | 2e-98 |
| 4452 | *cek1* | EAK91333 | 4e-29 | - | - | - | - | 1.152 | 6e-18 | 6.54 | 1.6e-121 | EAA49102 | 7e-35 | XP_328578 | 7e-36 |
| 1106 | *cpp1* | P43078 | 4e-12 | - | - | - | - | - | - | 6.91 | 3.8e-67 | - | - | XP_326107 | 2e-46 |
| 266 | *cst20* | AAB38875 | 9e-49 | AAL58841 | 2e-47 | - | - | 1.19 | 3e-35 | 2.9 | 1.1e-69 | AAP93639 | 2e-60 | XP_323213 | 1e-59 |
| 358 | *hog1* | Q92207 | 1e-57 | AAM26267 | 1e-67 | CAD28436 | 7e-75 | 1.125 | 6e-08 | 8.5 | 1.3e-45 | AAF09475 | 2e-74 | AAK83124 | 2e-72 |
| 985 | *nik1* | AAC72284 | 2e-35 | - | - | - | - | - | - | 1.55 | 1.3e-57 | EAA55991 | 3e-50 | AAB03699 | 1e-49 |

-: no homolog found. In all cases, a similarity score ≥51% was observed.
[1]Accession number from http://www.broad.mit.edu/annotation/fungi/coccidioides_immitis/;
[2]Accession number from http://www.genome.wustl.edu/blast/histo_client.cgi.
PbAEST = *P. brasilienses* assembled expressed sequence tags.

in resting macrophages. In mice the survival defect was only observed after the acute phase, when cellular immunity was prominent. These data suggest that the necessity for isocitrate lyase depends on the immune status of the host, and its activity is required for persistence in the chronic phase of infected mice, when apparently there is a nutrient-limiting environment (McKinney et al., 2000).

In a murine model of systemic candidiasis, the *C. albicans ICL1* mutant strain has its virulence extremely reduced. Mice infected with the wild-type fungus developed candidiasis and died after an average of 3 days; those infected with the *ICL1* mutant strain survived for much longer (at day 28, 7 of 10 mice were still alive) (Lorenz and Fink, 2001). Thus, differently from *M. tuberculosis*, isocitrate lyase is required for full virulence in *C. albicans*. Further evidence of the contribution of glyoxylate cycle to the virulence potential of *C. albicans* was shown by Fradin et al. (2003). By using genomic arrays and a cDNA subtraction protocol, the transcriptional response of *C. albicans* to human blood was assessed. The authors found several differentially expressed genes, including those encoding for isocitrate lyase and malate synthase, suggesting that the glyoxylate cycle may be required for adaptation and survival in the bloodstream, an essential step for systemic candidiasis. Since hematogenic dissemination is also observed in experimental and human PCM (Brummer et al., 1993; Franco et al., 1993), we may speculate a similar role for the glyoxylate cycle in *P. brasiliensis.*

The enzymes of the glyoxylate cycle seem very promising as drug targets, since this pathway is absent in humans and is necessary for the virulence of two major human pathogens. The crystal structure of isocitrate lyase from *M. tuberculosis* has been determined without ligand and in complex with two inhibitors (3-bromopyruvate and 3-nitropropionate), what may lead to novel antibiotics (Sharma et al., 2000).

Besides the genes encoding for enzymes of the glyoxylate cycle, we were able to find in *P. brasiliensis* transcriptome other metabolism-related genes possibly implicated in virulence, including those involved in lipid (*NMT1* and *FAS2*), nucleotide (*ADE2*) and glucose metabolism (*TPS1*). Several eukaryotic proteins involved in cellular growth and signal transduction require the catalytic activity of the enzyme myristoyl-CoA:protein N-myristoyltransferase encoded by the *NMT1* gene. The disruption of this gene carried out in two pathogenic fungi, *C. neoformans* and *C. albicans*, resulted in temperature-sensitive myristic acid auxotroph strains that were unable to survive within the immunosuppressed mouse model of infection (Lodge et al., 1994; Weinberg et al., 1995). Similarly, *C. albicans* strains, in which the fatty acid synthase α-subunit gene (*FAS1*) was disrupted, required lipids (myristic and stearic acids) *in vitro* and were less virulent in a murine model of systemic candidiasis (Zhao et al., 1997). Enzymes involved in nucleotide metabolism have also been shown to be required for fungi survival *in vivo*. The *ADE2* gene encodes a phosphoribosylaminoimidazole carboxylase required for *de novo* purine biosynthesis, providing adenine and guanine nucleotide precursors to DNA synthesis and other key events in cell metabolism. Null mutants for *ADE2* in *C. albicans* and *C. neoformans* are adenine auxotrophs *in vitro* and showed a significant decrease in the *in vivo* growth rate (Perfect et al., 1993; Donovan et al., 2001). Furthermore, a *C. albicans ade2/ade2* strain had impaired growth in human serum, unless it was supplemented with exogenous adenine, suggesting a role for the *ADE2* gene in *C. albicans* survival in humans (Donovan et al., 2001). *TPS1* encodes a trehalose-6-phosphate synthase necessary to the accumulation of trehalose, a disaccharide, and to control the influx of glucose into the cell. Disruption of the *C. albicans TPS1* gene impairs growth on glucose at certain temperatures, as well as formation of hyphae and

infectivity in a mouse model (Zaragoza et al., 1998). Since the enzymes of the trehalose biosynthetic pathway are absent in humans, it is reasonable to consider them potential targets for the design of specific therapeutic agents. Taken together, the results suggest that nutrient availability may be limiting during fungal systemic infection (Navarro-García et al., 2001).

## CELL-WALL RELATED GENES

The cell wall of *P. brasiliensis* is a complex structure composed of lipids, proteins and polysaccharides (Kanetsuna et al., 1969). This composition, primarily of polysaccharides, varies according to the morphological phase of the fungus (Kanetsuna and Carbonell, 1970). Mycelium to yeast (M-Y) transition is characterized by a three-fold increase in chitin content and also by a change of glucose polymer glucoside bonds, arranged only as β-1,3-glucan in the M phase and mainly as α-1,3-glucan in the pathogenic Y form (San-Blas and San-Blas, 1977). Indirect evidence has shown that α-glucan may be an important virulence factor for *P. brasiliensis*: a) virulence of different isolates correlates to the levels of α-glucan, b) extended *in vitro* culture of virulent isolates leads to lowered cell wall α-glucan levels and loss of virulence in various animal models, and c) α-glucan may have a protective role against digestive enzymes of phagocytic cells (San-Blas, 1985). α-Glucan is also a cell wall component of many fungal pathogens and has been implicated in the virulence of *B. dermatitidis* (Hogan and Klein, 1994) and *H. capsulatum* (Klimpel and Goldman, 1988). The mechanism by which α-glucan contributes to the virulence of fungi is not well understood; however, it has been suggested that its outer layer localization in the cell wall (Kanetsuna and Carbonell, 1970; San-Blas and San-Blas, 1977) may avoid the immunostimulatory effects posed by β-glucan (Figueiredo et al., 1993; Anjos et al., 2002).

Our group has cloned and sequenced a β-glucan-synthase gene (Pereira et al., 2000), and an ortholog of the α-glucan-synthase gene (*AGS1*) of *H. capsulatum* was identified in *P. brasiliensis* transcriptome. Only recently a direct evidence for the role of α-glucan in the virulence of a fungal pathogen has been addressed. Rappleye et al. (2004), via RNA interference (RNAi), silenced *AGS1* expression in *H. capsulatum*. As shown by the determination of macrophage killing *in vitro*, yeasts transformed with the *AGS1*-RNAi plasmid were significantly less virulent when compared to yeasts transformed with the vector alone. In addition, *AGS1*-RNAi yeasts were defective in their ability to colonize murine lungs after intranasal infection. The development of drugs that target the biosynthesis of α-glucan, which is absent in humans, may lead to a more specific and efficient antifungal treatment, not only for histoplasmosis, but also for many other fungal diseases caused by etiologic agents that rely on α-glucan to express their full virulence.

The chitin content in the cell wall of *P. brasiliensis* yeast form, which colonizes the human host, is three times higher than in the mycelial cell wall (San-Blas and San-Blas, 1977). We have identified two sequences ortholog to *C. albicans* chitin synthase 3 (*CHS3*) and glucosamine-6-phosphate acetyltransferase (*GNA1*) genes in the *P. brasiliensis* transcriptome. These two *C. albicans* genes were found to be strongly correlated to virulence, since mutations in *CHS3* and *GNA1* genes resulted in impaired virulence and host survival, respectively (Bulawa et al., 1995; Mio et al., 2000).

*O*-linked mannosyl residues attached to proteins are indispensable for cell wall integrity and normal morphogenesis in fungi, mediating *C. albicans* adhesion and colonization of host

tissues (Timpel et al., 2000). Besides the genes discussed above, we have also identified in *P. brasiliensis* transcriptome two sequences related to *C. albicans* mannosyl transferase (*PMT1*) and α-1,2-mannosyltransferase (*MNT1*) genes, which are also implied in fungal virulence (Timpel et al., 1998; Buurman et al., 1998).

In *C. albicans*, the morphological transition between the hypha and the yeast forms, an essential attribute for virulence, is dependent on the environment pH in a regulatory pathway mediated by the *Aspergillus nidulans pac*C gene homologue (*PPR2*; Ramon et al., 1999). *C. albicans* 1,3-β-glucanosyltransferases involved in cell wall synthesis, Phr1p and Phr2p, are differentially expressed in response to ppr2 regulation: Phr1p is expressed at alkaline pH and Phr2p at acidic conditions (Muhlschlegel and Fonzi, 1997). A *PHR1*-deficient strain is avirulent in a systemic mouse model, while the *PHR2* mutant is virulent. On the other hand, the *PHR2* mutant presents a considerably attenuated virulence in a rat vaginitis model and the *PHR1*-deficient strain cannot cause this type of infection (De Bernardis et al., 1998). These data suggest that the ability of *C. albicans* to react to different pH environments is important to its pathogenicity. This may also be true for *P. brasiliensis*, since it also presents *pac*C, Phr1p and Phr2p homologues.

## DETOXIFICATION-RELATED GENES

The facultative intracellular life style of *P. brasiliensis* has to be compatible with the microenvironment imposed by phagocytic cells in the host. Upon phagocytosis, macrophages expose a variety of toxic molecules to the pathogens, including reactive oxygen intermediates (ROI) generated by the phagocyte NADPH oxidase system, and reactive nitrogen intermediates (RNI) generated by the inducible nitric oxide synthase (iNOS) (Nathan and Shiloh, 2000; Missall et al., 2004a). In activated human and murine macrophages, the fungicidal mechanisms of *P. brasiliensis* cells depend on iNOS-generated RNI, since inhibitors of iNOS activity blocked this process (Brummer et al., 1989; Moscardi-Bacchi et al., 1994; Bocca et al., 1998; Gonzalez et al., 2000). In addition, iNOS2 gene-disrupted mice are highly susceptible to *P. brasiliensis* infection (Nascimento et al., 2002). In contrast, the role of ROI in host protection against PCM is less clear. *P. brasiliensis* is relatively resistant to killing by ROI *in vitro* (Schaffner et al., 1986; Brummer et al., 1988b) and can replicate within non-activated macrophages, where the oxygen radicals can be expected to have a more important role in fungistasis, since RNI production is not significant. A possible explanation for these observations is that *P. brasiliensis* might possess efficient mechanisms to neutralize the NADPH oxidase-dependent oxidative burst products. In agreement with this hypothesis, we were able to find orthologs of virulence genes related to the detoxification of oxidative radicals: Cu/Zn superoxide dismutase (*SOD1*), thiol peroxidase (*TSA1*) and alternative oxidase (*AOX1*). In regard to this phenotype, we were also able to identify a peroxisomal and a cytoplasmic catalase, previously described by our group (accession numbers: AF428076 and AY494834, respectively; Moreira et al., 2004).

Superoxide dismutases (SODs) are highly conserved metalloenzymes that detoxify endogenously generated superoxide radical anions by degrading them to dioxygen and hydrogen peroxide ($H_2O_2$); the latter being also a toxic radical that is subsequently degraded mainly by catalases (Barbior, 2000; Missall et al., 2004a). The removal of superoxide effectively blocks secondary reactions that would lead to the formation of reactive hydroxyl radical and peroxinitrite, which are highly toxic radicals to virtually all biological macromolecules (Barbior, 2000;

Missall et al., 2004a). Superoxide anion is a normal by-product of aerobic respiration and is also produced in abundance by phagocytic cells, being the first intermediate in the oxidative burst generated in the phagosome (Fridovich et al., 1995). Consequently, SODs are important for the survival of intracellular pathogens during infection of macrophages and neutrophils. SODs can be complexed with iron, manganese, and copper plus zinc. The cytoplasmic and, to a lesser extent, the peroxisomal localization of Cu, Zn-SOD in eukaryotic cells, and in the periplasmic space of some prokaryotes, suggest a role in protection from exogenous oxidative stress. Indeed, a protective role of Cu, Zn-SOD has been shown not only for bacterial, but also for fungal pathogens (Farrant et al., 1997; Wilks et al., 1998; Battistoni et al., 1998; Hwang et al., 2002; Cox et al., 2003).

In order to elucidate the putative role of Cu, Zn-SOD in *C. albicans* virulence, its coding gene, *SOD1*, was disrupted (Hwang et al., 2002). The mutant *sod1/sod1* had impaired hyphal growth and was more sensitive *in vitro* to menadione, a chemical agent that generates superoxide radicals, but was not sensitive to hydrogen peroxide. Interestingly, when cells of *C. albicans* lacking *SOD1* were treated with a sublethal concentration of menadione, they showed an adaptive response comparable with that of wild-type cells. Thus, anti-oxidant enzymes or factors other than Cu/Zn-SOD may have a role in the adaptive response of *C. albicans* to superoxide radicals. In fact, three additional CuZn-containing superoxide dismutases, *SOD4, SOD5*, and *SOD6* were identified in this microorganism (Martchenko et al., 2004). At least one of them, *SOD5*, was found to be necessary for the virulence of *C. albicans* in a mouse model of infection. The role of *SOD1* in the virulence of *C. albicans* was further addressed by evaluating the ability of the *SOD1* null mutant to survive the fungicidal attack of a macrophage cell-line and its survival in an immunocompetent mouse model of infection. In both circumstances, *C. albicans* cells lacking *SOD1* showed attenuated virulence, suggesting a protective role of CuZn-containing superoxide dismutases against oxidative stresses imposed by the host (Hwang et al., 2002). Similar results were obtained in virulence studies carried out with *C. neoformans*. Cox et al. (2003) showed that a *sod1/sod1* mutant strain had decreased SOD activity, slower intracellular growth within both murine and human macrophages and was attenuated in virulence in nasal inhalation-infected mice. The deletion of *SOD1* gene in *C. neoformans* var. *gattii* resulted not only in decreased SOD activity and virulence, but also in defects in the expression of defined virulence factors for *C. neoformans*, i.e., laccase, urease and phospholipase (Narasipura et al., 2003). Thus, activity of these virulence factors, coupled to protection from oxidative stress, may contribute to the colonization and disease establishment by *C. neoformans*. Noteworthy, orthologs of genes encoding urease and phospholipase were found in *P. brasiliensis* transcriptome and will be discussed below.

Thiol peroxidases, or peroxiredoxins, are ubiquitous peroxidases whose active site is a thiol group of a conserved N-terminal cysteine. These enzymes degrade peroxides, providing protection against oxidative damage (Rhee et al., 2001). Five thiol peroxidases have been characterized in *S. cerevisiae*, three of them (Tsa1p, Tsa2p and Ahp1p) located in the cytoplasm, with Tsa1p considered the main antioxidant enzyme against hydrogen peroxide (Park et al., 2002). In the pathogenic fungus *C. neoformans*, SAGE (serial analysis of gene expression) and proteomics analysis showed that the *TSA1* gene and its encoding product were highly induced when the fungus was grown at 37ºC (Steen et al., 2002; Missal et al., 2004b). Since growth at the host temperature is essential for the pathogenesis of *C. neoformans*, Missal et al. (2004b) evaluated the effects of *TSA1* gene disruption in two mouse models of cryptococcosis. When

compared to reconstituted and wild-type strains, the *tsa1/tsa1* strain had its survival significantly reduced both in the tail vein and inhalation models of infection. In addition, a link between oxidative and nitrosative stress pathways was demonstrated, since the *tsa1/tsa1* mutant was not only sensitive to $H_2O_2$, but also to nitric oxide *in vitro*. Another *C. neoformans* gene (*AOX1*), which encodes an alternative oxidase, is also induced in response to high temperature (Akhter et al., 2003). Alternative oxidase is assumed to have important metabolic and antioxidant roles in several microorganisms (Vanlerberghe and McIntosh, 1997; Veiga et al., 2003). Although not present in *S. cerevisiae*, this enzyme is found in many pathogenic fungi besides *C. neoformans*, including *C. albicans*, *H. capsulatum* and *A. fumigatus* (Huh and Kang, 1999, 2001; Johnson et al., 2003). The role of alternative oxidase in *C. neoformans* pathogenesis was evaluated in a recent study employing a null mutant strain for *AOX1*, which showed a high susceptibility to peroxide stress *in vitro* and decreased survival in an inhalational murine model (Akhter et al., 2003).

Detoxification of $H_2O_2$ by catalase is also regarded as a protective factor for bacteria and fungi within phagocytic cells of the host (Kawasaki et al., 1997; Wysong et al., 1998; Manca et al., 1999; Basu et al., 2004). The *C. albicans CTA1* null mutant had its catalase activity significantly lowered when compared to the parental strain, being more sensitive to human neutrophils damage. In addition, it was almost avirulent in a systemic mouse model (Wysong et al., 1998). In contrast, the finding of a protective role for catalase in experimental aspergillosis seems controversial (Chang et al., 1998; Paris et al., 2003). The disruption of three catalase genes in *A. fumigatus*, one expressed only by conidia (*CATA*) and two by mycelia (*CAT1*, and *CAT2*), resulted in mutants with no catalase activity but only with slightly increased sensitivity to $H_2O_2$. Moreover, mutants of both, conidium and mycelium-specific catalases, were as sensitive to killing by phagocytic cells as the wild-type strains. However, only the mycelium mutants showed a delayed infection in the rat model of aspergillosis, when compared to infection by the wild-type strain. These results indicate that conidial catalase is not a virulence factor and that mycelial catalases may have a protective role against oxidative molecules produced by the host (Paris et al., 2003). Our group has identified and cloned a peroxisomal catalase of *P. brasiliensis* (Moreira et al., 2004). Interestingly, the protein and mRNA levels increased during the transition from mycelium to the pathogenic yeast phase and during exposure *in vitro* to $H_2O_2$, suggesting a putative role of this catalase in the virulence of *P. brasiliensis*.

## SECRETED FACTORS AND OTHER DETERMINANTS

The secretion of hydrolytic enzymes, such as proteinases and phospholipases, is thought to increase virulence in invasive fungi (Hube et al., 1998; Ghannoum, 2000). These extracellular hydrolytic enzymes disrupt host cell membranes and extracellular matrices, thus contributing to dissemination and tissue invasion. It is well established that PCM is characterized by fungal dissemination, with involvement of any organ or system (Brummer et al., 1993; Franco et al., 1993), and it is also known that *P. brasiliensis* invades endothelial cells (Hanna et al., 2000; Mendes-Giannini et al., 2004). In this sense, the finding of an ortholog of *C. albicans* phospholipase B (*PLB1*) in *P. brasiliensis* transcriptome is of great importance.

Phospholipases are a class of enzymes that specifically hydrolyze one or more ester linkages in glycerophospholipids. Besides the hydrolase activity, phospholipase B, differently from phospholipase A, C, and D, also shows lysophospholipasetransacylase activity (Leidich et

al., 1998; Ghannoum, 2000). Indirect evidence that phospholipase secretion is a virulence determinant of *C. albicans* was shown by Ibrahim et al. (1995). The authors, using nine human blood isolates, revealed that the level of phospholipase activity secreted by each particular isolate was directly correlated with its pathogenicity in a murine model of candidiasis. In addition, only extracellular phospholipase activity was predictive of mortality when compared with other virulence factors, including proteinase production, adherence, germination, growth rate, and ability to damage endothelial cells. Since this study used genetically unrelated candidal strains, the differences observed in virulence could be ascribed to factors other than phospholipases. The phospholipase B (*PLB1*) gene of *C. albicans* was cloned and characterized, enabling the construction of genetically defined *PLB1*-deficient mutants by targeted gene disruption (Leidich et al., 1998). *PLB1*-deficient strains had no defect in growth, morphology, or adherence. However, the ability to penetrate host cell monolayers was significantly impaired when compared to that of the parental strain, suggesting a role of phospholipase B in candidal virulence, possibly by enzymatic degradation of the phospholipid constituents of the host cell membrane, leading to an increased dissemination to hosts' death. Indeed, in a murine model of disseminated candidiasis the parental strain caused the death of all infected mice within 9 days. Conversely, 60% of mice infected with a *PLB1*-disrupted strain were alive at day 15 post-infection (Leidich et al., 1998). The contribution of phospholipase to fungal virulence is also recognized in *C. neoformans*. Santangelo et al. (1999) demonstrated that the major lipid components of lung surfactant correspond to optimal substrates for *C. neoformans* phospholipases, suggesting an important role of this enzyme during early lung infection. A similar role may be of importance in PCM, since the infection is also acquired by inhalation. A specific evaluation of the contribution of *PLB1* in *C. neoformans* virulence was achieved with its disruption (Cox et al., 2001). Both, in the mouse inhalational model and the rabbit meningitis model, the absence of *PLB1* caused an evident susceptibility of the fungus to the host.

Another virulence-related secreted enzyme whose putative gene was found in the *P. brasiliensis* transcriptome is urease. This enzyme catalyzes the hydrolysis of urea, generating carbon dioxide ($CO_2$), and ammonia ($NH_3$), which reacts with water to form $NH_4OH$, a strong base. Thus, under physiological conditions, this reaction can result in a pH increase. Urease is found in plants, bacteria and fungi (Mobley et al., 1995). This enzyme has been shown to be an important virulence factor for *Helicobacter pylori*, the main causative agent of gastric and duodenal human ulcers (Lee et al., 1993; Mera, 1995). If urease activity is biochemically or genetically impaired, the colonization ability of human strains of *H. pylori* is reduced in experimental models, most probably due to the inability to raise the pH within the highly acid gastric mucosa (Eaton and Krakowka, 1994; Tsuda et al., 1994). Besides the role of urease in increasing microenvironmental pH, *H. pylori*-derived urease has been shown to activate monocytes, to induce the secretion of inflammatory cytokines, and to act as a chemotactic factor for leukocytes (Craig et al., 1992; Mai et al., 1992).

The urease gene was identified and cloned from two human pathogenic fungi, *Coccidioides immitis* and *C. neoformans* (Yu et al., 1997; Cox et al., 2000). However, urease activity is identified in several fungi, including *P. brasiliensis* (data not shown). In order to assess a putative role of urease in *C. neoformans* virulence, its encoding gene (*URE1*) was disrupted (Cox et al., 2000). Strains lacking *URE1* gene had no differences regarding phenoloxidase activity and capsule size when compared with the parental strain. When the survival of these strains was evaluated by means of coloning-forming unit counts, in the central nervous system

rabbit model of infection, no differences were found. Conversely, in both, the murine intravenous and inhalational infection models, mice infected with the *ure1/ure1* strain lived longer than mice infected with the parental strain. These results suggest that urease activity is involved in the pathogenesis of cryptococcosis, probably in a species and/or infection site manner.

## CONCLUDING REMARKS

The transcriptome of *P. brasiliensis*, a dimorphic fungus responsible for a severe systemic mycosis in Latin America, has provided important information about this microorganism's physiology. Comparative analyses of PbAESTs with DNA sequences of other fungi allowed us to identify several putative virulence genes in *P. brasiliensis*, which were grouped into five classes. These encompass metabolism-, cell wall-, detoxification-related genes, secreted factors, and other determinants. The results presented in this report could be used as a starting point for further studies in order to better understand the molecular biology of this important human pathogen.

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