Molecular biology of the dimorphic fungi *Paracoccidioides* spp

Mark H.J. Sturme[1], Rosana Puccia[2], Gustavo H. Goldman[3,4], Fernando Rodrigues[1]

[1] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[2] Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, São Paulo, SP, Brazil

[3] Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil

[4]Laboratorio Nacional de Ciencia e Tecnologia do Bioetanol (CTBE), Campinas, Brazil

Corresponding author:

Dr. Fernando Rodrigues

Life and Health Sciences Research Institute (ICVS),

School of Health Sciences,

University of Minho,

Campus de Gualtar, 4710-057, Braga, Portugal

Key words: *Paracoccidioides brasiliensis*, GP43, CDC42, transcriptional profiling, molecular tools, pathogenic fungi

Abstract

Paracoccidioides spp, herein commonly referred as *P. brasiliensis*, is the etiological agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis endemic in Latin America. Many aspects of the biology of *P. brasiliensis* remain unknown, in particular its ecology and the apparent lack of a sexual reproduction stage in its life cycle. This review will highlight the current knowledge on the genetics and genomics of *P. brasiliensis*, its most important putative virulence factors and the challenges for developing genetic tools in this organism. *P. brasiliensis* is a dimorphic ascomycete fungus belonging to the order *Onygenales*, family *Ajellomycetaceae*. *P. brasiliensis* pathogenic yeast form is characterized by a multi-budding and -nucleate nature , with a high polymorphic cellular shape. Successful infection and dissemination by *P. brasiliensis* requires initial interaction of the fungus with host cells. The fungus has to adhere to host cells after which internalization of the fungus takes place. Gp43 is a 43-kDa glycoprotein that participates in the interaction with the host at different levels. There are very few putative virulence factors described in *P. brasiliensis*, among them an extracellular phospholipase B, a 32-kDa haloacid dehalogenase PbHAD32 that was shown to bind laminin, fibrinogen, and fibronectin, and to be important for initial adhesion to pulmonary epithelial cells, the pigment melanin, and the Rho-like GTPase Cdc42. The morphological transition of *P. brasiliensis* from mycelium to the yeast form is a key process for the infectivity of the fungus. There are several transcriptional profiling studies addressing which genes have increased or decreased mRNA accumulation during mycelium-to-yeast transitions. Functional genomics studies in *P. brasiliensis* have been hampered by the absence of efficient molecular techniques that enable targeted gene inactivation in this fungus. However, an optimized *Agrobacterium tumefaciens*-mediated transformation method has been developed and was used to knock-down the Rho-like GTPase Cdc42 and the HAD-type hydrolase *PbHAD32*. A challenge for the future remains the development of mutagenesis methods that allow for the creation of targeted insertional gene mutants *Paracoccidioides* spp. The complete genome sequencing of three isolates of *Paracoccidioides* species provides the opportunity to perform more complete evaluations of the transcriptomic and proteomic data, and to understanding the biology and virulence of these important pathogenic fungi.

1. Introduction

Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis endemic in Latin America (Brummer *et al.*, 1993). An estimated 10 million people are thought to be infected in the endemic area (Brummer *et al.*, 1993), and an annual incidence rate of 1-3 per 100.000 inhabitants and mean mortality rate of 0.14 per 100.000 is estimated for Brazil (Restrepo *et al.*, 2001). The disease is mainly observed in agricultural workers and there is a clear gender bias for males, with reported male:female PCM incidence ratios around 10:1 in endemic regions (Brummer *et al.*, 1993). *P. brasiliensis* shows a thermally dimorphic phenotype, whereby it switches from the non-pathogenic mycelium form at ambient temperatures to the pathogenic multiple-budding yeast form when exposed to temperatures similar to those of the mammalian host. Infection of the host is thought to occur via the inhalation of infective airborne propagula like conidia or mycelia from the environment. Inhaled propagules then differentiate in the lungs into the pathogenic yeast form, after which the fungus disseminates to other organs of the host (McEwen *et al.*, 1987).

Many aspects of the cellular and molecular biology of *P. brasiliensis* remain unknown, in particular its ecology, sexual reproduction, multi-budding and –nucleate nature. A very high frequency of *P. brasiliensis* infections has been found in different species of armadillos. The fungus has been isolated in 75-100% of animals captured in PCM hyper-endemic regions (Bagagli *et al.*, 2006) (Richini-Pereira *et al.*, 2009). Many *P. brasiliensis* strains have been isolated from armadillos, while only a few were isolated from soil, penguins and bat feces (Matute *et al.*, 2006; Teixeira *et al.,* 2009). Analysis of Random Amplified Polymorphic DNA (RAPD), *PbGP43* gene and ribosomal internal transcribed spacer (ITS) sequences of clinical isolates and isolates from armadillos showed that *P. brasiliensis* isolates from both origins often were highly similar, indicating that humans, in particular agricultural workers, might acquire the fungus from contact with soil from armadillo habitats (Sano *et al.*, 1999; Restrepo *et al.*, 2001; Hebeler-Barbosa *et al.*, 2003).

This review will highlight the current knowledge on the molecular biology of *P. brasiliensis*, its most important putative virulence factors and the challenges for developing genetic tools in this organism.

2. Phylogeny and cryptic speciation

P. brasiliensis is a dimorphic ascomycete fungus belonging to the order *Onygenales*, family *Ajellomycetaceae*. This family also includes the anamorphic genera *Blastomyces, Emmonsia* and *Histoplasma*, and all members are saprobic and pathogenic vertebrate-associated taxa (Untereiner *et al.*, 2004). Four phylogenetic clades have been described for *P. brasiliensis*: S1, PS2, PS3 and the recently defined new clade "Pb01" (proposed as the new species *Paracoccidioides lutzii*) (Teixeira *et al.*, 2009). Recombination analysis indicated that the fungus recombines in nature (Matute *et al*, 2006), but a teleomorphic form of *P. brasiliensis* has not yet been isolated. Nonetheless, sexual reproduction cycle is anticipated based on the presence of two idiomorphic mating type loci (*MAT1-1* and *MAT1-2*) and the observed 1:1 distribution of these MAT loci in a large panel of *P. brasiliensis* strains (Torres *et al.*, 2010). In fact, heterothallic strains with opposite mating loci are expected to undergo sexual reproduction, but, in many fungi, this often requires prolonged co-culturing under very specific conditions. Moreover, comparative genomics of *P. brasiliensis* with other fungi revealed the presence of all components of the pheromone-signaling cascade of the mating process with the exception of the pheromone-response scaffold protein Ste5, and the cyclin-dependent kinase inhibitor Far1 (unpublished results). Gene expression of the *MAT* loci was confirmed in several *P. brasiliensis* strains, and mating assays produced structures resembling fruiting bodies on malt agar (Torres *et al.*, 2010). However, these structures did not contain asci or ascospores (Torres *et al.*, 2010), which might indicate that the production of competent sexual structures still need additional stimuli. Altogether, it seems that *P. brasiliensis* has all the molecular machinery needed for matting and it is critical to identify, in the next future, the correct signals controlling sexual development, which will allow enormous advances on the molecular genetic studies of this termodimorphic fungus.

P. brasiliensis yeast form is characterized by a multi-budding and -nucleate nature , with a high polymorphic cellular shape (Figure 1; Almeida *et al.*, 2006; Feitosa *et al.*, 2003), while conidia are uninucleated structures (McEwen *et al.*, 1987). Earlier studies using pulsed field gel electrophoresis, DNA hybridization, and microfluorometry established the possible existence of haploid and diploid (or aneuploid) isolates of the fungus by comparing clinical and environmental isolates from different geographic areas (Cano *et al.*, 1998; Feitosa *et al.*, 2003). Further studies by flow cytometry, of different *P. brasiliensis* strains, confirmed the uninucleate cellular DNA content and showed the remarkable variability of DNA content per cell due to the multi-nucleate nature of the fungus (Almeida *et al.*, 2007). These studies

indicated that *P. brasiliensis* genome sizes ranged from 23.3 to 35.5 Mb for the yeast form and 30.2 Mb for conidia, indicative of a haploid DNA content (Cano *et al.*, 1998; Feitosa *et al.*, 2003; Almeida *et al.*, 2007). Genome sequences of three *P. brasiliensis* strains (Pb01, Pb03, and Pb18) have recently been released by the *Paracoccidioides brasiliensis* Sequencing Project at the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). The sequences indicated five chromosomes, 40-75 kb of mitochondrial DNA and nuclear genomic DNA sizes of 29-33 Mb, which confirms the earlier studies.

4. Identification of genes which have their mRNA accumulation modulated during dimorphic transition

The morphological transition of *P. brasiliensis* from mycelium to the yeast form is a key process for the infectivity of the fungus (Figure 2). There are several transcriptional profiling studies addressing which genes have increased or decreased mRNA accumulation during mycelium-to-yeast transitions (Felipe *et al.*, 2005; Nunes *et al.*, 2005; Bastos *et al.*, 2007; Parente *et al.*, 2008; Pereira *et al.*, 2009), as well as during the conidia-to-yeast transition (Garcia *et al.*, 2009). A *P. brasiliensis* biochip carrying sequences of 4,692 genes from this fungus was used to monitor gene expression at several time points of the mycelium-to-yeast morphological shift (from 5 to 120 h) (Nunes *et al.*, 2005). The results revealed a total of 2,583 genes that displayed statistically significant modulation in at least one experimental time point. Among the identified gene homologues, some encoded enzymes involved in amino acid catabolism, signal transduction, protein synthesis, cell wall metabolism, genome structure, oxidative stress response, growth control, and development (Nunes *et al.*, 2005). The expression pattern of 20 genes was independently verified by real-time reverse transcription-PCR, revealing a high degree of correlation between the data obtained with the two methodologies. One gene, encoding 4-hydroxyl-phenyl pyruvate dioxygenase (4-HPPD), was highly overexpressed during the mycelium-to-yeast differentiation, and the use of NTBC [2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione], a specific inhibitor of 4-HPPD activity, as well as that of NTBC derivatives, was able to inhibit growth and differentiation of the pathogenic yeast phase of the fungus in vitro (Nunes *et al.*, 2005). *P. brasiliensis* can grow as a prototroph for organic sulfur as a mycelial

(non-pathogenic) form, but it is unable to assimilate inorganic sulfur as a yeast (pathogenic) form. Temperature and the inability to assimilate inorganic sulfur are the single conditions known to affect *P. brasiliensis* mycelium-to-yeast (M–Y) dimorphic transition (da Silva Ferreira *et al*., 2006). For a comprehensive evaluation of genes that have their expression modulated during the M–Y transition in different culture media, a large-scale analysis of gene expression was performed using a microarray hybridization approach (da Silva Ferreira *et al*., 2006). Ninety-five percent of the genes in the microarray are mainly responding to the temperature trigger, independently of the media where the M–Y transition took place (da Silva Ferreira *et al*., 2006). These authors suggest that although *P. brasiliensis* cannot use inorganic sulfur as a single sulfur source to initiate both M–Y transition and Y growth, the fungus can somehow use both organic and inorganic pathways during these growth processes.

In another study, a genomic DNA microarray, covering approximately 25% of the genome (12,000 elements) of the organism was constructed and used to identify genes and gene expression patterns during growth *in vitro* (Monteiro *et al.*, 2009). To examine gene expression, mRNA was extracted and amplified from mycelial or yeast cultures grown in semi-defined medium for 5, 8 and 14 days. Principal components analysis and hierarchical clustering indicated that yeast gene expression profiles differed greatly from those of mycelia, especially at earlier time points, and that mycelial gene expression changed less than gene expression in yeasts over time. Genes upregulated in yeasts were found to encode proteins shown to be involved in methionine/cysteine metabolism, respiratory and metabolic processes (of sugars, amino acids, proteins and lipids), transporters (small peptides, sugars, ions and toxins), regulatory proteins and transcription factors (Monteiro *et al.*, 2009). Mycelial genes involved in processes such as cell division, protein catabolism, nucleotide biosynthesis and toxin and sugar transport showed differential expression. Transposable elements and components of respiratory pathways tended to increase in expression with time, genes encoding ribosomal structural proteins and protein catabolism tended to sharply decrease in expression over time, particularly in yeast (Monteiro *et al.*, 2009).

To identify genes specifically expressed during infection some studies have isolated Expression Sequence Tags (ESTs) from infected mice (Bailão *et al.*, 2006; Costa *et al.*, 2007). One study showed that during liver infection genes involved in utilization of multiple carbon sources were activated, which included glucose and glyoxylate cycle substrates. In addition, genes for nitrogen metabolism and biosynthesis, as well as lipid biosynthesis were highly expressed. This suggests that nitrogen and lipid compounds are probably not easily obtained from the host, while the availability of carbohydrates for energy maintenance is not limited

(Costa *et al.*, 2007). A second study found that many genes related to melanin biosynthesis, iron acquisition and cell defense were specifically higher expressed during infection in a mouse model, while yeast transcripts upregulated during exposure to human blood were mainly related to cell wall remodeling/synthesis (Bailão *et al.*, 2006).

The complete sequencing of the genome (see above) will provide the opportunity to perform more complete evaluations of the transcriptomic and proteomic data.

5. Tools for genetic manipulation of *P. brasiliensis*

Functional genomics studies in *P. brasiliensis* have been hampered by the absence of efficient molecular techniques that enable targeted gene inactivation in this fungus. Established methods to introduce DNA into fungi are based on protoplast transformation, electroporation, or lithium acetate transformation, while more recalcitrant fungi have been transformed using biolistic approaches (Ruiz-Diez, 2002). More recent approaches have successfully applied *Agrobacterium tumefaciens*-mediated transformation (ATMT) to yeast (*Saccharomyces*), filamentous fungi (*Aspergillus, Fusarium*) and dimorphic fungal pathogens like *Histoplasma capsulatum*, *Blastomyces dermatitidis, Coccidioides immitis* and *Penicillium marneffei* (de Groot *et al.*, 1998; Kummasook *et al.*, 2010; Michielkse *et al.*, 2005; Sullivan *et al.*, 2002). This method takes advantage of the natural capacity of the soil bacterium *A. tumefaciens* to transfer part of its Ti plasmid DNA (the T-DNA) to a eukaryote host, after which the T-DNA randomly integrates into the host genome. For transformation a so-called binary vector system is used, in which the T-DNA and the virulence region (responsible for T-DNA formation and transport) of the Ti plasmid are placed on two separate plasmids. This allows for genetic manipulation of the binary vector containing the T-DNA (Hoekema *et al.*, 1983). The T-region of the Ti plasmid is surrounded by a 24-bp left and right border repeat, which are essential as the *cis*-acting signal for the DNA transfer system to target cells. All other sequences of the natural T-DNA can however be deleted and replaced by other (non-homologous) DNA sequences.

ATMT in fungi is, in general, a highly efficient and simple method, which has allowed advances in functional genetic studies in many species that previously were difficult to transform. The fact that the T-DNA integrates randomly into genomes makes ATMT an efficient tool for random insertional mutagenesis studies, as was shown for the dimorphic fungi *H. capsulatum* and *B. dermatitidis* (Gauthier *et al.*, 2010; Nemecek *et al.*, 2006).

Transformation of *P. brasiliensis* has proven to be more difficult, as transformation efficiencies from ATMT were low (Leal *et al.*, 2004), while electroporation resulted in transformants with low mitotic stability (Soares *et al.*, 2005). An optimized ATMT method with increased transformation efficiencies of two-fold was obtained by manipulating cell recovery and co-culture drying conditions (Almeida *et al.*, 2007), which resulted in a mitotically stable homokaryon progeny with a single gene copy integration. Moreover, the application of such technique allowed the expression of green fluorescent protein (GFP), showing the feasibility of new developments in gene expression systems into *P. brasiliensis*. In fact, the application of this powerful toolkit will allow the visualization of structural organization and dynamic processes in *P. brasiliensis*, by direct observations of living cells expressing fluorescently tagged proteins. However, it is of major relevance to undertake efforts to ensure that tagged genes are not over expressed by taking advantage of the new know-how coming from the genome sequences of *P. brasiliensis*, particularly, promoter sequences and regulatory elements, and eventually look for the feasibility of the Tet-On and Tet-Off inducible gene expression systems.

A gene expression approach was also exploited in an antisense RNA (aRNA) technology that allows for targeted down-regulation of gene expression (Almeida *et al.*, 2009; Hernández *et al.*, 2010). In this way, several mutants were produced with a large range of ranking of expression down-regulation of the Rho-like GTPase Cdc42 within two different *P. brasiliensis* strains (Almeida *et al.*, 2009). Such technology was also applied to downregulate the HAD-type hydrolase *PbHAD32* (Hernández *et al.*, 2010). These studies showed for the first time that targeted silencing of genes encoding important virulence factors can be achieved in *P. brasiliensis*, as described to above, and signifies the clear value of the aRNA technology for elucidating the function of putative virulence factors of *P. brasiliensis*.

6. GP43 and putative virulence factors

Successful infection and dissemination by *P. brasiliensis* requires initial interaction of the fungus with host cells. The fungus has to adhere to host cells after which internalization of the fungus takes place. Gp43 is a 43-kDa glycoprotein that participates in the interaction with the host at different levels. It is the main diagnostic and prognostic antigen so far characterized in *P. brasiliensis* (Puccia *et al*., 1986). Anti-gp43 monoclonal antibodies have immunotherapeutic potential in mice (Bruissa-Filho *et al*., 2008), while vaccination with gp43

elicits vigorous interferon-g protective response especially due to P10, a 15 aminoacid-long conserved T-cell epitope (reviewed in Travassos *et al*., 2008). In addition, gp43 has adhesive properties to extracellular matrix proteins that might help fungal dissemination [Hanna *et al*., 2000; Mendes-Giannini *et al*., 2004; Vicentini *et al*., 1994).

Pb*GP43* has been studied under many different aspects since its characterization and the accumulated information has been extremely useful to unravel novel features of *Paracoccidioides* molecular genetics. The Pb*GP43* ORF is 1,329-pb long and contains a 78-bp intron (Cisalpino *et al*., 1996). Translation results in a precursor protein of 416 amino acids, including a leader peptide of 35 residues. The gp43 sequence belongs to the glycosyl hydrolases family 5, with 50% identity with fungal exo-β-1,3-glucanases; however, it apparently lacks enzymatic activity probably because a catalytic NEP motif (Cutfield *et al*., 1999; Mackenzie *et al*., 1997) is mutated to NKP in isolates from the three *P. brasiliensis* phylogenetic groups (Cisalpino *et al*., 1996; Morais *et al*., 2000; Matute *et al*., 2006).

Polimorphism in the Pb*GP43* ORF was suggested by Sano *et al*. (1999) and fully characterized by Morais *et al*. (2000), which helped to explain diversity in gp43 isoelectric points (pI) (Puccia *et al*., 1986; Moura-Campos *et al*., 1995). A Maximum-likelihood phylogenetic tree suggested the existence of genetic groups: highly polymorphic and phylogenetically distant Pb*GP43* genotype A translates into a peculiarly basic gp43 detected previously in one *P. brasiliensis* isolate (Moura-Campos *et al*., 1995). Basic gp43 is typical of cryptic species PS2 defined in Matute *et al*. (2006) multilocus study, where Pb*GP43* exon 2 was the most informative locus. Isolates within a major independent species S1 contain genotypes B, D or E, as extensively reviewed by Puccia *et al*. (2008). Most substitutions are non-synonymous and several are under selective pressure (Matute *et al*., 2008).

The Pb*GP43* transcript starts at positions -25, -33, and -35, as mapped similarly in four *P. brasiliensis* isolates by primer extension (Carvalho *et al*., 2005). Comparison of 3' RACE amplicons revealed that within a range of 37 bp there were 11 poly(A) cleavage sites within two main clusters of possible alternative poly(A) (Rocha *et al*., 2009b). These data resulted from comparison of 56 cloned 3' UTR sequences from ten isolates of *P. brasiliensis* and also showed that the 3' UTR sequence is highly conserved.

At the other end of the gene, 2,047 nucleotides of the 5' intergenic region from the Pb339 isolate were fully characterized (Rocha *et al*., 2009b). Comparisons with other isolates were carried out by PCR amplicon size polymorphism and at the sequence level with Pb3, Pb18 and Pb01 Broad Institute genomes. Size polymorphism partially correlated with *P.*

brasiliensis phylogenetic groups: PS2 isolates yielded a shorter amplicon (about 1,500 bp) than most S1 representatives (about 2,000 bp). The -2,047 fragment, which is about 95% identical between Pb18 and Pb339, is quite peculiar because it is composed of three similar tandem repeats of about 500 bp preceded upstream by 442 bp (Rocha *et al*., 2009b). In Pb3, one tandem repeat is missing. The amount of gp43 culture fluids can vary among isolates (Moura-Campos *et al*., 1995) and both transcriptional and post-transcriptional mechanisms are apparently responsible for these differences (Carvalho *et al*., 2005). In a controlled study carried out in defined medium, the number of accumulated Pb*GP43* transcripts in Pb339 was about 1,000-fold higher than in Pb18 and 129-fold higher than in Pb3, but these differences could not be justified by a missing 5' intergenic repeat (Rocha *et al*., 2009b). They could be due, however, to polymorphisms, mRNA stability and/or 3' UTR regulators.

Earlier studies had shown seven substitution sites in the proximal -326 bp of the promoter region (Carvalho *et al*., 2005). Electrophorectic mobility shift assays (EMSA) with a series of probes covering this region identified protein binding oligonucleotides between -134 to -103 and -255 to -215. These fragments contain three substitution sites characteristic of *P. brasiliensis* PS2 isolates and mutation at -230 seemed to alter binding affinity. The transcription elements involved are speculative, but NIT2 is apparently active (Rocha *et al*., 2009a). In addition, gene reporter assays in *Aspergillus nidulans* suggested that the first -480 bp were sufficient to promote basal levels of Pb*GP43* transcripts and also modulation with ammonium sulfate (Rocha *et al*., 2009a). The involvement of NIT2-like binding motifs in transcription modulation of the Pb*GP43* gene was suggested by the presence four clusters of NIT2-like sites, four of which were positive in EMSA. Similar negative modulation with ammonium sulfate and glucose in Pb3, Pb18 and Pb339, in addition to the finding to an upstream oligonucleotide in a beta-glucosidase gene identical to a protein-binding fragment of Pb*GP43*, suggest that although gp43 is a non-functional b-1,3-exoglucanase, transcription regulation could be partially similar (Rocha *et al*, 2009a, 2009b).

The ortologue to gp43 in *P. lutzii* Pb01 (PAAG 05770.1), is only 80% identical, does not have any *N*-glycosylation site or conserved P10, and catalytic NEP is preserved, suggesting that it might be an active glucanase. The immunological identity with gp43 is questionable, since sera from PCM patients from central-western Brazil, where virtually all *P. lutzii* samples have been isolated, present low rates of positivity in diagnostic tests using extracellular antigens from Pb339 (Batista *et al*., 2009), where the main antigenic component is gp43 (Camargo *et al*., 1988). In order to address this point, the gp43 ortogue from Pb01 is

currently being expressed in bacteria and *Pichia pastoris* (unpublished results), following expression of other gp43 isoforms (Carvalho *et al*., 2009). On the other hand, an anti-gp43 protective antibody MAb32 (Puccia and Travassos, 1991), which recognizes a conserved epitope encoded in exon 1 (Bruissa-Filho *et al*., 2009), was also protective against infection with Pb01 (unpublished results).

Isolates Pb2, Pb3 and Pb4, which belong to PS2 group, evoked milder experimental PCM in B10.A mice than Pb18 and other representative isolates from the main species S1 (Carvalho *et al*., 2005). However the role played by differences in the gp43 sequence in the outcome of disease is unknown. On the other hand, the role played by gp43 as a virulence factor has never been genetically proven. Recently, using the anti-sense strategy standardized by Almeida *et al*. (2009), successful knock-down mutants from Pb339 have been obtained that hardly express any gp43 (unpublished results). These mutants are currently being tested for virulence *in vivo*.

Activity of the extracellular phospholipase B (PLB) also plays a role in *P. brasiliensis* virulence. PLB activity and *plb1* gene expression were increased when *P. brasiliensis* and alveolar macrophages were cocultured in the presence of pulmonary surfactant (a phospholipid-rich PLB substrate). This coincided with reduced adherence to and increased internalization by these macrophages. Increased PLB activity in the presence of pulmonary surfactant might lead to a greater release of substrates for lipid synthesis and leukotriene production, which in turn act as suppressors of the innate immune response, as confirmed by the observed reduced expression levels of the cytokines TNF-α and IL-1β genes. PLB therefore seems to play a role in fungal evasion via interference with the immune response of macrophages (Soares *et al.*, 2010).

The 32-kDa haloacid dehalogenase PbHAD32 was shown to bind to several ECM proteins such as laminin, fibrinogen, and fibronectin, and to be important for initial adhesion to pulmonary epithelial cells expressing these ECMs (González *et al.*, 2005, 2008). Gene silencing of *PbHAD32* gene expression further confirmed that *PbHAD32* plays a role in binding of *P. brasiliensis* to human epithelial cells, and in addition showed that *PbHAD32* is important for *P. brasiliensis* virulence in a mouse model of infection (Hernández *et al.*, 2010). The fact that *PbHAD32*-silenced yeast cells showed an altered cell morphology with more elongated buds could play a role in the reduced adhesion that was observed.

Another type of virulence factor is the pigment melanin, which is common in many dimorphic fungi. Phagocytosis of melanized *P. brasiliensis* yeast cells is reduced, and in

addition melanized yeast cells show an increase resistance to reactive oxygen species that might be produced by the host cells (da Silva *et al.*, 2006; Silva *et al.*, 2009). Melanins are negatively charged and hydrophobic polymers, and as such their presence in fungal cell walls might alter cell surface charge and hydrophobicity, and thereby might inhibit phagocytosis (da Silva *et al.*, 2006; Nosanchuk and Casadevall, 1997). Moreover, melanin interferes with the binding of several antibodies resulting in an overall reduction in the internalization of melanized cells.

Finally, the Rho-like GTPase Cdc42 was shown to have an effect on virulence. Gene silencing of *PbCDC42* resulted in yeast cells with a reduced cell size and fewer buds per cell, and a more homogeneous cellular morphology overall. *In vitro* phagocytosis of these *PbCDC42*-silenced yeast cells by macrophages was increased, and moreover, these cells were less virulent in a mouse model of infection (Almeida *et al.*, 2009). This role of *PbCDC42* in maintaining enlarged, multiple-budding and heterogeneously sized *P. brasiliensis* yeast cells might promote host evasion (Figure 3). Furthermore, in fungi *CDC42* is a central regulator that orchestrates several cellular functions such as cell division, cell polarity, cytoskeleton remodeling and hyphal development (Johnson, 1999; Wang, 2009), and might therefore also regulate the expression of other proteins involved in the infection process.

7. Future prospects: where do we go from here?

A challenge for the future remains the development of mutagenesis methods that allow for the creation of targeted insertional gene mutants in *P. brasiliensis*. In closely related dimorphic fungi such as *H. capsulatum* and *B. dermatitidis*, gene inactivation by homologous recombination in early studies was often frustrated by ectopic integration of transformed DNA at multiple loci, resulting in random DNA duplications, deletions or rearrangements. This might be due to the presence of dominant illegitimate recombination events (by non-homologous end-joining) that override homologous recombination. In addition, transforming DNA can be maintained on extra-chromosomal elements in *Histoplasma spp.*, which obstructs DNA integration (24, 54, 55). Despite these aspects, allelic replacement by homologous recombination of genes was found to be straight-forward in *Blastomyces dermatitidis* (Brandhorst *et al.*, 1999), while for *Histoplasma capsulatum* these problems could be circumvented by using a combination of telomeric linear vectors and a two-step genetic selection procedure (Woods and Goldman, 1992). Attempts for mutagenesis by homologous

recombination in *P. brasiliensis* have not been reported and it is not known if *P. brasiliensis* is able to form linear plasmids as observed in *Histoplasma spp*. A feasible approach for creating gene deletion mutants might be to down-regulate the activity of non-homologous end-joining (NHEJ) pathways to increase the frequency of homologous recombination events that take place. In several filamentous fungi like *Sordaria macrospora*, *Aspergillus fumigatus*, *Penicillium chrysogenum* and *Botrytis cinerea* this has been achieved by aRNA gene silencing of the Ku70 and/or Ku80 genes, which encode key proteins involved in NHEJ (Krappmann *et al.*, 2006; Pöggeler and Kück, 2006; Choquier *et al.*, 2008;; Snoek *et al.*, 2009). Such a directed mutagenesis tool would facilitate functional studies of genes implicated in virulence and morphogenesis, in particular those newly identified in microarray studies.

Acknowledgments

We would like to thank Fundação para a Ciência e Tecnologia, Lisbon, Portugal (Grant No.: PTDC/BIA-MIC/108309/2008), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

References

Almeida, A. J., Cunha, C., Carmona, J.A:, Sampaio-Marques, B., Carvalho, A., Malavazi, I., Steensma, H. Y., Johnson, D.I., Leão, C., Logarinho, E., Goldman, G.H., Castro, A.G., Ludovico, P., and Rodrigues, F. 2009. Cdc42p controls yeast-cell shape and virulence of *Paracoccidioides brasiliensis*. Fungal Genetics and Biology 46, 919-926.

Almeida, A. J., Martins, M., Carmona, J.A., Cano, L.E., Restrepo, A. Leão, C., and Rodrigues, F. 2006. New insights into the cell cycle profile of *Paracoccidioides brasiliensis*. Fungal Genetics and Biology 43, 401-409.

Almeida, A. J., Matute, D.R., Carmona, J.A., Martins, M., Torres, I., McEwen, J.G., Restrepo, A., Leão, C., Ludovico, P., and Rodrigues, F., 2007. Genome size and

ploidy of *Paracoccidioides brasiliensis* reveals a haploid DNA content: flow cytometry and GP43 sequence analysis. Fungal Genetics and Biology 44, 25-31.

Bagagli, E., Bosco, S.M., Theodoro, R.C., and Franco, M., 2006. Phylogenetic and evolutionary aspects of *Paracoccidioides brasiliensis* reveal a long coexistence with animal hosts that explain several biological features of the pathogen. Infect Genet Evol. 6, 344-351.

Bailão, A. M., Schrank, A., Borges, C.L., Dutra, V., Walquíria, I., Molinari-Madlum, E.E., Felipe, M.S., Soares Mendes-Giannini. M.J., Martins, W.S., Pereira, M., and Alemida Soares, M.C., 2006. Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. Microbes and Infection 8, 2686-2697.

Bastos, K. P., Bailão, A.M., Borges, C.L., Faria, F.P., Felipe, M.S., Silva, M.G., Martins, W.S., Fiúza, R.B., Pereira, M., and Soares, C.M., 2007. The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals novel and induced genes potentially associated to the dimorphic process. BMC Microbiology 10, 29.

Batista, J.J., de Camargo, Z.P., Fernandes, G.F., Vicentini, A.P., Fontes, C.J., Hahn, R.C., 2010. Is the geographical origin of a *Paracoccidioides brasiliensis* isolate important for antigen production for regional diagnosis of paracoccidioidomycosis? Mycoses 53, 176-180

Brandhorst, T. T., Wüthrich, M., Warner, T., and Klein, B., 1999. Targeted gene disruption reveals an adhesin indispensable for pathogenicity of *Blastomyces dermatitidis*. Journal of Experimental Medicine 189, 1207-1216.

Brummer, E., Castaneda, E., and Restrepo, A., 1993. Paracoccidioidomycosis: an Update. Clinical Microbiology Reviews 6, 89-117.

Buissa-Filho, R., Puccia, R., Marques, A.F., Pinto, F.A., Munoz, J.E., Nosanchuk, J.D., Travassos, L.R., and Taborda, C.P., 2008. The monoclonal antibody against the major diagnostic antigen of *Paracoccidioides brasiliensis* mediates immune protection in infected BALB/c mice challenged intratracheally with the fungus. Infection and Immunity 76, 3321-3328.

Camargo, Z.P., 2008. Serology of paracoccidioidomycosis. Mycopathologia 165, 289-302.

Camargo, Z., Unterkircher, C., Campoy, S.P., and Travassos, L.R., 1998. Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. Journal of Clinical Microbiology 26, 2147-2151.

Cano, M. I., Cisalpino, P. S., Galindo, I., Ramírez, J.L., Mortara, R.A., and da Silveira, J.F., 1998. Electrophoretic karyotypes and genome sizing of the pathogenic fungus *Paracoccidioides brasiliensis*. Journal of Clinical Microbiology 36, 742-747.

Carvalho, K. C., Ganiko, L., Batista, W.L., Morais, F.V. E. R. Marques, G. H. Goldman, M. F. Franco, and R. Puccia, 2005. Virulence of *Paracoccidioides brasiliensis* and gp43 expression in isolates bearing known PbGP43 genotype. Microbes and Infection 7, 55-65.

Carvalho, K.C., Ganiko, L., Batista, W.L., Morais, F.V., Marques, E.R., Goldman, G.H., Franco, M.F., and Puccia, R., 2005. Virulence of *Paracoccidioides brasiliensis* and gp43 expression in isolates bearing known Pb*GP43* genotype. Microbes and Infection 7, 55-65.

Choquer, M., G. Robin, P. Le Pêcheur, C. Giraud, C. Levis, and M. Viaud, 2008. Ku70 or Ku80 deficiencies in the fungus *Botrytis cinerea* facilitate targeting of genes that are hard to knock out in a wild-type context. FEMS Microbiology Letters 289, 225-232.

Cisalpino, P.S., Puccia, R., Yamauchi, L.M., Cano, M.I., da Silveira, J.F., and Travassos, L.R., 1996. Cloning, characterization, and epitope expression of the major diagnostic antigen of *Paracoccidioides brasiliensis*. Journal of Biological Chemistry 271, 4553-4560.

Costa, M., C. L. Borges, A. M. Bailão, G. V. Meirelles, Y. A. Mendonça, S. F. Dantas, F. P. de Faria, M. S. Felipe, E. E. Molinari-Madlum, M. J. Mendes-Giannini, R. B. Fiuza, W. S. Martins, M. Pereira, and C. M. Soares, 2007. Transcriptome profiling of *Paracoccidioides brasiliensis* yeast-phase cells recovered from infected mice brings new insights into fungal response upon host interaction. Microbiology 153, 4194-4207.

Cutfield, S.M., Davies, G.J., Murshudov, G., Anderson, B.F., Moody, P.C., Sullivan, P.A., and Cutfield, J.F., 1999. The structure of the exo-beta-(1,3)-glucanase from *Candida*

albicans in native and bound forms: relationship between a pocket and groove in family 5 glycosyl hydrolases. Journal of Molecular Biology 294, 771-783.

da Silva Ferreira, M.E., Marques. E.R., Malavazi, I., Torres, I., Restrepo, A., Nunes, L.R., de Oliveira, R.C., Goldman, M.H.S., and Goldman, G.H., 2006. Transcriptome analysis and molecular studies on sulfur metabolism in the human pathogenic fungus *Paracoccidioides brasiliensis*. Molecular Genetics and Genomics 276, 450–463.

da Silva, M. B., A. F. Marques, J. D. Nosanchuk, A. Casadevall, L. R. Travassos, and C. P. Taborda, 2006. Melanin in the dimorphic fungal pathogen *Paracoccidioides brasiliensis*: effects on phagocytosis, intracellular resistance and drug susceptibility. Microbes and Infection 8, 197-205.

de Groot, M. J., P. Bundock, P. J. Hooykaas, and A. G. Beijersbergen, 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nature Biotechnology 16:839-842.

Feitosa Ldos, S., P. S. Cisalpino, M. R. dos Santos, R. A. Mortara, T. F. Barros, F. V. Morais, R. Puccia, J. F. da Silveira, and Z. P. de Camargo, 2003. Chromosomal polymorphism, syntenic relationships, and ploidy in the pathogenic fungus *Paracoccidioides brasiliensis*. Fungal Genetics and Biology 39, 60-69.

Felipe, m.s.s., Andrade, R.V., Arraes, F.B.M., Nicola, A.M., Maranhão, A.Q.M., Torres, F.A.G., Silva-Pereira, I., Poças-Fonseca, M.J., Campos, E.G., Moraes, L.M.P., Andrade, P.A., Tavares, A.H. F. P., Silva, S.S., Kyaw, C.M., Souza, D.P., PbGenome Network, Pereira, M., Jesuíno, R.S. A., Andrade, E.V., Parente, J.A., Oliveira, G.S., Barbosa, M.S., Martins, N.F., Fachin, A.L., Cardoso, R.S., Passos, G.A.S., Almeida, N.F., Walter, M.E.M.T., Soares, C.M.A., Carvalho, M.J.A., and Brígido, M.M., 2005. Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. The Journal of Biological Chemistry 280, 24706–24714.

Flavia Popi, A. F., Lopes, J.D. , and Mariano, M. 2002. GP43 from *Paracoccidioides brasiliensis* inhibits macrophage functions. An evasion mechanism of the fungus. Cell Immunol 218:87-94.

Garcia, A. M., O. Hernandez, B. H. Aristizabal, L. A. De Souza Bernardes, R. Puccia, T. W. Naranjo, G. H. Goldman, M. H. Goldman, L. E. Cano, A. Restrepo, and J. G. McEwen. 2009. Gene expression analysis of *Paracoccidioides brasiliensis* transition from conidium to yeast cell. Medical Mycology 30:1-9.

Gauthier, G. M., T. D. Sullivan, S. S. Gallardo, T. T. Brandhorst, A. J. Vanden Wymelenberg, C. A. Cuomo, G. Suen, C. R. Currie, and B. S. Klein. 2010. SREB, a GATA transcription factor that directs disparate fates in *Blastomyces dermatitidis* including morphogenesis and siderophore biosynthesis. PLoS Pathogens 6:e1000846.

Gesztesi, J.L., Puccia, R., Travassos, L.R., Vicentini, A.P., de Moraes, J.Z., Franco, M.F., and Lopes, J.D., 1996. Monoclonal antibodies against the 43,000 Da glycoprotein from *Paracoccidioides brasiliensis* modulate laminin-mediated fungal adhesion to epithelial cells and pathogenesis. Hybridoma 15, 415-422.

González, A., E. Caro, C. Muñoz, A. Restrepo, A. J. Hamilton, and L. E. Cano. 2008. *Paracoccidioides brasiliensis* conidia recognize fibronectin and fibrinogen which subsequently participate in adherence to human type II alveolar cells: involvement of a specific adhesin. Microbial Pathogenesis 44:389-401.

González, A., B. L. Gómez, S. Diez, O. Hernández, A. Restrepo, A. J. Hamilton, and L. E. Cano. 2005. Purification and partial characterization of a *Paracoccidioides brasiliensis* protein with capacity to bind to extracellular matrix proteins. Infection and Immunity 73:2486-2495.

Hanna, S.A., Monteiro da Silva, J.L., Giannini, M.J., 2000. Adherence and intracellular parasitism of *Paracoccidioides brasiliensis* in Vero cells. Microbes and Infection 2, 877-884.

Hebeler-Barbosa, F., F. V. Morais, M. R. Montenegro, E. E. Kuramae, B. Montes, J. G. McEwen, E. Bagagli, and R. Puccia. 2003. Comparison of the Sequences of the Internal Transcribed Spacer Regions and PbGP43 Genes of Paracoccidioides brasiliensis from Patients and Armadillos (Dasypus novemcinctus). Journal of Clinical Microbiology 41:5735–5737.

Hernández, O., A. J. Almeida, A. Gonzalez, A. M. Garcia, D. Tamayo, L. E. Cano, A. Restrepo, and J. G. McEwen. 2010. A 32-kilodalton hydrolase plays an important role in *Paracoccidioides brasiliensis* adherence to host cells and influences pathogenicity. Infection and Immunity 78:5280-5286.

Hoekema, A., P. R. Hirsch, P. J. J. Hooykaas, and R. A. Schilperoort. 1983. A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179-180.

Hogan, L. H., and B. S. Klein. 1997. Transforming DNA integrates at multiple sites in the dimorphic fungal pathogen *Blastomyces dermatitidis*. Gene 186:219-226.

Johnson, D. I. 1999. Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. Microbiology and Molecular Biology Reviews 63:54-105.

Krappmann, S., C. Sasse, and G. H. Braus. 2006. Gene targeting in *Aspergillus fumigatus* by homologous recombination is facilitated in a nonhomologous end- joining-deficient genetic background. Eukaryotic Cell 5:212-215.

Kummasook, A., C. R. Cooper, and N. Vanittanakom. 2010. An improved *Agrobacterium*-mediated transformation system for the functional genetic analysis of *Penicillium marneffei.* Medical Mycology 48:1066-1074.

Leal, C. V., B. A. Montes, A. C. Mesa, A. L. Rua, M. Corredor, A. Restrepo, and J. G. McEwen. 2004. *Agrobacterium tumefaciens*-mediated transformation of *Paracoccidioides brasiliensis*. Medical Mycology 42:391-395.

Mackenzie, L.F., Brooke, G.S., Cutfield, J.F., Sullivan, P.A., and Withers, S.G., 1997. Identification of Glu-330 as the catalytic nucleophile of *Candida albicans* exo-beta-(1,3)-glucanase. Journal of Biological Chemistry 272, 3161-3167.

Martin, K., McDougall, B.M., McIlroy, S., Chen, J., and Seviour, R.J., 2007. Biochemistry and molecular biology of exocellular fungal beta-(1,3)- and beta-(1,6)-glucanases. FEMS Microbiology Reviews 31, 168-192.

Matute, D. R., J. G. McEwen, R. Puccia, B. A. Montes, G. San-Blas, E. Bagagli, J. T. Rauscher, A. Restrepo, F. Morais, G. Niño-Vega, and J. W. Taylor. 2006. Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. Molecular Biology and Evolution 23:65-73.

McEwen, J. G., V. Bedoya, M. M. Patiño, M. E. Salazar, and A. Restrepo. 1987. Experimental murine paracoccidiodomycosis induced by the inhalation of conidia. Journal of Medical and Veterinary Mycology 25:165-175.

McEwen, J. G., B. I. Restrepo, M. E. Salazar, and A. Restrepo. 1987. Nuclear staining of *Paracoccidioides brasiliensis* conidia. Journal of Medical and Veterinary Mycology 25.

Mendes-Giannini, M.J., Andreotti, P.F., Vincenzi, L.R., da Silva, J.L., Lenzi, H.L., Benard, G. Zancopé-Oliveira, R., de Matos Guedes, H.L., and Soares, C.P. 2006.: Binding of extracellular matrix proteins to *Paracoccidioides brasiliensis*. Microbes and Infection 8, 1550-1559.

Michielse, C. B., P. J. Hooykaas, C. A. van den Hondel, and A. F. Ram. 2005. *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Current Genetics 48:1-17.

Monteiro, J.P., Clemons, K.V., Mirels, L.F., Coller, Jr, J.A., Wu, T.D., Shankar, J., Lopes, C.R., and Stevens, D.A., 2009. Genomic DNA microarray comparison of gene expression patterns in *Paracoccidioides brasiliensis* mycelia and yeasts *in vitro*. Microbiology 155, 2795–2808.

Morais, F.V., Barros, T.F., Fukada, M.K., Cisalpino, P.S., and Puccia, R., 2000. Polymorphism in the gene coding for the immunodominant antigen gp43 from the pathogenic fungus Paracoccidioides brasiliensis. Journal of Clinical Microbiology 38, 3960-3966.

Nemecek, J. C., M. Wüthrich, and B. S. Klein. 2006. Global control of dimorphism and virulence in fungi. Science 312:583-588.

Nosanchuk, J. D., and A. Casadevall. 1997. Cellular charge of *Cryptococcus neoformans*: contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. Infection and Immunity 65:1836-1841.

Nunes, L. R., R. Costa de Oliveira, D. B. Leite, V. S. da Silva, E. dos Reis Marques, M. E. da Silva Ferreira, D. C. Ribeiro, L. A. de Souza Bernardes, M. H. Goldman, R. Puccia, L. R. Travassos, W. L. Batista, M. P. Nóbrega, F. G. Nobrega, D. Y. Yang, C. A. de Bragança Pereira, and G. H. Goldman. 2005. Transcriptome analysis of *Paracoccidioides brasiliensis* cells undergoing mycelium-to-yeast transition. Eukaryotic Cell 4:2115-2128.

Parente, J. A., C. L. Borges, A. M. Bailão, M. S. Felipe, M. Pereira, and C. M. de Almeida Soares. 2008. Comparison of transcription of multiple genes during mycelia transition to yeast cells of *Paracoccidioides brasiliensis* reveals insights to fungal differentiation and pathogenesis. Mycopathologia 165:259-273.

Pereira, M., A. M. Bailão, J. A. Parente, C. L. Borges, S. M. Salem-Izacc, and C. M. Soares. 2009. Preferential transcription of *Paracoccidioides brasiliensis* genes: host niche and time-dependent expression. Memorias do Instituto Oswaldo Cruz 104:486-491.

Pöggeler, S., and U. Kück. 2006. Highly efficient generation of signal transduction knockout mutants using a fungal strain deficient in the mammalian ku70 ortholog. Gene 378:1-10.

Puccia, R., Schenkman, S., Gorin, P.A., and Travassos, L.R.:, 1986. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infection and Immunity 53, 199-206.

Puccia, R., McEwen, J.G., and Cisalpino, P.S., 2008. Diversity in *Paracoccidioides brasiliensis*. The Pb*GP43* gene as a genetic marker. Mycopathologia 165, 275-287.

Moura-Campos, M.C., Gesztesi, J.L., Vincentini, A.P., Lopes, J.D., Camargo, Z.P., 1995. Expression and isoforms of gp43 in different strains of *Paracoccidioides brasiliensis*. Journal of Medicine and Veterinary Mycology 33, 223-227.

Restrepo, A., J. G. McEwen, and E. Castañeda. 2001. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? Medical Mycology 39:233-241.

Richini-Pereira, V. B., S. M. Bosco, R. C. Theodoro, L. Barrozo, S. C. Pedrini, P. S. Rosa, and E. Bagagli. 2009. Importance of xenarthrans in the eco-epidemiology of *Paracoccidioides brasiliensis*. BMC Research Notes 2:228.

Rocha, A.A., Malavazi, I., Goldman, G.H., and Puccia, R., 2009a. Transcription regulation of the Pbgp43 gene by nitrogen in the human pathogen *Paracoccidioides brasiliensis*. Fungal Genetics and Biology 46, 85-93.

Ruiz-Díez, B. 2002. Strategies for the transformation of filamentous fungi. Journal of Applied Microbiology 92:189-195.

Rocha, A.A., Morais, F.V., and Puccia, R., 2009b. Polymorphism in the flanking regions of the PbGP43 gene from the human pathogen Paracoccidioides brasiliensis: search for protein binding sequences and poly(A) cleavage sites. BMC Microbiology 9, 277.

Sano, A., R. Tanaka, K. Yokoyama, M. Franco, E. Bagagli, M. R. Montenegro, Y. Mikami, M. Miyaji, and K. Nishimura. 1999. Comparison between human and armadillo *Paracoccidioides brasiliensis* isolates by random amplified polymorphic DNA analysis. Mycopathologia 143:165-169.

Schmaler-Ripcke, J., V. Sugareva, P. Gebhardt, R. Winkler, O. Kniemeyer, T. Heinekamp, and A. A. Brakhage. 2009. Production of pyomelanin, a second type of melanin, via the tyrosine degradation pathway in *Aspergillus fumigatus*. Applied and Environmental Microbiology 75:493-503.

Silva, M. B., L. Thomaz, A. F. Marques, A. E. Svidzinski, J. D. Nosanchuk, A. Casadevall, L. R. Travassos, and C. P. Taborda. 2009. Resistance of melanized yeast cells of *Paracoccidioides brasiliensis* to antimicrobial oxidants and inhibition of phagocytosis using carbohydrates and monoclonal antibody to CD18. Memorias do Instituto Oswaldo Cruz 104:644-648.

Snoek, I. S., Z. A. van der Krogt, H. Touw, R. Kerkman, J. T. Pronk, R. A. Bovenberg, M. A. van den Berg, and J. M. Daran. 2009. Construction of an hdfA *Penicillium chrysogenum* strain impaired in non-homologous end-joining and analysis of its potential for functional analysis studies. Fungal Genetics and Biology 46:418-426.

Soares, D. A., R. V. de Andrade, S. S. Silva, A. L. Bocca, S. M. Soares Felipe, and S. Petrofeza. 2010. Extracellular *Paracoccidioides brasiliensis* phospholipase B involvement in alveolar macrophage interaction. BMC Microbiology 10:241.

Soares Rde, B., T. A. Velho, L. M. De Moraes, M. O. Azevedo, C. M. Soares, and M. S. Felipe. 2005. Hygromycin B-resistance phenotype acquired in *Paracoccidioides brasiliensis* via plasmid DNA integration. Medical Mycology 43:719-723.

Sullivan, T. D., P. J. Rooney, and B. S. Klein. 2002. *Agrobacterium tumefaciens* integrates transfer DNA into single chromosomal sites of dimorphic fungi and yields homokaryotic progeny from multinucleate yeast. Eukaryotic Cell 1:895-905.

Teixeira, M. M., R. C. Theodoro, M. J. de Carvalho, L. Fernandes, H. C. Paes, R. C. Hahn, L. Mendoza, E. Bagagli, G. San-Blas, and M. S. Felipe. 2009. Phylogenetic analysis reveals a high level of speciation in the *Paracoccidioides* genus. Molecular Phylogenetics and Evolution 52:273-283.

Torres, I., A. M. García, O. Hernández, A. González, J. G. McEwen, A. Restrepo, and M. Arango. 2010. Presence and expression of the mating type locus in *Paracoccidioides brasiliensis* isolates. Fungal Genetics and Biology 47:373-380.

Travassos, L.R., Rodrigues, E.G., Iwai, L.K., and Taborda, C.P., 2008. Attempts at a peptide vaccine against paracoccidioidomycosis, adjuvant to chemotherapy. Mycopathologia 165, 341-352.

Untereiner, W. A., J. A. Scott, F. A. Naveau, L. Sigler, J. Bachewich, and A. Angus. 2004. The *Ajellomycetaceae*, a new family of vertebrate-associated *Onygenales*. Mycologia 96:812-821.

Vicentini, A. P., J. L. Gesztesi, M. F. Franco, W. de Souza, J. Z. de Moraes, L. R. Travassos, and J. D. Lopes. 1994. Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. Infection and Immunity 62:1465-1469.

Wang, Y. 2009. CDKs and the yeast-hyphal decision. Current Opinion in Microbiology 12:644-649.

Woods, J. P., and W. E. Goldman. 1992. *In vivo* generation of linear plasmids with addition of telomeric sequences by *Histoplasma capsulatum*. Molecular Microbiology 6:3603-3610.

Worsham, P. L., and W. E. Goldman. 1990. Development of a genetic transformation system for *Histoplasma capsulatum*: complementation of uracil auxotrophy. Molecular and General Genetics 221:358-362.

Fig. 1 - Multiple budding and multinucleate nature of the *P. brasiliensis* yeast form. Confocal microscopy observation of PI and FITC-ConA double-stained yeast cells. White bars correspond to 5 µm.

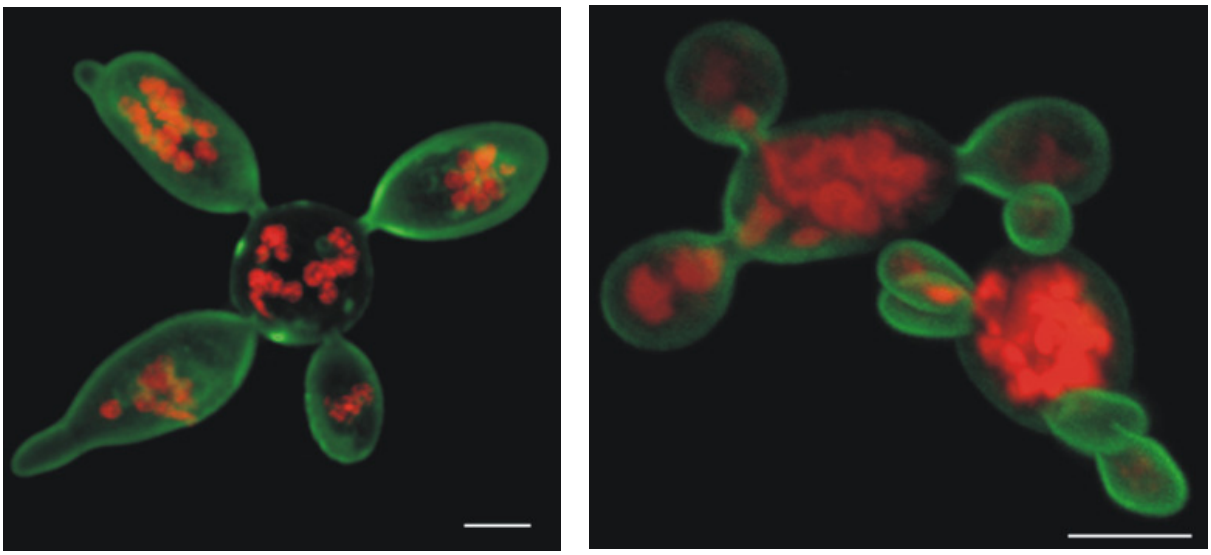

Fig. 2 - The cell size and multiple budding cell morphology of *P. brasiliensis* affects phagocytosis by macrophages, thereby promoting host evasion. Confocal microscopy observation using DAPI to visualize nuclei, α-tubulin-antibody and FITC-ConA staining. Scale bar corresponds to 10 µm.

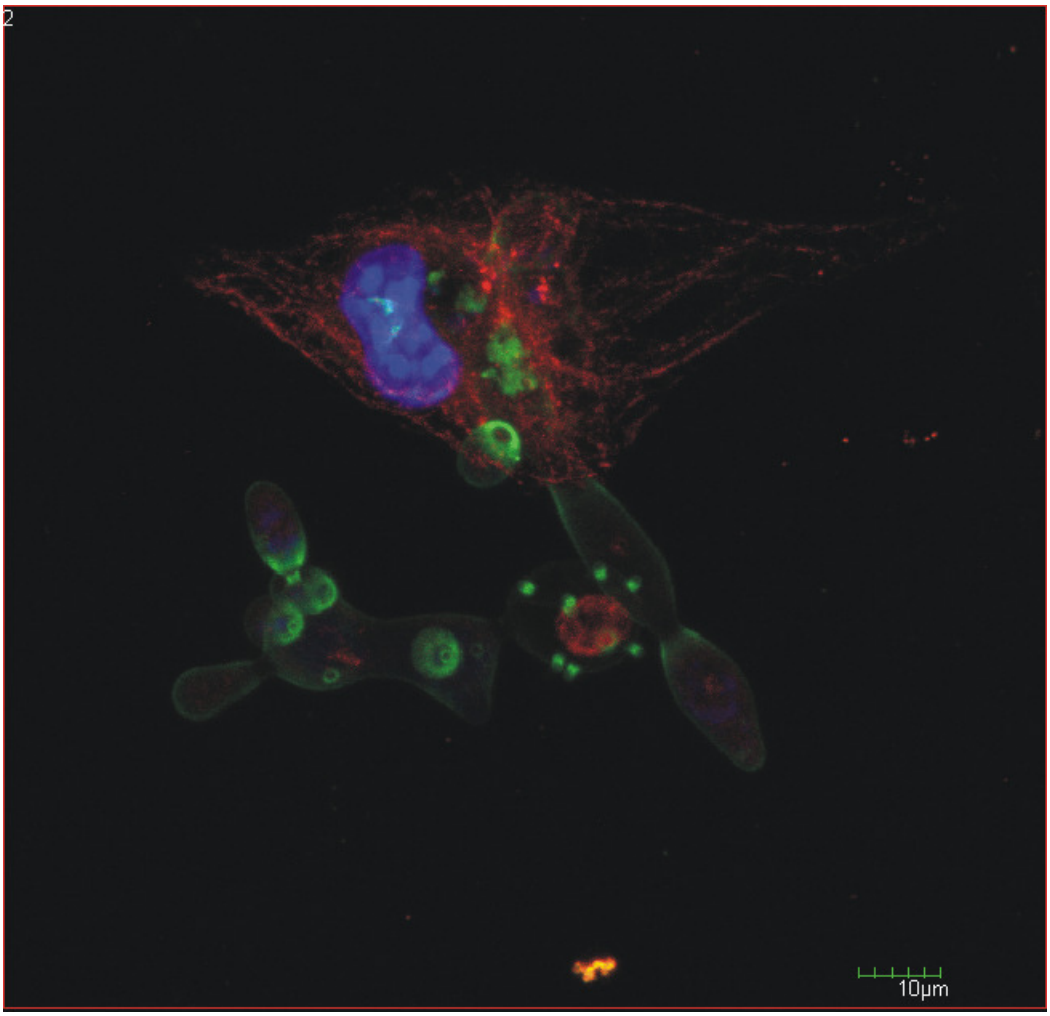

Fig. 3 - Morphology of *P. brasiliensis* cells growing in liquid culture, which were induced to undergo mycelium-to-yeast transition by an increase in temperature from 26ºC to 37ºC. The cellular forms observed were hyphae (0 days), differentiating mycelium (3 days), transforming yeast (5 days), yeast (10 days). Black bars correspond to 5 µm.