Universidade do Minho
Escola de Ciências da Saúde

Agostinho João Ramalho de Almeida

Cellular and molecular studies on the dimorphic pathogenic fungus *Paracoccidioides brasiliensis*

Estudos celulares e moleculares no fungo dimórfico patogénico *Paracoccidioides brasiliensis*

Tese de Doutoramento
Ciências da Saúde – Ciências Biológicas e Biomédicas

Trabalho efectuado sob a orientação de:
Doutor Fernando José dos Santos Rodrigues
Professor Auxiliar da Escola de Ciências da Saúde,
Universidade do Minho, Braga, Portugal

Doutora Maria Cecília de Lemos Pinto Estrela Leão
Professora Catedrática da Escola de Ciências da Saúde,
Universidade do Minho, Braga, Portugal

Março de 2007

DECLARAÇÃO

Nome: Agostinho João Ramalho de Almeida

Endereço electrónico: ajalmeida@ecsaude.uminho.pt

Telefone: +351 914086067

Número do Bilhete de Identidade: 11333504

Título dissertação:

Cellular and molecular studies of the dimorphic pathogenic fungus *Paracoccidioides brasiliensis*

Orientador:

Doutor Fernando Rodrigues

Doutora Cecília Leão

Ano de conclusão: 2007

Designação do Ramo de Conhecimento do Doutoramento:

Ciências da Saúde – Ciências Biológicas e Biomédicas

Universidade do Minho, 13/03/2007

Assinatura: _____

A tese de doutoramento aqui apresentada foi desenvolvida no âmbito do projecto "Estudos celulares e moleculares do fungo patogénico *Paracoccidioides brasiliensis*: dimorfismo e a sua regulação por hormonas esteróides" financiado pela Fundação para a Ciência e Tecnologia (FCT) com a referência POCTI/ESP/45327/2002, bem como uma bolsa de doutoramento com a referência SFRH/BD/8655/2002.

ACKNOWLEDGMENTS

Para a realização deste trabalho recebi o contributo de várias instituições e pessoas sem as quais não teria sido possível a sua concretização. Nesse sentido, gostaria de expressar a minha gratidão e reconhecimento a todos aqueles que directa ou indirectamente me apoiaram, salientando não só o apoio institucional mas principalmente o humano:

Ao Prof. Doutor Fernando Rodrigues, muito obrigado por ter continuado a acreditar em mim e no projecto, mesmo contra as evidências. Não quero deixar de lembrar a ambição e o incentivo incutido durante os últimos anos, bem como a orientação, disponibilidade e contribuição científica na revisão de todos os trabalhos.

O Instituto de Ciências da Vida e da Saúde (ICVS), Escola de Ciências da Saúde da Universidade do Minho, nomeadamente a Prof. Doutora Cecília Leão, na qualidade de directora e orientadora deste trabalho. O meu muito obrigado por me ter acolhido anos atrás, permitindo que desse o meu pequeno contributo na construção desta instituição.

Os laboratórios *Medical and Experimental Mycology* e *Cellular and Immunogenetics* da *Corporación para Investigaciones Biológicas* (CIB), Medellín, Colômbia, nas pessoas da Prof. Doutora Luz Cano e Prof. Doutor Juan McEwen a quem agradeço, principalmente, a amizade, mas também a oportunidade de colaboração, o apoio institucional e a disponibilização de meios técnicos.

La Doctora Angela Restrepo. Fica aqui expressa a minha eterna gratidão pelo incansável apoio e a fé que sempre depositou na minha pessoa. Muito obrigado por ter contribuído decisivamente na minha formação, tanto profissional como humana.

A Prof. Doutora Paula Ludovico por me ter visto nascer no laboratório e por me ter ensinado que a vida é muitas vezes como uma partida de xadrez.

O Prof. Doutor Jorge Pedrosa e o Prof. Doutor Gil Castro. Obrigado pelo encorajamento dispensado.

A todos os meus colegas do ICVS, em particular a Kikas, a Sandra, o Rui, o Tó e os antigos companheiros de bancada do I&D1. Muito obrigado pelo apoio e bons momentos.

A todos os meus amigos de laboratório com quem batalhei ao longo destes anos. Especialmente para o Agostinho, a Belém, o Bruno, a Cristina, o Gustavo, a Margarida e o Nuno fica o meu eterno agradecimento pelo companheirismo e espírito de equipa e por terem estado presentes nos bons e maus momentos. Uma pequena menção também para os três Gustos, a Magda, a Martinha e o Egídio por terem aparecido quando mais precisava.

A todos os meus amigos da CIB e Colômbia, particularmente o Roberto, a Ana Maria, o Orville, o Angel, o Daniel e a Vicky, por me terem recebido como um igual.

A todos os meus amigos de curso e afins com os quais tenho tido a sorte de manter contacto. Um obrigado muito especial para a João e o Herlânder, o Tarroso, a Carla, o Nicolae e a Marta pela sua enorme amizade, e o Gonçalo por me ter transmitido a sua vontade de viver.

Aos amigos do Douro, especialmente o Joca e a Matilde. Obrigado por me terem salvo tantas vezes do laboratório. A vossa amizade será sempre incontornável para mim.

Aos meus sogros e cunhadas que sempre me fizeram sentir em casa e parte da família. Obrigado por tudo!

Os meus queridos pais, irmãos e sobrinhos. A vitória neste trajecto também vos pertence pelo carinho, incentivo, paciência, educação e, acima de tudo, alegria dispensados ao longo destes últimos 28 anos. A minha eterna gratidão por nunca terem deixado de acreditar em mim.

Para a Jenny, que deu rumo à minha vida. Sem ti não creio que teria chegado ao fim deste percurso. Obrigado por fazeres de mim uma melhor pessoa.

Para os meus Pais, que sempre deram tudo pelos filhos, a Paula e o Luís por nunca terem desistido de mim... e para ti, Jenny...

CELLULAR AND MOLECULAR STUDIES ON THE DIMORPHIC PATHOGENIC FUNGUS *PARACOCCIDIOIDES BRASILIENSIS*

ABSTRACT

Paracoccidioides brasiliensis, the etiological agent of paracoccidioidomycosis, is a multinuclear thermal dimorphic fungus that switches from the environmental mycelial/conidial non-pathogenic form to the pathogenic multiple budding yeast. The general aim of the research developed within this thesis was the development of molecular tools to elucidate unclear biological phenomena in *P. brasiliensis* and the study of its genome content, in particular the interaction between DNA replication, multinuclear segregation and multiple budding, and cellular differentiation. In order to fulfil these objectives, we initially developed a flow cytometric (FCM) technique for cell cycle profile analysis based on high resolution measurements of nuclear DNA. The direct application of this method to yeast cells of ten *P. brasiliensis* isolates, grown in a batch system, revealed a genome size ranging from 26.3±0.1 Mb (26.9±0.1 fg) to 35.5±0.2 Mb (36.3±0.2 fg) per uninucleated cell. This analysis together with the evaluation of the intra-individual variability of a highly polymorphic *P. brasiliensis* gene, *GP43*, and taking into account the previously described average karyotype-determined genome size, showed that all analysed isolates presented a haploid, or at least aneuploid, DNA content. Moreover, no association was detected between genome size/ploidy and the clinical-epidemiological features of the studied isolates.

In addition, we show that exponentially growing cells in poor-defined or rich-complex nutritional environments present an increased percentage of daughter cells in accordance with its multiple budding. However, during the stationary growth-phase cell cycle progression in rich-complex medium was characterized by an accumulation of cells with higher DNA content or pseudohyphae-like structures, whereas in poor-defined medium arrested cells mainly displayed two DNA contents. Moreover, the anti-microtubule drug benomyl was shown to induce an accumulation of cells presenting high and varying DNA contents. Altogether, our data suggest that, depending on the growth conditions, *P. brasiliensis* possesses alternative cell division control mechanisms to manage multiple budding and its multinucleated nature.

In an attempt to understand the molecular basis of *P. brasiliensis* multiple budding, we isolated *PbCDC42*, a Rho-like GTPase encoding gene, which restore growth of the *CDC42* null mutant in *S. cerevisiae.* The expression of *PbCDC42* in *Saccharomyces cerevisiae* leads to the formation of

both abnormal and multiple budded cells, indicating a deregulation of the spatial control of cell division and suggesting that Pbcdc42p may play an important role in the regulation of *P. brasiliensis* multiple budding.

In addition, we have developed a molecular toolbox for the dimorphic fungus *P. brasiliensis*, specifically an efficient transformation and a gene expression system. *Agrobacterium tumefaciens*-mediated transformation (ATMT) revealed to be possible in this fungus where cellular recovery and air drying of *A. tumefaciens*:*P. brasiliensis* mixtures are crucial parameters for high efficiencies. Overall data indicate a transformation efficiency of 78±9 transformants/co-cultivation (5±1 transformants/10^6 target cells). *P. brasiliensis GFP*-expressing isolates were obtained by the use of this methodology. Furthermore, we demonstrated single gene copy integration per genome and generation of homokaryon progeny, relevant for the future use in targeted mutagenesis and linking mutations to phenotypes.

In summary, the knowledge resulting from the work developed shed light on *P. brasiliensis* cell cycle progression and interaction between multiple budding and nuclear replication/division. In addition, the molecular tools herein developed, as well as their future prospects, have opened hopeful perspectives for studies on *P. brasiliensis* biological processes.

ESTUDOS CELULARES E MOLECULARES NO FUNGO DIMÓRFICO PATOGÉNICO *PARACOCCIDIOIDES BRASILIENSIS*

RESUMO

Paracoccidioides brasiliensis, o agente etiológico da paracoccidioidomicose, é um fungo termodimórfico multinucleado que sofre uma complexa transformação morfológica, passando da fase micelar/conidial não patogénica (a temperaturas ambientais) para a levedura patogénica multi-gemulante (a temperaturas do hospedeiro). A investigação realizada no âmbito desta tese teve como principiais objectivos desenvolver ferramentas moleculares para elucidar processos biológicos ainda pouco esclarecidos e estudar o conteúdo genómico, a interacção entre a replicação de DNA, a segregação multinuclear e a gemulação múltipla, e a diferenciação celular em *P. brasiliensis*. Nesse sentido, optimizámos um protocolo de citometria de fluxo baseado no conteúdo de DNA nuclear para a análise do perfil de ciclo celular de células leveduriformes. A aplicação directa desta metodologia a células leveduriformes de *P. brasiliensis* permitiu determinar o conteúdo em DNA de dez isolados distintos, variando entre 26,3±0,1 Mb (26,9±0,1 fg) e 35,5±0,2 Mb (36,3±0,2 fg) por célula leveduriforme uninucleada. A ploidia de vários isolados de *P. brasiliensis* foi também determinada através da comparação do tamanho do genoma, avaliado por citometria de fluxo, com a variabilidade intra-individual do gene altamente polimórfico *GP43* de *P. brasiliensis*. Os nossos resultados indicaram que todos os isolados estudados possuem um conteúdo em DNA haploide, ou pelo menos aneuploide, não tendo sido encontrada nenhuma associação entre o tamanho do genoma/ploidia e as características clínicas ou ambientais dos isolados.

Através da análise do perfil de ciclo celular de células leveduriformes de *P. brasiliensis* verificámos que células na fase exponencial de crescimento em meios pobre-definido e rico-complexo apresentam uma elevada percentagem de células filhas em concordância com a multi-gemulação do fungo. No entanto, durante a fase estacionária de crescimento a progressão do ciclo celular em meio rico-complexo é caracterizada pela acumulação de células com conteúdos em DNA mais elevados ou estruturas do tipo pseudohifa, contrariamente ao que ocorre em meio pobre-definido em que as células apresentam maioritariamente dois conteúdos em DNA distintos. Por outro lado, foi demonstrado que o fungicida benomil induz uma paragem de progressão do ciclo celular caracterizada pela acumulação de células com um conteúdo em DNA

elevado e variável. No seu conjunto, estes resultados sugerem que, dependendo das condições de crescimento, *P. brasiliensis* poderá apresentar mecanismos de controlo alternativos durante o crescimento celular de modo a regular a gemulação múltipla e a sua natureza multinuclear.

Com o objectivo de elucidar a base molecular que regula a gemulação múltipla de *P. brasiliensis*, isolámos o gene *PbCDC42*, que codifica uma Rho GTPase, e demonstrámos que complementa funcionalmente a delecção total de *CDC42* em *Saccharomyces cerevisiae*. A expressão de *PbCDC42* em *S. cerevisiae* resulta tanto na formação de células anormais como de células multi-gemulantes, indicando que parece ocorrer uma desregulação do controlo espacial da divisão celular e sugerindo que Pbcdc42p poderá desempenhar um papel importante na regulação da gemulação múltipla de *P. brasiliensis*.

Adicionalmente, desenvolvemos um conjunto de ferramentas moleculares para o estudo de células leveduriformes de *P. brasiliensis*, especificamente um sistema eficiente de transformação mediada por *Agrobacterium tumefaciens* (TMAT) e um sistema de expressão genética. Os nossos dados revelaram que a recuperação celular e a secagem das misturas de co-cultivo de *Agrobacterium*:*P. brasiliensis* são essenciais para a TMAT, resultando numa eficiência de transformação de 78±9 transformantes/co-cultivo (5±1 transformantes/10^6 células alvo). Foram, também, construídos isolados de *P. brasiliensis* que expressam *GFP* através da inserção do gene *GFP* sob o controlo de distintos promotores de vários fungos. Demonstrámos, ainda, que ocorre a integração de apenas uma cópia do gene em estudo aquando da TMAT, um facto relevante para o uso desta metodologia na mutagénese direccionada e na associação de mutações específicas ao fenótipo que induzem.

Em suma, o conhecimento proveniente da execução destes trabalhos representa um passo importante na elucidação da progressão do ciclo celular da fase leveduriforme de *P. brasiliensis* e da interacção entre a gemulação múltipla e replicação/divisão nuclear. As ferramentas moleculares aqui desenvolvidas, assim como as suas perspectivas futuras, serão relevantes para o esclarecimento de processos biológicos em *P. brasiliensis*, bem como para a criação de novas linhas de investigação.

TABLE OF CONTENTS

OBJECTIVES AND OUTLINE OF THE THESIS

The work presented throughout this thesis was developed in the context of the project **Cellular and molecular studies in the pathogenic fungus *Paracoccidioides brasiliensis*: dimorphism and its regulation by steroid hormones** coordinated by Doutor Fernando Rodrigues, a research line of the Infection Diseases Domain of the Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, funded by the *Fundação para a Ciência e a Tecnologia* (Grant Number: POCTI/ESP/45327/2002). The majority of the work developed in this thesis had the collaboration of Jenny Carmona, a technician associated with the funded project.

The initial objectives of the research conducted in the scope of this thesis aimed to (i) investigate the genome content and the multinucleated nature of *P. brasiliensis* yeast cells, (ii) develop a molecular toolbox for *P. brasiliensis*, and (iii) evaluate the molecular bases of cellular differentiation, specifically its characteristic multiple budding trait. Prior to the prosecution of the proposed objectives I received training on basic microbiology procedures for handling *P. brasiliensis* under the supervision of Professora Doutora Angela Restrepo at the Medical and Experimental Mycology Group of the *Corporación Investigaciones Biológicas* in Medellín, Colombia. Since this fungus belongs to the Group 3 of Biological Agents, the majority of the work was carried out in a Biosafety Level 3 Laboratory (BSL3), for which I undertook specific training and evaluation by the director of the BSL3 of the ICVS, Professor Doutor Jorge Pedrosa.

Chapter 1 consists of a general introduction, presenting a review of the current knowledge and up-to-date literature on the etiological agent *P. brasiliensis* and the systemic mycosis it causes, paracoccidioidomycosis. Its main purpose is to prepare the reader for the following chapters, particularly in what refers to the lack of information on certain topics of *P. brasiliensis* biology and molecular tools and the principle aims of this thesis.

Chapter 2 focuses on the analysis of cell cycle progression of *P. brasiliensis* yeast cells under different environmental conditions using a flow cytometric (FCM) technique optimized for cell cycle profile analysis based on high resolution measurements of nuclear DNA. Our findings indicate that *P. brasiliensis* may possess alternative control mechanisms during cell growth to manage multiple budding and its multinucleated nature. This is the first report regarding the study of *P. brasiliensis* characteristic cellular division process in accordance with its nuclear DNA content. I have optimized the FCM protocol and evaluated *P. brasiliensis* batch culture growth

with technical assistance of Margarida Martins during the initial steps of FCM protocol development.

Chapter 3 illustrates an accurate and reliable approach to study poorly understood biological parameters in *P. brasiliensis*, such as genome size and ploidy. The genome size of ten isolates was determined using the FCM protocol optimized in Chapter 2; ploidy assessment was conducted by comparing genome sizing by FCM with the previously described average haploid size obtained from electrophoretic karyotyping and the analysis of intra-individual variability of a highly polymorphic and single copy *P. brasiliensis* gene. Data presented throughout this chapter provides new knowledge on *P. brasiliensis* genetics, important for future research in basic cellular/molecular mechanisms and for the development/design of molecular techniques in this fungus. I have evaluated the genome size and ploidy of all ten isolates with technical assistance of Margarida Martins for the analysis of certain strains and Daniel Matute during sequence analysis.

Chapter 4 illustrates the development of a molecular toolbox for the study of *P. brasiliensis*, particularly an efficient transformation and a gene expression system. Data presented in this chapter substantially improves the available techniques for genetic transformation and reports for the first time the generation of stable homokaryon progeny and *GFP*-targeted *P. brasiliensis* isolates. Overall, this work describes relevant information regarding the future genetic manipulation of *P. brasiliensis*, such as targeted mutagenesis or regulation of gene expression. I have directly contributed to the majority of the tasks included in this chapter.

Chapter 5 focuses on the elucidation of *P. brasiliensis* cellular differentiation, particularly the study of its distinctive multiple budding phenotype. Data is presented concerning the isolation and characterization of a *P. brasiliensis* homolog to *Saccharomyces cerevisiae* Rho-like GTPase Cdc42p (Pbcdc42p) and its possible role in the regulation of spatial organization, namely bud emergence and growth. This is the first report implicating a specific *P. brasiliensis* protein and its putative function in the control of multiple budding cellular division of this dimorphic pathogenic fungus. I have directly contributed to the prosecution of the majority of the tasks included in this chapter with technical assistance of Cristina Cunha and Belém Marques.

In Chapter 6, concluding remarks are presented bringing together Chapter 2, 3, 4 and 5 in the context of the initially proposed objectives. Furthermore, future perspectives in *P. brasiliensis* investigation are depicted, particularly in what refers to the further elucidation of the fungus' characteristic multiple budding and multinucleated feature and its implications in pathogenesis.

CHAPTER 1 – GENERAL INTRODUCTION

To start this introduction a brief history of the thermally dimorphic fungus Paracoccidioides brasiliensis *sounds imperative. This fungus was firstly described by Adolfo Lutz in 1908 as the etiological agent of pseudococcidial hyphoblastomycosis, for differentiation of coccidioidomycosis and hyphoblastomycosis, a term commonly used to designate all diseases caused by dimorphic fungi (Lacaz, 1994a). Later on, in 1912, Alfredo Splendore authored the first publication on histopathological and mycological examinations of the so-called Brazilian blastomycosis or South American blastomycosis. Just in 1930, Floriano de Almeida officially "baptized" the etiological agent of the emerging disease as* P. brasiliensis; *nonetheless, the terminology paracoccidioidomycosis was only officially recognized in 1971 during the first symposium on the subject held in Medellin, Colombia.*

PARACOCCIDIOIDES BRASILIENSIS, THE ETIOLOGICAL AGENT

MORPHOLOGY

As other thermodimorphic fungi, *P. brasiliensis* presents distinct cellular morphology solely depending on the growth temperature. This fungus grows with a yeast form (known as pathogenic) in host tissues, as well as in cultures at 36-37°C, whereas at temperatures below 28°C it develops as mycelia (non-pathogenic form) (Restrepo-Moreno, 2003). Macroscopically, the yeast form is characterized by soft, wrinkled, and cream colored colonies with a cerebriform aspect. Generally, cellular growth is only evident after long periods, being macroscopically detected upon 3 to 4 days for isolates with fast growth rates (Figure 1). Microscopically, the yeast form presents multinucleated cells with variable sizes (4 to 40 μm), a thick two-layered refractile cell wall (200 to 600 nm) and a cytoplasm containing lipid droplets. The cell wall is mainly composed by α-1,3-glucan (and to a much lesser extent β-1,3-glucan) that in its outermost layer has a slime surface of complex mucopolysaccharides, while internally it presents an elevated chitin composition (comprising 43% of the cell wall) (San-Blas and San-Blas, 1994). *P. brasiliensis* yeast cells' most distinctive feature is its "pilot wheel" appearance that results from a multiple budding cell division process, i.e., a mother cell surrounded by numerous small blastoconidia (2 to 4 μm). Nonetheless, single cells with one bud, odd-like morphologies and transitional forms (cells arranged in short chains or chalice-, balloon-shaped or broken cells) are also common when *P. brasiliensis* yeast cells are subcultured for prolonged periods (Brummer *et al.*, 1993; Queiroz-Telles, 1994).

On the other hand, *P. brasiliensis* mycelial form grows more slowly (≈20 days of incubation) at temperatures ranging from 19 to 28°C (Restrepo-Moreno, 2003). Colonies are typically white to tan, small, irregular, and covered by short aerial mycelia. Microscopically, hyphae are thin (1 to 3 μm), hyaline, multinucleated and septated (Figure 1). The mycelial cell wall is also composed of two layers (80 to 150 nm), the outermost made of β-1,3-glucan fibrils and the innermost of rigid chitin fibrils (at reduced levels – 13% of the cell wall – when compared to the yeast form) (Queiroz-Telles, 1994; San-Blas and San-Blas, 1994). When cultured under specific nutritional deprivation conditions (reduced carbohydrate content and low temperatures), certain isolates give rise to diverse types of propagules such as arthroconidia or pedunculated and single-celled conidia (Figure 1C) (Brummer *et al.*, 1993; Samsonoff *et al.*, 1991). Conidia are small sized (3.5 to 5 μm) structures, uninucleated,

structurally equipped to survive environmental stress (Edwards *et al.*, 1991) and also exhibit thermal dimorphism, giving rise either to mycelia or yeast (McEwen *et al.*, 1987b).

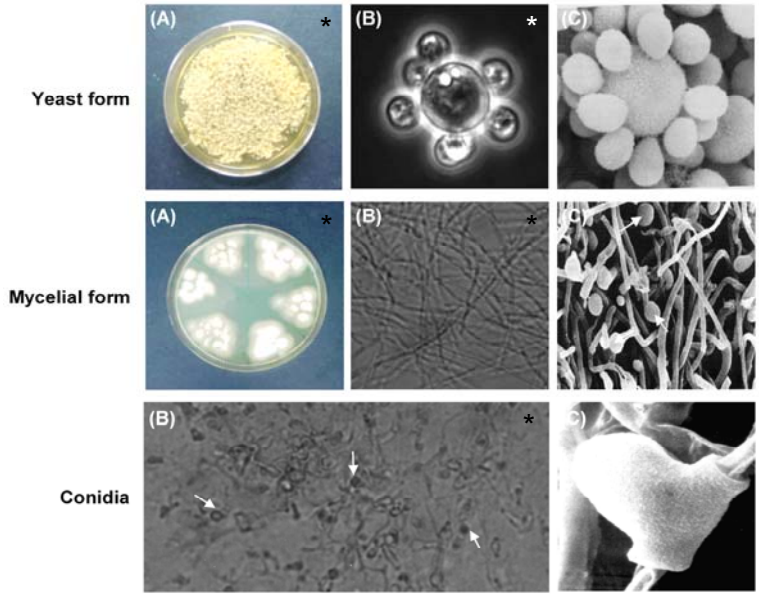

Figure 1 – *P. brasiliensis* yeast, mycelial and conidial forms. (A) Macroscopic features. (B) Bright field microscopy analysis. (C) Scanning electron microscopy analysis (white arrows indicate intercalary conidia) (adapted from Wanke and Londero, 1994; **(*)** A. J. Almeida *et al.*, unpublished data).

In accordance with other fungi, the dimorphism of *P. brasiliensis* has been extensively studied. Particularly, the switch between temperatures above or below 28°C triggers in this fungus morphologic alterations consistent with the yeast or mycelial form. When mycelia are exposed to temperatures above 28°C, the first observed alteration is the development of intercalary or lateral swellings that increase in size and eventually produce multiple buds (Figure 2) (Brummer *et al.*, 1993). Further reports showed that isolated conidia cultured at 36°C round up at 36 h and produce lateral buds at 48 h, while the characteristic multiple budding appeared only after 132 h (Restrepo *et al.*, 1986). The morphological transition in *P. brasiliensis* is accompanied by extensive modifications in the composition of the cell wall. Specifically, the mycelial-to-yeast transition is accompanied by the switch in glucan polymer linkage in the cell wall from β-1,3-glucan to α-1,3-glucan, not only the quantity but also the spatial arrangement of these polysaccharides (San-Blas and San-Blas, 1977; San-Blas and San-Blas, 1994). Interestingly, work performed by San-Blas and co-workers (1976) on a *P. brasiliensis* mutant indicated that the earlier steps of mycelia to yeast transition (swelling of hyphal segments to give rise to yeast-like structures) is independent of α-1,3-glucan synthesis, whereas it is essential for later stages, namely those involved in liberation from hyphae and propagation of yeast cells. Furthermore, the cell membrane lipid composition also changes during

the shift from the two forms, particularly glycosphingolipids (Toledo *et al.*, 1995). Previous studies have suggested that certain modifications in the composition of *P. brasiliensis* cell membrane are required to regulate the activity of synthases (glucan and chitin synthases) and hydrolases (glucanases and chitinases) in the assembly of the cell wall (Borges-Walmsley *et al.*, 2002). Although several efforts have been made to better understand the morphologic alterations, particularly those depending on the temperature, the lack of molecular tools to study this fungus has drastically hampered this line of research.

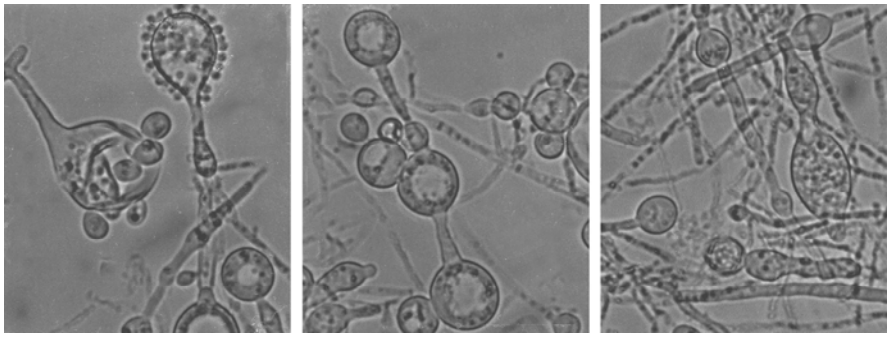

Figure 2 – Mycelia to yeast morphological transition of *P. brasiliensis* at 36°C (A. J. Almeida, *et al.*, unpublished data).

TAXONOMY

Classic systematics typically included *P. brasiliensis* within the now-abandoned higher anamorph taxa the phylum Deuteromycota (imperfect fungi) and the class Hyphomycetes due to the lack of identification of sexual structures (San-Blas *et al.*, 2002). However, with the recent molecular typing techniques, molecular epidemiological and population studies have overridden constraints in precise taxonomic classification. Phylogenetic studies have classified *P. brasiliensis* in the phylum Ascomycota, order Onygenales, family Onygenaceae together with other dimorphic fungi such as *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Coccidioides immitis*, although the latter is rather distant to the others (Bagagli *et al.*, 2006). Deeper molecular epidemiological analysis developed by Matute and co-workers presented consistent evidences that *P. brasiliensis* is in fact a complex of at least three correlated phylogenetic species, S1, PS2 and PS3 (Matute *et al.*, 2006).

ECOLOGY

The identification of the mico-niche together with a fully description of the habitat-specificities of a pathogenic fungus is of utmost importance to understand its pathophysiology. Habitat

assessments commonly afford a critical understanding of the fungus autoecology (spore dispersal, reproductive ecology, factors limiting its distribution and abundance in its native habitat). Nonetheless, the exact environmental niche where *P. brasiliensis* may occur is yet to be determined. The term *reservarea*, initially conceptualized by Borelli (1972), was applied to define the natural habitat of the fungus, where all the factors conductive to infection exist. Although there are differences between the ecological characteristics of the endemic regions (such as the altitude and the rainfall of the Colombian and Brazilian *reservareas*), the temperature, the presence of rivers, and the rain forest type of vegetation are always present (Bagagli *et al.*, 2003; Restrepo-Moreno, 1994). On the other hand, several lines of evidence have pointed to that soil, with particular conditions, might be the *P. brasiliensis reservarea*. These evidences arise mostly from animal and physiopathologic studies (Restrepo *et al.*, 2001, Bagagli *et al.*, 2006). However, one should stress that only sporadically this fungus has been isolated from different sources such as soil (Albornoz, 1971; Negroni, 1966; Silva-Vergara *et al.*, 1998), dog food contaminated with soil in a farm located in an area of high humidity in Brazil (Ferreira *et al.*, 1990), and penguin excreta in Antarctica (Gezuele, 1989). One possible explanation for the failure to efficiently isolate *P. brasiliensis* from the soil might be explained by its slow growth and restricted ability to compete or survive in the presence of natural soil microbiota (Bagagli *et al.*, 2006). In fact, the difficulty to isolate *P. brasiliensis* mycelial form together with the tendency of the yeast pathogenic form to present prolonged latency periods in the human host (Restrepo, 2000) provide circumstantial evidence towards an adaptive capacity for a host-associated life cycle (Bagagli *et al.*, 2006). Of note, is that *P. brasiliensis* has already been isolated from animals with or without symptoms of the disease, namely dogs and armadillo (Ricci *et al.*, 2004; Bagagli *et al.*, 1998; Bagagli *et al.*, 2003; Corredor *et al.*, 2005; Naiff *et al.*, 1986; Silva Vergara and Martinez, 1998; Silva-Vergara *et al.*, 2000). Naiff and co-workers (1986) were the first to isolate *P. brasiliensis* from the nine-banded armadillo *Dasypus novemcinctus* in Brazil. More recently, the presence of the pathogen was also detected in the naked-tailed armadillo *Cabassous centralis* in Colombia (Corredor *et al.*, 2005). Additionally, high frequencies of infection (75-100% of captured nine-banded armadillos) were demonstrated in a hyperendemic region in Brazil (Botucatu, São Paulo) (Bagagli *et al.*, 1998; Bagagli *et al.*, 2003). Although the species *D. novemcinctus* presents a decreased cell-mediated immunity, the nature of its relationship with *P. brasiliensis* – commensal or parasitic – still remains unclear (Bagagli *et al.*, 2006). *P. brasiliensis* was collected from both young and older animals of either sex, however without visible manifestations of PCM (Bagagli *et al.*, 1998; Bagagli *et al.*, 2003). Besides presenting no

distinguishable virulence or molecular features from clinical isolates, the fungal specimens isolated from *D. novemcinctus* were detected in granuloma within lymph nodes, liver and lungs, possibly indicating a condition of active disease (Bagagli *et al.*, 1998; Bagagli *et al.*, 2003; Hebeler-Barbosa *et al.*, 2003b; Hebeler-Barbosa *et al.*, 2003a; Sano *et al.*, 1998; Sano, 1998; Silva Vergara and Martinez, 1998; Silva-Vergara *et al.*, 2000). Taking into account that nine-banded armadillos are soil-digging animals and lack migration habits with short home range, one would expect to isolate *P. brasiliensis* mycelial form from the mammalian's natural habitat. Despite several efforts, this still remains unsuccessful even though PCR amplification of *P. brasiliensis* DNA was shown positive on burrow soil samples and faeces of naturally infected armadillos (Bosco *et al.*, 2005; Theodoro *et al.*, 2005). Altogether, increasing evidence suggest that *P. brasiliensis* fungal infections of humans and armadillos occur in the agricultural and forest microenvironment that they share. Nevertheless, further studies are still required to elucidate the definitive role that these animals play in *P. brasiliensis* natural life cycle, a crucial assessment for the future implementation of prophylactic measures against infection of the most important systemic mycosis in Latina America (Bagagli *et al.*, 2006).

PARACOCCIDIOIDOMYCOSIS

EPIDEMIOLOGY: Demographics-Geographical Distribution

Paracoccidioidomycosis (PCM) is restricted to Latin America where it is distributed from Mexico (23°N) to Argentina (34°S), although not all countries within these latitudes have reported cases (e.g., Nicaragua or Chile) (Restrepo *et al.*, 2001) (Figure 3). PCM has been detected in 14 Latin American countries, with the highest incidence in Brazil (80%) followed by Venezuela and Colombia (Brummer *et al.*, 1993). Throughout the endemic region with 90 million habitants as many as ten million individuals can be infected, although the majority does not present an active form of the disease. However, PCM is not distributed homogenously within the endemic territories, but rather tend to concentrate around *reservareas* (Brummer *et al.*, 1993). The most recent estimates indicate that in areas of high endemicity, such as Brazil, the annual incidence rate of active disease is 1-3 per 100000 inhabitants and a mean mortality rate of 0.14 per 100000; on the other hand, a 30-year survey in Colombia indicated lower and fluctuating incidence rates (0.05-0.22 per 100000 inhabitants) (Restrepo *et al.*, 2001; Wanke and Londero, 1994). Moreover, several PCM

cases have also been reported outside the endemic region, ranging from the United States to Europe and Asia (Ajello and Polonelli, 1985; Brummer *et al.*, 1993; Kamei *et al.*, 2003; Van Damme *et al.*, 2006), although in every case the patient had previously lived, or at least visited, one of the endemic countries. Nonetheless, as PCM is not contagious from person to person and due to the absence of epidemic outbreaks, several aspects of the epidemiology of PCM still remain unclear.

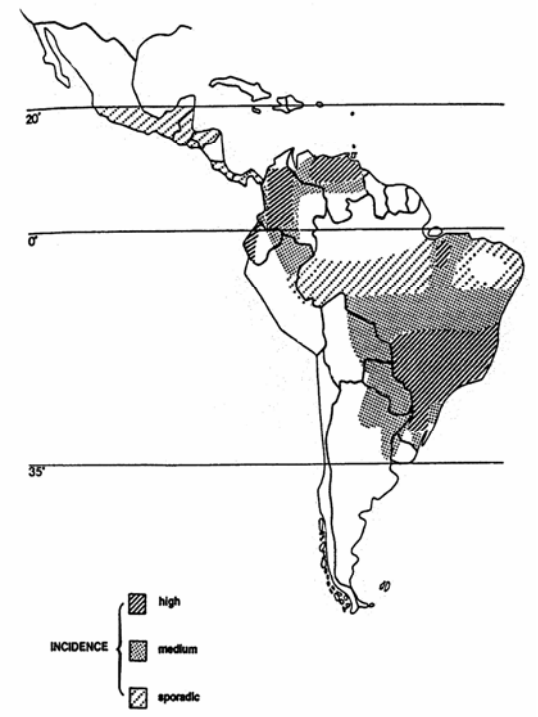

Figure 3 – Geographic distribution of PCM in Central and South America (Wanke and Londero, 1994).

The accurate determination of where and when the infection takes place has also been impaired by the disease's prolonged incubation time. In what regards the demographics of PCM, epidemiological studies indicate that a significant part of the recorded cases occur in immunocompetent individuals, although immunocompromised patients have also been shown to present active disease (Restrepo-Moreno, 2003). In addition, clinical data regarding PCM suggest that host susceptibility to *P. brasiliensis* is related to certain host-related factors, particularly its genetic and hormonal background. In fact, host genetic factors have been proven to influence the progression of infection by *P. brasiliensis*. Different congenic strains of mice infected intraperitoneally with *P. brasiliensis* yeast cells were shown to present distinct resistant/susceptibility patterns (Calich *et al.*, 1994). More recently, it was reported that macrophage lines carrying a mutated form of *Nramp1* (which controls the production of reactive nitrogen intermediates) have a diminished ability to phagocytose conidia

and a lower mannose receptor expression (a C-type lectin that recognizes mannose receptors of a wide spread spectrum of microorganisms). These authors proposed the possible involvement of differential genetic backgrounds in the resistance/susceptibility to *P. brasiliensis* infection (Jimenez *et al.*, 2006). In addition, a frequency analysis of polymorphisms within the tumour necrosis factor-alpha (G/A at position -308) and interleukin-10 (G/A at position -1082) genes of PCM patients suggested that these allele variants might be implicated in the mechanisms of resistance/susceptibility to PCM infection, as proven for cytokine genes in other diseases (Bozzi *et al.*, 2006). Due to the fact that interbreeding is common among the populations of the endemic regions, the influence of race in PCM infection is difficult to assess. Lacaz and co-workers stated that immigrants that have more recently arrived at the areas of endemicity usually develop a more severe form of disease, possibly indicating a greater susceptibility to *P. brasiliensis* (Lacaz *et al.*, 2002). However, studies performed on the potential role of human leukocyte antigens in acquiring PCM infection showed no evidence of a specific association (Dias *et al.*, 2000).

PCM infection, detected by skin tests with paracoccidioidin antigen, is more commonly observed in farmers and rural workers between the third and fourth decades of their life (Restrepo-Moreno, 1994). Relevantly, PCM is rarely observed in children and young adults (3 and 10% of the reported cases) but is more frequently diagnosed in male adults (30 to 60 years of age) (Brummer *et al.*, 1993). In fact, while no significant differences exist between genders in *P. brasiliensis* subclinical or juvenile infection (Brummer *et al.*, 1993), active chronic PCM disease is much more frequent in men than in women, with an overall ratio of 14:1 in the total endemic region, indicating that disease progression seems to be blocked in females, a phenomenon also proven in a mice experimental model of PCM (Aristizabal *et al.*, 1998; Aristizabal *et al.*, 2002; Restrepo-Moreno, 2003).

According to the natural history of PCM, once in contact with *P. brasiliensis* infectious propagules the infection may become dormant with the formation of quiescent foci with viable yeast cells (Restrepo-Moreno, 2003). Endogenous reactivation may occur depending on intrinsic host determinants such as debilitating disease, chronic alcoholism, malnutrition, smoking, and immunosuppression; however, the precise reasons that underlie this phenomenon are still unclear (Restrepo-Moreno, 2003). Contrary to other fungal diseases, PCM does not have a marked opportunistic character, although it has been infrequently detected in patients with distinct immunosuppressive diseases (Marques and Shikanai-Yasuda, 1994). For example, the AIDS epidemic did not increase incidence rates of PCM even though it is of extreme social impact in

the areas of endemicity. A review of all the reported cases of PCM-HIV accounted as a total of 79 with a notable increase in the last years, although the real number is probably larger (Benard and Duarte, 2000). This low incidence has been thought to be related to the fact that HIV infection is more prevalent amongst urban populations, whereas *P. brasiliensis reservareas* appear to occur mainly in relatively isolated rural communities (Marques and Shikanai-Yasuda, 1994). Intriguingly, since the *P. brasiliensis* infection would be acquired during the past and the manifestation of it resulting from endogenous activation, one would expect the presentation of the chronic form of PCM; nonetheless, PCM clinical manifestations when co-infected with HIV resemble those of the severe juvenile form (Benard and Duarte, 2000).

VIRULENCE FACTORS

Virulence factors may be defined as specific attributes of a pathogenic microorganism that enhances its aptitude to invade the host and grow under hostile conditions, thus supporting the infection process. In fungal pathogens, these virulence attributes allow the pathogen to adhere, colonize and disseminate while eluding the host immune response mechanisms (Mendes-Giannini *et al.*, 2000). Up to date, distinct virulence factors of *P. brasiliensis* have been described. As itself, the thermal dimorphism presented by this fungus has been identified as an important virulence factor as it allows the pathogen to adapt and survive in host tissues (Camargo and Franco, 2000; San-Blas *et al.*, 2002). Several studies have specifically associated altered morphology patterns to a distinct virulence profile (Kurokawa *et al.*, 2005; Villar and Restrepo, 1989).

As referred previously, epidemiological data has long demonstrated that the active form of postpuberty PCM has a sex bias, with a higher incidence in men than in women (Restrepo-Moreno, 2003). Restrepo and colleagues initially presented evidence proving that estrogens (17β-estradiol) selectively inhibit the mycelium-to-yeast transition in *P. brasiliensis* (Restrepo *et al.*, 1984). A 60 kDa molecular mass estrogen-binding protein was identified in the cytosol of both the mycelial and yeast form; it was suggested that once in contact with its estrogen hormone ligand, the protein-ligand complex modulated protein expression and ultimately the morphological conversion (Clemons *et al.*, 1989; Stover *et al.*, 1986). Moreover, the inhibitory action of 17β-estradiol on the conidia to yeast transition in female mice was clearly blocked the initial morphological switch that is crucial for the establishment and dissemination of the disease (Aristizabal *et al.*, 1998; Aristizabal *et al.*, 2002). More recently, transcriptome profile analysis detected a preferentially expressed gene in the pathogenic yeast form that most likely encodes

the previously identified estradiol binding protein (Felipe *et al.*, 2005). Although evidence is presented explaining the innate resistance of females to PCM, the precise molecular events that underlie this particular phenomenon in *P. brasiliensis* still remain unknown.

P. brasiliensis yeast cell wall components such as galactomannan and α-1,3-glucan have also been characterized as important virulence factors. During the morphological transition from mycelia to yeast a spatial and quantity rearrangement is observed with a switch from β-1,3-glucan to α-1,3-glucan (San-Blas *et al.*, 2002). San-Blas and co-workers (1977) were the first to describe that *P. brasiliensis* mutants lacking this particular compound presented a decreased virulence. These authors showed that multiple sub-culturing of *P. brasiliensis* yeast form reduces α-1,3-glucan content in the cell wall associated to a gradual loss of virulence, whereas animal passage seems to restore it to near normal levels (San-Blas and San-Blas, 1977). In addition, since the host does not produce α-glucanases capable of degrading the cell wall, the high α-1,3-glucan content in the yeast cells might elude this particular host defence response. More recently, it has been suggested that after animal passage the gain-of-virulence of *P. brasiliensis* might also be associated to other restored factors involved in the pathogenicity of the fungus, such as size and polymorphism of the cells (Brummer *et al.*, 1990; Svidzinski *et al.*, 1999). The cell wall component galactomannan, one of the most immunogenic molecules in fungal cell walls, has also been shown to vary in content in different isolates. A higher concentration of galactomannan was detected in clinical isolates from unifocal PCM cases while isolates from disseminated PCM presented lower values (San-Blas *et al.*, 1984).

The 43 kDa immunodominant glycoprotein Gp43 has also been described as an important virulence determinant in *P. brasiliensis*. This exocellular glycoprotein is homolog to fungal β-1,3-glucanases, but is not enzymatically active and its true biological activity is still under evaluation (Carvalho *et al.*, 2005; Cisalpino *et al.*, 1996). Nonetheless, extracellular proteolytic activity in *P. brasiliensis* has been documented for another yeast cell wall component. A serine-thiol proteinase was shown to cleave basal membrane proteins, such as laminin and fibronectin, and suggested as important to facilitate host tissue invasion (Puccia *et al.*, 1998). Gp43 has been characterized as an adhesin and shown to bind to extracellular laminin and enhance pathogenicity in a hamster model of infection (Vicentini *et al.*, 1994). The role of Gp43 in microbial adherence was further elucidated by the inhibition of the adherence of *P. brasiliensis* yeast cells to different cell lines by treatment with anti-Gp43 patient serum and monoclonal antibodies against this glycoprotein (Gesztesi *et al.*, 1996; Hanna *et al.*, 2000). Gp43 was also reported as an important evasion mechanism for the installation

of primary infection as demonstrated by the inhibition of phagocytosis and fungicidal ability of macrophages of resistant and susceptible mice (Popi *et al.*, 2002). In fact, André and colleagues (2004) also demonstrated that laminin binding to *P. brasiliensis* yeast cells decreased pathogenicity, particularly the interaction with extracellular proteins. Additionally, studies on DNA vaccination in a murine model showed that protective immunity against *P. brasiliensis* can be prompted by the challenge with the *GP43* gene (Pinto *et al.*, 2000). More recently, the regulation of *GP43* expression and virulence of *P. brasiliensis* isolates were evaluated (Carvalho *et al.*, 2005). Data presented by these authors suggested that isolates with a particular *GP43* genotype were less virulent and that its expression is regulated not only at the transcriptional level, but also at the protein and/or secretion level.

Other *P. brasiliensis* molecules that interact with host cells and can ultimately influence pathogenesis have been identified. The presence of two proteins – a 19 and a 32 kDa mass protein – were detected in the cell wall of *P. brasiliensis* yeast cells that interact with extracellular matrix (ECM) proteins, such as laminin, fibronectin and fibrinogen (González *et al.*, 2005a; González *et al.*, 2005b). These authors also showed that host adhesion molecules are up-regulated during infection of mice with conidia and participate in the pathogenesis of experimental PCM (González *et al.*, 2005c). In addition, a 30 kDa protein was isolated from *P. brasiliensis* yeast cells and presented adhesin-like properties as demonstrated by binding to laminin but not to other ECM components (Andreotti *et al.*, 2005). Paracoccin, the second most recognized component by serum-antibodies from patients with PCM, was identified as a GlcNAc-binding lectin that interacts with laminin on the ECM and stimulates the release of immunological mediators previously described to play an important function in PCM (Coltri *et al.*, 2006). Recent studies have also evaluated the putative role of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), overexpressed in *P. brasiliensis* yeast parasitic form, in the adherence to microbial cell ligands of the host cells (Barbosa *et al.*, 2006). GAPDH was shown to localize to the outermost layer of the cell wall and interact with ECM proteins, such as laminin and fibronectin, triggering host responses during the early stages of infection. Furthermore, in a different study the same authors proved that when invading epithelial mammalian cells, *P. brasiliensis* induces cytoskeletal rearrangement and apoptosis which most likely aids this fungal pathogen to evade the host immune system and present a more efficient dissemination (Mendes-Giannini *et al.*, 2004). Melanin, a multifunctional polymer, has also been indicated as a virulence factor in *P. brasiliensis*. It was shown that *P. brasiliensis* conidia and yeast cells produces melanin both in culture and during murine infection (Gómez *et al.*, 2001). In

addition, the presence of fungal melanin enhanced *P. brasiliensis* resistance to antifungal compounds and protected the fungus from phagocytosis (Silva *et al.*, 2006).

Different molecules have been associated to *P. brasiliensis* virulence. The 61 kDa monofunctional catalase P was proved to be overexpressed in the yeast parasitic form and to be induced by the addition of exogenous H_2O_2; these data may suggest a putative role for protection of *P. brasiliensis* against oxygen radicals during infection (Moreira *et al.*, 2004). A member of the flavodoxin-like protein family, PBY20, was detected as high levels of expression in the yeast form; in view of the fact that other members of this protein family are closely related to heat shock and oxidative stress response in distinct biological systems it was suggested that PBY20 might play a role in intracellular detoxification in the infection pathway of *P. brasiliensis* (Daher *et al.*, 2005). In addition, an intracellular calcium modulator from *P. brasiliensis*, calmodulin, has been implicated as a putative virulence factor as drug inhibitors of the Ca^{2+}/calmodulin signalling pathways were able to hinder the mycelium-to-yeast transition (Carvalho *et al.*, 2003).

CLINICAL FORMS

As stated before, the precise manner in which *P. brasiliensis*, a facultative intracellular microorganism, infects the human host is still not clearly understood particularly due to the lack of knowledge on the accurate ecological niche of the fungus. Epidemiological and experimental evidence suggest that natural infection begins with the inhalation of conidia or mycelial fragments and deposition in the distal region of the lungs (Restrepo-Moreno, 2003). According to *in vivo* assays performed on intranasally infected mice, conidia phagocytosed by alveolar macrophages morphologically switch to the yeast pathogenic form within only a few hours (12-18), followed by dissemination and induction of a progressive disease resembling the human chronic form of PCM (McEwen *et al.*, 1987a). Depending on host factors or strain-level virulence differences, individuals exposed to *P. brasiliensis* may develop an asymptomatic infection (i.e., contagion with no clinical manifestations) or can give rise either to subclinical or an active form of the disease with a gamut of clinical manifestations (Restrepo-Moreno, 2003).

Subclinical infection. Several reports have presented data that prove the existence of a subclinical *P. brasiliensis* infection. Skin testing reactivity to paracoccidioidin was detected in healthy residents of endemic regions (Brummer *et al.*, 1993). Clinical evidence has also been documented detailing previous contact of the patient with the fungus without the development of PCM (Severo *et*

al., 1979). Moreover, the fungus has been isolated from partially calcified lesions in histopathological studies of individuals evaluated for other conditions (Angulo-Ortega, 1972).

Active disease. PCM generally occurs in immunocompetent individuals under two main distinguishable clinical forms, the acute or sub-acute form (juvenile type), and the chronic form (adult type). The clinical manifestation of the disease is contingent upon the patients' immune response, hormonal balance and age (Restrepo-Moreno, 2003). Overt PCM is a disease that may involve any organ and resemble other clinical conditions, thus complicating diagnosis.

The acute or sub-acute clinical form is the severest (higher mortality rates) and less frequent type and affects children and young adults irrespective of gender, representing only 3 to 5% of all cases (Restrepo-Moreno, 2003). It is characterized by rapidly progressing from the primary lesion (weeks to months), by lymphatic or lympho-haematogenous dissemination to the reticulo-endothelial system with marked involvement and hypertrophy of spleen, liver, lymph nodes, and bone marrow (Figure 4). The lungs are rarely the primary focus as there are no special clinical or radiological manifestations, even though the fungus can usually be isolated from pulmonary secretions (de Mattos *et al.*, 1991; Restrepo *et al.*, 1989). This form of PCM is characterized by a severely depressed cell mediated immune response, while the humoral immune response is at normal levels. Histopathology usually reveals macrophage infiltration and the frequent formation of loose granuloma with large numbers of actively multiplying *P. brasiliensis* yeast cells (Borges-Walmsley *et al.*, 2002).

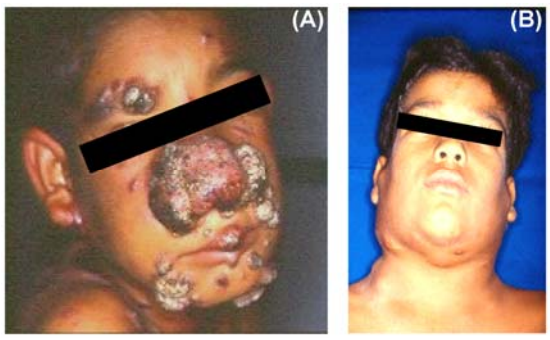

Figure 4 – Acute or juvenile form of PCM. (A) Patient with extensive mucocutaneous lesions. (B) Patient with multiple lymph node involvement (*Corporación para Investigaciones Biológicas*', CIB, archives).

The chronic form of the disease is manifested in more than 90% of the documented PCM cases (Restrepo-Moreno, 2003). Contrary to the juvenile type, this form of PCM evolves slowly (months to years) and mainly occurs in adult males. Most patients (90%) presenting the chronic form of

the disease show evident pulmonary manifestations. Approximately 25% of the cases feature the lungs as being the only clinically affected organ (unifocal form); however, the majority of cases are characterized by an asymptomatic pulmonary involvement followed by dissemination by the bronchial, lymphatic or lympho-haematogenic routes with multi-organ involvement (multifocal form). Secondary lesions are more commonly observed in the mucous membranes (particularly the oropharyngeal mucosa) and the skin with the formation of ulcers, followed by involvement of the lymph nodes, spleen and adrenals (Figure 5). However, involvement of any organ may occur (e.g., ocular, central nervous system, bones, and genital and testicular lesions) (Montenegro and Franco, 1994). Respiratory symptoms are non-specific, often minor and do not correspond to the extensive lung involvement frequently revealed by X-rays (Restrepo-Moreno, 2003). Furthermore, the chronic form can be sub-divided into *mild*, *moderate* or *severe* according to the degree of dissemination, the clinical findings and the patient's general condition. Cellular immunity is preserved in the unifocal forms or depressed in the multifocal forms, whereas the humoral host response is variable (Brummer *et al.*, 1993). Also, in this particular form of PCM, the host immune response leads to the formation of compact granulomas that present low counts of *P. brasiliensis* yeast cells (Borges-Walmsley *et al.*, 2002).

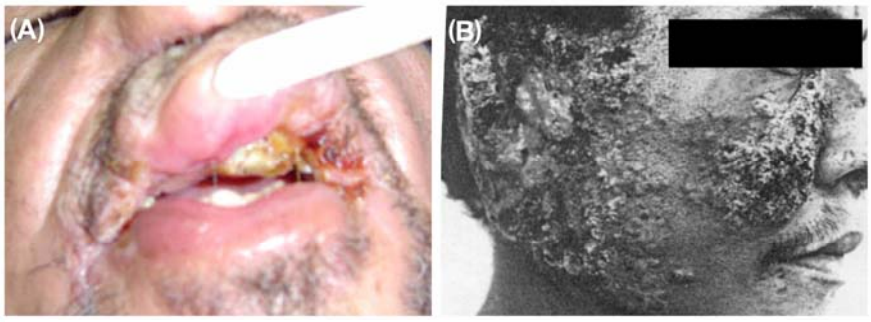

Figure 5 – Chronic or adult form of PCM. (A) Patient with mucocutaneous lesions. (B) Patient with extensive skin involvement and ulcerated lesions (adapted from CIB's archives).

Regardless of the organ involved, in most patients PCM heals by pulmonary fibrosis leading to the development of fibrotic sequelae that can drastically hamper respiratory functions, thus interfering with the well-being of the patient (Tobón *et al.*, 2003). Fibrosis is the result of an active progressive pulmonary infection that initiates concomitantly with the inflammatory process and the presence of leukocyte infiltrates, and later on progresses and consolidates during granuloma formation (Montenegro and Franco, 1994). As a result, this strong inflammatory response to an active and progressive pulmonary infection ultimately transforms the lungs into a structure with an impaired capability for proper gaseous exchange (Restrepo-Moreno, 2003).

DIAGNOSIS

Direct examination and histopathology. Upon the supposition of a *P. brasiliensis* infection, the foremost diagnostic test to confirm PCM involves direct examination of clinical samples allowing the visualization of the causative agent. Detection of fungal elements can be carried out in a number of clinical specimens, such as sputum, bronchoalveolar lavage fluids, the outer edge of ulcers, tissue biopsies, among others (Brummer *et al.*, 1993). *P. brasiliensis* yeast cells are often visualized as oval to round cells of varying size some of which may present a multiple budding morphology. Even though the sensitivity of direct examination is generally high (85 to 100%), distinct forms of *P. brasiliensis* (short chains, single-budded cells and odd-like morphologies) may still be mistaken for other fungal infections (Lacaz, 1994a; Lacaz, 1994b; Restrepo-Moreno, 2003). Staining procedures to detect fungal elements include KOH, calcofluor and immunofluorescence (Figure 6). Furthermore, histopathological preparations such as haematoxylin and eosin, methenamine-silver or periodic acid-Schiff are very useful for the evaluation of biological samples (Restrepo-Moreno, 2003). In biopsy material, these staining techniques reveal an inflammatory reaction characterized by the presence of granulomas centred on yeast cells, some of which have been phagocytosed. The granulomatous foci usually present infiltration of polymorphonuclear leukocytes (neutrophils), macrophages, mononuclear cells, and multinucleated giant cells (Brummer *et al.*, 1993).

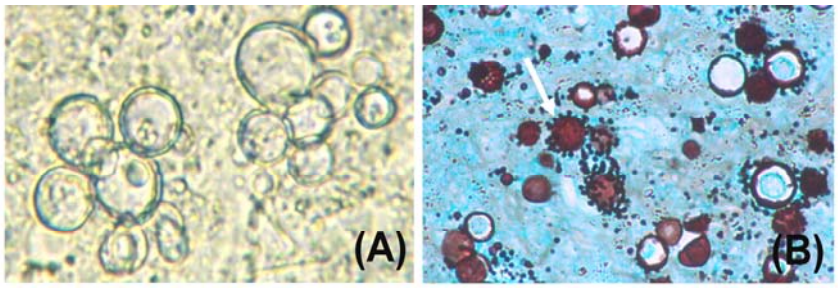

Figure 6 – Analysis of fresh clinical specimens of PCM by detection of *P. brasiliensis* yeast cells. (A) Direct examination using 10% KOH. (B) Histopathological analysis of biopsy material stained with methenamine silver (white arrow indicates *P. brasiliensis* multiple budding yeast cell) (adapted from CIB's archives).

Culture. The isolation of the causative agent of a particular fungal disorder is essential to confirm accurate identification and that the infection is indeed active. *P. brasiliensis* isolation from clinical samples can be carried out using selective and non-selective media. Antibiotic drugs and mold growth inhibitors are usually supplemented to modified Sabouraud (Mycosel) medium to restrain

growth of contaminants (Brummer *et al.*, 1993). *P. brasiliensis* colonies appear after 20 days of incubation at room temperature (19-24°C) with a variety of colonial forms; however, cultures should be kept for at least two months before confirming a negative result (Figure 7). Since the mycelial form is not distinctive, dimorphism must be demonstrated by subculturing at 37°C (Restrepo-Moreno, 2003). Furthermore, when specimens are bacteria-free, media without antibiotics and a 36°C incubation temperature may also be employed. Overall, the fungal recovery rates by culture are around 80% (Lacaz *et al.*, 2002).

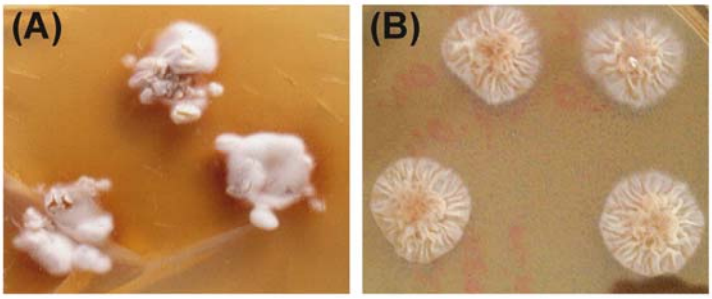

Figure 7 – Culturing of *P. brasiliensis* from clinical samples. (A) Mycelial colonies with short aerial hyphae at room temperature; and (B), yeast colonies with a characteristic cerebriform texture at 36°C (adapted from CIB's archives).

Immunodiagnosis. Immunodiagnostic techniques are of considerable value in the diagnosis of PCM due to the wide range of clinical presentations and the time-consuming procedures for the isolation of *P. brasiliensis* from clinical specimens. Since PCM patients are usually immunocompetent, they are generally not deficient in antibody production. In fact, about 90% of the individuals with clinical disease present specific antibodies at the time of diagnosis (Mendes-Giannini *et al.*, 1994). Consequently, several sensitive serological tests have been developed, particularly for the titration of circulating antibodies, and are extremely valuable both for the diagnosis and prognosis of PCM. However, antigens prepared from *P. brasiliensis* vary in quality depending on the strain, fungus growth phase and culture media. Moreover, the use of complex mixtures of undefined antigens from diverse cellular components (e.g., cell wall preparations, cytoplasm or culture filtrates) has impaired the standardization of antigenic source as well as resulting in cross-reactivity with other fungal antigens (Brummer *et al.*, 1993). In this sense, attempts have been made to use serological assays for the detection of antibodies against chemically characterized and/or recombinant *P. brasiliensis* antigens. In fact, advances in molecular biology have allowed the production of reproducible and defined antigenic proteins through gene cloning and sequencing. The antigens Gp43, Gp70, Pb27, Hsp60 and Hsp87 are the most frequently used for PCM diagnosis (Camargo and Franco, 2000; Cunha *et al.*, 2002; Diez *et al.*, 2002; Marques da Silva *et al.*,

2003; Marques da Silva *et al.*, 2004; Ortiz *et al.*, 1998). The most commonly applied serological testing methodologies are complement fixation (CF), agar-gel immunodiffusion (ID), double ID, immunoelectrophoresis (IE), counter-immunoelectrophoresis (CIE), enzyme linked immunosorbent assay (ELISA), indirect immunofluorescence (IF), dot-blot and immunoblot; depending on the test, sensitivity and specificity varies from 65 to 100% (Brummer *et al.*, 1993; Mendes-Giannini *et al.*, 1994; Restrepo-Moreno, 2003).

Contrary to serological assays, the reactive paracoccidioidin skin test has no value as a diagnostic tool. Nonetheless, this test can be used to determine the incidence of subclinical infection in the general population of endemic regions (Restrepo-Moreno, 2003). Moreover, during therapy skin testing may be a useful tool for monitoring the immune status of the patient since the conversion from a non-reactive to a positive reaction usually signals a good prognosis for PCM (Brummer *et al.*, 1993).

Molecular diagnosis. During the last decade, molecular-based techniques have been developed to provide alternative strategies for the diagnosis of PCM and override the often time-consuming conventional methods. DNA probes have been developed for the rapid identification of *P. brasiliensis* in mycelial and yeast cultures as well as in biopsies from oral cavity lesions of PCM patients and guinea pigs inoculated with the fungus (de Brito *et al.*, 1999; Sandhu *et al.*, 1997). Furthermore, several molecular biological tools have been applied to detect specific *P. brasiliensis* DNA sequences, such as polymerase chain reaction (PCR), nested PCR, PCR-enzyme immunoassay, real time PCR (RT-PCR) and loop-mediated isothermal amplification (LAMP) (Bialek *et al.*, 2000; Gomes *et al.*, 2000; Lindsley *et al.*, 2001; Motoyama *et al.*, 2000; San-Blas *et al.*, 2005; Semighini *et al.*, 2002). Within these studies, different DNA templates were applied to detect *P. brasiliensis* from distinct sources: DNA from clinical and environmental isolates; sputum and cerebrospinal fluid from PCM patients; tissues samples from mice intranasally infected with conidia. The *GP43* and ribosomal DNA genes have been the most commonly targeted *P. brasiliensis* sequences for the detection of the fungus in clinical samples. On the other hand, the *GP27* gene has been applied to screen for *P. brasiliensis* in artificially contaminated soils and in tissues of armadillos naturally infected with the fungus, although it has not been used for the evaluation of human tissue samples (Diez *et al.*, 1999). Moreover, microsatellites in the genome of *P. brasiliensis* have recently been detected and proposed as genetically associated elements with the potential to discriminate clinical isolates in accordance with virulence profiles (Nascimento *et al.*, 2004).

Overall, the development of these molecular tools may play an important role not only in PCM diagnosis, when patients present negative serologic reactions due to low concentration of the antibody and/or the antigen, but also for epidemiological and ecological studies of *P. brasiliensis*.

THERAPY

In the absence of therapy PCM is a fatal disease. Treatment of PCM, using fungistatic rather than fungicidal drugs, is usually prolonged with the majority of patients receiving therapy for 6 months to two years (Tobón *et al.*, 2003). Globally, therapeutic strategies may involve an initial attack with sulphonamides (either alone or combined with trimethoprim), amphotericin B, or azole derivatives, followed by a maintenance treatment (Mendes *et al.*, 1994).

Sulphonamides were the first effective antifungal drugs used against PCM (since 1940) (Brummer *et al.*, 1993); however, the combination with other drugs (e.g., trimethoprim) yields better results. Up to date these combinations are still applied, not only due to low cost, its easy administration to the patient, and lack of major toxicity, but also because when used properly they present an efficiency of 70% during the initial phases of treatment (Mendes *et al.*, 1994). The long period of drug administration required (up to 5 years), the frequent relapses (25%) during maintenance therapy, the elevated number of cases of acquired resistance (15%), and the high morbidity even after prolonged treatment are the limitations of this type of therapeutic strategy (Restrepo-Moreno, 2003).

Amphotericin B was introduced for PCM treatment almost 2 decades after sulphonamides and was proven more effective (Mendes *et al.*, 1994). It is usually reserved for severe cases of PCM due to its high toxicity and the need for intravenous administration. Additionally, a combination with sulphonamides or azoles should be included during the maintenance treatment. Remission of the majority of symptoms occurs in about 70% of the cases, but approximately 25% of the patients relapse (Restrepo-Moreno, 2003).

The most important advance in PCM management was the introduction of azole derivatives in 1978, as it allows ambulatory therapy with reduced courses of treatment, toxicity and relapses (Brummer *et al.*, 1993; Tobón *et al.*, 2003). In the case of ketoconazole, approximately 90% of the patients administered with this drug responded positively in the first 3 to 6 months (only 8% were non-responsive to treatment). 3 years after therapy about 11% of the patients relapsed (Restrepo-Moreno, 2003). The triazole itraconazole was introduced in 1982 and also presented high efficiency (98%) during a 3 to 12 month treatment period, but an even lower relapse rate

(less than 5%). Another triazole, fluconazole, has also been used in some patients; however, the high doses and long treatment periods necessary to avoid relapses primarily recommend the use of ketoconazole or itraconazole.

TRENDS IN MOLECULAR STUDIES OF *P. BRASILIENSIS*

In the last few years, the main stream of *P. brasiliensis* related research fields have been mostly directed towards the study of host-pathogen interactions and morphogenesis. The recent transcriptome profile analysis of both mycelial and yeast forms, carried out by expressed tag sequencing, yielded important information on the differential expression pattern of both morphologies and identified new candidates that might play an important role in the fungus' virulence (Andrade *et al.*, 2005; Bailao *et al.*, 2006; Felipe *et al.*, 2005; Goldman *et al.*, 2003; Marques *et al.*, 2004; Nunes *et al.*, 2005). However, the understanding of specific protein function that triggers cellular/molecular virulence mechanisms has been impaired by the absence of tractable molecular techniques or the precise knowledge on *P. brasiliensis* genetics.

P. BRASILIENSIS GENETICS

Several questions regarding *P. brasiliensis* genetics are still poorly clarified. The fact that *P. brasiliensis* is refractory to classic cytogenetic techniques initially hampered the investigation of the chromosome number and organization. Moreover, and even though orthologues for *Saccharomyces cerevisiae* transcription factors (such as *MAT*, *MCM1*, and *NsdD*) implicated in ascomycete sexual reproduction have been recently identified in *P. brasiliensis*, the lack of an accurately described sexual phase also complicated classic genetic approaches to study this fungus (Felipe *et al.*, 2005; San-Blas *et al.*, 2002).

As in other pathogenic fungi, the advance in pulsed-field gel electrophoresis (PFGE) methodologies allowed the karyotype analysis of *P. brasiliensis*. To date, eighteen clinical and six environmental isolates were evaluated regarding chromosome size and number (Cano *et al.*, 1998; Feitosa *et al.*, 2003; Montoya *et al.*, 1997; Montoya *et al.*, 1999). Altogether, these studies reported the identification of ten distinct karyotype profiles and the presence of 4 to 5 chromosomal bands of variable molecular weight, ranging from 2 to 10 Mb, independently of the origin of the isolate. It has been suggested that chromosomal length polymorphism may have

originated from genetic rearrangements, translocations or deletions. In fact, genomic plasticity and the fluidity of chromosomal organization is a well known trait of pathogenic fungi that may have important implications in the control of gene expression, genetic variability, and evolution, and explain certain clinical-epidemiological features of the studied isolate (Carr and Shearer, 1998; Pan and Cole, 1992; Perfect *et al.*, 1989; Thrash-Bingham and Gorman, 1992). Nonetheless, one must not discard that the difference in electrophoretic mobility patterns may have resulted from the application of distinct technical parameters during the migration of chromosomal molecules (Cano *et al.*, 1998). *P. brasiliensis* genome size was also estimated from the sum of the average molecular weights of chromosomes ranging from 23 to 31 Mb (Cano *et al.*, 1998; Feitosa *et al.*, 2003; Montoya *et al.*, 1997; Montoya *et al.*, 1999). These data are similar to those reported for the dimorphic fungi *Histoplasma capsulatum* and *Coccidioides immitis* but lower than that of *Cryptococcus neoformans* (Carr and Shearer, 1998; Pan and Cole, 1992; Perfect *et al.*, 1989). In addition, work performed by Cano *et al.* (1998) and Feitosa *et al.* (2003) using 4,6-diamino-2-phenylindole (DAPI)-nuclei staining revealed that the studied *P. brasiliensis* isolates presented a variable average number of nuclei (2-8 per cell) and genome size (46-61 Mb) as determined by microfluorometric analysis of DAPI-labelled nuclei. By comparing these data with the genome size determined by the average sum of the molecular weights of chromosome-sized DNA molecules separated by PFGE, the authors suggested the existence of naturally occurring haploid, diploid and aneuploid isolates. However, the possibility of incorrect assessments concerning ploidy was considered not only due to limitations in separating homologous chromosomes during electrophoretic karyotyping, but also due to the possible variations of nuclear DNA content during the cell cycle (Feitosa *et al.*, 2003).

Feitosa and co-workers (2003) have also addressed the genome structure and organization of *P. brasiliensis* by mapping nine homologous genes to the chromosomes of twelve clinical isolates from different endemic regions, using Southern blot hybridization techniques. The results indicated the existence of intraspecific variability, thus suggesting the occurrence of chromosomal rearrangements, a feature usually observed in fungi lacking a meiotic cycle that can provide means for genetic variation in natural isolates. Furthermore, three syntheny groups were defined containing two or three genes: group I, *PbMDJ1* (MDJ1-like protein gene), *PbLON* (ATP-dependent proteinase gene), and clone 11 (putative mannosyltransferase gene); group II, *GP43*, *RANBP* (Ran binding protein homologue gene), and *NAG* (putative N-acetyl-β-D-glycosaminidase); group III, *28S rRNA* and *CHS1* (chitin synthase gene). These data suggest that

a certain degree of similarity in the genome organization of the studied isolates exists, regardless of the detected chromosomal length polymorphisms. In summary, data regarding molecular karyotype of *P. brasiliensis* is crucial for the future construction of physical maps and the detection of genetic markers that might be linked to important phenotypic features of this pathogenic fungus.

P. brasiliensis gene content has also been evaluated on two distinct occasions. Goldman and co-workers indicated that this fungus contains between 10000 and 15000 genes (Goldman *et al.*, 2003). On the other hand, a study by Reinoso and colleagues by sequencing 50 kb of genomic DNA revealed a density of one gene per 3.5-4.5 kb, suggesting the existence of 7500 to 9000 genes in *P. brasiliensis* total genome (Reinoso *et al.*, 2003). Moreover, these same authors have randomly sequenced several *P. brasiliensis* genes from a partial genomic library with an average insert size of 8 kb (Reinoso *et al.*, 2005). Data obtained during this study has given a new insight regarding gene structure (e.g., promoter regions and introns), providing a liable identification of both genes and intergenic sequences. However, large-scale genome DNA sequencing is necessary to further investigate basic molecular regulatory mechanisms.

Although relevant biological issues have already been addressed, fundamental questions concerning *P. brasiliensis* genetic composition still remain unanswered, namely the correct assessment of natural ploidy levels and its possible significance in the regulation of basic cellular and molecular mechanisms in pathogenesis.

GENETIC TRANSFORMATION SYSTEMS IN *P. BRASILIENSIS*

Fungal genetic transformation has long been an essential molecular tool for gene isolation/disruption and expression analysis, thus linking a specific protein function inside a cell to its coding DNA (Magee *et al.*, 2003). Up to date, only two reports have briefly described *P. brasiliensis* genetic transformation, either by electroporation or *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Leal *et al.*, 2004; Soares *et al.*, 2005).

ATMT is a highly successful insertional mutagenesis technique that has been extensively used in yeast (*S. cerevisiae*), filamentous fungi (of the genus *Aspergillus*) and dimorphic fungal pathogens (*Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*) (Abuodeh *et al.*, 2000; Beijersbergen *et al.*, 2001; Bundock *et al.*, 1995; McClelland *et al.*, 2005; Sullivan *et al.*, 2002). This transformation system is based on the natural infecting properties of the plant pathogen *A. tumefaciens* that is capable of randomly transferring DNA into

the genome of the host. Fungal ATMT is generally characterized by its high efficiency and simplicity; however, the same was not observed in *P. brasiliensis* (Leal *et al.*, 2004; Michielse *et al.*, 2005). Leal and co-workers (2004) proved that Hygromycin B resistant (Hyg^R) clones could be obtained by insertion of foreign DNA into *P. brasiliensis* yeast cells, but the developed protocol presented low transformation efficiency (34 Hyg^R clones per co-cultivation; 3.4 Hyg^R clones/10^6 target cells). In addition, within the tested Hyg^R transformants all showed mitotic stability, maintaining the same level of resistance to the antibiotic after serial subculturing in non-selective medium.

It has also been demonstrated that *P. brasiliensis* is susceptible to transformation via electroporation, a technique previously described for other fungi (Soares *et al.*, 2005). Plasmid DNA integration after electroporation resulted in 8 Hyg^R clones/μg plasmid DNA, but with only 8% of mitotic stability.

The data presented by these two studies proved that the genetic manipulation of this fungus is indeed possible. Nevertheless, both works resulted in low transformation efficiency or mitotic stability and lacked a more extensive evaluation of experimental parameters to optimize and ultimately obtain an efficient genetic transformation system for *P. brasiliensis*. The successful generation of a higher number of stable homokaryon progeny is essential to pursue targeted insertional mutagenesis and screening of mutants and associated phenotype. The development of such a tool is imperative for future molecular genetic studies in *P. brasiliensis*.

TRANSCRIPTOME PROFILE OF *P. BRASILIENSIS* DIMORPHISM

Thermodimorphism represents a crucial step in *P. brasiliensis* pathogenicity, but the biochemical events that control the morphological transition of this human pathogen are still poorly defined. The use of *in silico* expressed sequence tags (EST) subtraction and cDNA microarray has been essential for the study of organisms that do not have available a conclusive genetic research history. In this sense, recent breakthrough studies directed towards the evaluation of *P. brasiliensis* transcriptome profile have taken advantage of these techniques and identified differentially expressed genes in its pathogenic and non-pathogenic form, yielding relevant molecular-level information (Felipe *et al.*, 2003; Felipe *et al.*, 2005; Goldman *et al.*, 2003; Marques *et al.*, 2004). While work performed by Felipe *et al.* (2005) indicated that their transcriptome analysis covered 80% of the expected total gene number (≅8000), Goldman *et al.* (2003) suggested that their analysis represented 30-50% (from a total of 10000-15000 genes).

The recognition of cell specific expression patterns and the discovery of homologous genes to other extensively studied eukaryotic systems (e.g., *S. cerevisiae*, *Candida albicans* and *Aspergillus fumigatus*) has provided a more comprehensive view of diverse biological phenomena such as general cellular metabolism, cell signaling pathways, oxidative stress response, growth control and development and host-pathogen interactions. For example, genes implicated in evolutionarily conserved processes as the cell cycle and the machinery involved in the flow of genetic information have been annotated in comparison with their counterparts from *S. cerevisiae*. Among others, orthologues proteins involved in kinase activity (e.g., cell cycle cyclin-dependent kinase Cdc28p), origin recognition complex partners (e.g., pre-replicative ATP-binding protein Cdc6p), mini-chromosome maintenance complex (e.g., ATP-dependent helicase Cdc46p), DNA polymerization (e.g., DNA Polymerase Pol δ), spindle pole body duplication (e.g., Cdc31p), polar growth (e.g., Rho-like GTPase Cdc42p) and cytokinesis (e.g., septin ring component Cdc10p) were identified through computational analysis of transcriptome data (Felipe *et al.*, 2005).

The study of the expression of genes responsible for morphogenesis during *P. brasiliensis* switch from the non-pathogenic to the pathogenic form may, nevertheless, be impaired by the fact that it is a temperature-dependent phenomenon, and thus is necessarily accompanied by the expression of cellular responses required for physiological adaptation to higher temperatures. Marques and co-workers addressed the possible influence of batch culture growth conditions, namely rich and poor nutritional environments, on gene expression of mycelia and yeast cells at 26°C and 37°C, respectively; twenty genes were shown to be overexpressed in the pathogenic yeast form, independently of the culture media (Marques *et al.*, 2004). Later on, a large-scale gene expression analysis was carried out using high throughput microarray technology in order to compare mRNA expression during the mycelium-to-yeast transition in both rich and poor culture medium (Nunes *et al.*, 2005). This work also revealed the overexpression of several genes in the course of the morphological switch independently of the growth conditions. Interestingly, data were also presented regarding a potential antifungal target for *P. brasiliensis* yeast form, an overexpressed gene encoding a 4-hydroxyl-phenyl pyruvate dioxygenase (4-HPPD). The use of NTBC [2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione] and derivatives, specific inhibitors of 4-HPPD activity, inhibited both growth and differentiation of yeast cells *in vitro*, thus opening the door for the use of these substances as new chemotherapeutic agents against PCM.

More recently, a study was carried out with the objective of further elucidating *P. brasiliensis* dimorphism, particularly in what refers to sulfur requirements for growth (Ferreira *et al.*, 2006). *P. brasiliensis* yeast form inability to assimilate inorganic sulfur has been previously described (Paris *et al.*, 1985). By studding 79 isolates of distinct geographical distribution, Ferreira and co-workers (2006) confirmed that in the absence of organic sulfur compounds (e.g., methionine, cysteine, or cystine) yeast cells cannot grow, suggesting that it is in fact a general genetic trait of this fungus. However, the results obtained during the course of this work indicated that genes involved in the metabolic pathways of both organic sulfate assimilation (*CDI1*, cysteine dioxygenase, and *MEP1*, methionine permease) and inorganic sulfur mobilization and storage (*CHS1*, choline sulfatase, *ASP1*, 5'-adenylsulfate kinase, and *SUR1*, sulfite reductase) were overexpressed either during the mycelium-to-yeast transition or the yeast form. These data point to the possibility that the yeast form can activate sulfur organic and inorganic metabolic pathways even though the fungus is not capable of initiating morphological transition or grow in the presence of inorganic sulfur as a single sulfur source. The implications of these findings in the regulation of *P. brasiliensis* dimorphism remain, however, unclear.

The differential gene expression in *P. brasiliensis* has also been evaluated in the context of host interacting conditions, thus allowing the identification of putative gene candidates that the pathogen may express during adaptation (Bailao *et al.*, 2006). Two distinct experimental parameters were taken under consideration, a mouse model of infection and human blood to mimic the hematological route of infection. While during infection, *P. brasiliensis* yeast cells particularly upregulated genes involved in iron acquisition, melanin synthesis and cell defense mechanisms, when seeded in blood an accumulation of mRNA of genes related to cell wall remodeling and synthesis was observed.

Altogether, the overview of *P. brasiliensis* expression profiles in diverse experimental conditions has given the scientific community a broader insight into basic biological features that have so far been elusive due to the absence of extensive molecular investigations on this human pathogen. Nevertheless, the study of protein function of key players in particular cellular events (e.g., pathogenesis) has been greatly compromised by the absence of efficient molecular tools for genetic transformation or gene expression modulation.

CHAPTER 2 – NEW INSIGHTS INTO THE CELL CYCLE PROFILE OF *PARACOCCIDIOIDES BRASILIENSIS*

The results presented over this chapter were published:

(i) in international peer reviewed journals,

Almeida, A.J., Martins, M., Carmona, J.A., Cano, L.E., Restrepo, A., Leão, C., Rodrigues, F. (2006) New insights into the cell cycle profile of *Paracoccidioides brasiliensis*. *Fungal Genet Biol*, 43(6):401-9.

(ii) in conference proceedings,

Almeida, A.J., Martins, M., Carmona, J., Ludovico, P., Restrepo, A., Cano, L., Leão, C., Rodrigues, F. (2005) Cell cycle and ploidy analysis of *Paracoccidioides brasiliensis*, at the *XIII Jornadas de Biologia de Leveduras Professor Nicolau van Uden*, in Vila Real, Portugal (*oral communication*).

Almeida, A.J., Martins, M., Carmona, J.A., Cano, L.E., Restrepo, A., Leão, C., Rodrigues, F. (2005) New insights into the cell cycle profile of *Paracoccidioides brasiliensis*, at the IX International Meeting on Paracoccidioidomycosis, in Águas de Lindóia, Brazil. *Marcello Franco award* (*poster and oral presentation*).

Almeida, A.J., Martins, M., Carmona, J.A., Torres, I., McEwen, J.G., Restrepo, A., Leão, C., Rodrigues, F. (2005) New insights into the cell cycle profile of *Paracoccidioides brasiliensis* in its pathogenic yeast form at the 5th Virtual Mycological Congress (*presentation as an invited author*).

Almeida, A.J., Martins, M., Carmona, J.A., Cano, L.E., Restrepo, A., Leão, C., Rodrigues, F. (2005) New insights into the cell cycle profile of *Paracoccidioides brasiliensis* pathogenic yeast form, at the National Congress of Microbiology, "MICROBIOTEC 2005", in Póvoa do Varzim, Portugal (*poster presentation*).

Almeida, A.J., Martins, M., Carmona, J.A., Torres, I., McEwen, J.G., Restrepo, A., Leão, C., Rodrigues, F. (2006) Cell cycle progression of *Paracoccidioides brasiliensis* pathogenic form under distinct environmental conditions, at the American Society for Microbiology Conference on Dimorphic Fungal Pathogens, in Denver, Colorado, USA (*poster presentation*).

INTRODUCTION

P. brasiliensis yeast cells are generally characterized by the pilot's wheel appearance, i.e., a multiple budding mother cell surrounded peripherally by daughter cells (Restrepo-Moreno, 2003). Both mycelia and yeast cells feature multiple nuclei, whereas conidia – supposedly the natural infectious propagules – present only a single nucleus (San Blas, 1986). Even though knowledge concerning several aspects of the biology of *P. brasiliensis* has increased, little attention has been given so far to issues such as the regulation of the cell cycle and the interaction between DNA replication, nuclei segregation and budding. The control mechanisms that underlie cell growth and nuclear/cellular division of the eukaryotic model system *Saccharomyces cerevisiae* have been extensively studied. During normal growth conditions, this single budding yeast progresses to a specific "start" point in the G_1 phase of the cell cycle which precedes the triggering of three cell cycle milestones, namely initiation of budding, initiation of DNA synthesis and spindle-body duplication as a primary step of nuclear division (Hartwell *et al.*, 1974; Hartwell, 1974). Interestingly, cell cycle control mechanisms different than those of *S. cerevisiae* have been described in other microorganisms, such as bacteria from the archaeal genus *Sulfolobus* or the opportunistic fungus *Cryptococcus neoformans* (Bernander, 1998; Ohkusu *et al.*, 2001; Takeo *et al.*, 2004; Hjort and Bernander, 2001). These microorganisms seem to possess alternative checkpoints for the regulation of cell cycle progression, specifically in what concerns DNA synthesis and cell division.

The main goal of this work was to analyze the cell cycle profile of *P. brasiliensis* yeast cells under different environmental conditions, for which we have optimized a flow cytometric (FCM) technique based on nuclear DNA content, using SYBR Green I, a probe with high affinity for double stranded DNA (Fortuna *et al.*, 2000). Cell cycle progression was evaluated along different phases of growth in batch cultures, namely in defined and complex media. Furthermore, the effect of benomyl on such profile was also analyzed. Methyl benzimidazole-2-yl-carbamate, the active component of this drug, has been shown to disturb microtubule assembly in *S. cerevisiae*, inducing mitotic chromosome loss (Rodrigues *et al.*, 2003) and cause metaphase arrest with blocking of the cells in the G_2/M phases of the cell cycle (Wood, 1982a; Wood, 1982b).

MATERIAL AND METHODS

Strains and Media. *P. brasiliensis* yeast cells, strain ATCC 60855, a Colombian isolate, were maintained at 36°C by periodic subculturing in slanted tubes with brain heart infusion (BHI) solid media

(1.5% wt/vol agar) supplemented with 1% glucose. For the assays carried out in this study, yeast cells were routinely grown in both BHI and modified synthetic McVeigh Morton (MMcM) (Restrepo and Jimenez, 1980) liquid medium, at 36°C with aeration on a mechanical shaker (200 rpm).

Microscopic count methods and cell viability assays. Direct microscopic counts of yeast cell suspensions were carried out using bright-field microscopy and Neubauer counting chamber procedures (cells were considered individually for count methods). Cell viability (colony-forming ability) was measured by plating serial dilutions of yeast cultures onto BHI plates supplemented with 1% glucose and colony counting after 15 days of incubation at 36°C.

Cell cycle analysis. *P. brasiliensis* yeast cells were cultured in BHI liquid medium supplemented with 1% glucose to an early stage of the exponential phase of growth. When indicated, cells were harvested, washed with distilled sterile water and suspended in MMcM medium. Samples were collected at defined culture times by centrifugation (4000×*g* for 5 min at 4°C). An overnight fixation was carried out with 70% ethanol (vol/vol) at 4°C, a procedure that does not perturb the formation of dsDNA/SYBR Green I complexes. After fixation, cells were harvested, washed and suspended at 1×10^7 cells/ml in 1 ml of sodium citrate buffer (50 mM; pH 7.5), an optimal staining condition for this fluorochrome (Fortuna *et al.*, 2000). The suspensions were then subjected to three consecutive ultrasound (Sonics Vibra-cell; Newton, CT, USA) pulses at 40 W for 1 s, with an interval of 1 to 2 s between each pulse. After sonication, cell suspensions were treated for 30, 60 and 75 min at 50°C with RNase A (0.25, 0.5 and 0.75 mg/ml) and/or with proteinase K (0.5, 1, 1.5 and 2 mg/ml). To facilitate formal validation of the optimization process, a cell concentration of 1×10^7 cells/ml was stained overnight at 4°C with SYBR Green I (Molecular Probes, Eugene, Or, USA) at a final concentration of 25× throughout all assays. For titration procedures, cell staining was carried out with SYBR Green I at final concentrations ranging from 0.01× to 100×. Before FCM analysis, Triton® X-100 (Sigma-Aldrich, St Luis, MO, USA) was added to samples at a final concentration of 0.25 % (vol/vol).

Flow cytometry measurements. All FCM experiments were performed on an EPICS XL-MCL (Beckman-Coulter Corporation, Hialeah, Fl, USA) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The green fluorescence was collected through a 488 nm blocking filter and a 550 nm/long-pass dichroic with a 525 nm/band-pass. A minimum of 30,000 cells per sample was acquired at low flow rate and an acquisition protocol was defined to measure forward

scatter (FS) and side scatter (SS) on a four-decade logarithmic scale and green fluorescence (FL1) on a linear scale. Offline data were analyzed with the Multigraph software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0 and statistical analysis was carried out with the Windows Multiple Document Interface for Flow Cytometry 2.8 (WinMDI 2.8). Cell sorting was performed on a MoFlo® high speed cell sorter (DakoCytomation; Fort Collins, CO) configured with two lasers for 488nm and 633nm excitation simultaneously. For cell sorting assays, *P. brasiliensis* yeast cell samples, isolate ATCC 60855, were prepared as described above for cell cycle analysis. Both FS LOG and FL1 measurements were carried out in order to define subpopulations for cell sorting. The purity of cell preparations was >80%.

Epifluorescence microscopy analysis. Epifluorescence microscopy was performed on a Zeiss Axioskop epifluorescence microscope fitted with 10× eyepieces and 40× and 100× (oil immersion) objectives and equipped with a Carl Zeiss AxioCam (HR/MR). Due to lower sensitivity of this technique comparatively to FCM, SYBR Green I cells staining was carried out with a final concentration of 80×.

Benomyl treatment. *P. brasiliensis* yeast cells were cultured in MMcM liquid medium to a stationary phase of growth. Cells were then harvested, washed with distilled sterile water and suspended in MMcM liquid medium in order to obtain a final cell number of about 3×10^6 cells/ml. Benomyl solutions were prepared in dimethyl sulfoxide (DMSO) in such a manner that the final concentration of this organic solvent in the culture medium never exceeded 1% (vol/vol), a concentration that on our preliminary analysis revealed no effect on cell growth or viability. Treatments with different concentrations of the antifungal agent were performed at 36°C with aeration on a mechanical shaker (200 rpm). Samples for cell cycle analysis, cell viability assays and direct microscopic counts were collected at the times indicated in Results.

Reproducibility of the results and statistical analysis. All experiments were repeated at least three times with duplicate samples. The data regarding FCM protocol optimization are from one representative experiment, while data from cell cycle profile analysis are reported as mean values (standard deviation ≤10%). Statistical analysis regarding treatment of *P. brasiliensis* yeast cells with benomyl was carried out by Two-way ANOVA using the GraphPad Prism Software version 4.00. Percentage of individual cell subpopulations were compared between treated and untreated conditions at all intervals indicted in Results.

RESULTS

The present study focused on the characteristics of the cell cycle profile of *P. brasiliensis* yeast cells during batch culturing and under the effects of benomyl, an antifungal drug known to promote a cell cycle arrest in the G2/M phases of *S. cerevisiae* (Wood, 1982a; Wood, 1982b). Taking into consideration the proposed objectives and specific traits of this pathogenic fungus, we optimized a previously described protocol for *S. cerevisiae* and *Zygosaccharomyces bailii* based on SYBR Green I staining of dsDNA that provides high resolution DNA measurements by single-cell analysis of large samples (Fortuna *et al.*, 2000). As described below, an array of experiments was carried out to optimize staining conditions, using decreases in the half-peak coefficient of variation (HPCV), a statistical factor that inversely correlates with a homogenous and selective staining of dsDNA, as an indicator of protocol improvements for high-resolution DNA measurements (Fortuna *et al.*, 2000).

PROTOCOL DEVELOPMENT FOR CELL CYCLE ANALYSIS BY FCM OF *P. BRASILIENSIS* YEAST CELLS

In order to characterize the cellular population of *P. brasiliensis* batch cultures by FCM, a light scatter analysis of forward scatter (FS LOG) and side scatter (SS LOG) was performed, parameters that are correlated to cell size and cell complexity, respectively. Yeast cell samples were collected at early stages of the exponential and stationary phases of growth. In line with observations from bright-field microscopy (Figure 1, I), the biparametric analysis of *P. brasiliensis* stationary phase yeast cells correlating relative size and complexity revealed a vast heterogeneity in both parameters. The physical scattergram (Figure 1, II) disclosed two major subpopulations: a subpopulation (A) most likely consisting of smaller and less complex cells with a more homogeneous morphology, and a

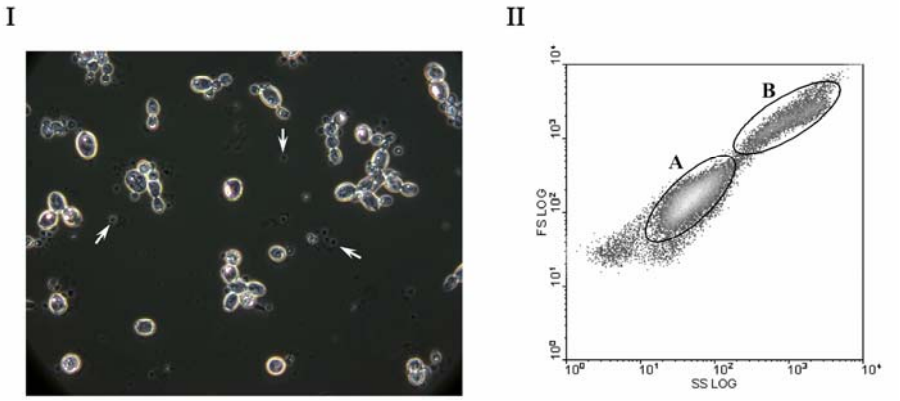

Figure 1 – *P. brasiliensis* stationary phase yeast cells in MMcM liquid medium. (I) Light microscopy analysis (dark field) of distinct cellular morphologies identified during batch culture (white arrows indicate small daughter cells). (II) Dot plot of logarithmic forward scatter (FS LOG) *versus* logarithmic side scatter (SS LOG) of a light scatter analysis of unstained *P. brasiliensis* yeast cells; (A) and (B) represent subpopulations with low and high relative size and complexity, respectively.

subpopulation (B) with higher scatter values probably representing multiple budding cells or cell aggregates. Similar results were obtained with exponential growing cells (data not shown). The flow cytometric analysis of ethanol-fixed *P. brasiliensis* yeast cells revealed a low specificity for SYBR Green I staining, with the appearance of poorly discriminated cell subpopulations with heterogeneous mean fluorescence intensities (Figure 2, I-A). Under these conditions, the cytometric pattern did not present the characteristics of a cell cycle profile, in accordance with the observation of cytoplasmic diffuse fluorescence by epifluorescence microscopy (Figure 2, II-A). Therefore, a series of pre-treatments with RNase A and proteinase K were carried out as described in Material and Methods Section. The pre-treatment of cell suspensions with 0.75 mg/ml RNase A for 60 min at 50°C led to the discrimination of two major homogeneous subpopulations of cells with different DNA content (R_1 and R_2) and a subpopulation of cells presenting a high and varying mean fluorescence intensity (R_3) (Figure 2, I-B).

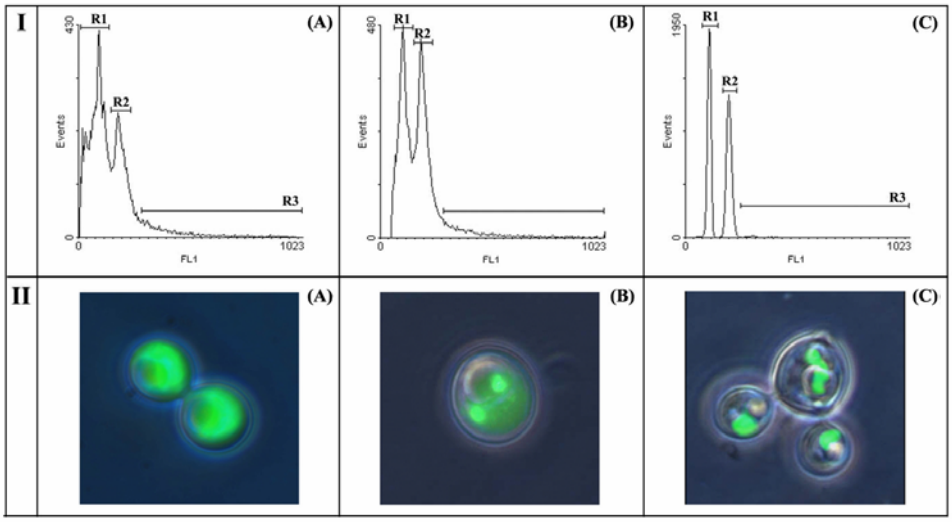

Figure 2 – *P. brasiliensis* yeast cells grown in MMcM batch cultures to the stationary phase, subjected to SYBR Green I staining. (A) Without pre-treatment; (B) treated with 0.75 mg/ml of RNase for 60 min at 50°C; (C) treated sequentially with RNase A as in (B) and 1 mg/ml of proteinase K for 60 min at 50°C. (I) Monoparametic histograms of green fluorescence (FL1); (II) epifluorescence microscopy analysis (overlap of bright field and green fluorescence).

Along with diminished HPCV values (Table 1), epifluorescence microscopy revealed that RNase A pre-treatment led to a more selective SYBR Green I staining of dsDNA, although some non-specific cytoplasmic distribution was still visible (Figure 2, II-B). An increase in peak resolution was observed when cells were firstly treated with RNase A as above and subsequently with proteinase K (1 mg/ml; 60 min at 50°C) (Figure 2, I-C; Table 1), but not with a single proteinase K pre-treatment (data not shown). In addition, two new subpopulations were identified, R_4 and R_5, even though they represented very low percentages of the total population of cells and were not

always discriminated (Figure 2, I-C). As shown by Figure 2 II-C, epifluorescence microscopy further corroborated selective nuclear SYBR Green I staining. The evaluation of individual subpopulations also revealed that R_2, R_4 and R_5 each present, 2, 3 and 4 fold increases in the mean fluorescence intensity of R_1 (Table 1).

Table 1 – Analysis of the cell cycle profile of *P. brasiliensis* stationary yeast cells grown in MMcM batch cultures, labeled with SYBR Green I: R_1, R_2, R_3, R_4 and R_5 are subpopulations with different nuclear DNA contents. Shown are population size (Events, %), mean fluorescence intensity (MFI) and half peak of coefficient of variation (HPCV, %) of each subpopulation.

	R1			R2			R3			R4			R5		
	Events	MFI	HPCV	Events	MFI	HPCV	Events	MFI	HPCV	Events	MFI	HPCV	Events	MFI	HPCV
Non-treated	48.9	95.0	25.0	38.6	193.2	22.3	10.4	592.8	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
RNase A[a]	44.5	101.8	10.7	43.1	198.1	11.5	9.8	575.5	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
RNase A + Proteinase K[b]	47.1	100.4	4.5	43.5	201.0	5.2	8.4	553.1	*nd*	2.1	301.6	*nd*	0.7	402.3	*nd*

[a] Treatment with 0.75 mg/ml RNase A for 60 min at 50° C.

[b] Sequential treatment with 0.75 mg/ml RNase A and 1 mg/ml proteinase K for 60 min each at 50°C.
nd Non-detected.

To assess the specificity of DNA labeling after RNase A and Proteinase K pre-treatments, *P. brasiliensis* yeast cells were treated overnight with DNase before and after staining. The complete loss of green fluorescence in both conditions confirmed the DNA specific labeling (data not shown). In order to achieve a saturating mean fluorescence intensity associated with a minimum non-specific labeling, *P. brasiliensis* yeast cells, treated with RNase A and Proteinase K, were stained with SYBR Green I at concentrations ranging from 0.01× to 100× (data not shown). Considering the array of tested SYBR Green I concentrations, 25× per 1×10^7 cells/ml was chosen as an optimal dye-to-cell concentration ratio.

CHARACTERIZATION OF CELL MORPHOLOGY AND NUCLEI CONTENT OF *P. BRASILIENSIS* YEAST CELLS

With the aim of characterizing the cellular morphology and nuclei content of the various subpopulations discriminated during cell cycle profile analysis of *P. brasiliensis* yeast cells, samples were collected during the exponential phase of growth in BHI batch culture and submitted to cell cycle analysis. We then proceeded with physical separation by cell sorting in accordance with specific parameters, namely relative size (FS LOG) and green fluorescence intensity (FL1 LOG), and selective gates were defined corresponding to R_1, R_2 and R_3 subpopulations (due to technical limitations subpopulations R_4 and R_5 were not individualized (Figure 3, I). FCM of sorted subpopulations revealed high purity separations: cells from R_1

achieved 97.3%, cells from R_2 80.5% and cells from R_3 87.4%. Epifluorescence microscopy analysis of sorted cells showed that R_1 subpopulation was composed by cells of reduced size containing only one nucleus (Figure 3, II-A). On the other hand, R_2 consisted of uninucleated and binucleated cells slightly larger than those in R_1 (Figure 3, II-B). Regarding R_3, this subpopulation enclosed a heterogeneous group of cells concerning cell morphology and nuclei number (Figure 3, II-C): both multiple budding cells with a variable number of nuclei as well as single cells with 3 or more nuclei were observed.

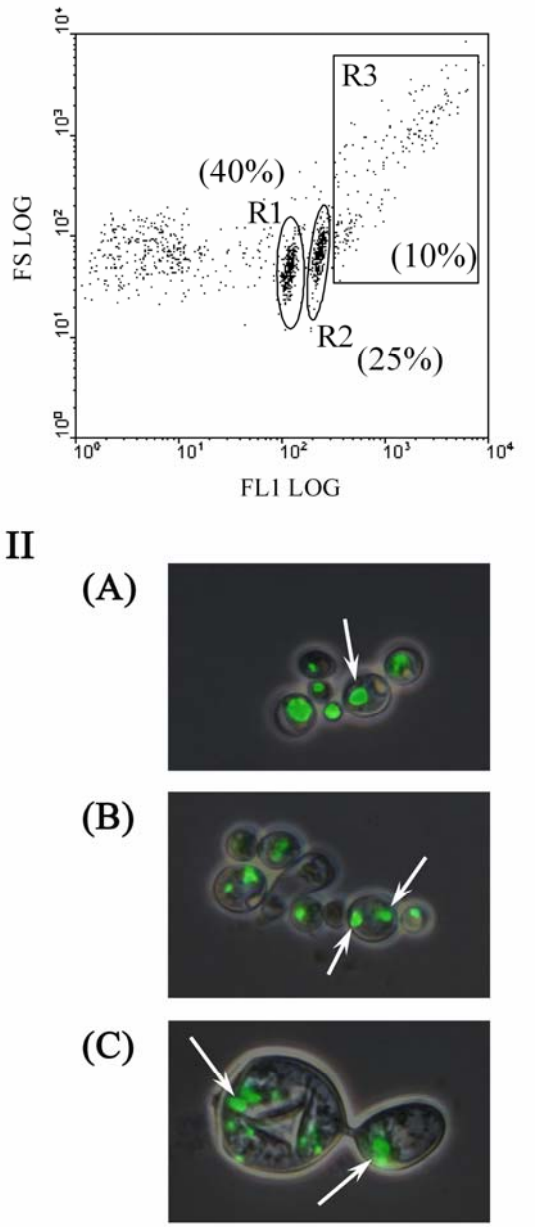

Figure 3 – *P. brasiliensis* yeast cells in the exponential phase of growth in BHI medium subjected to cell cycle analysis. (I) Representative dot plot of forward scatter (FS LOG) *versus* green fluorescence intensity (FL1 LOG) with selective gates defined around subpopulations R_1, R_2 and R_3 prior to cell sorting (cell percentage is represented in brackets). (II) Epifluorescence microscopy analysis of *P. brasiliensis* yeast cells after cell sorting (overlap of bright field and green fluorescence): cells sorted from subpopulation R_1 (A), R_2 (B) and R_3 (C) (white arrows indicate SYBR Green I nuclear staining).

BATCH CULTURE GROWTH OF *P. BRASILIENSIS* YEAST CELLS IN DEFINED AND COMPLEX MEDIUM SHOW DIFFERENTIAL PATTERNS OF CELL CYCLE PROGRESSION

P. brasiliensis cells were cultured in batch system in defined (MMcM) and complex (BHI) media to assess specific differences of cell cycle profile during growth in poor and rich nutritional environment conditions. Samples were collected during growth and subjected to direct microscopic counts together with the FCM analysis of the nuclear DNA content of individual cells. The growth curve of *P. brasiliensis* in MMcM medium showed that the exponential phase occurred between 12 and 60 h ($\mu = 0.025 \pm 0.004$ h^{-1}) while the stationary phase was initiated between 60 and 72 h (Figure 4A). During the transition of the lag to the exponential phase the percentage of cells in subpopulation R_1 increased from 50.0% to 69.8% of the total population (Figure 4B). Whereas the percentage of cells in subpopulation R_1 remained approximately constant during the exponential phase, it continuously decreased up to 42.2% through the stationary phase. In contrast, the percentage of cells in subpopulation R_2 (10%) remained unaltered until the late exponential phase, followed by an increase to 43.1% during the stationary phase. Between the end of the lag phase and the early exponential phase (24 h) the percentage of cells in subpopulation R_3 diminished from 37.8% to 17.6%. This proportion was maintained during the exponential phase and partially during the stationary phase up to 96 h, decreasing to 11.4% at 120 h. According to both cytometric physical parameters and epifluorescence microscopy observations, a batch culture of *P. brasiliensis* yeast cells in the stationary phase of growth in MMcM medium, is characterized by the predominance of subpopulations R_1 and R_2, mostly represented by smaller uninucleated cells with a lower DNA content (Figure 4C).

Regarding *P. brasiliensis* yeast cells cultured in batch system in BHI medium, the growth rate was only slightly higher than in MMcM medium ($\mu = 0.040 \pm 0.008$ h^{-1}). However, *P. brasiliensis* grown in BHI medium displayed a prolonged exponential phase (between 24 and 84 h) followed by a transition to the stationary phase occurring between 96 and 108 h (Figure 5A). During the transition from the lag to exponential phase of growth (until 24 h) an increase in subpopulation R_3 to 29.8% was observed (Figure 5B). Throughout the exponential phase of growth, the percentage of cells in subpopulation R_1 increased from 32.8 to 71.2%, whereas at a later stationary phase a decrease to 50.9% was detected. On the other hand, subpopulation R_2 was characterized by a decrease – 16.1 to 8.5% – from the beginning of the exponential phase (24 h) to the early stationary phase (120 h) followed by an augment to 13.4% at 180 h. Subpopulation R_3 diminished from 29.8 to 6.5% until the early stationary phase, then increasing

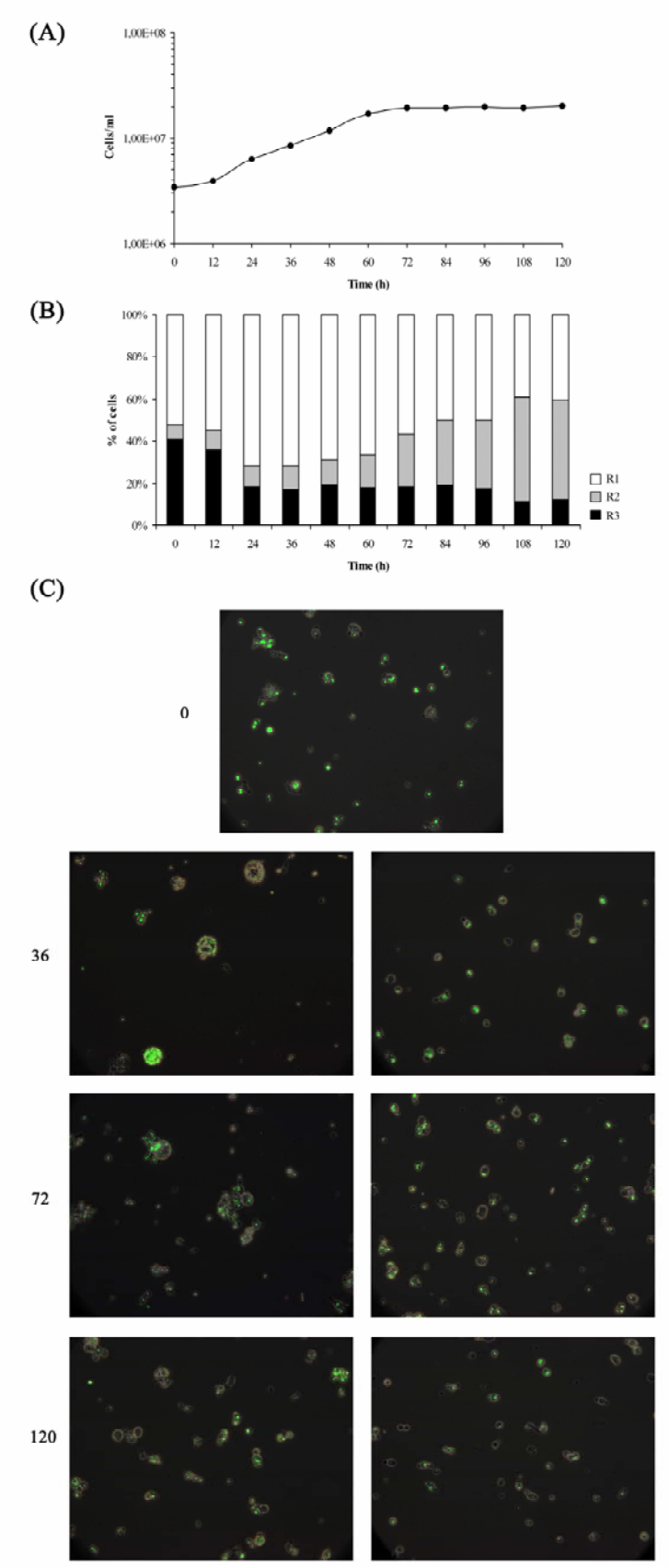

Figure 4 – Batch growing culture of *P. brasiliensis* yeast cells in MMcM medium. (A) Representative plot of a growth curve obtained by direct microscopic counts along time. (B) Cell cycle profile presented as the percentage of cells in each subpopulation (R_1, R_2 and R_3). (C) Epifluorescence microscopy analysis of different cellular morphologies detected at distinct phases of batch culture growth (0, 36, 72 and 120 h).

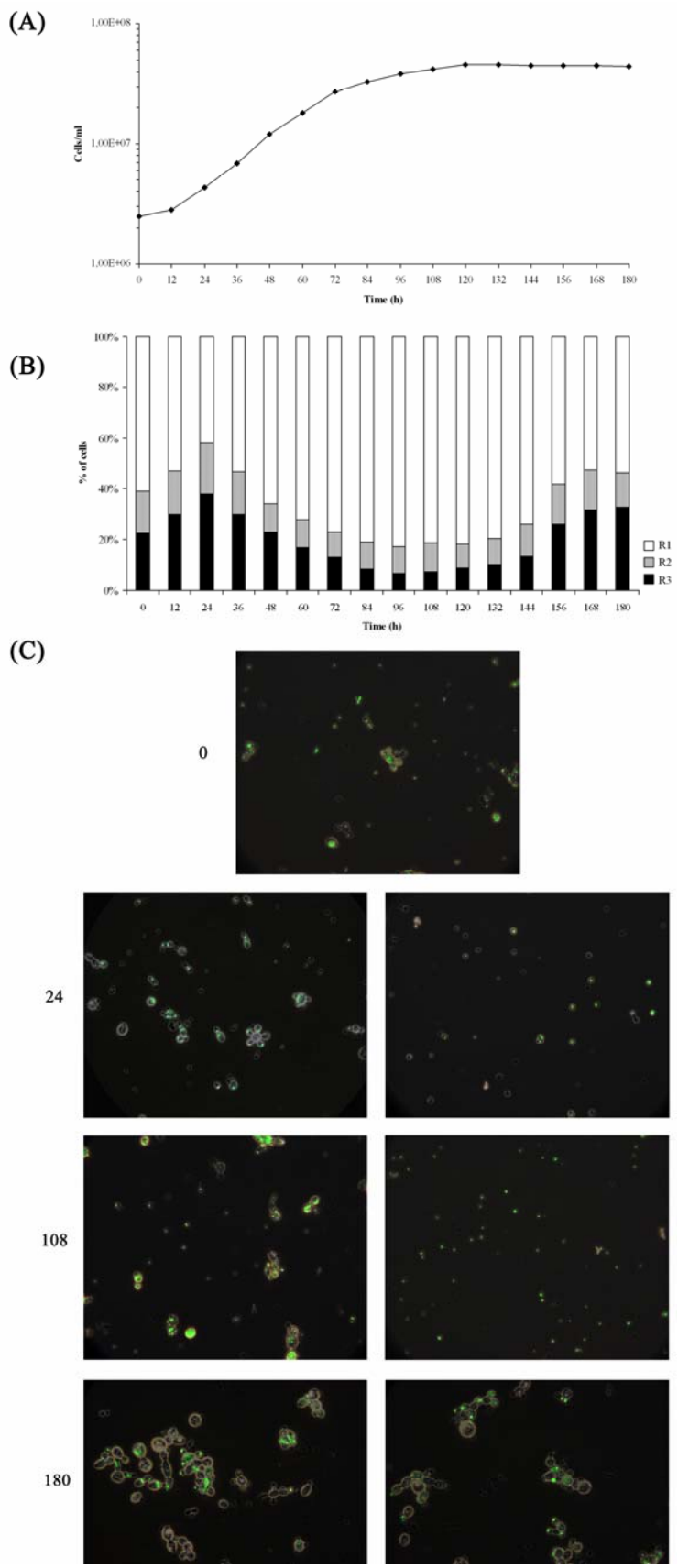

Figure 5 – Batch growing culture of *P. brasiliensis* yeast cells in BHI medium. (A) Representative plot of a growth curve obtained by direct microscopic counts along time. (B) Cell cycle profile presented as the percentage of cells in each subpopulation (R_1, R_2 and R_3). (C) Epifluorescence microscopy analysis of different cellular morphologies detected at distinct phases of batch culture growth (0, 24, 108 and 180 h).

to 27.5% at 180 h. Additionally, microscopy analysis showed that cells growing in BHI batch cultures presented distinct features such as pseudohyphae-like structures rather than the "typical" multiple budding cells present in cultures grown in MMcM medium (Figure 5C).

THE FUNGICIDE BENOMYL TRIGGERS AN ARREST IN THE CELL CYCLE PROGRESSION OF *P. BRASILIENSIS* YEAST CELLS

With the intention of disturbing cell cycle progression of *P. brasiliensis*, we selected the fungicide benomyl since in other fungi it induces mitotic chromosome loss (Rodrigues *et al.*, 2003) and cell cycle arrest in the G_2/M phases (Jacobs *et al.*, 1988; Wood, 1982a; Wood, 1982b). Yeast cells were grown with a range of benomyl concentrations (from 5 to 250 μg/ml) and samples were collected at intervals for cell cycle profile analysis. Cell viability assays determined by colony forming ability indicated that a final concentration up to 100 μg/ml of benomyl did not result in survival changes (data not shown). In comparison with untreated controls, no differences were observed (P >0.05) during the first 16 h of treatment in the cell cycle profile of *P. brasiliensis* yeast cells exposed to 100 μg/ml benomyl (Figure 6). However, after 24 h benomyl-treated cells

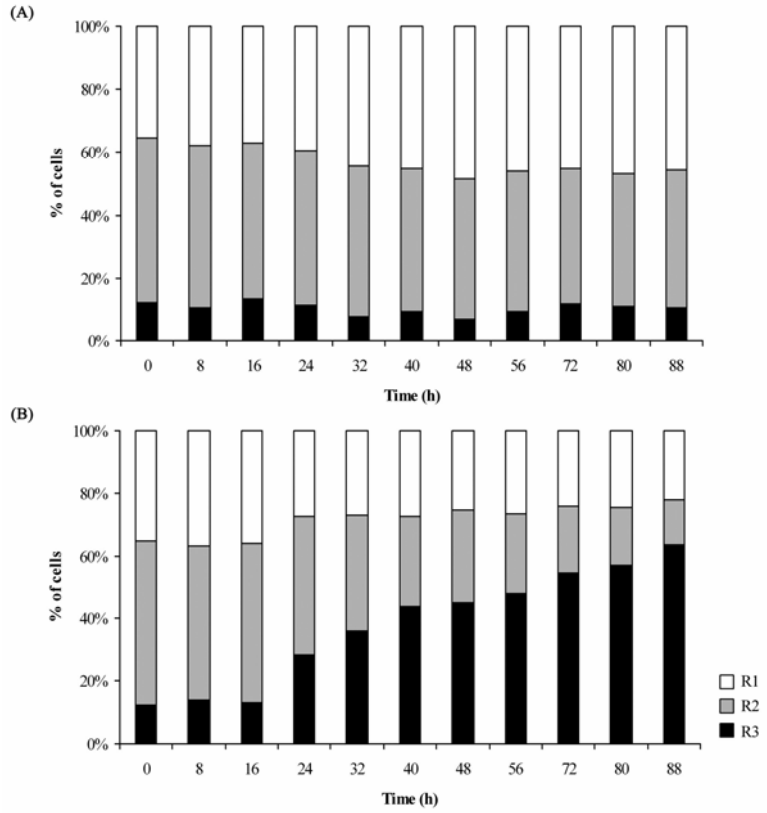

Figure 6 – Cell cycle profile of *P.* brasiliensis yeast cells growing in MMcM medium, presented as the percentage of cells in each subpopulation (R_1, R_2 and R_3) in (A) absence (control with 1% DMSO) and (B) presence of 100 μg/ml of benomyl.

showed an increase in the percentage of cells in subpopulation R_3 and a decrease in subpopulations R_1 and R_2 (Figure 6B). Throughout the remaining period of treatment (24 h to 88 h), significant differences were detected between all subpopulations of treated and untreated cells ($P < 0.005$). At 88 h, the percentage of benomyl-treated cells in subpopulations R_1, R_2 and R_3 was 23.4%, 14.1% and 60.6%, respectively, whereas the untreated control presented 44.5%, 42.9% and 12.2% of the same subpopulations (Figure 6). We also found a dose-dependent effect of the fungicide regarding the arrest of cell cycle progression of *P. brasiliensis* yeast cells (data not shown).

DISCUSSION

The main objective of this study was to evaluate the cell cycle profile of the pathogenic yeast form of the dimorphic fungus *P. brasiliensis*. In order to achieve this goal we optimized a flow cytometric technique for the analysis of nuclear DNA content, thus allowing the evaluation of cell cycle progression of *P. brasiliensis* yeast cells growing under different environmental conditions.

A major constraint regarding the application of flow cytometry for the analysis of *P. brasiliensis* yeast cells is the cell population heterogeneity which not only presents distinct multiple budding yeast cells but also cells with odd morphologies; additionally, accumulation of debris in its culture media hampers the potential approach of flow cytometric studies (Queiroz-Telles, 1994; Restrepo-Moreno, 2003). Nonetheless, our results show that the cytofluorimetric analysis of this pathogenic fungus is practicable, indicating that the settings for an optimal cell cycle profile analysis of *P. brasiliensis* yeast cells based on nuclear DNA content are: i) overnight cell fixation with 70% ethanol (vol/vol) at 4 °C; ii) ultrasonication of 1×10^7 cells/ml (sodium citrate buffer; 50 mM; pH 7.5) with 3 ultrasound pulses at 40 W for 1 s, with an interval of 1 to 2 s between each pulse; iii) a subsequent pre-treatment, at 50°C, with RNase A (0.75 mg/ml) and proteinase K (1 mg/ml) for 60 min each; iv) overnight staining at 4°C with SYBR Green I (25×) and v) addition of Triton (0.25 %; vol/vol) before acquisition by flow cytometry. Our results demonstrate that treatment with RNase A and proteinase K are essential for probe targeting of dsDNA with SYBR Green I. As other fungi, *P. brasiliensis* seems to have high levels of RNA thus hampering selective dsDNA staining (Futcher, 1993; Winson and Davey, 2000). Moreover, besides eliminating cell debris, proteinase K facilitates the access of fluorochrome to dsDNA and reduces nonspecific binding due to its important proteolytic activity against histones (Dien *et al.*, 1994).

Bearing in mind that *P. brasiliensis* yeast cell populations are extremely heterogeneous, half-peak coefficient of variation (HPCV) values ranging between 4.5 and 5.1% indicate an accurate and reliable dsDNA staining. Furthermore, the direct application of this method to *P. brasiliensis* yeast cells grown in a batch system discriminated subpopulations with both homogenous and heterogeneous mean fluorescence intensity. In fact, up to four different cellular subpopulations with homogeneous mean fluorescence intensity were distinguished (R_1, R_2, R_4 and R_5) indicating one to four fold of genomic DNA content, characteristic of multiple budding and/or polynucleated cells. In view of these results, a query prior to cell cycle profile analysis under distinct growth conditions was to describe cell morphology and nuclei content of each individual subpopulation. In accordance with epifluorescence microscopy analysis (Figure 3, II), cells within R_1 most likely represent small uninucleated daughter cells. Regarding R_2, this subpopulation encloses both uninucleated and binucleated cells that may correspond to cells in different phases of the cell cycle. In addition, it is also possible that some of the cells in R_2 presenting two nuclei correspond not to dividing cells but to cells of multinucleated nature. On the other hand, cells with higher mean fluorescence intensity that belong to subpopulation R_3 correspond to cells with an elevated number of nuclei per individual cell, multiple budding cells and/or aggregates. These findings are consistent with the extensively described multinucleate and multiple budding nature of *P. brasiliensis* yeast cells (Restrepo-Moreno, 2003).

During batch growth cultures in defined (Figure 4) or complex (Figure 5) nutritional environments, a differential cell cycle progression of *P. brasiliensis* yeast form was observed. Contrary to what would be expected, a low percentage of cells with higher mean fluorescence intensity (subpopulation R_3), characteristic of dividing cells, was observed during the exponential growth-phase in both media, although to a lesser extent in BHI. At the same time, an increase of cells in subpopulation R_1 was detected. An explanation for this behaviour might be that, contrary to "single" budding yeasts in which one mother cell gives rise to two daughter cells, a *P. brasiliensis* yeast mother cell may give rise to numerous progeny due to its typical multiple budding (Queiroz-Telles, 1994; Restrepo-Moreno, 2003). It is likely that these variations in the cell cycle profile of exponentially growing cells result from a large increase in small daughter cells accompanying a higher growth rate. However, during the stationary phase of growth distinct cell cycle profiles were detected in both environments. In complex medium, we observed an increase in the percentage of cells in subpopulation R_3 associated with the occurrence of pseudohyphae-like structures and cells with higher DNA content at later stages of this growth phase (Figure 5).

These results may suggest that de-regulation of the normal processes that coordinate DNA replication, nuclei segregation and cell division occurs under rich nutrition-environmental conditions. On the other hand, in defined medium a decline in R_1 and R_3 subpopulations was detected in parallel with an increase in subpopulation R_2 in a stationary growth-phase. Consequently, approximately 90% of the total population consisted of two major subpopulations having 1*n* and 2*n* DNA content (R_1 and R_2, respectively), although the majority of the cells presented 1 nucleus and a much more homogenous morphology (Figure 4). The stationary phase of growth of a microbial population is generally characterized by an arrest of the great majority of cells in G_0/G_1 phases of the cell cycle with a lower and unique DNA content (Herman, 2002). However, several reports indicate the existence of microorganisms that do not follow these same rules that usually coordinate cellular and nuclear division during cell growth. As demonstrated for *P. brasiliensis* yeast cells during this work, Archaeobacteria from the genus *Sulfolobus* and the pathogenic fungus *C. neoformans* have also been shown to present two fully replicated genomes in a stationary-phase culture, demonstrating some level of independence between DNA duplication and cell division (Bernander, 1998; Hjort and Bernander, 2001; Takeo *et al.*, 2004). Incidentally, commitment to budding in *C. neoformans* cells revealed not to be directly related to DNA synthesis or critical cell size requirement as in the yeast model system *S. cerevisiae*, since large unbudded G_2 cells are produced during the transition to the stationary phase of growth (Hartwell, 1974; Ohkusu *et al.*, 2001). Additionally, the fungal pathogen *Candida albicans* was shown to regulate cell cycle in order to modulate cell shape while nuclear division was maintained also indicating alternative control mechanisms (Sudbery *et al.*, 2004).

To further contribute to the elucidation of the differential patterns of cell cycle progression of *P. brasiliensis* yeast cells, this work also focused on the action of an antifungal drug during batch culture growth. Our results show that treatment with benomyl induces an arrest in the cell cycle profile (Figure 6) and accumulation of cells in R_3 subpopulation. These results suggest that even though benomyl progressively blocks nuclear division of *P. brasiliensis* yeast form, treated cells retain their DNA replication capacity, concurring with the effect it induces in other fungi (Rodrigues *et al.*, 2003; Wood, 1982a; Wood, 1982b). However, *P. brasiliensis* yeast cells accumulate in stages characterized by higher DNA content, rather than the G_2/M phases of the cell cycle, consistent with its particular multinuclear and cellular division.

The methodology herein developed has allowed us to evaluate the cell cycle profile of *P. brasiliensis* yeast form growing under different environmental conditions. Even though this flow

cytometric technique does not ascertain specific cell cycle phases, taken together our findings seem to show that this fungus may possess alternative control mechanisms during cell growth in order to manage its characteristic multiple budding and multinucleated nature. Nevertheless, future studies are required for the clarification of the biological phenomena that regulate nuclear/cellular division of *P. brasiliensis*.

CHAPTER 3 – GENOME SIZE AND PLOIDY OF *PARACOCCIDIOIDES BRASILIENSIS* REVEALS A HAPLOID DNA CONTENT: FLOW CYTOMETRY AND *GP43* SEQUENCE ANALYSIS

The results presented over this chapter were published:

(i) in international peer reviewed journals:

Almeida, A.J., Matute, D., Carmona, J.A., Martins, M., Torres, I., McEwen, J.G., Restrepo, A., Leão, C., Ludovico, P., Rodrigues, F. (2007) Genome size and ploidy of *Paracoccidioides brasiliensis* reveals a haploid DNA content: flow cytometry and GP43 sequence analysis. *Fungal Genet Biol*, 44(1):25-31.

(ii) in conference proceedings,

Martins, M., Almeida, A.J., Rodrigues, F., Leão, C. (2004) *Paracoccidioides brasiliensis*: development of molecular genetic tools, at the 11th International Congress on Yeasts, in Rio de Janeiro, Brazil (*oral communication*).

Almeida, A.J., Martins, M., Carmona, J., Ludovico, P., Restrepo, A., Cano, L., Leão, C., Rodrigues, F. (2005) Cell cycle and ploidy analysis of *Paracoccidioides brasiliensis*, at the *XIII Jornadas de Biologia de Leveduras Professor Nicolau van Uden*, in Vila Real, Portugal (*oral communication*).

Almeida, A.J., Martins, M., Carmona, J.A., Torres, I., McEwen, J.G., Restrepo, A., Leão, C., Rodrigues, F. (2005) *Paracoccidioides brasiliensis* genome size and ploidy, at the IX International Meeting on Paracoccidioidomycosis, in Águas de Lindóia, Brazil (*poster and oral presentation*).

INTRODUCTION

Even though during the last decade molecular approaches have allowed a broader insight into *P. brasiliensis* genomic organization, definitive conclusions are far from being achieved (Felipe *et al.*, 2005). The absence of a recognized teleomorphic stage and the fact that this fungus is relatively unamenable in what refers to basic cytogenetic analysis has contributed to the lack of substantial data regarding *P. brasiliensis* genetic composition (San-Blas *et al.*, 2002). Consequently, several studies have employed different techniques, such as pulsed-field gel electrophoresis (PFGE) and microfluorometry (Cano *et al.*, 1998; Feitosa *et al.*, 2003; Montoya *et al.*, 1999; Montoya *et al.*, 1997), identifying four or five chromosomes – with 2 to 10 Mb – in both clinical and environmental isolates. Additionally, the existence of chromosomal polymorphism, a characteristic previously reported in other pathogenic fungi, has also been reported (Morais *et al.*, 2000; Pan and Cole, 1992; Perfect *et al.*, 1989; Thrash-Bingham and Gorman, 1992). The different sizes of chromosomal DNA molecules separated by PFGE allowed the estimation of *P. brasiliensis* genome size as 23-31 Mb (Cano *et al.*, 1998; Feitosa *et al.*, 2003; Montoya *et al.*, 1999; Montoya *et al.*, 1997). However, results regarding microfluorometric analysis revealed that some of these isolates presented twice the genome content (46-61 Mb) suggesting that the nuclei of *P. brasiliensis* yeast cells could be diploid, although without discarding the possibility of haploid or even aneuploid isolates (Cano *et al.*, 1998; Feitosa *et al.*, 2003). These studies, though reporting important information on *P. brasiliensis* genome size and ploidy, have not fully clarified these issues. On one hand, the determination by PFGE of genome size in a microorganism whose ploidy is still unknown may lead to erroneous conclusions due to limitations when separating homologous chromosomes (Torres-Guerrero, 1999). Moreover, inferring cellular ploidy on the basis of nuclear DNA content that varies along the different phases of the cell cycle may also result in incorrect assessments (Feitosa *et al.*, 2003).

In the present work a flow cytometric (FCM) protocol (Almeida *et al.*, 2006) was applied to determine the genome size per uninucleated *P. brasiliensis* yeast cell and conidia. The ploidy state of several isolates was also assessed by comparing data from genome sizing by FCM with the previously described electrophoretic karyotype (Feitosa *et al.*, 2003; Montoya *et al.*, 1997; Montoya *et al.*, 1999). Additionally, we evaluated the intra-individual variability of the highly polymorphic *P. brasiliensis* gene (*GP43*) that encodes the main antigenic component, an

exocellular glycoprotein of 43 KDa (Cisalpino *et al.*, 1996; Morais *et al.*, 2000), further supporting our analysis of *P. brasiliensis* ploidy state.

MATERIALS AND METHODS

Microorganisms and culture media. *P. brasiliensis* clinical and environmental isolates are listed in Table 1 and were provided by the *Corporación para Investigaciones Biológicas*' (Medellín, Colombia) culture collection. Yeast cells were maintained at 36°C by periodic subculturing in slanted tubes with brain heart infusion (BHI) solid media (1.5% wt/vol agar) supplemented with 1% glucose. For subsequent assays, yeast cells were cultured in the modified synthetic McVeigh Morton (MMcM) (Restrepo and Jimenez, 1980) liquid medium at 36°C with aeration on a mechanical shaker (200 rpm). Stock mycelial culture was routinely grown in slanted tubes with modified MMcM solid medium at 18°C. *P. brasiliensis* conidia from strain ATCC 60855 were collected and dislodged in accordance with a previously described technique (Restrepo *et al.*, 1986).

Table 1 – *P. brasiliensis* isolates analyzed during this study.

Isolate identification	Country of isolation	Source	Citation
18	Brazil	Chronic PCM	(Teixeira *et al.*, 1987)
ATCC 32069	Brazil	Chronic PCM	(Restrepo-Moreno and Schneidau, Jr., 1967)
Ibiá	Brazil	Soil	(Silva-Vergara *et al.*, 1998)
T10B1	Brazil	Armadillo	(Hebeler-Barbosa *et al.*, 2003)
ATCC 60855	Colombia	Chronic PCM	(Gomez *et al.*, 2001)
11762	Colombia	Chronic PCM	(Montoya *et al.*, 1997)
29068	Colombia	Chronic PCM	(Montoya *et al.*, 1997)
30878	Colombia	Chronic PCM	(Montoya *et al.*, 1997)
Penguin	Uruguay	Penguin	(Gezuele, 1989)
300	Venezuela	Soil	(de Albornoz, 1971)

Saccharomyces cerevisiae haploid strain BY4742, Euroscarf acc. no. Y10000 (MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) and diploid strain BY4743, Euroscarf acc. no. Y23146 (Mat α/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YBR011c::kanMX4/YBR011c) were maintained in yeast extract-peptone-dextrose (YEPD) (Sambrook *et al.*, 1998) solid media (2% wt/vol agar) at 26°C. Both strains were grown for experimental procedures in YEPD liquid medium at 26°C on a mechanical shaker (160 rpm).

Cell cycle analysis and estimation of genome size. *P. brasiliensis* yeast cells of all isolates were grown in MMcM batch culture to the stationary phase of growth. Yeast cells and conidia suspensions were harvested by centrifugation (3000×*g* for 5 min at 4°C) and fixed overnight with 70% ethanol (vol/vol) at 4°C. Prior to sample treatment, conidia suspensions were additionally washed with 1% Tween® 80 (vol/vol) and subjected to sonication (four ultrasound pulses at 40 W for 2 s, with an interval of 1 to 2 s between pulses) for removal of clumps and excessive debris, a consequence of the method applied to dislodge and collect conidia (Restrepo *et al.*, 1986). Cell samples were collected and subjected to cell cycle analysis by flow cytometry (FCM) as previously described (Almeida *et al.*, 2006). *S. cerevisiae* haploid and diploid strains were grown in YEPD liquid medium to mid-log phase or under nutrient starvation conditions and cell samples were collected and subjected to cell cycle analysis as described by Fortuna and co-workers (Fortuna *et al.*, 2000).

The genome size estimated by FCM analysis was converted to mass of DNA using the formula reported by Dolezel and co-workers: genome size (bp) = $(0.978 \times 10^9) \times$ DNA content (pg) (Dolezel *et al.*, 2003).

Flow cytometry. All FCM experiments were performed on an EPICS XL-MCL (Beckman-Coulter Corporation, Hialeah, Fl, USA) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. A minimum of 30000 cells per sample were acquired at low flow rate and an acquisition protocol was defined to measure forward scatter (FS LOG) and side scatter (SS LOG) on a four-decade logarithmic scale and green fluorescence (FL1) on a linear scale. Offline data were analyzed with the Multigraph software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0 and the Windows Multiple Document Interface for Flow Cytometry 2.8 (WinMDI 2.8).

Microscopic count methods and epifluorescence microscopy analysis. Direct microscopic counts of *P. brasiliensis* yeast cells and conidia suspensions were carried out using bright-field microscopy and Neubauer counting chamber procedures. Epifluorescence microscopy was performed on a Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany) epifluorescence microscope fitted with 10× eyepieces and 40× and 100× (oil immersion) objectives and equipped with a Carl Zeiss AxioCam (HR/MR). Due to lower sensitivity of this technique

comparatively to FCM, SYBR Green I (Molecular Probes, Eugene, Or, USA) cell staining of conidia was carried out with a final concentration of 80×.

PCR amplification, cloning and sequencing. A fragment of 521 bp from the *GP43* locus, specifically exon 2, was obtained by PCR amplification of genomic DNA from eight randomly selected clones of *P. brasiliensis* 18, 300, ATCC 32069, Ibiá and T10B1. Total DNA was extracted from the yeast culture of each isolate with protocols using glass beads (van Burik *et al.*, 1998) or maceration of frozen cells (Morais *et al.*, 2000). The following primers were used: 5' CCAGGAGGCGTGCAGGTGTCCC 3' and 5' GCCCCCTCCGTCTTCCATGTCC 3'. PCR was conducted in a 25 µl reaction volume (2.5 µl of 10X PCR buffer, 1.5 µl of $MgCl_2$, 2.5 µl of 25 µM dNTP, 0.5 µl of each 10 µM primer, 0.5 µl of 0.5U *PfuTurbo*© DNA Polymerase (Stratagene, Cedar Creek, TX. USA), 250 ng of DNA template, and 18.75 µl of sterile distilled water). The reactions were carried out for 1 cycle of 5 min at 95°C followed by 35 cycles of 30 s at 95°C for denaturation, 1 min at 51.5°C for annealing, and 1 min at 72°C for extension followed by 1 cycle of 5 min at 72°C. The PCR product was separated by electrophoresis on a 1.5% agarose gel and the band was excised and purified using the QIAquick gel purification kit (Qiagen, Valencia, CA, USA). Purified PCR products were cloned with TOPO TA Cloning© Kit for sequencing (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Recombinant plasmid DNA was isolated with QIAprep© 96 Turbo Miniprep Kit (Qiagen). DNA sequencing was performed with BigDye™ Terminator Cycle Sequencing Kit v3.0 (Applied Biosystems, Foster City, CA, USA) and run on an Applied Biosystems ABI3100 automated DNA sequencer. Each purified PCR fragment was sequenced in both directions to ensure accuracy. Sequence data collected from both strands were aligned manually and examined with Sequence Navigator v. 1.0.1 (Applied Biosystems). The sequences obtained during this study have been deposited in the GenBank database under the numbers DQ364074-DQ364113.

Statistics. Data are reported as the mean±standard deviation (SD) of at least three independent assays. Mann-Whitney test regarding the genome size of *P. brasiliensis* strain ATCC 60855 conidia and yeast cells was performed using GraphPad Prism Software version 4.00 for Windows (San Diego, CA, USA).

RESULTS

GENOME SIZE OF *PARACOCCIDIOIDES BRASILIENSIS* YEAST FORM

Aiming to determining *P. brasiliensis* genome size, a flow cytometric (FCM) protocol for cell cycle profile analysis (Almeida *et al.*, 2006) was applied to ten different clinical and environmental isolates (Table 1). As expected from our previous studies, this technique discriminates various cellular subpopulations with different DNA content, namely R_1, R_2 and R_3 (Figure 1A exemplifies the case of *P. brasiliensis* 29068). All tested isolates presented half-peak coefficient of variation (HPCV) of R_1 lower than 7% (data not shown), indicating high resolution DNA measurements and establishing a direct correlation between mean green fluorescence intensity and the amount of DNA in each cell (Rodrigues *et al.*, 2003).

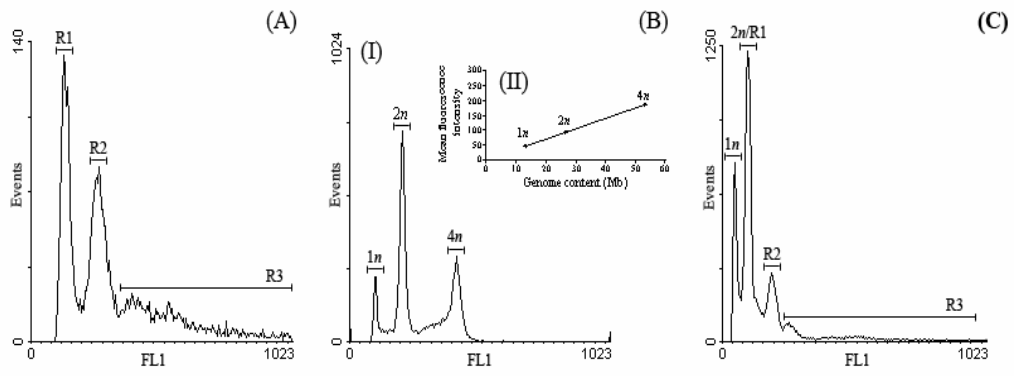

Figure 1 – Representative cell cycle analysis histograms of: (A) *P. brasiliensis* 29068 yeast cells grown in MMcM medium to the stationary phase of growth (discrimination of subpopulations R_1, R_2 and R_3); (B) (I) mixed cell populations of *S. cerevisiae* haploid and diploid strains grown to the exponential phase of growth in YEPD medium and (II) graph shows typical standard curve relating mean fluorescence intensity of the peaks 1*n*, 2*n* and 4*n* of *S. cerevisiae* strains and the theoretical amount of DNA per cell; and (C) mixed cell populations of *P. brasiliensis* 29068 yeast cells and *S. cerevisiae* haploid strain.

As references for direct DNA estimation, we used both *Saccharomyces cerevisiae* haploid and diploid strains, isogenic to the previously sequenced yeast S288C with a haploid genome of 13.5±2.5 Mb (Goffeau *et al.*, 1996). A concurrent cell cycle analysis of *S. cerevisiae* haploid and diploid strains revealed three distinct peaks (Figure 1B-I), corresponding to 1*n*, 2*n* and 4*n* DNA contents, where the mean green fluorescence intensity of each peak was directly correlated to the amount of DNA (Mb) of its corresponding cell subpopulation (Figure 1B-II, r^2>0.999). The analysis of different ratios of mixed cell populations of each *P. brasiliensis* isolate and *S. cerevisiae* haploid strain was then used to determine single-cell DNA content (Figure 1C). The genome size of each *P. brasiliensis* isolate was estimated in accordance with R_1 subpopulation of the cell cycle profile, previously characterized as being composed by uninucleated cells (Almeida

et al., 2006). The average amount of DNA per cell was determined as ranging between 26.3±0.1 and 35.5±0.2 Mb (Table 2) and was converted to mass of DNA content (Dolezel *et al.*, 2003), varying from 26.9±0.1 to 36.3±0.2 fg/yeast uninucleated cell (Table 2).

Table 2 – Genome size (Mb) and DNA content (fg) per uninucleated cell of *P. brasiliensis* isolates estimated by flow cytometry of SYBR Green I-stained cells.

Microorganisms	MFI±SD [a]	Genome size ±SD (Mb)	DNA content ±SD (fg) [c]
S. cerevisiae			
BY4742	48.2±1.5	13.25±0.4 [b]	13.5±0.4
BY4743	94.6±2.5	26.5±0.7 [b]	27.1±0.7
P. brasiliensis			
18	95.7±0.3	26.3±0.1	26.9±0.1
ATCC 32069	119.2±3.8	32.8±1.0	33.5±1.2
Ibiá	108.9±0.6	29.9±0.2	30.6±0.2
T10B1	129.1±0.7	35.5±0.2	36.3±0.2
ATCC 60855			
Yeast	113.9±1.9	31.3±0.5	32.0±0.5*
Conidia	109.9±2.8	30.2±0.8	30.9±0.8*
11762	126.5±2.0	34.8±0.5	35.5±0.6
29068	116.4±3.1	32.0±0.9	32.7±0.9
30878	117.1±4.2	32.3±1.2	33.0±1.2
Penguin	112.2±5.6	30.9±1.5	31.5±1.6
300	128.3±1.4	35.3±0.4	36.1±0.4

[a] Mean fluorescence intensity (MFI) of cells in G_0/G_1 phases (*S. cerevisiae* strains) or R_1 subpopulation (*P. brasiliensis* isolates) of the cell cycle profile.

[b] Theoretical genome size of *S. cerevisiae* haploid and diploid strains (Goffeau *et al.*, 1996).

[c] DNA content, in fentograms, calculated accordingly with Dolezel and co-workers (Dolezel *et al.*, 2003).

*No significant differences detected between *P. brasiliensis* strain ATCC 60855 conidial and yeast form ($P>0.05$).

GENOME CONTENT OF *P. BRASILIENSIS* CONIDIA

P. brasiliensis conidia are uninucleated structures generally accepted to be the natural infectious form of this dimorphic fungus and its morphological switch to the pathogenic yeast leads to the appearance of multinucleated cells, raising questions on the occurrence of ploidy shifts during this transition (McEwen *et al.*, 1987; Aristizabal *et al.*, 1998). In this sense, we have adapted the protocol for cell cycle profile analysis of yeast cells (Almeida *et al.*, 2006) to determine the genome size of these structures in *P. brasiliensis* ATCC 60855. Epifluorescence microscopy analysis revealed specific and homogenous nuclear staining (Figure 2A), confirming the

uninucleated feature of *P. brasiliensis* conidia. Moreover, the treatment of SYBR Green I-stained conidia with DNase I led to the loss of green fluorescence, indicating specific DNA labeling (data not shown). FCM evaluation indicated high peak resolution with a HPCV lower than 7% (data not shown), allowing the correct estimation of the DNA content per conidia (Figure 2B-I). By mixed cell staining with *S. cerevisiae* haploid reference strain (Figure 2B-II), the DNA content per conidia was determined as 30.2±0.8 Mb, corresponding to a mass of DNA of 30.9±0.8 fg per conidia (Table 2). No significant differences were detected between the DNA content of *P. brasiliensis* pathogenic yeast phase and the infectious propagules ($P > 0.05$).

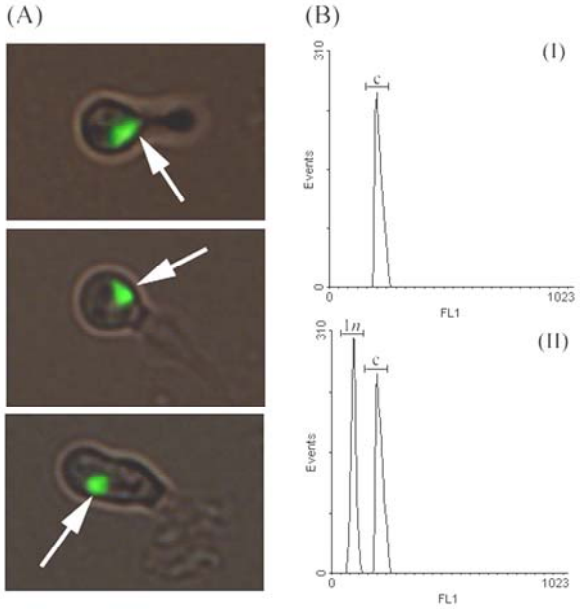

Figure 2 – *P. brasiliensis* conidia, strain ATCC 60855, submitted to cell cycle analysis. (A) Epifluorescence microscopy analysis (overlap of bright field and green fluorescence); white arrows indicate SYBR Green I nuclear staining. (B) Representative green fluorescence (FL1) histogram of (I) conidia cells (c) and (II) mixed cell populations of conidia cells (c) and *S. cerevisiae* haploid strain under nutrient starvation (1*n*).

P. BRASILIENSIS PLOIDY

Ploidy level is generally defined by the number of copies of each individual set of chromosomes per nucleus, thus an organism carrying one or two sets of nuclear chromosomes is classified as haploid or diploid, respectively, whereas aneuploidy is characterized by a non-integer ploidy number (Zeyl, 2004). In this sense, we have compared the DNA content of uninucleated yeast cells determined by FCM (Table 2), with the average haploid genome size estimated by pulsed field gel electrophoresis (PFGE) (Feitosa *et al.*, 2003; Montoya *et al.*, 1997; Montoya *et al.*, 1999) of seven clinical and environmental *P. brasiliensis* isolates, thus establishing a ploidy ratio that infers the ploidy state of the studied organism (Carr and Shearer, Jr., 1998). *P. brasiliensis*

29068, Ibiá and Penguin presented a ploidy ratio of approximately 1.0, compatible with a haploid DNA content (Table 3). In contrast, for *P. brasiliensis*, 18, 11762, 30878 and ATCC 32069 the ploidy ratio was slightly higher (1.1), indicating a haploid, or at least an aneuploid, DNA content (Table 3).

Table 3 – Comparison of the genome size of different *P. brasiliensis* isolates determined by flow cytometry (FCM) with data regarding the chromosome-sized DNA separated by pulsed-field gel electrophoresis (PFGE) (Feitosa *et al.*, 2003; Montoya *et al.*, 1997; Montoya *et al.*, 1999).

Isolate identification	Genome size ±SD (Mb) [a]	∑ of PFGE bands (Mb) [b]	Ploidy ratio [c]
18	26.3±0.1	23.3	1.1
ATCC 32069	32.8±1.0	28.9	1.1
Ibiá	29.9±0.2	29.7	1.0
11762	34.8±0.5	33.0	1.1
29068	32.0±0.9	30.5	1.0
30878	32.3±1.2	30.5	1.1
Penguin	30.9±1.5	29.7	1.0

[a] Genome size, in megabases, of *P. brasiliensis* isolates estimated by FCM.

[b] Sum of *P. brasiliensis* chromosomal bands separated by PFGE, in megabases (Feitosa *et al.*, 2003; Montoya *et al.*, 1997; Montoya *et al.*, 1999).

[c] Ratio of the genome size estimated by FCM and ∑ of PFGE bands.

On the other hand, the cellular ploidy state can also be molecularly determined by the identification of intra-individual variability in genes that are known to be single copy and highly polymorphic. Therefore, the gene encoding the *P. brasiliensis* glycoprotein Gp43 was evaluated assuming that if the isolates were diploid or polyploid, certain variability would be detected within these sequences (Matute *et al.*, 2006; Morais *et al.*, 2000). Following this line of thought, we sequenced eight randomly selected clones containing the exon 2 of *GP43* gene for each *P. brasiliensis* isolate, namely 18, 300, ATCC 32069, Ibiá and T10B1 (GenBank database numbers DQ364074-DQ364113). To infer ploidy, we calculated the recovery probability for only one of the alleles under the assumption that all the alleles present the same frequency. Therefore, if *P. brasiliensis* cells were haploid the recovery probability would be equal to the allelic frequency (1.00) since only one allele would exist. In the case of being diploid, the allelic frequency would be 0.50 and the probability of not recovering one of the alleles, in spite of their existence, 0.50 to the 8th power (3.90×10^{-3}). If *P. brasiliensis* were triploid, then the allelic frequency would be 0.33 and the recovery probability would be 1.52×10^{-4}. According to these data, only one allele seems to exist within each studied isolate indicating that they are most likely haploid.

DISCUSSION

The main goal of our work was to obtain new insights regarding *P. brasiliensis* genome size and ploidy. We studied ten *P. brasiliensis* isolates, originally isolated from both clinical and environmental specimens, from four distinct endemic areas of paracoccidioidomycosis (Brazil, Colombia, Uruguay and Venezuela) (Restrepo and Tobón, 2005). Furthermore, representatives of all three recently identified species (S1, PS2 and PS3) are present within the studied isolates (Matute *et al.*, 2006).

Recently, our group applied a flow cytometric (FCM) protocol to characterize the cellular morphology and nuclei content of the various subpopulations discriminated during cell cycle profile analysis of *P. brasiliensis* yeast cells (Almeida *et al.*, 2006). This technique, besides being less time-consuming and analyzing larger samples, characterizes the various subpopulations discriminated during cell cycle analysis, being therefore not only complementary to other DNA content quantification methods but also further informative concerning genome size and ploidy (Dolezel and Bartos, 2005). Thus, we were able to estimate the DNA content in accordance with the FCM analysis of uninucleated cells, specifically subpopulation R_1 (Figure 1A). Our results indicate that *P. brasiliensis* uninucleated yeast cells present a genome size similar to those previously determined by the summation of the chromosomes length (23 to 31 Mb), with a low intraspecific variability (Table 2), as described elsewhere (Cano *et al.*, 1998; Feitosa *et al.*, 2003; Montoya *et al.*, 1999; Montoya *et al.*, 1997). Moreover, we estimated the DNA content of *P. brasiliensis* conidia, strain ATCC 60855, and compared it with the yeast form results (30.9±0.8 and 32.0±0.5, respectively) ($P>0.05$). These data seem to exclude the occurrence of ploidy shift during morphogenesis from the infectious propagules to its pathogenic yeast form, an important feature of *P. brasiliensis* virulence traits; however, additional studies must be conducted to completely rule out this hypothesis.

Taking under consideration that ploidy is an essential genetic feature that underlies significant cytological and physiological characteristics (Zeyl, 2004), the cellular ploidy level of several *P. brasiliensis* isolates was assessed. Results regarding genome sizing of seven *P. brasiliensis* isolates by FCM were used as complementary data to those obtained through electrophoretic karyotyping (Feitosa *et al.*, 2003; Montoya *et al.*, 1997; Montoya *et al.*, 1999) and a ploidy ratio was defined as the DNA content per uninucleated cell per haploid genome size (Table 3). Data presented throughout this report showed that all analyzed isolates feature a ploidy ratio between

1.0 and 1.1. Although these results are nearer to a haploid DNA content, as in other pathogenic fungi aneuploidy must also be taken under consideration (Carr and Shearer, Jr., 1998; Feitosa *et al.*, 2003; Lengeler *et al.*, 2001; Torres-Guerrero, 1999). Nevertheless, the genome size estimated by FCM includes DNA external to the nucleus (e.g., mitochondrial DNA), conversely to that determined by the summation of chromosomal molecules (Hijri and Sanders, 2004). Since *P. brasiliensis* yeast cells are characterized by presenting an increased number of mitochondria, a slight overestimation would be expected (Queiroz-Telles, 1994). Furthermore, the ploidy state of five *P. brasiliensis* isolates, three of which included in the previous analysis – 18, ATCC 32069 and Ibiá –, was inferred by evaluating the intra-individual variability of the *GP43* gene, which encodes an exocelullar glycoprotein and the major antigenic component in this pathogenic fungus (Cisalpino *et al.*, 1996). The *GP43* gene was selected not only because it is highly polymorphic, particularly exon 2, but also because it is single copy and no known paralogs have yet been reported, thus ruling out the presence of a homologous sequence derived from gene duplication within the genome (Morais *et al.*, 2000). No intra-individual variations were detected, even though some variation was observed among different isolates, as described elsewhere (Matute *et al.*, 2006). The results indicated that only one allele seems to be present per individual, thus pointing to a haploid DNA content of all isolates. Interestingly, *P. brasiliensis* isolates 18, ATCC 32069 and Ibiá were previously described as diploid, conflicting with our results from both approaches applied for ploidy assessment (Cano *et al.*, 1998; Feitosa *et al.*, 2003). In fact, data reported by Feitosa and co-workers (2003) showed that eight out of twelve isolates were diploid, whereas our results indicate that all isolates presented a haploid, or at least, an aneuploid DNA content. Nevertheless, in those studies the genome size was attained by microfluorometry of stained nuclei and as such subjected to variations in nuclei number per cell or nuclear DNA content due to uncharacterized nuclear cell cycle phase of this dimorphic pathogenic fungus. Furthermore, Morais and co-workers (2000) reported that isolate 18 possessed two genotypic forms of *GP43* based on the distribution of nucleotide polymorphisms, indicating that this isolate, among others, could be diploid (thirteen out of seventeen isolates were suggested as diploid). However, these results might also be explained by errors inherent to the applied methodology since polymorphisms were detected as alterations in only one nucleotide and always at distinct sites within the same isolate (Morais *et al.*, 2000). In addition, while these authors analyze only two PCR *GP43* fragments we evaluated eight fragments from each single isolate further validating our findings. Overall, our results clearly show that a

significant amount of *P. brasiliensis* cells in batch culture present a haploid, or at least aneuploid, DNA content. However, one should take into account that our analysis of genome size and ploidy was based on the uninucleated cell subpopulation presenting the lowest DNA content (R_1), within a total population of 30,000 cells. In this sense, it is not possible to discard the presence of a small number of cells with distinct ploidy levels within the total cell population, a phenomenon that has already been reported for the pathogenic fungus *Cryptococcus neoformans* (Hata *et al.*, 2000).

This study provides information that addresses fundamental questions of *P. brasiliensis* biology, namely genome size and ploidy, an important asset for the development/design of molecular techniques (e.g., gene disruption and/or over-expression) and the future genetic manipulation of this human dimorphic pathogen. As stated beforehand, we analyzed isolates that are greatly diverse not only in the source of origin (clinical and environmental isolates) and country of isolation, but also in respect to distinctly recognized species of this pathogenic fungus (Matute *et al.*, 2006). Even though no association was detected between genome size/ploidy and the clinical-epidemiological features of the studied isolates, one cannot discard the importance of these parameters in the regulation of basic cellular and molecular mechanisms, particularly *P. brasiliensis* pathogenesis. Nonetheless, a wide range analysis of a higher number of isolates concerning the incidence and phenotype of the disease is necessary to evaluate possible differences among virulence and genome size and/or ploidy state.

CHAPTER 4 – TOWARDS A MOLECULAR GENETIC SYSTEM FOR THE PATHOGENIC FUNGUS *PARACOCCIDIOIDES BRASILIENSIS*

The results presented over this chapter were submitted in an international peer reviewed journal:

Almeida, A.J., Carmona, J.A., Cunha, C., Carvalho, A., Rappleye, C.A., Goldman, W.E., Hooykaas, P.J., Leão, C., Ludovico, P., Rodrigues, F. (2007) Towards a molecular genetic system for the pathogenic fungus *Paracoccidioides brasiliensis*. (*manuscript under revision*).

INTRODUCTION

The development of efficient genetic transformation systems for fungi has been crucial for establishing a link between *in vitro* analysis of DNA and its *in vivo* function (Magee *et al.*, 2003). Classical genetic tools as electroporation, protoplasting and cell permeabilization with lithium acetate were initially developed for the transformation of several non-pathogenic fungi (Ruiz-Diez, 2002). Alternative methods such as biolistics and *Agrobacterium tumefaciens*-mediated transformation (ATMT) led to the further expansion of the range of fungal species that could be transformed (Beijersbergen *et al.*, 2001; Bundock *et al.*, 1995). ATMT systems take advantage of a natural plant transformation process brought about by *A. tumefaciens*, a bacterium that causes a tumorous growth in plants known as crown gall (de Groot *et al.*, 1998). This plant pathogen carries a 200-kbp tumour-inducing (Ti) plasmid that contains the transferred-DNA (T-DNA), a DNA segment that is randomly inserted into the plant genome during infection. Transfer is mediated by the proteins encoded by the *vir* genes (located in the Virulence region of the Ti plasmid), which are specifically activated by plant phenolic compounds such as acetosyringone (Michielse *et al.*, 2005). The T-DNA is surrounded by a 24 bp border repeat in the Ti plasmid. It has been found that the T-DNA is still delivered by the virulence system to plant cells when present in the chromosome or on a separate plasmid (Hoekema *et al.*, 1984). This binary system has led to the development of binary vectors, which lack the T-DNA genes that are naturally present on the T-DNA, which have a marker gene for selection of transformed host cells and which can be used for cloning in *E. coli* (Hellens *et al.*, 2000). Fungal ATMT presents advantages over other methods due to its high efficiency and simplicity, avoiding time-consuming steps and specialized equipment as well as the fact that it mostly leads to single-copy T-DNA integration (Michielse *et al.*, 2005). Moreover, this technique has been applied to a diversity of fungal starting material (yeast cells, hyphae, conidia, and protoplasts) providing a valuable tool for the study of dimorphic fungal human pathogens such as *Coccidioides immitis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* (Abuodeh *et al.*, 2000; McClelland *et al.*, 2005; Sullivan *et al.*, 2002).

P. brasiliensis is susceptible to both electroporation and ATMT (Leal *et al.*, 2004; Soares *et al.*, 2005). Even though these authors showed that hygromycin B resistant transformants could be obtained by insertion of foreign DNA into multinucleated yeast cells, the methods presented either low transformation efficiency or decreased mitotic stability, thus impairing the future

application of these methodologies. The present study aimed to contribute towards the development of a genetic toolbox for the recalcitrant fungus *P. brasiliensis* with both an efficient transformation system as well as a gene reporter system. In this sense, we took advantage of the well described ATMT system and evaluated several factors that influence its efficiency, such as co-cultivation conditions and host cell susceptibility for transformation. In addition, *P. brasiliensis* green fluorescent protein (Gfp)-targeted isolates were constructed by insertion of the *GFP* gene under the control of several promoters from different fungi. Real-time polymerase chain reaction, epifluorescence microscopy and flow cytometry analysis were applied to discriminate promoter strength together with the number of copies inserted per genome.

MATERIALS AND METHODS

Microorganisms and culture media. *Paracoccidioides brasiliensis* yeast cells, strain ATCC 60855, were maintained at 36°C by periodic subculturing in brain heart infusion supplemented with 1% glucose (BHI) solid media (1.5% wt/vol agar). For the assays carried out in this study, yeast cells were routinely grown in both BHI and modified synthetic McVeigh Morton (MMcM) (Restrepo and Jimenez, 1980) liquid medium, at 36°C with aeration on a mechanical shaker (200 rpm).

Agrobacterium tumefaciens strain LBA1100 (C58C1 with a disarmed octopine-type pTiB6 plasmid) (Beijersbergen *et al.*, 1992) was used as recipient for binary vectors. During optimization procedures of *Agrobacterium tumefaciens*-mediated transformation (ATMT), *A. tumefaciens* LBA1100 containing the binary vector pUR5750 (conferring kanamycin resistance in *A. tumefaciens* and *Escherichia coli*) was applied (de Groot *et al.*, 1998). *A. tumefaciens* was maintained at 28°C on Luria Bertani (LB) (Sambrook *et al.*, 1998) medium containing spectinomycin (250 μg/ml), rifampicin (20 μg/ml), and/or kanamycin for selective purposes (100 μg/ml). The filamentous fungi *Aspergillus flavus* MUM 00.29 and *Aspergillus fumigatus* MUM 98.02 were grown in yeast extract-peptone-dextrose (Sambrook *et al.*, 1998) liquid media for 36 h at 37°C with aeration on a mechanical shaker (160 rpm) for total DNA extraction procedures. *E. coli* XL-1-Blue strain grown at 37°C on LB medium supplemented with kanamycin (30 μg/ml) was used as host for plasmid amplification and cloning.

Plasmid construction. Plasmid DNA extraction, recombinant DNA manipulations and *E. coli* transformation procedures were performed as described elsewhere (Sambrook *et al.*, 1998). All vectors reported in this study for insertion of the green fluorescent protein gene (*GFP*) in *P. brasiliensis* by ATMT were constructed on the backbone of the binary vector pUR5750 and are presented in Table 1. Plasmid pUR5750 contains a transferred DNA (T-DNA) harboring an *E. coli* hygromycin B phosphotranseferase (*HPH*) gene driven by the *Aspergillus nidulans* glyceraldehyde 3-phosphate (*GPD*) promoter and transcriptional terminator (*TRPC*) from pAN7-1 (de Groot *et al.*, 1998). This vector was linearized with *Kpn*I and dephosphorylated with calf intestinal alkaline phosphatase (CIAP; Promega, Madison, WI, USA) to prevent self-ligation. The plasmids pCR35, pCR150, pCR313, and pCR314 where kindly provided by C. A. Rappleye (Department of Microbiology, Ohio State University, Columbus, Ohio, USA). These plasmids contain the *GFP* gene downstream from different *Histoplasma capsulatum* promoter regions: calcium-binding protein (*CBP1*), small ribosomal subunit (*RPS15*), large ribosomal subunit (*RPL1B*) and translation initiation factor (*SUI1*), respectively. In addition, regulatory elements from other pathogenic fungi were also tested by removal of the *CBP1* promoter region from pCR35 via digestion with *BamH*I and *Asc*I and insertion upstream to the *GFP* gene: alcohol dehydrogenase (*ADH*; *Aspergillus flavus*; Ac. no. L27433) and isocitrate lyase (*ISO*; *Aspergillus fumigatus* Ac. No. AJ620297). These promoters were obtained by PCR amplification with specific primers (Table 2) on total DNA of individual fungal species was extracted by maceration of frozen cells (Morais *et al.*, 2000). 200 ng of DNA were added to 50 μL final volume of PCR reaction mixture: reaction buffer 1×, 2 mM $MgCl_2$, 200 μM dNTP, 200 μM of each primer and 1 U *Taq* polymerase. Basic PCR reaction cycling conditions were 1 cycle at 94°C for 10 min, 40 cycles at 94°C for 15 s, 56°C for 30 s, 65°C for 120 s (depending on amplicon size), and 1 final cycle at 65°C for 10 min. After digestion with *BamH*I and *Asc*I the fragments were ligated into pCR35 digested with the same enzymes. The DNA fragments harboring individual promoter, *GFP*, and terminator regions were isolated by PCR on the various plasmids with specific primers (Table 1 and 2), followed by *Kpn*I digestion and ligation into the previously linearized pUR5750 binary vector. All constructs were confirmed by colony-PCR and diagnostic restrictive endonuclease treatment. The obtained binary vectors were mobilized to *A. tumefaciens* LBA1100 ultracompetent cells by electroporation as described by Dulk-Ras and Hooykaas (Dulk-Ras and Hooykaas, 1995) and transformants were isolated by kanamycin selection at 100 μg/ml.

Table 1 – Fungal promoter regions, plasmids and binary vectors used in this study.

Promoter regions	Fungal species	Plasmid	Binary vector
Calcium-binding protein (*CBP1*)	*H. capsulatum*	pCR35	pUR5750::P_{CBP1}-*GFP*
Small ribosomal subunit (*RPS15*)		pCR150	pUR5750::P_{RPS15}-*GFP*
Large ribosomal subunit (*RPL1B*)		pCR313	pUR5750::P_{RPL1B}-*GFP*
Translation initiation factor (*SUI1*)		pCR314	pUR5750::P_{SUI1}-*GFP*
Alcohol dehydrogenase (*ADH*)	*A. fumigatus*	pCR35::P_{ADH}-*GFP*	pUR5750::P_{ADH}-*GFP*
Isocitrate lyase (*ISO*)	*A. flavus*	pCR35::P_{ISO}-*GFP*	pUR5750::P_{ISO}-*GFP*

Table 2 – Primers used in this study for molecular cloning.

Target DNA for PCR amplification	Primer sequences (5'-3') [a]
A. flavus total nuclear DNA Alcohol dehydrogenase (*ADH*)	P1-cgggatcccgAAGCTTGACGTTTGACAGGG P2-ggcgcgccGAATTCAGTTACCAGGTCAC
A. fumigatus total nuclear DNA Isocitrate lyase (*ISO*)	P1-cgggatcccgGAAGGACAGGAACATTCGGG P2-ggcgcgccCATTGTGACAGGTATGAAGA
pCR35	
pCR150	
pCR313	P1-ggggtaccccGCGGATCACGGTATCGATGA
pCR314	P2-ggggtaccccTGAGATGGCAAGGAGCAACC
pCR35::P_{ADH}-*GFP*	
pCR35::P_{ISO}-*GFP*	
E. coli Hygromycin B phosphotranseferase (*HPH*)	P1-ATGCCTGAACTCACCGCGAC P2-TTCTACACAGCCATCGGTCC
P. brasiliensis GP43	P1-CGAAACATTGGGACACCTTT P2-CGATGACGACCCTCAGATTT
Green fluorescence protein (*GFP*)	P1-AGATACCTTACCCCGCGACT P2- CCTGTGTGTGAAGGAGCTGA

[a] Low caps indicate restriction enzyme sequence recognition sites.

P. brasiliensis *ATMT procedures*. *A. tumefaciens* LBA1100 carrying the desired binary vector was grown overnight on LB liquid medium with antibiotics in a water bath, at 28°C with shaker (180 rpm). 1 ml of the cell culture was spun down and washed with induction medium (IM) (Bundock *et al.*, 1995) with acetosyringone (Sigma, St. Louis, Mo, USA) (200 μM) and antibiotics. Bacterial cells were diluted in IM to an OD_{660nm} of 0.30, and re-incubated at 28°C until an OD_{660nm} of approximately 0.80.

P. brasiliensis yeast cells were grown in BHI or MMcM batch cultures to the exponential (48-60 h) and stationary (84-96 h) growth phase (Almeida *et al.*, 2006). *P. brasiliensis* yeast cell samples were centrifuged (4500×*g* for 15 min), washed with IM and adjusted to a final concentration of 1×10^8 cells/ml using direct microscopic counts (Neubauer counting chamber procedures). To test *P. brasiliensis* yeast cells sensitivity to Hygromycin B (HygB), cells were inoculated on BHI solid medium with HygB concentrations ranging from 50 to 300 μg/ml.

Different ratios of *A. tumefaciens* and *P. brasiliensis* cells were mixed to a final volume of 120 μl and inoculated onto sterile Hybond N membrane (Amersham Biosciences, Piscataway, NJ, USA) on solid IM for co-cultivation at 22, 25 and 28°C for 3 days. Alternatively, prior to incubation co-cultivation plates were air dried in a safety cabinet for different time periods (5, 10, 15, 20, 25, 30, 40, 50 and 60 min). Following co-cultivation, membranes were transferred to tubes with non-selective BHI liquid medium containing cefotaxime (200 μg/ml) for growth inhibition of *A. tumefaciens*, and cells were dislodged by aid of a spatula and vortexing for 1 min. The cell suspension was plated directly on BHI supplemented with HygB (50 μg/ml) and cefotaxime (200 μg/ml), or alternatively recovered for 48 h at 36°C, 200 rpm, before plating in selective media. Selection plates were incubated at 36°C for 15 to 20 days and monitored for colony forming ability. Transformation efficiency was determined by taking into account *P. brasiliensis* yeast cell concentration at the moment of co-cultivation and the number of hygromycin resistant (Hyg^R) colonies.

Transformant PCR. Randomly selected Hyg^R putative transformants were tested for the presence of the T-DNA by PCR amplification of the *HPH* gene using total DNA as template. Fungal DNA extractions were performed on 72 h cultures using mechanical disruption with glass beads as described elsewhere (van Burik *et al.*, 1998). 200 ng of DNA were added to 20 μL final volume reaction mixture defined by reaction buffer 1×, 2 mM $MgCl_2$, 200 μM dNTP, 200 μM of each primer and 1 U *Taq* polymerase. The PCR reaction cycling was as follows: 1 cycle at 94°C for 10 min, 40 cycles at 94°C for 15 s, 56°C for 30 s, 65°C for 60 s, and 1 final cycle at 65°C for 10 min. A 982 bp fragment was amplified using the primers indicated in Table 2.

Mitotic stability. A total of 208 Hyg^R *P. brasiliensis* colonies were randomly selected and serially transferred to non-selective BHI solid medium for at least three times. Following these

passages, transformants were serially inoculated on plates with non-selective and selective medium (50 μg/ml) for three consecutive rounds to examine mitotic stability.

Acivicin, azaserine and mizoribine treatment. *P. brasiliensis* yeast cells, strain 60855, were cultured in BHI liquid medium to the exponential phase of growth. Cells were harvested, washed and suspended in fresh medium in order to obtain a final cell number of about 1×10^6 cells/ml. Several concentrations of acivicin (25, 50, 100 and 200 μg/ml), azaserine (1, 5, 25, 50, 100 and 200 μg/ml), and mizoribine (25, 50, 100 and 200 μg/ml) were tested during drug treatments performed at 36°C with aeration on a mechanical shaker (200 rpm). Cell samples were collected at the times indicated in Results and subjected to cell viability assays and cell cycle analysis by flow cytometry as previously described (Almeida *et al.*, 2006). Cell viability was assayed by harvesting cells, washing (3×) and suspending in fresh medium followed by monitorization of growth by turbidimetry visualization. Specific treatment conditions were selected for ATMT experiments.

Flow cytometry measurements. Flow cytometric analysis of cell cycle progression of *P. brasiliensis* yeast cells after treatment with nucleotide synthesis inhibitors was performed on an EPICS XL-MCL (Beckman-Coulter Corporation, Hialeah, FI, USA) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. The green fluorescence was collected through a 488 nm blocking filter and a 550 nm/long-pass dichroic with a 525 nm/band-pass. A minimum of 30,000 cells per sample was acquired at low flow rate and an acquisition protocol was defined to measure forward scatter (FS) and side scatter (SS) on a four-decade logarithmic scale and green fluorescence (FL1) on a linear scale. Offline data were analyzed with the Windows Multiple Document Interface for Flow Cytometry 2.8 (WinMDI 2.8). Flow cytometric measurements of *P. brasiliensis* Gfp-targeted isolates were performed in a basic FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometer equipped with an argon-ion laser emitting a 488-nm beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter and a 550 nm/long-pass dichroic with a 530 nm/band-pass. A minimum of 50,000 cells per sample was acquired at low flow rate and an acquisition protocol was defined to measure forward scatter (FS) and side scatter (SS) on a four-decade logarithmic scale and green fluorescence (FL1) on a linear scale. Data were acquired and analyzed with CELLQuest PRO 4.0 (BD Biosciences).

Epifluorescence microscopy analysis. Epifluorescence microscopy was performed with an (Olympus, Melville, NY, USA) epifluorescence microscope equipped with a high-resolution DP70 digital camera and with an Olympus UPlanSApo 100X/oil objective, with a numerical aperture of 1.40.

Real-time polymerase chain reaction (RT-PCR). Total RNA and DNA from exponentially growing *P. brasiliensis* yeast cultures was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) standard procedures and heat shock treatment (20 min at 65°C followed by 60 min at -80°C) for cellular disruption. Total RNA (0.5-1 μg) was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Marnes La Coquette, France). For RT-PCR quantification, 2 μl of the reverse-transcribed RNA was used as template to amplify the *P. brasiliensis GP43* gene (195 bp) and the *GFP* gene (147 bp) using the primers indicated in Table 2 and the LightCycler FastStart DNA Master SYBR Green I (Roche, Nutley, NJ, USA). The thermal cycling conditions comprised an initial step at 94°C for 15 min, followed by 50 cycles at 94°C for 10 s, 60°C for 10 s and 72°C for 20 s, a melting step of 55°-95°C (0.5°C/s), and a final cooling at 40°C for 30 s. Real-time quantification was conducted on a LightCycler System (Roche) using threshold cycle (Ct) values for *GP43* transcripts as the endogenous reference. mRNA differential *GFP* expression levels were evaluated by normalizing *GFP* Ct values with the reference and comparing the ratio amongst the tested samples. Total DNA *GFP* gene copy number was determined with the standard curve method (Cts plotted against logarithm of DNA copy number) (Larionov *et al.*, 2005). Results were expressed as N-fold changes in target gene copies normalized to the *GP43* reference gene. For N-fold values between 0.7 and 1.3, the tested isolates were considered to harbour a single integrated copy of the target gene (Ferreira *et al.*, 2006a).

Reproducibility of the results and statistical analysis. Data are reported as the mean±standard error of the mean (SEM) of at least three independent assays. All statistical analysis was performed using the GraphPad Prism Software version 4.00. The One-way ANOVA Kruskal-Wallis test was used to compare the average number of Hyg^R colonies and transformation efficiencies of the following parameters: air drying of co-cultivation mixtures, co-cultivation temperature, bacteria to yeast ratios, and *P. brasiliensis* growth culture conditions. Gfp fluorescence (arbitrary units) and *GFP* gene expression level and copy number were also statistically analyzed. The data regarding flow cytometric cell cycle progression analysis of *P.*

brasiliensis yeast cells treated with purine synthesis inhibitor are from one representative experiment.

RESULTS

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION (ATMT) OF *PARACOCCIDIOIDES BRASILIENSIS* YEAST CELLS

Prior to experimental procedures for ATMT optimization, the Hygromycin B (HygB) minimum inhibitory concentration of *P. brasiliensis* yeast cells was determined. Our results showed that yeast growth was inhibited at a final concentration of HygB of 50 μg/ml, and was thus chosen for subsequent ATMT experiments (data not shown). In addition, for all controls performed no HygB natural resistant clones were obtained for *P. brasiliensis*. The initial transformation procedures were performed by co-cultivating *P. brasiliensis* yeast cells from exponentially batch growing cultures with *A. tumefaciens* (carrying the binary vector pUR5750) for 3 days at 25°C on induction medium (IM) with acetosyringone, an inducer of the *A. tumefaciens* virulence genes (Michielse *et al.*, 2005).

Co-cultivation parameters affect* P. brasiliensis *ATMT efficiency. To start establishing a protocol for ATMT of *P. brasiliensis*, we firstly tested the influence of cellular recovery of *P. brasiliensis* yeast cells on the transformation efficiency. Our data showed that in the absence of a 48 h incubation period in non-selective medium, no Hygromycin B resistant (Hyg^R) clones were obtained, demonstrating that this is a crucial step for *P. brasiliensis* ATMT. In addition, during these experiments, we observed that the length of time that the IM plates inoculated with the co-cultivation mixtures were left opened in the class II biosafety cabinet (with laminar flow) significantly changed the efficiency of transformation. Therefore, we evaluated what was the influence of air drying of IM plates with co-cultivation mixtures on the transformation efficiency. The results indicated that drying periods shorter than 25 min resulted in a significantly smaller number of transformants than exposure to the laminar flow for 30 min (Figure 1A). Drying for longer periods of time than 30 minutes again resulted in a much lower number of transformants (Figure 1A). Overall, these results clearly indicate that 30 min of air drying in a safety cabinet is essential for optimal ATMT of *P. brasiliensis*.

We also studied the influence of co-cultivation temperature on *P. brasiliensis* ATMT efficiency. As shown in Figure 1B a temperature of 25°C resulted in a significantly (P=0.019) higher average number of Hyg^R clones (153±60) than cocultivation at 22 and 28°C (19±11 and 4±3, respectively).

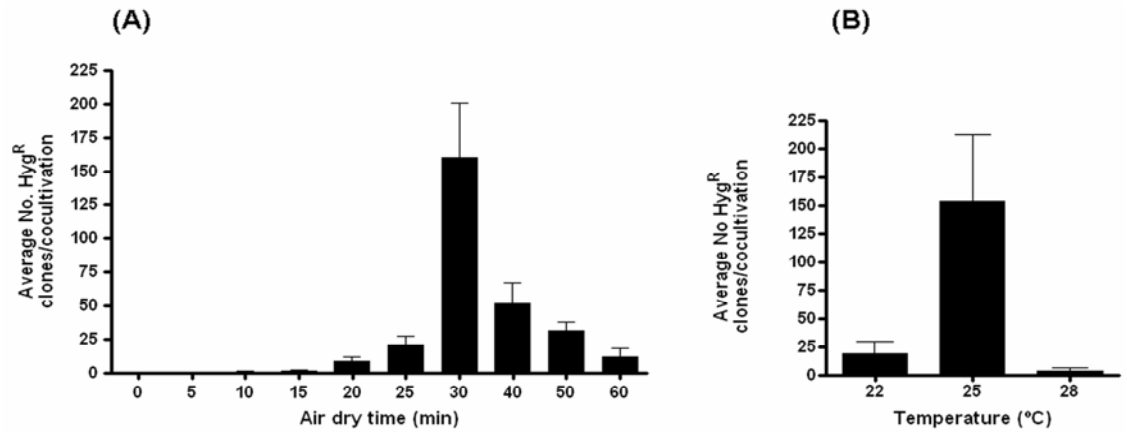

Figure 1 – Co-cultivation conditions affect *P. brasiliensis* ATMT outcome. (A) *P. brasiliensis* exponentially growing yeast cells in BHI batch cultures and *A. tumefaciens* cells mixtures were placed on membranes in co-cultivation plates and air dried for the given time periods, incubated for 3 days at 25°C and recovered 48 h in non-selective medium prior to HygB selection. (B) *A. tumefaciens* and *P. brasiliensis* yeast cells grown to exponential phase in BHI batch cultures were mixed together, air dried for 30 min, co-cultivated at 22, 25 and 28°C for 3 days, and recovered for 48 h in non-selective medium prior to HygB selection. Results represent the average number of Hyg^R clones per co-cultivation.

The ratio of *A. tumefaciens* to yeast cells during co-cultivation is also a well known key factor in the development of an ATMT system (Michielse *et al.*, 2005). Thus, several ratios of *Agrobacterium*:*P. brasiliensis* cells (10:1, 1:1, 1:5, and 1:10) were tested during the course of 25 transformation experiments (Table 3). Data analysis showed that an elevated concentration of fungal cells (1:5 and 1:10) leads to a higher average number of Hyg^R clones per co-cultivation (74±16 and 132±21, respectively) and the highest transformation frequencies per *Agrobacterium* donor. However, the calculation of transformation frequencies per recipient cell taught that ratios of 10:1 (11±3) and 1:1 (7±1) gave the highest transformation frequencies.

Host cell conditions influence* P. brasiliensis *competence for ATMT. With the intention of identifying higher host cell competence/susceptibility to *A. tumefaciens* infection, we evaluated the influence of the *P. brasiliensis* physiological status on ATMT. In accordance with previous studies by Almeida and co-workers (Almeida *et al.*, 2006) reporting a differential growth and cell cycle progression pattern of *P. brasiliensis* under distinct environmental conditions, yeast cell

Table 3 – *Agrobacterium tumefaciens*-mediated transformation of *P. brasiliensis* yeast cells by use of hygromycin selection.

Ratio Bacteria:Yeast	Hyg^R clones/ cocultivation (±SEM) [a]	Hyg^R clones/ 1×10^6 target cells (±SEM) [b]
10:1	64±16	11±3
1:1	40±8	7±1
1:5	74±16	3±1
1:10	132±21	2±1

[a] Number of Hygromycin B resistant (Hyg^R) clones obtained per co-cultivation (mean ± standard error of the mean).

[b] Transformation efficiency determined by counting the number of Hyg^R clones obtained per 1×10^6 target cells (mean ± standard error of the mean).

cycle progression pattern of *P. brasiliensis* under distinct environmental conditions, yeast cell samples were collected during the exponential and stationary growth phases in both rich (BHI) and poor (MMcM) nutritional environments and prepared for ATMT. As shown in Figure 2A, exponentially growing yeast cells in both media are susceptible to fungal transformation by *A. tumefaciens*, although to a greater extent in a rich nutritional environment. Conversely, stationary phase cells appear only to be susceptible to ATMT when grown in BHI batch cultures. Altogether, these data indicate that *P. brasiliensis* exponentially growing yeast cells in BHI medium are the most adequate candidates as hosts for ATMT under these particular experimental conditions.

The disruption of purine and pyrimidine synthesis has been previously demonstrated to cause hypersensitivity to ATMT in the yeast *Saccharomyces cerevisiae* and plant cell cultures (Roberts *et al.*, 2003). Therefore, we specifically aimed to evaluate the effect of blocking nucleotide biosynthesis on *P. brasiliensis* competence for ATMT. Addition of acivicin (100 and 200 μg/ml), azaserine (100 and 200 μg/ml) or mizoribine (50 and 100 μg/ml) to exponentially growing yeast cells of P. brasiliensis led to growth arrest as visualized by flow cytometric analysis (Figure 2B), but cell viability was not lost (data not shown). However, from the obtained transformation efficiencies one can conclude that growth arrest induced by these purine and pyrimidine synthesis inhibitors in *P. brasiliensis* yeast cells does not lead to an increase, but rather leads to a decrease in transformation competence (Figure 2C).

Characterization of* P. brasiliensis *Hyg^R isolates. In order to characterize the obtained transformants, we evaluated the mitotic stability of 208 randomly selected Hyg^R *P. brasiliensis*

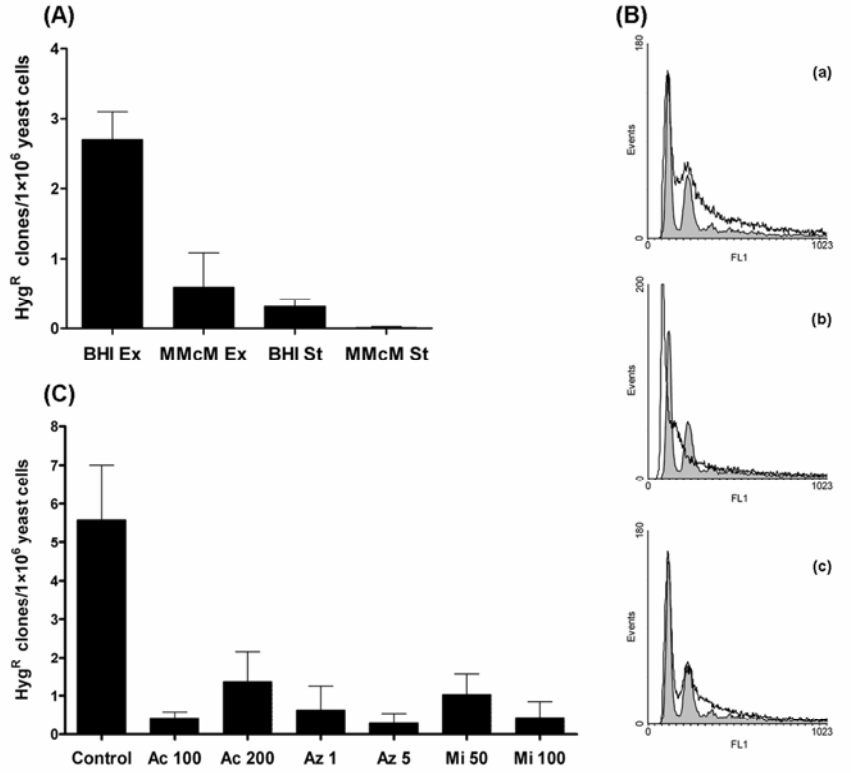

Figure 2 – Host cell growth conditions influence *P. brasiliensis* ATMT efficiency. (A) *P. brasiliensis* yeast cells were grown in rich (BHI) and poor (MMcM) nutritional environment to the exponential (48-60 h) and stationary (84-96 h) phase of growth and subjected to optimized co-cultivation procedures. (B) Exponentially growing *P. brasiliensis* yeast cells in BHI batch cultures subjected to 12 h treatments at 36°C with different purine and pyrimidine synthesis inhibitor concentrations: representative histograms of green fluorescence (FL1) of the cell cycle profile of *P. brasiliensis* yeast cells treated with (a) Acivicin (200 μg/ml), (b) Azaserine 5 μg/ml, and (c) Mizoribine (100 μg/ml) (grey area – untreated cells; white area – treated cells). (C) *P. brasiliensis* yeast cells treated with 100 and 200 μg/ml of Acivicin (Ac), 1 and 5 μg/ml of Azaserine (Az), and 50 and 100 μg/ml of Mizoribine (Mz) prior to transformation experiments. Results represent transformation efficiencies determined by dividing total number of Hyg^R clones by the initial number of *P. brasiliensis* cells in the co-cultivation mixture.

clones. Serial subcultures in non restrictive growth conditions revealed no differences in growth when compared to the wild-type strain. Following, three consecutive culture rounds, under non-restrictive conditions, the transformants were inoculated in HygB selective medium. The mitotic stability for each sequential round was 86, 80 and 80%, respectively. Furthermore, PCR amplification of the *hph* gene revealed the presence of the T-DNA in the total DNA isolated from 12 randomly isolated Hyg^R transformants (data not shown).

Altogether, our final assessments indicate that the most adequate settings for *P. brasiliensis* ATMT with high mitotic stability are: (i) co-cultivation of *A. tumefaciens* cells with *P. brasiliensis* exponentially growing yeast cells in BHI cultures in induction medium (IM) with acetosyringone (AS); (ii) air drying of IM plates inoculated with Hybond N membranes containing cell mixtures in

a safety cabinet for 30 min; (iii) incubation at 25°C for 3 days; and (iv) a 48 h recovery period in non-selective medium prior to Hygromycin B (HygB) selection.

CONSTRUCTION OF *P. BRASILIENSIS GFP*-TARGETED ISOLATES BY ATMT

P. brasiliensis yeast cells were subjected to fungal transformation by *A. tumefaciens* carrying binary vectors that harbor the *GFP* gene under the control of several regulatory elements from *Histoplasma capsulatum*, *Aspergillus flavus* and *Aspergillus fumigatus* (Table 1). After ATMT, 3 independent Hyg^R isolates were selected for each of the six different binary vectors for further analysis. All the chosen transformants were serially subcultured in BHI HygB selective and non-selective medium; they all displayed normal growth and an identical mitotic stability to that referred above. The *P. brasiliensis* exponentially growing transformed yeast cells in BHI batch cultures were then subjected to analysis of green fluorescence. Epifluorescence microscopy visualization (Figure 3) indicated a diffuse cellular Gfp localization in all the studied isolates in contrast with the *P. brasiliensis* yeast cells transformed with the empty vector (pUR5750).

Flow cytometric assessment was carried out to evaluate the green mean fluorescence intensity and the FS LOG (correlated to cell size), taking into account the budding pattern of *P. brasiliensis*. Individual *GFP*-targeted transformants of each studied promoter were analyzed independently in at least 3 independent experiments. This biparametric evaluation confirmed the epifluorescence microscopy analysis, revealing a subpopulation of smaller sized cells with low green mean fluorescence intensity and thus, the *GFP* expression analysis was performed using the mean fluorescence intensity of the subpopulation of larger and/or multiple budding cells. As shown in Figure 4, *GFP*-transformed isolates from the 6 tested promoter regions presented a significantly increased fluorescence when compared with the empty vector ($P < 0.001$), but no significant differences were detected between individual promoters ($P > 0.05$). However, our data also revealed the existence of a high variability even between replicas of the same *GFP*-targeted isolate of the individually tested promoters.

Furthermore, to evaluate *GFP* expression level and gene copy number we conducted real-time polymerase chain reaction (RT-PCR) on the 3 selected isolates of each studied promoter region. Threshold cycle (Ct) values of *GFP* were compared with the single copy endogenous reference, the *GP43* gene, *P. brasiliensis* main antigenic component (Almeida *et al.*, 2007). Melting curve analysis showed that all RT-PCR products corresponded to the targeted sequence (data not shown). Data presented in Table 4 show that there are no statistical differences ($P > 0.05$)

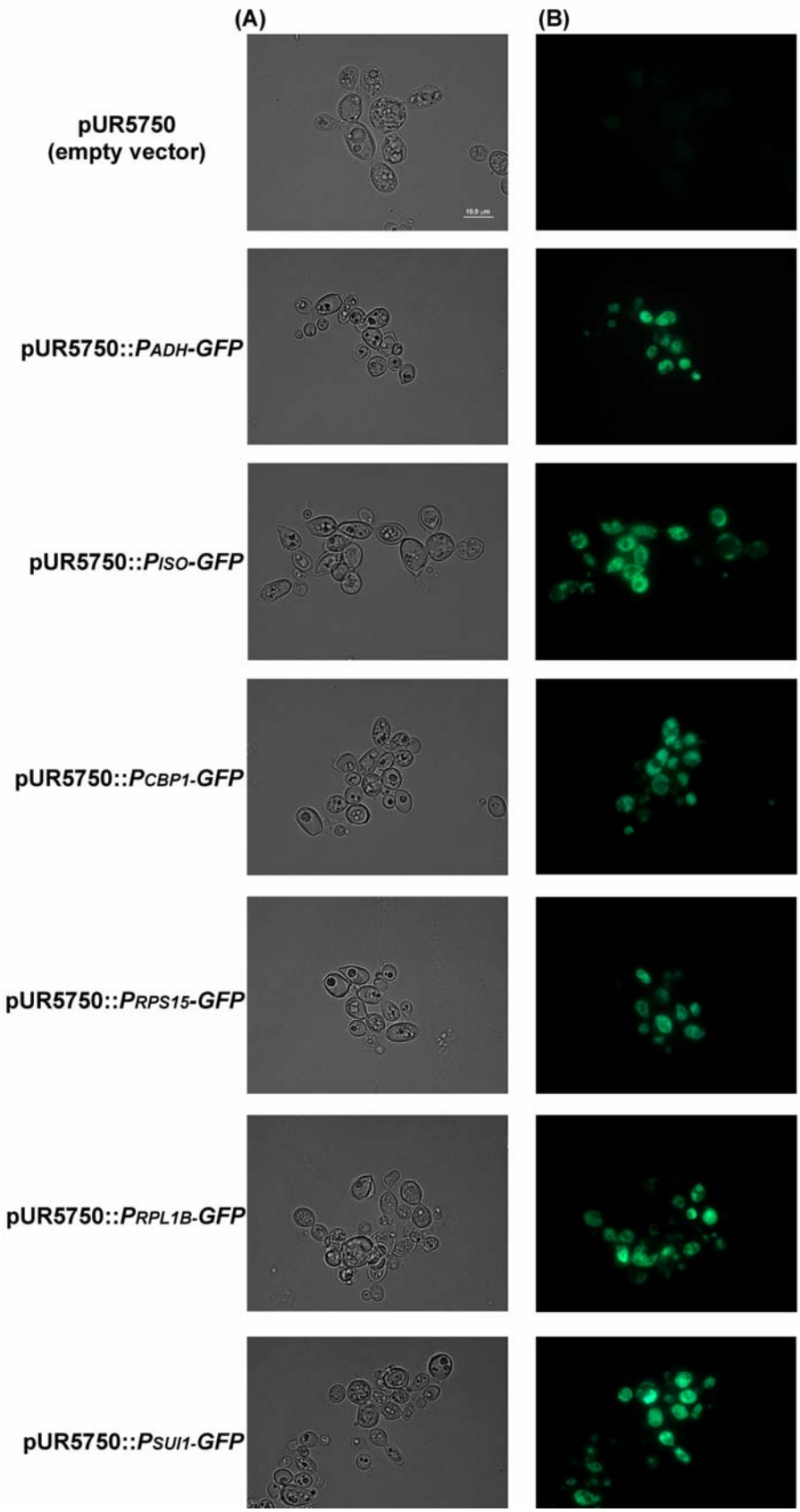

Figure 3 – Epifluorescence microscopy analysis of a randomly selected *P. brasiliensis GFP*-targeted isolate obtained by transformation of each one of the 6 tested promoter regions placed upstream of the *GFP* gene in the binary vector (A, bright field and B, green fluorescence). *P. brasiliensis* yeast cell samples were collected during the exponential growth phase in BHI batch cultures for Gfp visualization. *P. brasiliensis* yeast cells transformed with the empty vector (pUR5750) is used as control.

between *GFP* expression levels amongst the 6 promoters, correlating with flow cytometric analysis. The *GFP* copy number was also shown not to vary significantly ($P > 0.05$) with an N-fold of approximately 1, indicating that only a single copy of the target gene is inserted in the genome of *P. brasiliensis* isolates.

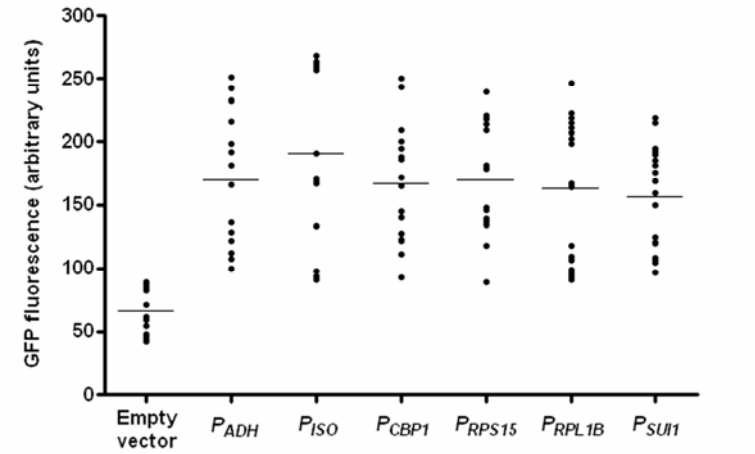

Figure 4 – Flow cytometric analysis of *P. brasiliensis GFP*-targeted isolates. *P. brasiliensis* exponentially growing yeast cells in BHI medium from 3 independent Hyg^R isolates transformed with the 6 distinct promoter regions were subjected to mean green fluorescence intensity analysis. *P. brasiliensis* yeast cells transformed with the empty vector (pUR5750) is used as control.

Table 4 – Gene expression levels and copy number of *GFP* inserted into *P. brasiliensis* yeast cells obtained with real-time polymerase chain reaction.

Promoter regions	Ct_{GP43}/Ct_{GFP} [a]	Gene copy number [b]
Alcohol dehydrogenase (*ADH*)	0.93	0.96
Isocytrate lyase (*ISO*)	0.96	0.89
Calcium-binding protein (*CBP1*)	1.10	1.01
Small ribosomal subunit (*RPS15*)	0.92	1.08
Large ribosomal subunit (*RPL1b*)	0.85	1.22
Translation initiation factor (*SUI1*)	1.01	0.94

[a] Average *GFP* expression levels evaluated by normalizing *GFP* Ct values with the reference *GP43*.

[b] Average *GFP* gene copy number was determined with the standard curve method and normalized with *GP43* (Larionov *et al.*, 2005).

DISCUSSION

Paracoccidioides brasiliensis has long been considered unamenable to molecular genetic studies (Bailao *et al.*, 2006). In this sense, the main goal of this work was to contribute for the

development of a molecular toolbox, particularly an efficient transformation and gene expression system for this recalcitrant dimorphic pathogenic fungus.

Agrobacterium tumefaciens-mediated transformation (ATMT) has been successfully applied to a variety of different fungal species and systems during the last decade due to its technical simplicity and efficiency (Michielse *et al.*, 2005). During this study, we aimed to improve the only existing report on *P. brasiliensis* ATMT (Leal *et al.*, 2004) by evaluating several factors, both co-cultivation and host cell conditions. Our assays show that cellular recovery in non-selective medium before antibiotic selection is essential for *P. brasiliensis* ATMT. To date, fungal transformation protocols mediated by *A. tumefaciens* do not include this experimental step, although it is an integral component of other genetic transformation systems of fungi (e.g., electroporation) (Sambrook *et al.*, 1998). This recovery period might be essential for T-DNA integration and/or hygromycin (*hph*) gene expression by host cell molecular machinery. In fact, reports concerning plant cell transformation by *A. tumefaciens* describe T-DNA integration as a limiting step (Gelvin, 2000). Moreover, *P. brasiliensis* yeast cells have been described to present particular requirements for optimal growth in batch cultures (Restrepo and Jimenez, 1980). In this sense, allowing cells to readapt in a rich medium subsequently to 3 days of culture in a poor nutritional environment as IM might increase its competence for growth on selective media. Furthermore, to our knowledge, this is the first report on the influence of air drying of mixtures prior to cocultivation of fungus and *A. tumefaciens*. Data herein presented clearly indicate that this is a crucial step in our particular experimental procedures. Due to the fact that this transformation system requires cell-to-cell contact for T-DNA transfer, the concentration of bacteria to yeast cell mixtures or the relevance of water activity might have some weight in the aptitude of *A. tumefaciens* for infection or the susceptibility of *P. brasiliensis* yeast cells to infection (Citovsky *et al.*, 2007). In addition, ATMT assisted by vacuum infiltration is applied by plant physiologists to enhance bacterial penetration into inter-cellular spaces by causing the air spaces between cells to decrease (Bechtold and Pelletier, 1998). Co-cultivation temperature is also an extensively covered parameter in fungal ATMT and its selection is usually balanced between biological imperatives of the effector and target cells. Up to date, optimal temperatures are usually defined between 22 and 25°C, although lower and higher values have been tested as well (Michielse *et al.*, 2005). Studies performed by Fullner and Nester showed that temperatures above 28°C are inhibitory to the T-DNA transfer machinery, thus preventing transformation (Fullner and Nester, 1996). In addition, one should consider the fact that being a dimorphic

fungus, *P. brasiliensis* is strictly regulated by temperature, undergoing a morphological transition from the yeast to the mycelial phase when shifted from 37°C to temperatures below 28°C (Restrepo and Tobón, 2005). Our results, indicating higher transformation efficiency at 25°C, correlate with these data, even though at 22 and 28°C Hygromycin B resistant (Hyg^R) transformants were also obtained. Another important variable in ATMT systems assessed throughout this work was the ratio between *A. tumefaciens* and *P. brasiliensis* yeast cells. Variability in this parameter is well documented and is generally dependent on the recipient fungal cells as well as the chosen *A. tumefaciens* strain. In fact, both the addition of an increased number of bacterial or fungal cells to co-cultivation mixtures may decrease or enhance transformation efficiency, depending on the system (Michielse *et al.*, 2005). Our results did not show a clear optimal condition for transformation. Whereas an elevated concentration of *P. brasiliensis* yeast cells (1:5 and 1:10) resulted in a higher average number of Hyg^R colonies per co-cultivation, lower fungal loads and higher number of bacterial cells (1:1 and 10:1, respectively) led to improved transformation efficiency in absolute values (Hyg^R colonies/10^6 target cells). The use of direct microscopic counts to adjust cell density prior to co-cultivation is probably concealing true transformation efficiencies due to the fact that *P. brasiliensis* yeast cells characteristically have multiple buds and the fact that batch growing cultures of this fungus present an unusually elevated number of dead cells (San-Blas and Cova, 1975). The fact that *P. brasiliensis* is a biosafety level three microorganism and has to be handled in a Biosafety Level 3 laboratory has hampered the viability assay by fluorescein-di-acetate and ethidium bromide double labelling (Restrepo *et al.*, 1982).

During the course of this work, we have also studied the influence of the physiological status of *P. brasiliensis* on its susceptibility to ATMT. Infection by *A. tumefaciens* also relies heavily on the cellular response of the host (Citovsky *et al.*, 2007). Reports on plant cells have already implicated specific cell cycle phases in host susceptibility to *A. tumefaciens* transformation (Villemont *et al.*, 1997). Therefore, we analyzed the *P. brasiliensis* competence to ATMT during specific growth phases and under distinct environmental conditions. Our results indicated that even though batch cultures growth in both rich and poor nutritional environments can lead to the formation of Hyg^R transformants, the efficiency is much higher for the first, while exponentially growing yeast cells are more susceptible to transformation than stationary phase cells. Additionally, nucleotide biosynthesis inhibition has been shown to enhance the transformation efficiency of plants, possibly related to a putative role for the transfer of purine pathway

intermediates between *A. tumefaciens* and host cells (Roberts *et al.*, 2003). Our results regarding purine and pyrimidine synthesis disruption revealed that, even though this induces a cell cycle arrest, it does not increase *P. brasiliensis* yeast cell competence to ATMT. Similarly, we have not seen an increase of ATMT to the yeast *Saccharomyces cerevisiae* upon the addition of purine synthesis inhibitors (Hooykaas *et al.*, 2006).

Altogether, our tested experimental procedures led to an overall transformation efficiency of 78±9 Hyg^R clones per co-cultivation (5±1 Hyg^R clones/10^6 target cells), higher than the ones reported previously, either for *P. brasiliensis* ATMT (34 Hyg^R clones per co-cultivation; 3.4 Hyg^R clones/10^6 target cells) or for plasmid DNA integration after electroporation (8 Hyg^R clones/μg plasmid DNA) (Leal *et al.*, 2004; Soares *et al.*, 2005). However, these previous results were based on data from only few functional experiments. Also it is important to note that transformants obtained via electroporation presented only 8% of mitotic stability in contrast with the transformants we obtained by ATMT (80% of mitotic stability).

Following optimization of the ATMT protocol, we intended to evaluate its relevance towards the development of a gene expression system in *P. brasiliensis* by generating stable green fluorescent protein *GFP*-targeted isolates. Gfp have been commonly used during the last decade as valuable fluorescent markers to study a large diversity of organisms; nonetheless prior to our study its application in *P. brasiliensis* was still unreported. Due to the scarce knowledge on genome sequences, specifically endogenous *P. brasiliensis* promoters, we evaluated *GFP* expression in yeast cells under the control of 6 regulatory control elements of several fungi. *Histoplasma capsulatum* promoter regions from calcium-binding protein (*CBP1*) (Rappleye *et al.*, 2004), small ribosomal subunit (*RPS15*), large ribosomal subunit (*RPL1B*) and translation initiation factor (*SUI1*) (C. A. Rappleye and W. E. Goldman, unpublished data) were previously used to study *GFP* expression. In addition, alcohol dehydrogenase (*ADH*; *Aspergillus flavus*) and isocitrate lyase (*ISO*; *Aspergillus fumigatus*), two well studied constitutive promoters, were also evaluated. Gfp visualization and expression were confirmed by microscopic observation, flow cytometric analysis and real-time polymerase chain reaction (RT-PCR) in isolates transformed with T-DNA harboring the *GFP* gene under the control of each promoter region. However, neither flow cytometry nor RT-PCR revealed any significant differences between the average *GFP* expression levels. As there was quite some variation in expression between different transformed cells containing the same construct, we also assessed gene copy number of the inserted *GFP* (Sullivan *et al.*, 2002). By comparison with the single copy *GP43* gene (Morais *et al.*, 2000), we

proved that only one copy of the *GFP* gene is invariably integrated into *P. brasiliensis* yeast cells as described for the majority of yeasts and fungi (Michielse *et al.*, 2005). Taking into account the multinucleated nature of *P. brasiliensis* (Almeida *et al.*, 2006) and the fact that gene copy number analysis was performed on isolates subjected to several rounds of selection one should consider the possibility that immediately upon ATMT heterokaryons may be formed. Nevertheless, Sullivan and co-workers demonstrated that ATMT of the dimorphic multinucleated fungus *Blastomyces dermatitidis* resulted in homokaryon progeny following only one round of growth in selective medium (Sullivan *et al.*, 2002). Moreover, *P. brasiliensis* multiple budding yeast cells seem to generate daughter cells with only one nucleus which most likely facilitates the generation of homokaryons after only a few rounds of selection (Almeida *et al.*, 2006).

Overall, the present work represents a step forward in the construction of a competent molecular toolbox for the study of *P. brasiliensis*. Even though the reported ATMT system does not lead to transformation efficiencies as high as those described for other yeasts and fungi it successfully generates stable homokaryon progeny, an essential trait to pursue targeted insertional mutagenesis and screening of mutants and associated phenotype. Additionally, by obtaining *GFP*-targeted isolates we demonstrate its potential as a gene expression system, thus opening the door for future studies on *P. brasiliensis* biology using fused reporter genes and providing a way to visualize gene expression *in vivo*.

CHAPTER 5 – EXPRESSION OF THE *PARACOCCIDIOIDES BRASILIENSIS CDC42* HOMOLOG IN *SACCHAROMYCES CEREVISIAE* TRIGGERS A MULTIPLE BUDDING PHENOTYPE

The results presented over this chapter:

(i) are under preparation for manuscript submission in an international peer reviewed journal,

Almeida, A.J., Cunha, C., Sampaio-Marques, B., Carmona, J.A., Carvalho, A., Steensma, H.Y., Johnson, D.I., Leão, C., Ludovico, P., Rodrigues, F. Expression of the *Paracoccidioides brasiliensis CDC42* homolog in *Saccharomyces cerevisiae* triggers a multiple budding phenotype.

(ii) were published in a conference proceeding,

Almeida, A. J., Cunha, C., Marques, B., Ludovico, P., Leão, C. and Rodrigues, F. (2006) Molecular bases for *Paracoccidioides brasiliensis* multiple budding cellular division: a role for the small Rho-like GTPase Cdc42p, at the XLI Congress of Portuguese Society of Microscopy, in Braga, Portugal (*poster presentation*).

INTRODUCTION

Paracoccidioides brasiliensis is a thermal dimorphic fungus that presents at 37°C a characteristic multiple budding yeast form (i.e., a yeast mother cell surrounded peripherally by daughter cells) associated with its multinucleated nature (Almeida *et al.*, 2006). However, to date the mechanisms that regulate nuclear/cellular division of *P. brasiliensis* are still unknown.

The budding yeast *Saccharomyces cerevisiae* has been extensively used to study cell division and growth. During vegetative growth, bud site localization and subsequent cell daughter growth is determined by specific "landmarks" imprinted on the plasma membrane close or opposite to the previous site of cytokinesis (axial and bipolar budding patterns, respectively) (Chant, 1994). "Landmark" molecules, such as the Budp proteins, are essential for marking and distributing sites for membrane growth, however deletion of *BUD* genes still leads to the formation of daughter cells at random surface locations (Nelson, 2003). On the other hand, the deletion of *CDC42*, encoding a protein of the Rho-like GTPase family, results in a isotropic growth of the mother cell, cell growth arrest at the G1 phase of the cell cycle without further progeny, and ultimately cell death (Johnson and Pringle, 1990). This highly conserved protein, initially identified during screening of temperature sensitive mutants, has been generally described as a pivotal molecule in the regulation of polarized growth of several eukaryotic cells (Johnson, 1999). Moreover, *S. cerevisiae* Cdc42p has also been associated with the coordination of bud emergence and growth, cell cycle progression, cytokinesis, actin cytoskeleton remodeling, pseudohyphal growth, vesicle dynamics and mating (Johnson, 1999; Nelson, 2003). Sccdc42p localizes to the cell periphery and internal membranes, but clusters at the incipient bud site during the G_1 phase of the cell cycle at the mother-bud neck region during and after cytokinesis and cell separation (Richman *et al.*, 2002).

Notably, site-directed and random mutagenesis of *ScCDC42* gene has led to the identification of a class of mutants that feature a hyperactive Sccdc42p, capable of overriding both temporal and spatial control of the budding process, resulting in a multiple budding phenotype similar to that observed in *P. brasiliensis* yeast cells (Caviston *et al.*, 2002; Richman and Johnson, 2000). Accordingly, this work was developed within the hypothesis that Cdc42p of *P. brasiliensis* could be involved in the multiple budding cellular division of this fungus. Due to the absence of efficient targeted mutagenesis techniques in *P. brasiliensis*, we have conducted our studies in the eukaryotic yeast model *S. cerevisiae*. In this sense, we have isolated *PbCDC42* that complements

the *S. cerevisiae* phenotype conferred by a *CDC42* null mutation. Characterization of cellular morphology, spatial organization of cell division, nuclei number and cell cycle progression along batch culture growth revealed that the isolated gene may contribute to *P. brasiliensis* multiple budding phenotypes. Furthermore, the expression of the *ScCDC42* gene in *P. brasiliensis* yeast cells does not overcome the functions of Pbcdc42p.

MATERIALS AND METHODS

Microorganisms and culture media. *Paracoccidioides brasiliensis* yeast cells, strain ATCC 60855, were maintained at 36°C by periodic subculturing in brain heart infusion supplemented with 1% glucose (BHI) solid media (1.5% wt/vol agar). For the assays carried out in this study, yeast cells were grown in BHI liquid medium, at 36°C with aeration on a mechanical shaker (200 rpm). *Saccharomyces cerevisiae CDC42/Δcdc42* (Euroscarf accession no. Y25138; Mat a/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YLR229c::kanMX4/YLR229c), was maintained in yeast extract-peptone-dextrose (YEPD) (Sambrook *et al*., 1998) solid media (2% wt/vol agar) at 26°C. For posterior assays cells were routinely grown on Yeast Nitrogen Base (YNB) selective medium supplemented with auxotrophic markers (50 histidine μg/ml, 300 leucine μg/ml, lysine 50 μg/ml, and 100 uracil μg/ml) and indicated carbon source (2% glucose or galactose). For selection purposes, gentamicin (G418) was used at a final concentration of 150 μg/ml. *Agrobacterium tumefaciens* strain LBA1100 (Beijersbergen *et al*., 1992) was used as recipient for the binary vector constructed in this study. Bacterial cells were maintained at 28°C on Luria Bertani (LB) (Sambrook *et al*., 1998) medium containing spectinomycin (250 μg/ml) and rifampicin (20 μg/ml). *Escherichia coli* XL-1-Blue strain grown at 37°C on LB medium supplemented with ampicillin (100 μg/ml) was used as host for plasmid amplification and cloning.

Cloning of P. brasiliensis PbCDC42. DNA and total RNA from exponentially growing *P. brasiliensis* yeast cultures were extracted using Trizol standard procedures (van Burik *et al*., 1998). PCR amplification on genomic DNA was performed using degenerate primers (Deg1 and Deg2, Table 1) designed based on protein sequence alignment of the Cdc42p from *S. cerevisiae* (GenBank accession number AAB67416), *Candida albicans* (ac. no. O14426), *Penicillium marneffei* (ac. no. AAK56917), *Drosophila melanogaster* (ac. no. AAD43793) and *Homo sapiens*

(ac. no. CAI19851). The sequence of the 734-bp PCR product (STAB VIDA, Oeiras, Portugal) was used to design 5 primers for *PbCDC42* isolation from the constructed cDNA library (P1-P5). Briefly, 1 μg of *P. brasiliensis* total RNA was used as template for the cDNA library construction (Smart cDNA Library Construction Kit, Clontech Laboratories, USA), following supplier recommendations. The obtained cDNA fragments were cloned into p426GALL, containing a promoter that is tightly repressed by glucose and moderated induced by galactose (Mumberg *et al.*, 1994), modified with a *Sfi*I restriction site within *BamH*I and *Xho*I sites of the polylinker region (p426GAL-*Sfi*I). For isolation of *PbCDC42* the internal *PbCDC42* primers P1-P5 were used together with 5' and 3' external p426GAL primers (P5' and P3') for PCR amplification on the cDNA library. The PCR fragments were sequenced (STAB VIDA) and assembled to obtain a final consensus sequence corresponding to the *PbCDC42* cDNA. Primers P1Pbcdc42 and P2Pbcdc42 were used to amplify the entire *PbCDC42* cDNA and the genomic fragment.

Nucleotide and protein sequence analysis. Sequence similarity searches were performed using BLAST (**http://www.ncbi.nlm.nih.gov/BLAST/**). Both nucleotide and protein sequence alignments were carried out with the DNAMAN Version 4.0 program (Lynnon BioSoft). Transcription regulatory elements were investigated using the DNAMAN program, while transcriptional start sites were sought out using the Neuronal Network Prediction Program (**http://www.fruitfly.org/seq_tools/promoter.html**). Protein sequence analysis was conducted at the PROSITE from ExPASy (**http://us.expasy.org/prosite/**).

Plasmid construction. Plasmid DNA extraction, recombinant DNA manipulations and *E. coli* transformation procedures were performed as described elsewhere (Sambrook *et al.*, 1998). The *PbCDC42* and *S. cerevisiae CDC42* (*ScCDC42*) (ac. no. U19027) coding sequences were amplified from cDNA and genomic DNA, respectively, with primer sets P3Pbcdc42-P4Pbcdc42 and P1Sccdc42-P2Sccdc42 designed with *Sfi*I restriction sites. The 680 and 576-bp amplicons were digested and inserted into the linearized p426GAL-*Sfi*I (p426GALL::*PbCDC42* and p426GALL::*ScCDC42*). Primer sets P3Pbcdc42-P4Pbcdc42 and P1Sccdc42 and P2Sccdc42 (Table 1) were designed with *Sfi*I restriction sites to isolate *PbCDC42* cDNA and introduce it into the linearized p426GAL-*Sfi*I (p426GALL::*PbCDC42*). Primers P5Pbcdc42, P6Pbcdc42, P3Sccdc42 and P4Sccdc42 engineered with *BamH*I and *Cla*I restriction sites were used for PCR reactions on p426GALL::*PbCDC42* and p426GALL::*ScCDC42*. The *BamH*I and *Cla*I digested 592

and 576-bp fragments were inserted into the plasmid pUG36 (ampR and *HIS3*) (Rodrigues *et al.*, 2001) under the control of the inducible promoter MET25. Following, both plasmids were digested with *Xba*I to remove the *yEGFP3* gene generating the plasmids pMET25::*PbCDC42* and pMET25::*ScCDC42*). The constructed plasmids were transformed into to *S. cerevisiae* diploid cells using lithium acetate experimental procedures as described elsewhere (Gietz and Woods, 2006). Sporolation, tetrad analysis and dissection of haploid segregants was performed using standard microbiology techniques (Sambrook *et al.*, 1998).

Table 1 – Primers used in this study for molecular cloning and real-time polymerase chain reaction (RT-PCR).

Primer sequences (5'-3')[a]
Deg1-GAYGGNGCNGTNGGNAARACN
Deg2-YTGNGTNARNGCNSWRCAYTC
P1-GAACTTGTTGGTGGTGTACG
P2-TATGATAGTGGTCGTAGACG
P3-AGATCCTTTGCCATACGGTC
P4-AGTTGGCCAAGCAGAAGATG
P5-TTCGAGAACGTCCGTGAGAA
P5'-CCCGGATTCTAGAACTAGTG
P3'-GGCGTGAATGTAAGCGTGAC
P1Pbcdc42-TTACGGCCGGGGGCTCCCCA
P2Pbcdc42-GAAGGGTTGGTGGGATCAGA
P3Pbcdc42-ggccattacggccTTATCCTGCTTTACATCCGC
P4Pbcdc42-ggccgcctcggccATTGTATTTTGTATCAAGGG
P5Pbcdc42-cgggatcccgCAAAATGGTTGTGGCTACTA
P6Pbcdc42-ccatcgatggTGGAAACGACTTAAAAGATG
P1Sccdc42-ggccattacggccATGCAAACGCTAAAGTGTGT
P2Sccdc42-ggccgcctcggccCTACAAAATTGCACATTTTT
P3Sccdc42-cgggatcccgATGCAAACGCTAAAGTGTGT
P4Sccdc42-ccatcgatggCTACAAAATTGCACATTTTT
P5Sccdc42-ggcgcgccATGCAAACGCTAAAGTGTGT
P6Sccdc42-ccgctcgagcggCTACAAAATTGCACATTTTT
P7Sccdc42-ggggtaccccGCGGATCACGGTATCGATGA
P8Sccdc42-ggggtaccccTGAGATGGCAAGGAGCAAC
RT-PCR
P7Pbcdc42-CGTTACATCCCCAGCATCTT
P8Pbcdc42-TCTTCTGCTTGGCCAACTTT
P9Sccdc42-GATGGTGCTGTTGGGAAAAC
P10Sccdc42-GGGTGGGGAAATAACACTGA
P1GP43-CGAAACATTGGGACACCTTT
P2GP43-CGATGACGACCCTCAGATTT
P1PDA-AGGCTACCCACGGAAAGAAT
P2PDA-CCAGCATCTTCGAGACAACA

[a] Low caps indicate restriction enzyme sequence recognition sites.

The plasmid pUR5750 (de Groot *et al.*, 1998) was used as binary vector for the insertion of the *ScCDC42* gene in *P. brasiliensis* by *A. tumefaciens*-mediated transformation (ATMT). This

plasmid contains a transferred DNA (T-DNA) harboring a Hygromycin B (HygB) resistance marker for selection in *P. brasiliensis* driven by the *Aspergillus nidulans* glyceraldehyde 3-phosphate promoter. The *ScCDC42* gene was initially inserted under the control of the calcium-binding protein (*CBP1*) promoter region in the plasmid pCR35 kindly provided by C. A. Rappleye (Department of Microbiology, Ohio State University, USA). *ScCDC42* was amplified from total DNA using primers with *Asc*I and *Xho*I restriction sites (P5Sccdc42 and P6Sccdc42). The obtained amplicon was double digested and ligated into pCR35 (pCR35::*ScCDC42*). A PCR was carried out on the plasmid pCR35::*ScCDC42* to isolate the DNA fragment harboring the *CBP1* promoter, *ScCDC42*, and terminator regions using specific primers with engineered *Kpn*I restriction sites (P7Sccdc42 and P8Sccdc42), followed by enzyme digestion and ligation into the previously linearized pUR5750. The obtained binary vector pUR5750::*ScCDC42* was mobilized to *A. tumefaciens* LBA1100 ultracompetent cells by electroporation as described previously (Dulk-Ras and Hooykaas, 1995) and transformants were isolated by kanamycin selection at 100 μg/ml.

Table 2 – Plasmids used during the course of this work.

Plasmids	Source
p426GALL	(Mumberg *et al.*, 1994)
p426GALL-*Sfi*I	This study
p426GALL::*PbCDC42*	This study
p426GALL::*ScCDC42*	This study
pUG36	(Rodrigues *et al.*, 2001)
pMET25::*PbCDC42*	This study
pMET25::*ScCDC42*	This study
pCR35	(Rappleye *et al.*, 2004)
pCR35::*ScCDC42*	This study
pUR5750	(de Groot *et al.*, 1998)
pUR5750::*ScCDC42*	This study

Photomicroscopic analysis. *S. cerevisiae* cells were grown in appropriate media for expression from GALL or MET promoters at the temperatures indicated in the Results. Cell samples were collected at defined time points, fixed in formaldehyde 3.7% for 1 h at 4°C, and washed and suspended in phosphate buffered saline (PBS), pH 7.2. Cellular morphology assays were performed by counting 300 budded cells after brief sonication. Visualization of bud scars was carried out by staining cells with 0.1 mg/ml Calcofluor (Sigma, St. Lois, MO, USA) for 20 min at room temperature. Samples were then washed and processed for examination of budding

patterns. For pulse-chase experiments, cells were grown to the mid-log phase, washed and suspended in PBS, and stained with 0.1 mg/ml FITC-conjugated Con A (Sigma) at room temperature in the dark for 15 min. Cells were then washed and resuspended in fresh media to follow up the deposition of new cell wall material. Finally, specific cell samples were stained for DNA with 4'-6-diamidino-2-phenylindole (DAPI) at a final concentration of 5 μg/ml for 20 min at 37°C. A total of 300 cells were examined for both budding pattern and nuclear localization assays. Photomicroscopic analysis was performed with an epifluorescence microscope equipped with a high-resolution DP70 digital camera and with an Olympus UPlanSApo 100X/oil objective, with a numerical aperture of 1.40 (Olympus, Melville, NY, USA).

For scanning microscopy (SEM) analysis, cell samples were collected during the late exponential growth phase, washed twice with distilled water and fixed for 22 h in 3% glutaraldehyde. Cells were then sequentially dehydrated in 60, 80 and 100% ethanol and processed for SEM observations.

Flow cytometry measurements and cell cycle analysis. All flow cytometric experiments were performed on an EPICS XL-MCL (Beckman-Coulter Corporation, Hialeah, FI, USA) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. *S. cerevisiae* cells were grown in liquid selective medium and cell samples were collected at defined times and subjected to cell cycle analysis as described elsewhere (Almeida *et al.*, 2007). A minimum of 30000 cells per sample were acquired at low flow rate and an acquisition protocol was defined to measure forward scatter (FS LOG) and side scatter (SS LOG) on a four-decade logarithmic scale and green fluorescence (FL1) on a linear scale. Offline data were analyzed with the Multigraph software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0 and the Windows Multiple Document Interface for Flow Cytometry 2.8 (WinMDI 2.8).

***ATMT of* P. brasiliensis.** *A. tumefaciens* LBA1100 carrying pUR5750::*ScCDC42* was grown overnight on LB liquid medium with antibiotics in a water bath, at 28°C with agitation (180 rpm). 1 ml of the cell culture was spun down and washed with induction medium (IM) (Bundock *et al.*, 1995) with acetosyringone (Sigma, St. Louis, Mo, USA) (200 μM) and antibiotics. Bacterial cells were diluted in IM to an OD_{660nm} of 0.30, and re-incubated at 28°C until an OD_{660nm} of approximately 0.80. *P. brasiliensis* yeast cells were grown in BHI batch cultures to the exponential growth phase. Cell samples were centrifuged (4500×*g* for 5 min), washed with IM and adjusted to a final

concentration of 1 × 10^8 cells/ml using direct microscopic counts (Neubauer counting chamber procedures). Different ratios of *A. tumefaciens* and *P. brasiliensis* cells were mixed to a final volume of 120 μl and inoculated onto sterile Hybond N membrane (Amersham Biosciences, Piscataway, NJ, USA) on solid IM for co-cultivation at 25°C for 3 days. Prior to incubation, co-cultivation plates with cellular mixtures were air dried in a safety cabinet for 30 min. Following co-cultivation, membranes were transferred to tubes with non-selective BHI liquid medium containing cefotaxime (200 μg/ml) for growth inhibition of *A. tumefaciens*, and cells were dislodged by aid of a spatula and vortexing for 1 min. The cell suspension was recovered for 48 h at 36°C, 200 rpm, before plating in selective media (HygB 50 μg/ml). Selection plates were incubated at 36°C for 15 to 20 days and monitored for colony forming ability. Randomly selected HygB resistant transformants, previously tested for mitotic stability, were selected for posterior assays.

Real-time polymerase chain reaction. Total RNA (0.5-1 μg) was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Marnes La Coquette, France). For RT-PCR quantification, 2 μl of the reverse-transcribed RNA was used as template for the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Nutley, NJ, USA) to amplify *S. cerevisiae CDC42* (234-bp) and *PDA* (177-bp) and *P. brasiliensis CDC42* (152-bp) *and GP43* (195-bp) using the primers indicated in Table 2. Real-time quantification was performed on a LightCycler System (Roche) using threshold cycle (Ct) values for *PDA* and *GP43* transcripts as the endogenous reference in *S. cerevisiae* and *P. brasiliensis*, respectively. mRNA differential *PbCDC42* and *ScCDC42* expression levels were evaluated by normalizing Ct values with the reference and comparing the ratio amongst the tested samples. Total DNA *PbCDC42* and *ScCDC42* gene copy number in *P. brasiliensis* was determined with the standard curve method (Cts plotted against logarithm of DNA copy number) (Larionov *et al.*, 2005). Results were expressed as N-fold changes in target gene copies normalized to the *GP43* reference gene. For N-fold values between 0.7 and 1.3, the tested isolates were considered to harbour a single integrated copy of the target gene (Ferreira *et al.*, 2006).

RESULTS

ISOLATION OF THE *PARACOCCIDIOIDES BRASILIENSIS PbCDC42* GENE

To isolate *CDC42* gene from *P. brasiliensis*, degenerate primers were designed from the highly conserved amino acid sequences DGAVGKT and ECSALTQ (positions 11-17 and 156-162, respectively, from Sccdc42p). A DNA fragment of 734-bp, instead of the expected 453-bp, was obtained from PCR on *P. brasiliensis* genomic DNA. The DNA sequence analysis of this fragment revealed an incomplete open reading frame (ORF) with high degree of similarity to *CDC42* ORFs from other organisms, and the presence of a 281-bp intronic region. In order to isolate the complete gene sequence together with the full length cDNA, a cDNA library of *P. brasiliensis* was constructed (materials and methods). First, the full length of the *PbCDC42* was isolated by PCR amplification of the constructed cDNA library using two set of primers (Table 1), one in the coding region (P1-P5) and another either in the promoter (P5') or terminator (P3') region of the expression plasmid. The sequence of the complete isolated cDNA fragment revealed a 579-bp ORF flanked by a 432-bp 5' untranslated region (5'UTR) and by a 315-bp 3' untranslated region (3'UTR) (Figure 1A) together with a long poly(A) tail (data not shown). To determine the transcribed genomic sequence of *PbCDC42* gene, the primers P1Pbcdc42 and P2Pbcdc42 (Table 1) were used. Sequence analysis of the obtained fragment revealed a three intron-containing gene (obeying the 5´-GT...AG-3´ acceptor and donor rule) with four exons (Daher *et al.*, 2005). Moreover, analysis of *P. brasiliensis* genome by quantitative real-time polymerase chain reaction (RT-PCR), using *GP43* as an internal control, revealed that *PbCDC42* is a single copy gene (with an N-fold of approximately 1, Table 5).

DEDUCED AMINO ACID SEQUENCE OF Pbcdc42p

The *PbCDC42* ORF encodes for a protein with 192 amino acids with a predicted molecular weight and pI of 21.36 kDa and 5.42, respectively. Sequence database search and cluster alignment showed an amino acid sequence identity ranking from 76 to 97% compared to several members of the Rho family of small GTPases (data not shown), being most closely related to the homologous gene from *Penicillium marneffei*. The major conserved domains proven necessary for the normal function of *Saccharomyces cerevisiae* counterpart (Johnson, 1999) are present in Pbcdc42p (Figure 2). The highly conserved GTP binding/hydrolysis domains were detected at positions 7-22, 57-64 and 113-120 within Pbcdc42p. Particularly, we identified a putative

ATP/GTP-binding site motif A (P-loop) – GDGAVGKT (12-19) – and the amino acid signature sequence TQXD (117-120) previously described as important for the association of Cdc42p to other effector molecules (Boyce *et al.*, 2001).

(A)

```
-412  TTACGGCCGG GGGCTCCCCA ACTGGCTTTC TCTCTCTTAT TCATCCTGTC ACGACCTCTG
-352  CTCACACAAG GGCTGCCCCA ATCTAGCTCA TCCAGGCCCA AATAGTCTTC GTCCACTCGC
-292  ACTTTCAACC TGCTTACCTC GCCCTTTGTA ACCCTCCGCT CTTTTCCTCG CGCATCCAAA
-232  TCTCTCTCTC ATCCCATCAT CCGACCACGA GTGTATTGCC GTGCCACCTG TTTCACCCAT
-172  TTCTATCTAA TCCTGCCATA TTCCATCGCT TCTCTACCGC ATCGACTCTT CTCAATCTTC
-112  CGTGAAAATC GCCCATCACA CACATCTTAT CCTAGTTGAA TCTTCTGTTT ATCCTGCTTT
-52   ACATCCGCAA TAGCCCTTTT TAGAGCTGTG TTTTCCTCCA CCAGAATCCA AA

 1    ATG GTT GTG GCT ACT ATC AAA TGC GTT GTG GTT GGA GAC GGT GCT GTC
      Met Val Val Ala Thr Ile Lys Cys Val Val Val Gly Asp Gly Ala Val

 49   GGC AAG ACA TGT CTA CTG ATT TCG TAC ACC ACC AAC AAG TTC CCC TCT
      Gly Lys Thr Cys Leu Leu Ile Ser Tyr Thr Thr Asn Lys Phe Pro Ser

 97   GAA TAC GTC CCG ACC GTC TTC GAT AAC TAT GCG GTT ACA GTG ATG ATC
      Glu Tyr Val Pro Thr Val Phe Asp Asn Tyr Ala Val Thr Val Met Ile

 145  GGA GAT GAG CCA TAT ACC CTG GGA CTC TTC GAT ACC GCA GGA CAA GAG
      Gly Asp Glu Pro Tyr Thr Leu Gly Leu Phe Asp Thr Ala Gly Gln Glu

 193  GAT TAT GAC CGT CTA CGA CCA CTA TCA TAC CCT CAA ACC GAT GTG TTT
      Asp Tyr Asp Arg Leu Arg Pro Leu Ser Tyr Pro Gln Thr Asp Val Phe

 241  CTC GTC TGT TTC TCC GTT ACA TCC CCA GCA TCT TTC GAG AAC GTC CGT
      Leu Val Cys Phe Ser Val Thr Ser Pro Ala Ser Phe Glu Asn Val Arg

 289  GAG AAA TGG TTT CCT GAG GTC CAT CAC CAC TGC CCA GGC GTT CCC TGC
      Glu Lys Trp Phe Pro Glu Val His His His Cys Pro Gly Val Pro Cys

 337  CTG ATT GTA GGC ACC CAA ACC GAC TTG CGC GAC GAC CCC AGC GTA CGA
      Leu Ile Val Gly Thr Gln Thr Asp Leu Arg Asp Asp Pro Ser Val Arg

 385  GAA AAG TTG GCC AAG CAG AAG ATG CAG CCC GTG CGA AAA GAG GAC GGT
      Glu Lys Leu Ala Lys Gln Lys Met Gln Pro Val Arg Lys Glu Asp Gly

 433  GAC CGT ATG GCA AAG GAT CTG GGT GCT GTC AAA TAC GTC GAG TGC TCC
      Asp Arg Met Ala Lys Asp Leu Gly Ala Val Lys Tyr Val Glu Cys Ser

 481  GCG TTG ACT CAA TAT AAA CTT AAG GAC GTG TTC GAT GAG GCA ATC GTC
      Ala Leu Thr Gln Tyr Lys Leu Lys Asp Val Phe Asp Glu Ala Ile Val

 529  GCT GCG CTG GAA CCA GCC CCA AGT AAG AAG CCT AAG TGT GTC ATC TTT
      Ala Ala Leu Glu Pro Ala Pro Ser Lys Lys Pro Lys Cys Val Ile Phe

 577  TAA
      ---

  +1  GTCGTTTCCA TATACTTCCC TTGATACAAA ATACAATAGA CCTATACGCA AAGCGTGGAA
 +61  TTCTAGCTTT GTCCGATTTT CGCTGTTTCG TTTGGTTGGG CGTGCGCACC CTAGATTCGG
+121  ACTCAACGTA CGTACAAACA TGGGCTCTAT TACGCAAAAG TTTTCCAGAT ATACACCAGA
+181  CAACGAGGGA GAAAGTGTTT CAATGGTCTG ATGGATGAAT TTGACGCCGA AATTTGGATT
+241  GGGGAAAGGG AAGATGGGCC GACATGGATT ATTATTTTAT CTCTTAAATC TGAATTCTGA
+301  TCCCACCAAC CCTTC
```

(B)

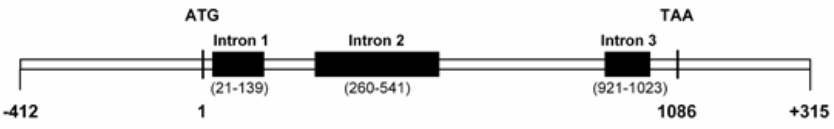

Figure 1 – Nucleotide and deduced amino acid sequence of the *P. brasiliensis* cDNA encoding the *CDC42* homolog. (A) The complete *PbCDC42* cDNA sequence contains a 579-bp open reading frame flanked upstream by a 432-bp 5' untranslated region and downstream by a 315-bp 3' untranslated region. (B) Schematic diagram of the 4 existing exons and 3 introns. Numbers in brackets indicate start and stop positions of the introns.

The effector domain of Pbcdc42p (28-53) was also shown to be highly conserved, presenting only three modifications when compared to Sccdc42p: Q29K, A32S, and D33E. Low identity was seen at the Rho insert (124-136) and membrane localization domains (186-192). As described for the Cdc42p family, Pbcdc42p possesses a putative prenyl group binding site (CAAX box) at the C-terminus (189-192) that presumably allows its association to the plasma membrane (Johnson, 1999). Additionally, Pbcdc42p sequence presents several putative protein kinase C and casein kinase II phosphorilation sites.

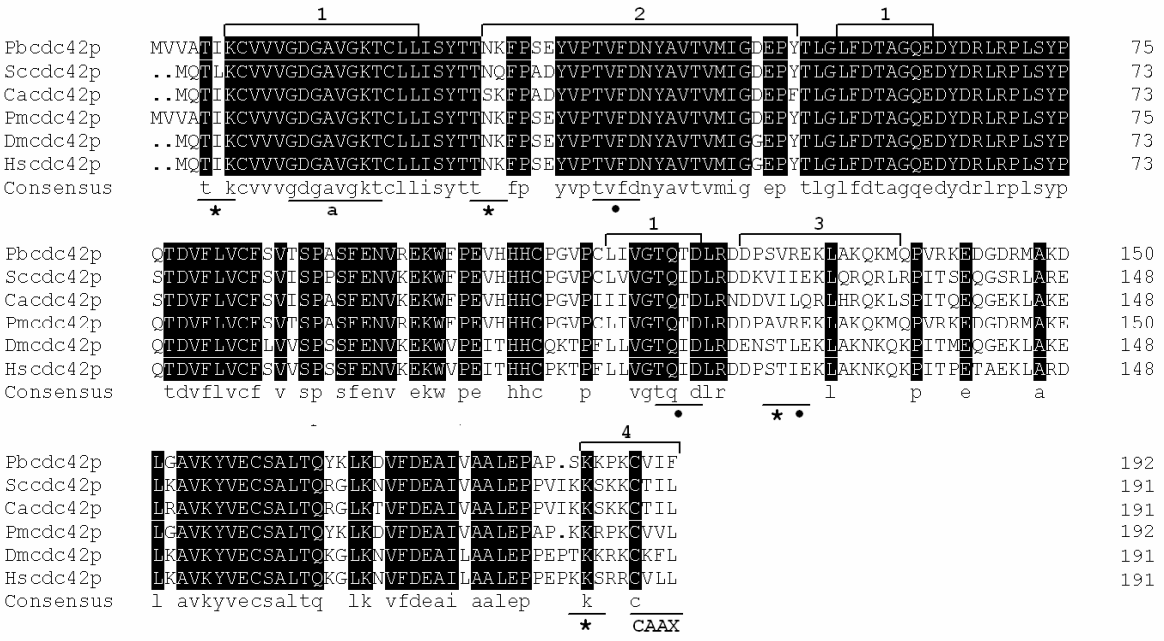

Figure 2 – Multiple sequence alignment of the deduced amino acid sequence of *P. brasiliensis CDC42* (Pbcdc42p), *S. cerevisiae CDC42* (Sccdc42p), *Candida albicans CDC42* (Cacdc42p), *Penicillium marneffei CDC42* (Pmcdc42p), *Drosophila melanogaster CDC42* (Dmcdc42p), and *Homo sapiens CDC42* (Hmcdc42p). (1) Included are the known functional domains: (1) GTP binding domains, (2) effector domain, (3) Rho insert domain, and (4) membrane localization domain. (a) Putative ATP/GTP-binding site motif A (P-loop), (*) putative protein kinase C phosphorylation sites, and (•) putative casein kinase II phosphorilation sites.

PbCDC42 EXPRESSION RESTORES CELL GROWTH OF *S. CEREVISIAE CDC42* NULL MUTANT

The absence of a functional Cdc42p in *S. cerevisiae* leads to a typical loss-of-function phenotype characterized by large round unbudded and unviable cells (Adams *et al.*, 1990; Johnson and Pringle, 1990). The rescue of cell growth at 37°C of temperature-sensitive cell-division-cycle mutant *cdc42*-1^{ts} (Miller and Johnson, 1997) by expression of *PbCDC42* cDNA revealed that the isolated sequence coded for a functional Cdc42p (data not shown). In addition, the heterozygous *S. cerevisiae CDC42*/Δ*cdc42* diploid strain transformed with either p426GALL::*PbCDC42* or the empty vector was subjected to sporulation and tetrad dissection. As expected, only two visible

colonies were obtained from each tetrad derived from cells transformed with the empty vector. On the other hand, for cells transformed with the plasmid p426GALL::*PbCDC42*, each of the 14 tetrads analyzed produced four viable spores, with a 2:2 segregation for G418 resistance and sensitivity. Further analyses revealed that the resistance to G418 co-segregated with the inability of cell growth on glucose media, caused by the glucose repression of the GALL promoter (data not shown). With the intention of characterizing cellular growth, specific growth rates at 26 and 37°C were determined for *S. cerevisiae CDC42* and Δ*cdc42* cells carrying either the *PbCDC42* (Figure 3) or *ScCDC42* (data not shown) genes. At 26°C, no differences were observed in respect to *ScCDC42* expression when compared to the expression of *PbCDC42*. Nonetheless, at 37°C Δ*cdc42*::GALL-*PbCDC42* cells presented a lower growth rate (0.166 h^{-1}) with a premature growth arrested (13.5 h) when compared to *CDC42*::GALL-*PbCDC42* cells (0.206 h^{-1}, 21.3 h). Altogether, these results showed that the *PbCDC42* gene driven by the GALL promoter is functionally complementing the *S. cerevisiae CDC42* null mutation, although only a partial complementation was observed at 37°C.

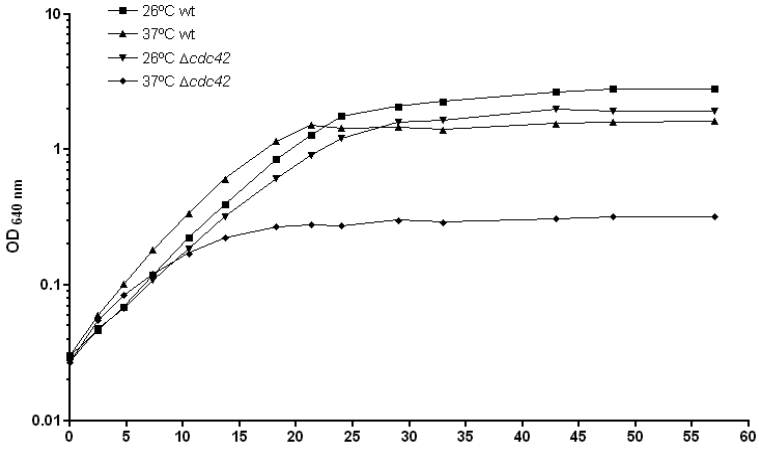

Figure 3 – Batch culture growth of *S. cerevisiae CDC42* (wt) and Δ*cdc42* cells transformed with p426GALL::*PbCDC42* in 2% galactose, YNB –Uracil liquid medium at 26 and 37°C. Representative plot of growth curves obtained by optical densitometry measurements (640 nm).

EXPRESSION OF *PbCDC42* IN *S. CEREVISIAE* INDUCES A MULTIPLE BUDDING PHENOTYPE

Four major morphologies could be seen following the expression of *PbCDC42* gene in either *wild type* or Δ*cdc42 S. cerevisiae*, at 26°C or 37°C: (i) single budded cells, (ii) multiple budding cells (more than one bud), (iii) abnormal cells (e.g., elongated cells or cells that with complete loss of polarity), and (iv) large unbudded cells (Figure 4). For quantitative analysis, we assessed cellular morphologies of both wild type and Δ*cdc42 S. cerevisiae* expressing *PbCDC42* at the distinct

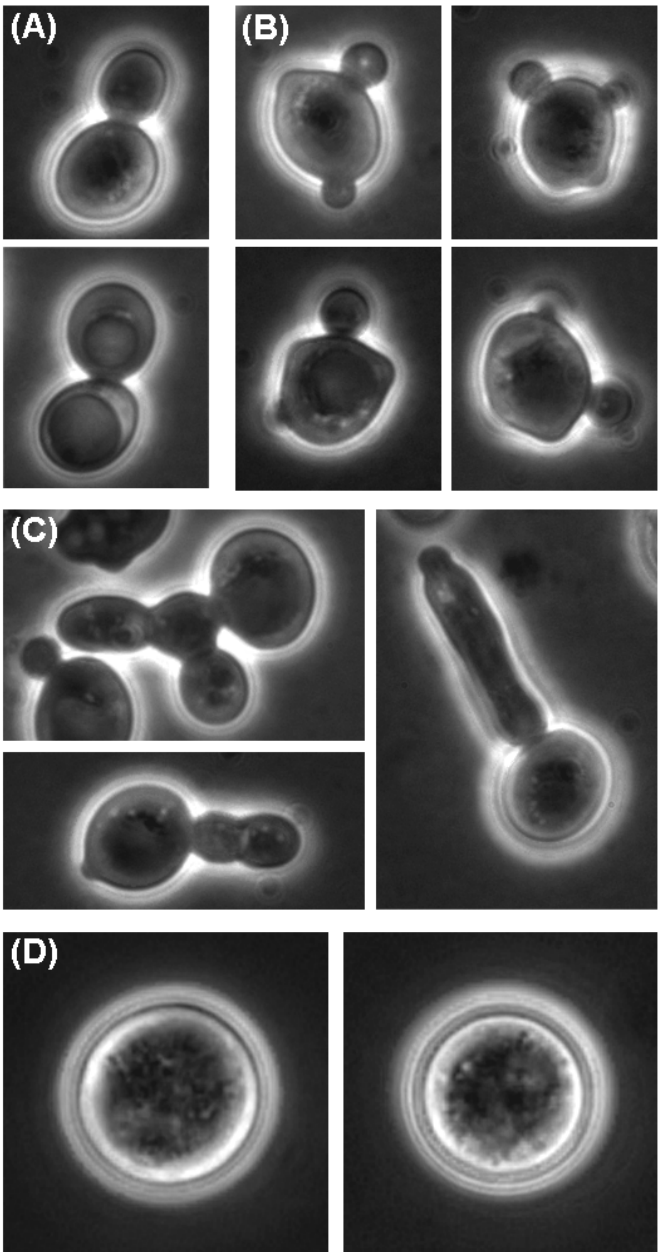

Figure 4 – Distinct cellular morphologies of *S. cerevisiae* Δ*cdc42* transformed with the *PbCDC42* gene. Included are representative images of (A) single budding cells, (B) multiple budding cells, (C) abnormal cells and (D) large round unbudded cells.

phases of batch culture growth (Figure 5). At both 26 and 37°C an increased number of abnormal cells was observed (≈50%) during growth of *CDC42*::GALL-*PbCDC42* (particularly at latter growth stages). Multiple budding cells were also observed at both temperatures, although at higher percentages in the early exponential phase at 37°C (≈10%). Regarding Δ*cdc42*::GALL-*PbCDC42* cells, at 26°C we observed a similar distribution of cellular morphologies during early phases of growth (early exponential and exponential). Nonetheless, during the late exponential and stationary growth phases, a small increase in the percentage of large round unbudded cells

was observed, although at 37°C a drastic increase of this cell population was observed (≈25% at 26°C and ≈97% at 37°C during the stationary phase). Moreover, *CDC42* and Δ*cdc42 S. cerevisiae* cells transformed with the vector carrying the endogenous *ScCDC42* gene (p426GALL::*ScCDC42*) also presented abnormal cells during batch culture growth, however to a much lesser extent (data not shown).

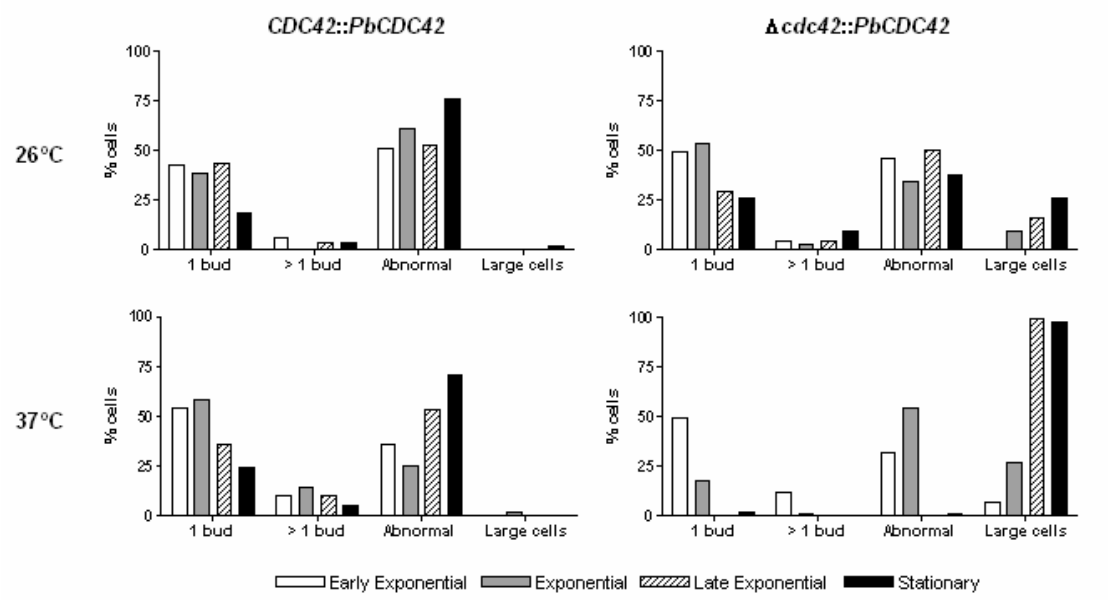

Figure 5 – Cellular morphology distribution patterns at distinct growth phases (percentage within a total of 300 budding cells) during batch culture growth of *S. cerevisiae CDC42* (wt) and Δ*cdc42* cells transformed with p426GALL::*PbCDC42* in 2% galactose, YNB –Uracil liquid medium at 26 and 37°C.

To discard the possibility that the observed morphological alterations could be due to *PbCDC42* overexpression (as well as *ScCDC42*) driven by the strong GALL promoter, a MET25 low expression promoter was used instead. Accordingly, quantitative RT-PCR analysis revealed that gene expression levels of *PbCDC42* under the control of MET25 in *S. cerevisiae* Δ*cdc42* cells were similar to those of the *ScCDC42* within its genomic environment (wild-type cells) (Table 3). At 19°C Δ*cdc42*::MET25-*PbCDC42* cells were characterized by a lower growth rate (0.181 h^{-1}) compared to 26, 30, 33 and 37°C (0.263, 0.275, 0.241, and 0.256 h^{-1}, respectively) (Figure 6A). Analysis of cellular morphologies in *S. cerevisiae ScCDC42* and Δ*cdc42* cells transformed with pMET25::*ScCDC42* or the empty vector showed over 90% of cells with only 1 bud at all temperatures (Figure 6B). Conversely, the expression of *PbCDC42*, driven by MET25 within Δ*cdc42* genetic background led to the formation of higher percentages of multiple budding cells (≈50%), rather than cells with abnormal morphology (≈10%), and a decreased number of large unbudded cells (Figure 6C).

Table 3 – Gene expression levels obtained by real-time polymerase chain reaction of the *ScCDC42* and *PbCDC42* genes in *S. cerevisiae* Δ*cdc42* cells driven by the GALL and MET25 promoter region.

	$\Delta Ct_{GALL}/\Delta Ct_{MET25}$	$\Delta Ct_{PbCDC42}/\Delta Ct_{ScCDC42}$	
	PbCDC42	**GAL**	**MET25**
***S. cerevisiae* Δ*cdc42* cells**	5.9	3.3	1.3

ScCDC42 and *PbCDC42* expression levels evaluated by normalizing Ct values with the internal reference *PDA*.

The multiple budding cells mainly presented 2 buds (only at 37°C ≈10% presented more than 2 buds). In addition, we observed that a number of buds were small and presented an underdeveloped morphology, resembling buds unable to complete cellular growth. During the late exponential phase at 26°C ≈34% of multiple budding cells presented underdeveloped buds compared to ≈8% at 37°C.

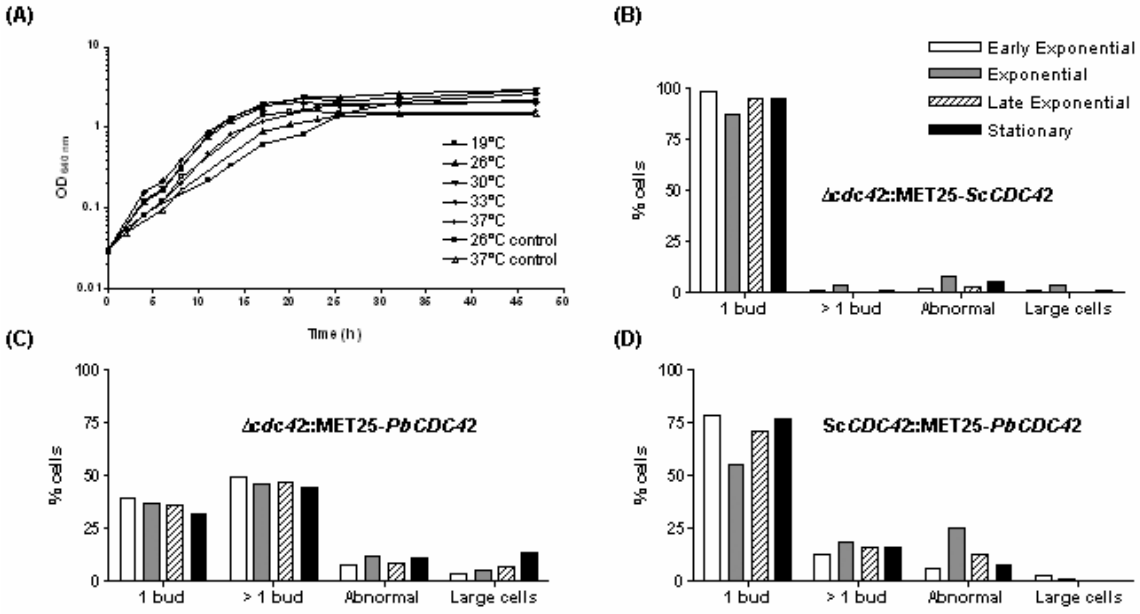

Figure 6 – Batch culture growth of *S. cerevisiae* Δ*cdc42*::MET25-*PbCDC42* cells in 2% glucose, YNB –Histidine liquid medium at 19, 26, 30, 33 and 37°C. (A) Representative plot of growth curves obtained by optical densitometry measurements (640 nm). The control represents *S. cerevisiae* Δ*cdc42* cells transformed with pMET::*ScCDC42*. Cellular morphology distribution patterns at distinct growth phases of batch culture growth at 37°C of (B) control cells, (C) *S. cerevisiae* Δ*cdc42*::MET25-*PbCDC42*, and (D) *S. cerevisiae CDC42*::MET25-*PbCDC42* (percentage within a total of 300 budding cells).

To evaluate the effect of the *P. brasiliensis* gene over *S. cerevisiae* counterpart, *PbCDC42* was expressed under the control of MET25 in *S. cerevisiae* wild type cells. The presence of the Pbcdc42p modifies to some extent normal cellular morphology of *S. cerevisiae* at temperatures ranging from 19 to 37°C (Figure 7 and Figure 6D).

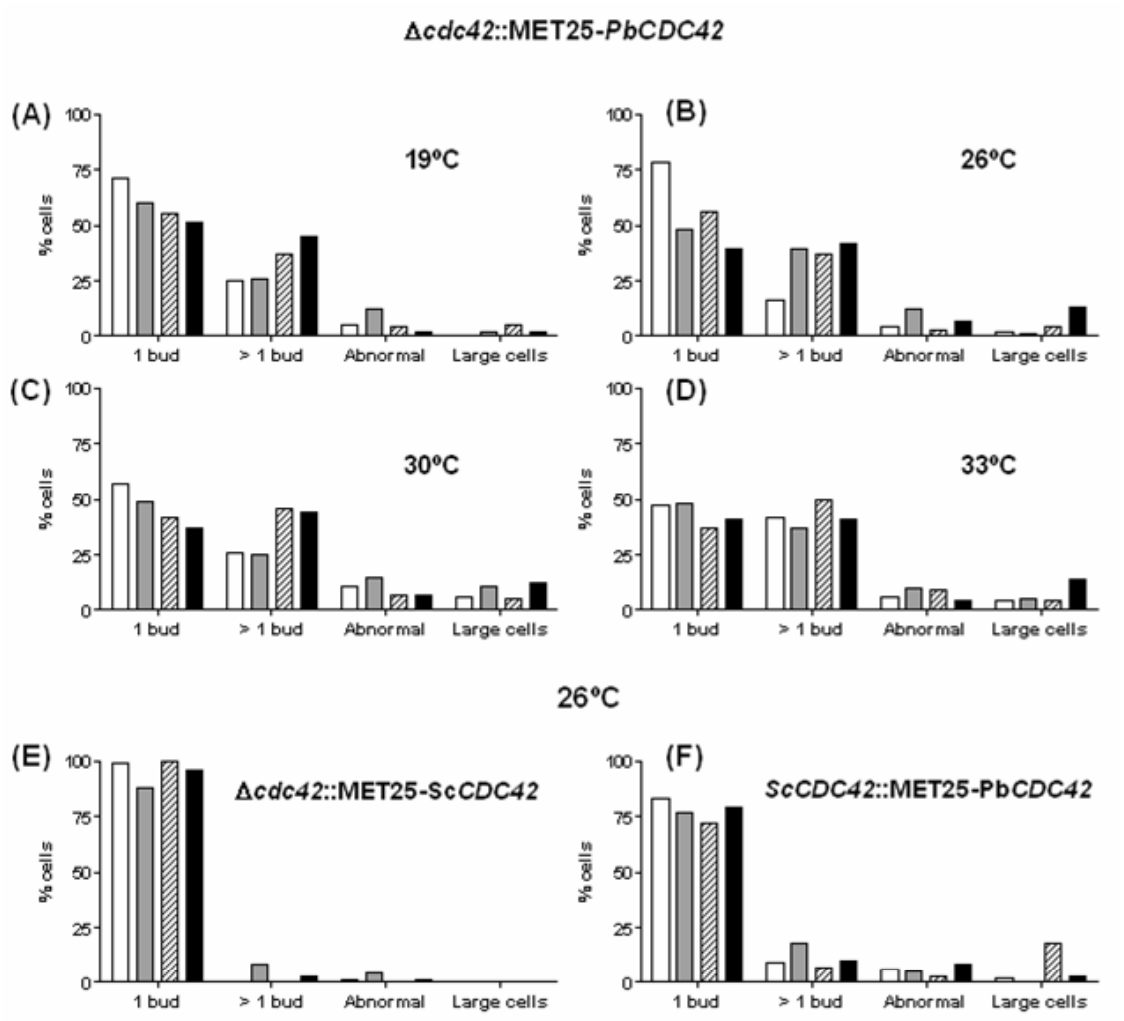

Figure 7 – Cellular morphology analysis of *S. cerevisiae* cells under batch culture growth in 2% glucose, YNB –Histidine liquid medium. Δ*cdc42*::MET25-*PbCDC42* cells grown at (A) 19°C, (B) 26°C, (C) 30°C, and (D) 33°C. (E) Δ*cdc42*::MET25-*ScCDC42* control cells grown at 26°C. (F) *ScCDC42*::MET25-*PbCDC42* cells grown at 26°C (percentage within a total of 300 budding cells).

THE Pbcdc42p OVERRIDES THE SPATIAL AND TEMPORAL CONTROL OF CELL DIVISION IN *S. CEREVISIAE* BUT DOES NOT DISRUPT DNA REPLICATION/SEGREGATION

Our results on cellular morphology analysis of *S. cerevisiae* expressing the *PbCDC42* gene suggested that at least part of the cells appeared to lack polarized selection for budding sites. Thus, we examined budding patterns both by staining bud scars with calcofluor (that specifically binds to chitin) and by performing scanning electron microscopy analysis in late exponential grown cells at the same temperatures used for morphologic analysis. As expected, ≈94% of the control cells (*S. cerevisiae* Δ*cdc42*::MET25-*ScCDC42* grown at 26°C) budded axially (Figure 8). Contrarily, over 55% of *S. cerevisiae* Δ*cdc42*::MET25-*PbCDC42* cells presented random bud site selection at all the studied temperatures (Table 4). *S. cerevisiae* wild type cells expressing

PbCDC42 also exhibited altered budding patterns at both 26 and 37°C, although to a lesser extent.

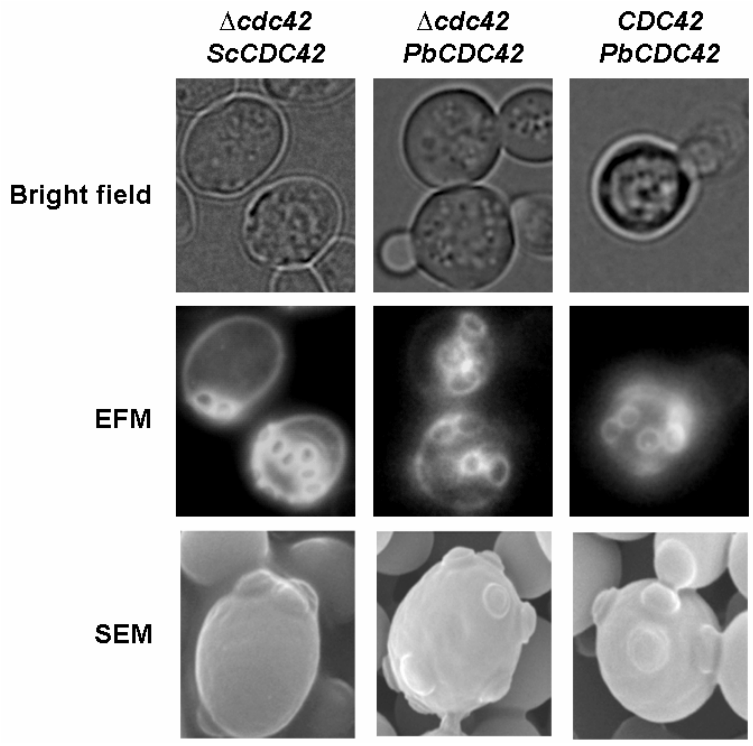

Figure 8 – Expression of *PbCDC42* in *S. cerevisiae* cells induces random bud site selection. Epifluorescence microscopy (EFM) and scanning electron microscopy (SEM) analysis of late exponentially growing *S. cerevisiae* cells from batch culture growth at 37°C: Δ*cdc42*::MET25-*ScCDC42*, Δ*cdc42*::MET25-*PbCDC42* and *CDC42*::MET25-*PbCDC42*.

Table 4 – Expression of *PbCDC42* in *S. cerevisiae CDC42* and Δ*cdc42* cells grown at 37°C. Shown are percentages of random budding at different phases of batch culture growth (percentage within a total of 300 cells).

	Δcdc42					***CDC42***	
Control 37ºC[a]	**19ºC**	**26ºC**	**30ºC**	**33ºC**	**37ºC**	**26ºC**	**37ºC**
6	58	58	57	56	55	20	27

[a] *S. cerevisiae* Δ*cdc42* cells complemented with the endogenous *ScCDC42* was used as control.

Taking into account that *P. brasiliensis* yeast form is characterized by a multiple budding and multinuclear morphology, we tested whether the observed morphological phenotypes, particularly multiple budding, were associated with alterations in nuclear replication and/or division. Early exponential, exponential, late exponential and early stationary grown *S. cerevisiae* Δ*cdc42*::MET25-*PbCDC42* cells, at 26 and 37°C, were stained for nuclear DNA with DAPI. Furthermore, pulse-labeling experiments with FITC-conjugated ConA were performed on

exponentially growing cells to monitor the formation of new buds together with nuclei visualization. Our results show that at both studied temperatures, ≈94% of the cells presented either one or two nuclei independently of its morphology or culture growth phase (Figure 9A). At 37°C, the majority of multiple budding cells presented growing buds with 1 nucleus contrary to cells grown at 26°C (showing non-enlarging buds without nuclear staining). DNA content analysis by flow cytometry revealed a differential cell cycle progression for the studied temperatures (Figure 9B). At 26°C cells mostly arrested with a single DNA content in the G_0/G_1 phases of the cell cycle when reaching the stationary growth phase, cells grown at 37°C progressively arrested with higher DNA contents.

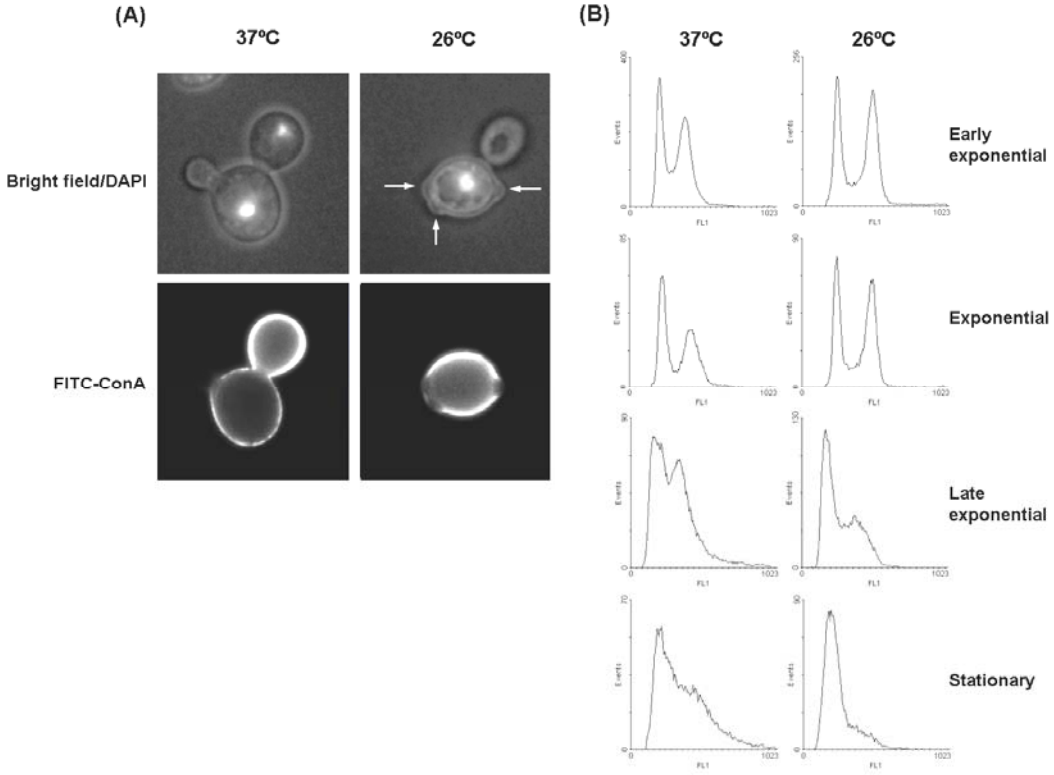

Figure 9 – *S. cerevisiae* Δ*cdc42*::MET25-*PbCDC42* cells grown in batch culture at 26 and 37°C. (A) Epifluorescence microscopy analysis of pulse-chased cells stained with FITC-ConA and DAPI (white arrows indicate underdeveloped buds). (B) Flow cytometric analysis of DNA content (representative monoparametic histograms of green fluorescence – FL1).

EXPRESSION OF *ScCDC42* IN *P. BRASILIENSIS* DOES NOT INDUCE AN ALTERED CELLULAR MORPHOLOGY

Although our results show that Pbcdc42p and Sccdc42p have identical/redundant function, all the data point for a gain of function of Pbcdc42p. In this sense, we further analyze the effect of the expression of the *ScCDC42* gene in *P. brasiliensis*. Three randomly selected *P. brasiliensis* clones, harboring the *ScCDC42* gene, were used for comparative morphologic analyses. Overall,

the results show that no significant differences in morphological features were detected during microscopic analysis of wild type and the three *P. brasiliensis* transformant cells grown in batch culture (Figure 10). Moreover, the expression levels for *ScCDC42* were lower than that for *PbCDC42* and only one copy of this exogenous gene was detected within *P. brasiliensis* genome (Table 5). The absence of morphologic alterations found in *P. brasiliensis* corroborates the effect of PbCdc42p observed in *S. cerevisiae* wild type cells.

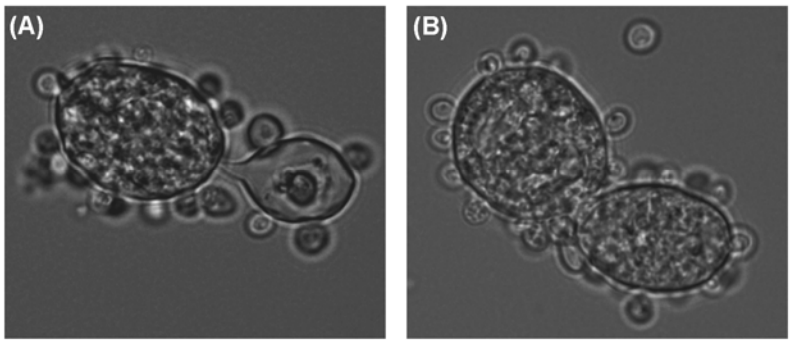

Figure 10 – *P. brasiliensis* yeast cells grown to the exponential phase of growth in BHI batch cultures. (A) Untransformed and (B) transformed cells with *ScCDC42*.

Table 5 – Gene expression levels and copy number obtained by real-time polymerase chain reaction of the *ScCDC42* and *PbCDC42* gene in *P. brasiliensis* yeast cells.

	PbCDC42		***ScCDC42***	
	$Ct_{GP43}/Ct_{PbCDC42}$ [a]	**Gene copy number** [b]	$Ct_{GP43}/Ct_{ScCDC42}$ [a]	**Gene copy number** [b]
Clone 1	1.1	1.2	0.7	0.8
Clone 2	1	1.2	0.7	0.7
Clone 3	1	1.2	0.7	1.2

[a] Average *PbCDC42* and *ScCDC42* expression levels evaluated by normalizing Ct values with the reference *GP43*.

[b] Average *PbCDC42* and *ScCDC42* gene copy number was determined with the standard curve method and normalized with *GP43* (Larionov *et al.*, 2005).

DISCUSSION

The dimorphic pathogenic fungus *Paracoccidioides brasiliensis* presents as its most distinctive feature a multiple budding phenotype. However, the molecular bases underlying this particular morphological trait are still unknown. The main objective of this study was to explore the role of the *P. brasiliensis* homolog to *S. cerevisiae CDC42* in the multiple budding cellular division process. This small Rho-like GTPase is a crucial protein in numerous cellular events of distinct

organisms (Johnson, 1999; Nelson, 2003). Studies on *cdc42* mutants have led to the identification of several distinct phenotypes regarding altered cell polarity and morphogenesis. Amino acidic point modifications both within and outside the effector's domain have been shown to induce a hyperactive Sccdc42p, overriding both spatial and temporal control of cell division characterized by the emergence of multiple buds during one mitotic cycle (Caviston *et al.*, 2002; Richman and Johnson, 2000). Mösch and co-workers (2001) also reported a group of single-amino-acid substitutions outside the effector domain that modify functional requirements for nitrogen-starved induced pseudohyphal growth of *S. cerevisiae* without affecting the regulation of cellular development in nutrient-rich conditions. Moreover, the same mutations in *Candida albicans* cause a defect in the budded-to-hyphae transition (Van den Berg *et al.*, 2004). None of these reported mutations were detected in Pbcdc42p. However, several modifications both outside and within important functional domains (namely, the Rho GTP binding, Rho Insert and effector domains) were identified and further research is needed to access their relevance in Pbcdc42p function including the molecular interaction with its effector partners. Particularly, 3 amino acids were shown to be altered in the effector domain in positions equivalent to that of Scdc42p (Q29K, A32S, and D33E), and thus their involvement in the regulation of *P. brasiliensis* multiple budding process should not be disregarded. In addition, the C-terminal CAAX box, vital for membrane localization and cell cycle specific clustering at polarized growth sites (Richman *et al.*, 2002), of Pbcdc42p presented several differences. In the context of *P. brasiliensis* multiple budding phenotype in which a mother cell may simultaneously present dozens of daughter cells, proper targeting of a functional Pbcdc42p may rely on a more stringent capacity to translocate from one intracellular site to another. Further studies using green fluorescent protein fusions may ascertain this hypothesis.

During the course of this work we show that *PbCDC42* functionally complements the lethal effect of the *CDC42* deletion in *S. cerevisiae*, with the appearance of distinct cellular morphologies, ranging from elongated to multiple budding cells. Although, previous studies have shown that altering Cdc42p levels can decisively modify the cellular morphology (Miller and Johnson, 1997), we show that the morphological alterations observed are not associated with increased levels of *PbCDC42* expression. Thus, as it has been described for several Sccdc42p mutant forms, Pbcdc42p induces in *S. cerevisiae* random bud site selection indicating a loss of spatial control of budding. Although initial assays seemed to indicate that the obtained morphological alterations were temperature independent, a deeper analysis suggested otherwise. At the stationary growth

phase, multiple budding cells at 26°C mainly seemed to present underdeveloped buds without nuclear staining, whereas at 37°C buds apparently showed normal growth with one distinguishable nucleus. Moreover, flow cytometric cell cycle analysis indicated that at 37°C cells accumulate with higher DNA contents. Overall, these results suggest that, rather than a defect in DNA replication at 26°C, Pbcdc42p function may be partially impaired, indicating a possible defect in the apical-isotropic switch (Richman *et al.*, 1999). In contrast, at 37°C Pbcdc42p seems to withstand higher functional capability and allow bud emergence and growth in tandem with the optimal growth temperature of *P. brasiliensis* yeast cells. Nonetheless, further studies are required to confirm this hypothesis.

To further elucidate the putative role of the Cdc42p in the multiple budding of *P. brasiliensis* yeast cells, we inserted the *ScCDC42* gene into *P. brasiliensis* genome by *Agrobacterium tumefaciens*-mediated transformation. Random single copy insertion of *ScCDC42* resulted in homokaryon progeny with only a single inserted gene. No differences in growth or morphology were observed. In tandem with these results, our data also showed that the expression of *PbCDC42* in *S. cerevisiae CDC42* cells induced both altered cellular morphology and random bud site selection.

This is the first report describing the function of an effector molecule involved in *P. brasiliensis* budding process. Our data clearly show an altered control of bud site selection, emergence and growth when expressing *PbCDC42* in *S. cerevisiae*. Previous work done in our laboratory has suggested that *P. brasiliensis* possesses alternative control mechanisms during cell growth in order to manage its characteristic multiple budding and multinucleated nature (Almeida *et al.*, 2006). In fact, besides its multiple budding phenotypes, *P. brasiliensis* yeast cells are also characterized by odd-like morphologies indicating a possible natural polarity control defect in this dimorphic fungus. Although Pbcdc42p seems to be an ideal candidate to study *P. brasiliensis* cellular division, the regulation of multiple budding and multinuclear divisions is likely to entail other key players. Our efforts are now directed towards the investigation of Pbcdc42p in its endogenous context. The down-regulation and the quest for interacting molecular partners in *P. brasiliensis* will undoubtedly shed new light on the relevance this protein might have in normal cell growth or even the morphogenetic switch and pathogenicity, as demonstrated for other fungi (Boyce *et al.*, 2001; Van den Berg *et al.*, 2004).

CHAPTER 6 – CONCLUDING REMARKS AND FUTURE PERSPECTIVES

For millennia, ubiquitous microorganisms as yeasts and filamentous fungi have enjoyed relatively good relations with humans. Despite their abundance and widespread distribution most specimens do not trigger disease in healthy people; however some are implicated with a broad spectrum of diseases varying from benign colonization to life-threatening situations (Ascioglu *et al.*, 2004). In fact, the pathogenicity of fungi is considered highly variable, ranging from opportunistic pathogens which usually cause infection in immunocompromised hosts (e.g., *Candida* and *Aspergillus* species) to highly pathogenic fungi capable of establishing an infection in all exposed individuals, such as the thermally dimorphic fungi *Histoplasma capsulatum*, *Coccidioides immitis* and *Paracoccidioides brasiliensis*.

P. brasiliensis is the etiological agent of paracoccidioidomycosis (PCM) the most important systemic mycosis in Central and South America (Restrepo-Moreno, 2003). In the last years, the transcriptional profiling of the dimorphic pathogenic fungus *P. brasiliensis* has provided a widespread genetic view of basic biology – ranging from metabolism to cell cycle – expanding the list of putative virulence factors or potential new drug targets (Andrade *et al.*, 2006; Bailao *et al.*, 2006; Felipe *et al.*, 2005; Ferreira *et al.*, 2006; Goldman *et al.*, 2003; Marques *et al.*, 2004). However, the absence of molecular tools for making gene disruptions in *P. brasiliensis* has hampered the functional testing of such candidates. The general aim of this thesis followed two main directives, on one hand to develop molecular tools to further elucidate biological phenomena and on the other to study *P. brasiliensis* genome content, the interaction between DNA replication, multinuclear segregation and multiple budding, and cellular differentiation.

In compliance with the primary objectives of this thesis, we developed a flow cytometric (FCM) technique for cell cycle profile analysis based on high resolution measurements of nuclear DNA (Chapter 2). The direct application of this method to *P. brasiliensis* yeast cells grown in a batch system discriminates cell subpopulations with both homogenous and heterogeneous mean fluorescence intensity, concurrent with its typical diverse cellular morphology and nuclei content. Also, we prove that this is an accurate and reliable methodology to determine the genome size of *P. brasiliensis* yeast cells and conidia, based on the DNA content of uninucleated daughter cells (Chapter 3), as demonstrated by cell sorting FCM analysis in Chapter 2. The ten studied isolates presented a genome size similar to those determined by pulsed-field gel electrophoresis (Cano *et al.*, 1998; Feitosa *et al.*, 2003; Montoya *et al.*, 1997; Montoya *et al.*, 1999). Furthermore, the ploidy of several *P. brasiliensis* isolates was assessed by comparing genome sizing by FCM with the described average haploid size obtained from electrophoretic karyotyping and by analyzing

the intra-individual variability of a highly polymorphic *P. brasiliensis* gene, *GP43*. Overall, the results showed that all analysed isolates presented a haploid, or at least aneuploid, DNA content, contrary to previous reports (Cano *et al.*, 1998; Feitosa *et al.*, 2003; Morais *et al.*, 2000), and no association was detected between genome size/ploidy and the clinical-epidemiological features of the studied isolates. In a global perspective, our findings on *P. brasiliensis* genetics/genomics provide information for the prosecution of new molecular techniques and the future research in basic cellular/molecular mechanisms of this fungus. With the advent of new data on phylogenetic and ecologic aspects of *P. brasiliensis* (Bagagli *et al.*, 2006) the screening of genome size and natural ploidy levels of numerous *P. brasiliensis* isolates may now be performed in tandem with the evaluation of clinical or environmental characteristics.

In addition, this FCM technique has allowed us to analyze the cell cycle progression of *P. brasiliensis* yeast cells under different environmental conditions and correlate, for the first time, fluctuations in DNA content with cellular morphology (Chapter 2). We show that exponentially growing cells in poor-defined or rich-complex nutritional environments present an increased percentage of daughter cells in accordance with the fungus' multiple budding. However, during the stationary growth-phase cell cycle progression in rich-complex medium was characterized by an accumulation of cells with higher DNA content or pseudohyphae-like structures, whereas in poor-defined medium arrested cells mainly displayed two DNA contents. Moreover, the fungicide benomyl was shown to induce an arrest of *P. brasiliensis* cell cycle with accumulation of cells presenting high and varying DNA contents, consistent with this fungus' unique pattern of cellular division. Altogether, our findings seem to suggest that depending on the growth conditions *P. brasiliensis* possesses alternative cell division control mechanisms to manage multiple budding and its multinucleated nature. The implications of these processes in growth during human host infection, although still an incognito, should not be overlooked. Our work has opened doors for the future investigation of the preliminary guidelines that regulate *P. brasiliensis* multiple budding, nuclear division and specific cell cycle phases, an important research field not only in basic biology but also to evaluate how this pathogenic fungus manages and/or benefits from these particular traits in the context of invasion of host tissues.

One potential application of the developed FCM protocol might be to study the distribution of cell cycle regulatory proteins, namely cyclins, within the distinct cellular subpopulations discriminated during cell cycle progression by nuclear DNA content analysis of a *P. brasiliensis* total batch culture population. In this sense, it will be possible to ascertain the cell's position in the cycle,

correlating it with the differential expression of cyclins during specific cell cycle phases, DNA content and cellular morphology of each previously sorted subpopulation.

Furthermore, we have made a first approach towards the understanding of the molecular basis of *P. brasiliensis* multiple budding phenotype. Our data clearly shows that *PbCDC42*, *P. brasiliensis* homolog of *Saccharomyces cerevisiae CDC42*, which encodes a Rho-like GTPase, has an important function in cell division control, particularly the regulation of bud emergence and growth and spatial organization (Chapter 5). As shown for certain mutant alleles of the *CDC42* gene in *S. cerevisiae* (Caviston *et al.*, 2002; Richman and Johnson, 2000), the expression of *PbCDC42* in this budding yeast leads to the formation of both abnormal and multiple budding cells. These results suggest that Pbcdc42p may play an important role in the regulation of *P. brasiliensis* multiple budding; however, the exact relevance of this molecule in cell division control, as well as other cellular events, in an endogenous context still remains unknown. In *S. cerevisiae* the Cdc42p GTPase signaling module is composed of distinct intervenient molecules such as guanine nucleotide exchange factors (GEF; e.g., Cdc24p), guanine nucleotide dissociation inhibitors (GDI; e.g., Rdi1p), GTPase-activating proteins (GAP; e.g., Rga1p), and several downstream effectors (e.g., Cla4p, Bni1p, Gic1p, and Gic2p) (Johnson, 1999). Three distinct control points for regulating Cdc42p activity and hierarchical pathway have been described, (i) the spatiotemporal recruitment of this protein to the plasma membrane and incipient bud site at specific cell cycle stages by Cdc24p and Budp "landmark" molecules; (ii) the balance between GTP/GDP bound active/inactive state by regulators of the guanine-nucleotide bound state (GEF, GDI, and GAP); (iii) and the control of the activity of downstream effectors (Nelson, 2003). The fact that active *S. cerevisiae cdc42* mutants can produce multiple random buds within the same mitotic cycle, although some merely present underdeveloped growth, indicates that these specific control points are bypassed during cellular growth (Nelson, 2003). Hypothetically, in *P. brasiliensis* a similar process may occur although one must consider the fact that in this pathogenic fungus a mother cell may simultaneously bud numerous normal growing daughter cells. The proper regulation and availability of interacting molecules of the Pbcdc42p's hierarchical regulatory pathway in accordance with this fact is in all likelihood also essential for controlling multiple budding. Currently, efforts are being made in our laboratory to silence gene expression of *Pbcdc42* in *P. brasiliensis* yeast cells and express *S. cerevisiae* Cdc42p, thus further contributing to study Pbcdc42p's role in multiple budding. The identification of putative interacting partners (using, for example, a two-hybrid system), both up- or down-stream in

Pbcdc42p's molecular pathway, will also be relevant in the elucidation of the mechanisms that rule this particular cellular division. In addition, to inquire on the relevance of Pbcdc42p in *P. brasiliensis* pathogenesis preliminary virulence studies in adult male mice, inoculating them with both *S. cerevisiae* transformed with *PbCDC42* and *P. brasiliensis* yeast cells with silenced *Pbcdc42* gene expression, may be conducted.

Data presented in Chapters 2, 3, and 5 have shed a new light on uncharted research fields in *P. brasiliensis*; nonetheless, to fully understand how DNA synthesis, nuclear segregation and budding/cell division are coordinated in this fungus extensive studies are still in need. Attending to these interconnecting phenomena the question if a strict regulation exists or not in tandem with the particular morphological traits of the fungus' yeast form arises. As shown in Chapter 2, cellular growth of this fungus seems to be dependent of the environmental nutritional status, presenting variations in cell size and form as well as in the number of buds that are produced. Our data also suggested that small daughter cells are uninucleated; however work developed in our laboratory (unpublished data) showed that during batch culture growth there is an abnormal accumulation of anucleated cells. The significance of these findings in the regulation of cell cycle events is still unclear. An interesting possibility might be to use isolated *P. brasiliensis* uninucleated haploid daughter cells by differential centrifugation, for time period pulse-chase experiments with fluorescein isothiocyanate labelled concanavalin A cell wall staining. Together with co-staining of actin filaments (e.g., phalloidin rhodamine or immunohistochemistry techniques) and nuclei (e.g., 4',6-diamidino-2-phenylindole) this would allow the follow up of both cellular and nuclear division. Following the same line of thought, the development of a nuclear green fluorescent protein (*NLS-GFP*) tagged *P. brasiliensis* isolate would also be of interest to ascertain *in vivo* nuclear division during time-lapse analysis. Moreover, the transient disruption of cytoskeletal structures of *P. brasiliensis* yeast cells using microtubule or actin depolymerising agents (e.g., nocodazole and latrunculin, respectively) may contribute to the knowledge on the regulation of nuclear and cellular division. In addition, we cannot disregard the possibility that altered regulation of the cellular/nuclear may impose advantages during the evasion of the host immune system; to clarify this hypothesis the investigation of cellular growth and characterization of nuclei content in the context of host tissue invasion is required.

As stated throughout this thesis, one of the major constraints regarding *P. brasiliensis* investigation is the absence of an efficient molecular toolbox that would allow the correlation between the studied gene expression profiles and protein function. Bearing this in mind, one of

the main objectives of this thesis was to develop an efficient transformation and gene expression system for *P. brasiliensis*. We have optimized an *Agrobacterium tumefaciens*-mediated transformation (ATMT) system for this fungus, evaluating parameters such as co-cultivation conditions and host cell susceptibility. The obtained transformation efficiencies were significantly higher than those reported for both ATMT and electroporation of *P. brasiliensis* (Leal *et al.*, 2004; Soares *et al.*, 2005). Nonetheless, distinct parameters of the developed transformation system may still be evaluated to increase efficiency, namely co-cultivation length, recovery time or the use of uninucleated yeast cells since *P. brasiliensis* multiple budding and multinucleate features is broadly considered one of its downfalls when using basic molecular biology techniques. Furthermore, *P. brasiliensis GFP*-expressing isolates were constructed by insertion of the *GFP* gene under the control of several fungal promoters. Real-time polymerase chain reaction (RT-PCR), epifluorescence microscopy and flow cytometry analysis revealed Gfp visualization for all studied promoters but without significant differences in fluorescence and gene expression levels. The identification and utilization of endogenous *P. brasiliensis* promoters will most likely increase *GFP* expression. What's more, the use of the extensive range of fluorescent tagging proteins for *in vivo* analysis is now feasible, expanding the possibilities in *P. brasiliensis* investigation. We also demonstrated single gene copy integration per genome and generation of homokaryon progeny, relevant for the future use in targeted mutagenesis and linking mutations to phenotypes.

Even though our proposed molecular tools are important for the research of *P. brasiliensis* in diverse fields, further developments are required in order to establish proficient genetic techniques for gene expression modulation and the study of functional genomics. At this point, two distinct methodologies are under consideration in our laboratory, both of which will be constructed on the backbone of *A. tumefaciens* binary vectors, thus taking advantage of the optimized ATMT system. With the aim of overriding the inexistence of suitable expression systems in *P. brasiliensis*, we intend to develop a tetracycline operator system, a strategy successfully used in other pathogenic fungi to modulate gene expression based on prokaryotic regulatory elements that respond to minute tetracycline concentrations without compromising the eukaryotic host (Garí *et al.*, 1997; Vogt *et al.*, 2005). RT-PCR for mRNA quantification and the use of fluorescence protein reporter as a gene target can be applied to evaluate this inducible promoter system. On the other hand, we also aim to develop a system for "knocking down" gene expression in *P. brasiliensis* using RNA-mediated interference (RNAi). Contrary to other allelic replacement strategies, RNAi "knocks-down" gene expression rather than "knocking-out" and has

proved to be an efficient genetic system for fungi whose homologous recombination machinery is still poorly described, such as *P. brasiliensis* (Rappleye *et al.*, 2004). Furthermore, *Neurospora crassa* orthologs for RNAi machinery were recently described in *P. brasiliensis*, opening perspectives for the successful establishment of this genetic strategy (Albuquerque *et al.*, 2005). Developmental procedures will be carried out using a *P. brasiliensis GFP*-targeted isolate developed in Chapter 4, as transformation of this reporter strain with a *GFP*-RNAi vector will allow gene silencing evaluation in a measurable fashion, essential for the validation of this technique. Additionally, as an initial candidate for gene silencing, we will use the previously identified *P. brasiliensis* 5' untranslated region of the orotidine-5'-phosphate decarboxylase gene (*PbrURA3*; AJ133782). Screening for selectable auxotrophic markers such as uracil is a priority for the development of consistent molecular genetic tools in this fungus that will allow the use of *PbrURA3* coding sequence to replace gene function on the developed strain.

In summary, the information resulting from the completion of this thesis has introduced a new line of research in *P. brasiliensis*, namely the study of the cell cycle, specifically the interaction between multiple budding and nuclear replication/division and its possible relevance in pathogenesis. In addition, the molecular tools herein developed, as well as their future prospects, have opened hopeful perspectives regarding the elucidation of the true meaning of the available information on the differential expression patterns of *P. brasiliensis* morphological transition in a biological context.

REFERENCES

Abuodeh,R.O., Orbach,M.J., Mandel,M.A., Das,A., and Galgiani,J.N. (2000) Genetic transformation of *Coccidioides immitis* facilitated by *Agrobacterium tumefaciens*. J Infect Dis 181: 2106-2110.

Adams,A.E., Johnson,D.I., Longnecker,R.M., Sloat,B.F., and Pringle,J.R. (1990) CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. J Cell Biol 111: 131-142.

Ajello,L. and Polonelli,L. (1985) Imported paracoccidioidomycosis: a public health problem in non-endemic areas. Eur J Epidemiol 1: 160-165.

Albornoz,M.B. (1971) Isolation of *Paracoccidioides brasiliensis* from rural soil in Venezuela. Sabouradia 9: 248-253.

Albuquerque,P., Baptista,A.J., Derengowsky,L.S., Procopio,L., Nicola,A.M., Arraes,F.B. *et al*. (2005) *Paracoccidioides brasiliensis* RNA biogenesis apparatus revealed by functional genome analysis. Genet Mol Res 4: 251-272.

Almeida,A.J., Martins,M., Carmona,J.A., Cano,L.E., Restrepo,A., Leao,C., *et al*. (2006) New insights into the cell cycle profile of *Paracoccidioides brasiliensis*. Fungal Genet Biol 43: 401-409.

Almeida,A.J., Matute,D.R., Carmona,J.A., Martins,M., Torres,I., McEwen,J.G. *et al*. (2007) Genome size and ploidy of *Paracoccidioides brasiliensis* reveals a haploid DNA content: Flow cytometry and GP43 sequence analysis. Fungal Genet Biol 44: 25-31.

Andrade,R.V., Da Silva,S.P., Torres,F.A., Pocas-Fonseca,M.J., Silva-Pereira,I., Maranhao,A.Q. *et al*. (2005) Overview and perspectives the transcriptome of *Paracoccidioides brasiliensis*. Rev Iberoam Micol 22: 203-212.

Andrade,R.V., Paes,H.C., Nicola,A.M., de Carvalho,M.J., Fachin,A.L., Cardoso,R.S. *et al*. (2006) Cell organization, sulphur metabolism and ion transport-related genes are differentially expressed in *Paracoccidioides brasiliensis* mycelium and yeast cells. BMC Genomics 7: 208.

André,D.C., Lopes,J.D., Franco,M.F., Vaz,C.A., and Calich,V.L. (2004) Binding of laminin to *Paracoccidioides brasiliensis* induces a less severe pulmonary paracoccidioidomycosis caused by virulent and low-virulence isolates. Microbes Infect 6: 549-558.

Andreotti,P.F., Monteiro da Silva,J.L., Bailao,A.M., Soares,C.M., Benard,G., Soares,C.P., *et al*. (2005) Isolation and partial characterization of a 30 kDa adhesin from *Paracoccidioides brasiliensis*. Microbes Infect 7: 875-881.

Angulo-Ortega,A. Calcification in paracoccidioidomycosis: are they the morphological manifestations of subclinical infections? Paracoccidioidomycosis. Proc First Pan Am Symp 129-133. 1972. Medellín, Colombia. Colombia Scient Publ.

Aristizabal,B.H., Clemons,K.V., Cock,A.M., Restrepo,A., and Stevens,D.A. (2002) Experimental *Paracoccidioides brasiliensis* infection in mice: influence of the hormonal status of the host on tissue responses. Med Mycol 40: 169-178.

Aristizabal,B.H., Clemons,K.V., Stevens,D.A., and Restrepo,A. (1998) Morphological transition of *Paracoccidioides brasiliensis* conidia to yeast cells: in vivo inhibition in females. Infect Immun 66: 5587-5591.

Ascioglu,S., Rex,J.H., de Pauw,B., Bennet,J.E., Bille,J., Crokaert,F. *et al.* (2004) Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 38: 1648-1649.

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.

Bagagli,E., Franco,M., Bosco,S.M., Hebeler-Barbosa,F., Trinca,L.A., and Montenegro,M.R. (2003) High frequency of *Paracoccidioides brasiliensis* infection in armadillos (Dasypus novemcinctus): an ecological study. Med Mycol 41: 217-223.

Bagagli,E., Sano,A., Coelho,K.I., Alquati,S., Miyaji,M., de Camargo,Z.P. *et al.* (1998) Isolation of *Paracoccidioides brasiliensis* from armadillos (*Dasypus noveminctus*) captured in an endemic area of paracoccidioidomycosis. Am J Trop Med Hyg 58: 505-512.

Bailao,A.M., Schrank,A., Borges,C.L., Dutra,V., Walquiria Inês Molinari-Madlum EE, Soares Felipe,M.S. *et al.* (2006) Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. Microbes Infect 8: 2686-2697.

Barbosa,M.S., Bao,S.N., Andreotti,P.F., de Faria,F.P., Felipe,M.S., dos Santos,F.L. *et al.* (2006) Glyceraldehyde-3-phosphate dehydrogenase of *Paracoccidioides brasiliensis* is a cell surface protein involved in fungal adhesion to extracellular matrix proteins and interaction with cells. Infect Immun 74: 382-389.

Bechtold,N. and Pelletier,G. (1998) In planta *Agrobacterium*-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. Methods Mol Biol 82: 259-266.

Beijersbergen,A., Bundock,P., Gouka,R.J., de Groot,M.J., and Hooykaas,P.J. *Agrobacterium*-mediated transformation of moulds, in particularly those belonging to the genus Aspergillus. [US6,255,115B1]. 03-07-2001.

Beijersbergen,A., Dendulkras,A., Schilperoort,R.A., and Hooykaas,P.J.J. (1992) Conjugative Transfer by the Virulence System of *Agrobacterium tumefaciens*. Science 256: 1324-1327.

Benard,G. and Duarte,A.J. (2000) Paracoccidioidomycosis: a model for evaluation of the effects of human immunodeficiency virus infection on the natural history of endemic tropical diseases. Clin Infect Dis 31: 1032-1039.

Bernander,R. (1998). Archaea and the cell cycle. Mol Microbiol 29: 955-961.

Bialek,R., Ibricevic,A., Aepinus,C., Najvar,L.K., Fothergill,A.W., Knobloch,J., *et al*. (2000) Detection of *Paracoccidioides brasiliensis* in tissue samples by a nested PCR assay. J Clin Microbiol 38: 2940-2942.

Borges-Walmsley,M.I., Chen,D., Shu,X., and Walmsley,A.R. (2002) The pathobiology of *Paracoccidioides brasiliensis*. Trends Microbiol 10: 80-87.

Bosco,S.M., Rosa,P.S., Simoes,L.B., Theodoro,R.C., Macoris,S.A., Pereira Junior,A.C. *et al*. (2005) Molecular detection of *Paracoccidioides brasiliensis* DNA in stool and tissue samples of naturally infected amadillos. Rev Inst Med Trop Sao Paulo 47 (Suppl. 14): 63.

Boyce,K.J., Hynes,M.J., and Andrianopoulos,A. (2001) The CDC42 homolog of the dimorphic fungus *Penicillium marneffei* is required for correct cell polarization during growth but not development. J Bacteriol 183: 3447-3457.

Bozzi,A., Pereira,P.P., Reis,B.S., Goulart,M.I., Pereira,M.C., Pedroso,E.P. *et al*. (2006) Interleukin-10 and tumor necrosis factor-alpha single nucleotide gene polymorphism frequency in paracoccidioidomycosis. Hum Immunol 67: 931-939.

Brummer,E., Castaneda,E., and Restrepo,A. (1993) Paracoccidioidomycosis: an update. Clin Microbiol Rev 6: 89-117.

Brummer,E., Restrepo,A., Hanson,L.H., and Stevens,D.A. (1990) Virulence of *Paracoccidioides brasiliensis*: the influence of in vitro passage and storage. Mycopathologia 109: 13-17.

Bundock,P., Dulk-Ras,A., Beijersbergen,A., and Hooykaas,P.J. (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J 14: 3206-3214.

Calich,V.L., Singer-Vermes,L.M., Russo,M., Vaz,C.A., and Burger,E. (1994) Immunogenetics in Paracoccidioidomycosis. In Paracoccidioidomycosis. Franco,M., Restrepo-Moreno,A., Lacaz,C.S., and del Negro,G.B. (eds). London: CRC Press, pp. 151-173.

Camargo,Z.P. and Franco,M. (2000) Current knowledge on pathogenesis and immunodiagnosis of paracoccidioidomycosis. Rev Iberoam Micol 17: 41-48.

Cano,M.I., Cisalpino,P.S., Galindo,I., Ramirez,J.L., Mortara,R.A., and da Silveira,J.F. (1998) Electrophoretic karyotypes and genome sizing of the pathogenic fungus *Paracoccidioides brasiliensis*. J Clin Microbiol 36: 742-747.

Carr,J. and Shearer,G. (1998) Genome size, complexity, and ploidy of the pathogenic fungus *Histoplasma capsulatum*. J Bacteriol 180: 6697-6703.

Carvalho,K.C., Ganiko,L., Batista,W.L., Morais,F.V., Marques,E.R., Goldman,G.H. *et al*. (2005) Virulence of *Paracoccidioides brasiliensis* and gp43 expression in isolates bearing known PbGP43 genotype. Microbes Infect 7: 55-65.

Carvalho,M.J., Jesuino,R.S., Daher,B.S., Silva-Pereira,I., de Freitas,S.M., Soares,C.M., *et al*. (2003) Functional and genetic characterization of calmodulin from the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis*. Fungal Genet Biol 39: 204-210.

Caviston,J.P., Tcheperegine,S.E., and Bi,E. (2002) Singularity in budding: a role for the evolutionarily conserved small GTPase Cdc42p. Proc Natl Acad Sci USA 99: 12185-12190.

Chant,J. (1994) Cell polarity in yeast. Trends Genet 10: 328-333.

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*. J Biol Chem 271: 4553-4560.

Citovsky,V., Kozlovsky,S.V., Lacroix,B., Zaltsman,A., Dafny-Yelin,M., Vyas,S. *et al.* (2007) Biological systems of the host cell involved in *Agrobacterium* infection. Cell Microbiol 9: 9-20.

Clemons,K.V., Feldman,D., and Stevens,D.A. (1989) Influence of oestradiol on protein expression and methionine utilization during morphogenesis of *Paracoccidioides brasiliensis*. J Gen Microbiol 135: 1607-1617.

Cock,A.M., Cano,L.E., Velez,D., Aristizabal,B.H., Trujillo,J., and Restrepo,A. (2000) Fibrotic sequelae in pulmonary paracoccidioidomycosis: histopathological aspects in BALB/c mice infected with viable and non-viable *Paracoccidioides brasiliensis* propagules. Rev Inst Med Trop Sao Paulo 42: 59-66.

Coltri,K.C., Casabona-Fortunato,A.S., Gennari-Cardoso,M.L., Pinzan,C.F., Ruas,L.P., Mariano,V.S. *et al.* (2006) Paracoccin, a GlcNAc-binding lectin from *Paracoccidioides brasiliensis*, binds to laminin and induces TNF-alpha production by macrophages. Microbes Infect. 8: 704-713.

Corredor,G.G., Peralta,L.A., Castano,J.H., Zuluaga,J.S., Henao,B., Arango,M. *et al.* (2005) The naked-tailed armadillo *Cabassous centralis* (Miller 1899): a new host to *Paracoccidioides brasiliensis*. Molecular identification of the isolate. Med Mycol 43: 275-280.

Cunha,D.A., Zancope-Oliveira,R.M., Sueli,M., Felipe,S., Salem-Izacc,S.M., Deepe,G.S., Jr., *et al.* (2002) Heterologous expression, purification, and immunological reactivity of a recombinant HSP60 from *Paracoccidioides brasiliensis*. Clin Diagn Lab Immunol 9: 374-377.

Daher,B.S., Venancio,E.J., de Freitas,S.M., Bao,S.N., Vianney,P.V., Andrade,R.V. *et al.* (2005) The highly expressed yeast gene pby20 from *Paracoccidioides brasiliensis* encodes a flavodoxin-like protein. Fungal Genet Biol 42: 434-443.

de Albornoz,M.B. (1971) Isolation of *Paracoccidioides brasiliensis* from rural soil in Venezuela. Sabouraudia 9: 248-253.

de Brito,T., Sandhu,G.S., Kline,B.C., Aleff,R.A., Sandoval,M.P., Santos,R.T. *et al.* (1999) In situ hybridization in paracoccidioidomycosis. Med Mycol 37: 207-211.

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

de Mattos,M.C., Mendes,R.P., Marcondes-Machado,J., Meira,D.A., Morceli,J., Pereira,P.C., *et al.* (1991) Sputum cytology in the diagnosis of pulmonary paracoccidioidomycosis. Mycopathologia 114: 187-191.

Dias,M.F., Pereira,A.C., Pereira,A., and Alves,M.S. (2000) The role of HLA antigens in the development of paracoccidioidomycosis. J Eur Acad Dermatol Venereol 14: 166-171.

Dien,B.S., Peterson,M.S., and Srienc,F. (1994). Cell-cycle analysis of *Saccharomyces cerevisiae*. Methods Cell Biol. 42 Pt B, 457-475.

Diez,S., Garcia,E.A., Pino,P.A., Botero,S., Corredor,G.G., Peralta,L.A. *et al*. (1999) PCR with *Paracoccidioides brasiliensis* specific primers: potential use in ecological studies. Rev Inst Med Trop Sao Paulo 41: 351-358.

Diez,S., Gomez,B.L., Restrepo,A., Hay,R.J., and Hamilton,A.J. (2002) *Paracoccidioides brasiliensis* 87-kilodalton antigen, a heat shock protein useful in diagnosis: characterization, purification, and detection in biopsy material via immunohistochemistry. J Clin Microbiol 40: 359-365.

Dolezel,J., Bartos,J., Voglmayr,H., and Greilhuber,J. (2003) Nuclear DNA content and genome size of trout and human. Cytometry A 51: 127-128.

Dulk-Ras,A. and Hooykaas,P.J. (1995) Electroporation of *Agrobacterium tumefaciens*. Methods Mol Biol 55: 63-72.

Edwards,M.R., Salazar,M.E., Samsonoff,W.A., Cano,L.E., Ostrander,G.A., and Restrepo,A. (1991) Electron microscopic study of conidia produced by the mycelium of *Paracoccidioides brasiliensis*. Mycopathologia 114: 169-177.

Feitosa,L.S., Cisalpino,P.S., dos Santos,M.R., Mortara,R.A., Barros,T.F., Morais,F.V. *et al*. (2003) Chromosomal polymorphism, syntenic relationships, and ploidy in the pathogenic fungus *Paracoccidioides brasiliensis*. Fungal Genet Biol 39: 60-69.

Felipe,M.S., Andrade,R.V., Arraes,F.B., Nicola,A.M., Maranhao,A.Q., Torres,F.A. *et al*. (2005) Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. J Biol Chem 280: 24706-24714.

Felipe,M.S., Andrade,R.V., Petrofeza,S.S., Maranhao,A.Q., Torres,F.A., Albuquerque,P. *et al*. (2003) Transcriptome characterization of the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis* by EST analysis. Yeast 20: 263-271.

Felipe,M.S., Torres,F.A., Maranhao,A.Q., Silva-Pereira,I., Pocas-Fonseca,M.J., Campos,E.G. *et al*. (2005) Functional genome of the human pathogenic fungus *Paracoccidioides brasiliensis*. FEMS Immunol Med Microbiol 45: 369-381.

Ferreira,I.D., Rosario,V.E., and Cravo,P.V. (2006a) Real-time quantitative PCR with SYBR Green I detection for estimating copy numbers of nine drug resistance candidate genes in *Plasmodium falciparum*. Malar J 5: 1.

Ferreira,M.E., Marques,E.R., Malavazi,I., Torres,I., Restrepo,A., Nunes,L.R. *et al*. (2006b) Transcriptome analysis and molecular studies on sulfur metabolism in the human pathogenic fungus *Paracoccidioides brasiliensis*. Mol Genet Genomics 276: 450-463.

Ferreira,M.S., Freitas,L.H., Lacaz,C.S., Del Negro,G.M., de Melo,N.T., Garcia,N.M. *et al*. (1990) Isolation and characterization of a *Paracoccidioides brasiliensis* strain from a dog food probably contaminated with soil in Uberlandia, Brazil. J Med Vet Mycol 28: 253-256.

Fortuna,M., Sousa,M.J., Corte-Real,M., Leão.C, Salvador,A., and Sansonetty,F. (2000) Cell Cycle Analysis of Yeast. In Current Protocols in Flow Cytometry. John Wiley & Sons, Inc., pp. 11.13.1-11.13.9.

Fullner,K.J. and Nester,E.W. (1996) Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. J Bacteriol 178: 1498-1504.

Futcher,B. (1993). Analysis of cell cycle in Saccharomyces cerevisiae. In The Cell Cycle - A Practical Appoach, P.Fantes and R.Broks, eds. (Oxford: Oxford Press), pp. 69-92.

Garí,E., Piedrafita,L., Aldea,M., and Herrero,E. (1997) A Set of Vectors with a Tetracycline-Regulatable Promoter System for Modulated Gene Expression in *Saccharomyces cerevisiae*. Yeast 13: 837-848.

Gelvin,S.B. (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51: 223-256.

Gesztesi,J.L., Puccia,R., Travassos,L.R., Vicentini,A.P., de Moraes,J.Z., Franco,M.F., *et al*. (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.

Gezuele,E. Aislamiento de *Paracoccidioides brasiliensis* sp de heces de pinguinode en la Antartida. (1989) Encuentro Internacional sobre Paracocidioidomicosis. Caracas.

Gietz,R.D. and Woods,R.A. (2006) Yeast transformation by the LiAc/SS Carrier DNA/PEG method. Methods Mol Biol 313: 107-120.

Goffeau,A., Barrell,B.G., Bussey,H., Davis,R.W., Dujon,B., Feldmann,H. *et al*. (1996) Life with 6000 genes. Science 274: 546, 563-546, 567.

Goldman,G.H., dos Reis,M.E., Duarte,R., de Souza Bernardes,L.A., Quiapin,A.C., Vitorelli,P.M. *et al*. (2003) Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. Eukaryot Cell 2: 34-48.

Gomes,G.M., Cisalpino,P.S., Taborda,C.P., and de Camargo,Z.P. (2000) PCR for diagnosis of paracoccidioidomycosis. J Clin Microbiol 38: 3478-3480.

Gómez,B.L., Nosanchuk,J.D., Diez,S., Youngchim,S., Aisen,P., Cano,L.E. *et al*. (2001) Detection of melanin-like pigments in the dimorphic fungal pathogen *Paracoccidioides brasiliensis* in vitro and during infection. Infect Immun 69: 5760-5767.

González,A., Gomez,B.L., Diez,S., Hernandez,O., Restrepo,A., Hamilton,A.J., *et al*. (2005a) Purification and partial characterization of a *Paracoccidioides brasiliensis* protein with capacity to bind to extracellular matrix proteins. Infect Immun 73: 2486-2495.

González,A., Gomez,B.L., Restrepo,A., Hamilton,A.J., and Cano,L.E. (2005b) Recognition of extracellular matrix proteins by *Paracoccidioides brasiliensis* yeast cells. Med Mycol 43: 637-645.

González,A., Lenzi,H.L., Motta,E.M., Caputo,L., Sahaza,J.H., Cock,A.M. *et al.* (2005c) Expression of adhesion molecules in lungs of mice infected with *Paracoccidioides brasiliensis* conidia. Microbes Infect 7: 666-673.

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

Hartwell,L.H. (1974). *Saccharomyces cerevisiae* cell cycle. Bacteriol Rev 38: 164-198.

Hartwell,L.H., Culotti,J., Pringle,J.R., and Reid,B.J. (1974). Genetic control of the cell division cycle in yeast. Science 183: 46-51.

Hata,K., Ohkusu,M., Aoki,S., Ito-Kuwa,S., Pienthaweechai,K., and Takeo,K. (2000) Cells of different ploidy are often present together in *Cryptococcus neoformans* strains. Nippon Ishinkin Gakkai Zasshi 41: 161-167.

Hebeler-Barbosa,F., Montenegro,M.R., and Bagagli,E. (2003a) Virulence profiles of ten *Paracoccidioides brasiliensis* isolates obtained from armadillos (*Dasypus novemcinctus*). Med Mycol 41: 89-96.

Hebeler-Barbosa,F., Morais,F.V., Montenegro,M.R., Kuramae,E.E., Montes,B., McEwen,J.G. *et al.* (2003b) Comparison of the sequences of the internal transcribed spacer regions and PbGP43 genes of *Paracoccidioides brasiliensis* from patients and armadillos (*Dasypus novemcinctus*). J Clin Microbiol 41: 5735-5737.

Hellens,R., Mullineaux,P., and Klee,H. (2000) Technical Focus: a guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci 5: 446-451.

Herman,P.K. (2002). Stationary phase in yeast. Curr Opin Microbiol 5: 602-607.

Hijri,M. and Sanders,I.R. (2004) The arbuscular mycorrhizal fungus *Glomus intraradice*s is haploid and has a small genome size in the lower limit of eukaryotes. Fungal Genet Biol 41: 253-261.

Hjort,K. and Bernander,R. (2001). Cell cycle regulation in the hyperthermophilic crenarchaeon *Sulfolobus acidocaldarius*. Mol Microbiol 40: 225-234.

Hoekema,A., Roelvink,P.W., Hooykaas,P.J., and Schilperoort,R.A. (1984) Delivery of T-DNA from the *Agrobacterium tumefaciens* chromosome into plant cells. EMBO J 11: 2485-2490.

Hooykaas,P.J., den Dulk-Ras,A., Bundock,P., Soltani,J., van,A.H., and van Heusden,G.P. (2006) Yeast (*Saccharomyces cerevisiae*). Methods Mol Biol 344: 465-473.

Jacobs,C.W., Adams,A.E., Szaniszlo,P.J., and Pringle,J.R. (1988). Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. J Cell Biol 107: 1409-1426.

Jimenez,M.P., Restrepo,A., Radzioch,D., Cano,L.E., and Garcia,L.F. (2006) Importance of complement 3 and mannose receptors in phagocytosis of *Paracoccidioides brasiliensis* conidia by Nramp1 congenic macrophages lines. FEMS Immunol Med Microbiol 47: 56-66.

Johnson,D.I. (1999) Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. Microbiol Mol Biol Rev 63: 54-105.

Johnson,D.I. and Pringle,J.R. (1990) Molecular characterization of CDC42, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. J Cell Biol 111: 143-152.

Kamei,K., Sano,A., Kikuchi,K., Makimura,K., Niimi,M., Suzuki,K. *et al*. (2003) The trend of imported mycoses in Japan. J Infect Chemother 9: 16-20.

Kurokawa,C.S., Lopes,C.R., Sugizaki,M.F., Kuramae,E.E., Franco,M.F., and Peracoli,M.T. (2005) Virulence profile of ten *Paracoccidioides brasiliensis* isolates: association with morphologic and genetic patterns. Rev Inst Med Trop Sao Paulo 47: 257-262.

Lacaz,C.S. (1994a) Historical evolution of the knowledge on paracoccidioidomycosis and its etiological agent, *Paracoccidioides brasiliensis*. In Paracoccidioidomycosis. Franco,M., Lacaz,C.S., Restrepo-Moreno,A., and del Negro,G.B. (eds). London: CRC Press, pp. 1-7.

Lacaz,C.S. (1994b) Mycological diagnosis. In Paracoccidioidomycosis. Franco,M., Lacaz,C.S., Restrepo-Moreno,A., and del Negro,G.B. (eds). London: CRC Press, pp. 339-344.

Lacaz,C.S., Porto,E., Martins,J.E., Heins-Vaccari,E.M., and de Melo,N.T. (2002) Paracoccidioidomicose. In Tratado de Micologia Medica Lacaz. Lacaz,C.S., Porto,E., and Martins,J.E. (eds). São Paulo: Sarvier Publishers, pp. 639-729.

Larionov,A., Krause,A., and Miller,W. (2005) A standard curve based method for relative real time PCR data processing. BMC Bioinformatics 6: 62.

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

Lengeler,K.B., Cox,G.M., and Heitman,J. (2001) Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. Infect Immun 69: 115-122.

Lindsley,M.D., Hurst,S.F., Iqbal,N.J., and Morrison,C.J. (2001) Rapid identification of dimorphic and yeast-like fungal pathogens using specific DNA probes. J Clin Microbiol 39: 3505-3511.

Magee,P.T., Gale,C., Berman,J., and Davis,D. (2003) Molecular genetic and genomic approaches to the study of medically important fungi. Infect Immun 71: 2299-2309.

Marques da Silva,S.H., Colombo,A.L., Blotta,M.H., Lopes,J.D., Queiroz-Telles,F., and Pires De,C.Z. (2003) Detection of circulating gp43 antigen in serum, cerebrospinal fluid, and bronchoalveolar lavage fluid of patients with paracoccidioidomycosis. J Clin Microbiol 41: 3675-3680.

Marques da Silva,S.H., Grosso,D.M., Lopes,J.D., Colombo,A.L., Blotta,M.H., Queiroz-Telles,F., and Camargo,Z.P. (2004) Detection of *Paracoccidioides brasiliensis* gp70 circulating antigen and follow-up of patients undergoing antimycotic therapy. J Clin Microbiol 42: 4480-4486.

Marques,E.R., Ferreira,M.E., Drummond,R.D., Felix,J.M., Menossi,M., Savoldi,M. *et al.* (2004) Identification of genes preferentially expressed in the pathogenic yeast phase of *Paracoccidioides brasiliensis*, using suppression subtraction hybridization and differential microarray analysis. Mol Genet Genomics 271: 667-677.

Marques,S.A. and Shikanai-Yasuda,M. (1994) Paracoccidioidomycosis associated with immunosuppression, AIDS, and cancer. In Paracoccidioidomycosis. Franco,M., Lacaz,C.S., Restrepo-Moreno,A., and del Negro,G.B. (eds). London: CRC Press, pp. 393-410.

Matute,D.R., McEwen,J.G., Puccia,R., Montes,B.A., San-Blas,G., Bagagli,E. *et al.* (2006) Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. Mol Biol Evol 23: 65-73.

McClelland,C.M., Chang,Y.C., and Kwon-Chung,K.J. (2005) High frequency transformation of *Cryptococcus neoformans* and *Cryptococcus gattii* by *Agrobacterium tumefaciens*. Fungal Genet Biol 42: 904-913.

McEwen,J.G., Bedoya,V., Patino,M.M., Salazar,M.E., and Restrepo,A. (1987a) Experimental murine paracoccidioidomycosis induced by the inhalation of conidia. J Med Vet Mycol 25: 165-175.

McEwen,J.G., Restrepo,B.I., Salazar,M.E., and Restrepo,A. (1987) Nuclear staining of *Paracoccidioides brasiliensis* conidia. J Med Vet Mycol 25: 343-345.

Mendes,R.P., Negroni,R., and Arechavala,A. (1994) Treatment and control of cure. In Paracoccidioidomycosis. Franco,M., Lacaz,C.S., Restrepo-Moreno,A., and del Negro,G.B. (eds). London: CRC Press, pp. 373-392.

Mendes-Giannini,M.J., del Negro,G.B., and Siqueira,A.M. (1994) Serodiagnosis. In Paracoccidioidomycosis. Franco,M., Lacaz,C.S., Restrepo-Moreno,A., and del Negro,G.B. (eds). London: CRC Press, pp. 345-363.

Mendes-Giannini,M.J., Hanna,S.A., da Silva,J.L., Andreotti,P.F., Vincenzi,L.R., Benard,G. *et al.* (2004) Invasion of epithelial mammalian cells by *Paracoccidioides brasiliensis* leads to cytoskeletal rearrangement and apoptosis of the host cell. Microbes Infect 6: 882-891.

Mendes-Giannini,M.J., Taylor,M.L., Bouchara,J.B., Burger,E., Calich,V.L., Escalante,E.D. *et al.* (2000) Pathogenesis II: fungal responses to host responses: interaction of host cells with fungi. Med Mycol 38 Suppl 1: 113-123.

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

Miller,P.J. and Johnson,D.I. (1997) Characterization of the *Saccharomyces cerevisiae cdc42*-1ts allele and new temperature-conditional-lethal cdc42 alleles. Yeast 13: 561-572.

Montenegro,M.R. and Franco,M. (1994) Pathology. In Paracoccidioidomycosis. Franco,M., Lacaz,C.S., Restrepo-Moreno,A., and del Negro,G.B. (eds). London: CRC Press, pp. 131-150.

Montoya,A.E., Alvarez,A.L., Moreno,M.N., Restrepo,A., and McEwen,J.G. (1999) Electrophoretic karyotype of environmental isolates of *Paracoccidioides brasiliensis*. Med Mycol 37: 219-222.

Montoya,A.E., Moreno,M.N., Restrepo,A., and McEwen,J.G. (1997) Electrophoretic karyotype of clinical isolates of *Paracoccidioides brasiliensis*. Fungal Genet Biol 21: 223-227.

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*. J Clin Microbiol 38: 3960-3966.

Moreira,S.F., Bailao,A.M., Barbosa,M.S., Jesuino,R.S., Felipe,M.S., Pereira,M., *et al*. (2004) Monofunctional catalase P of *Paracoccidioides brasiliensis*: identification, characterization, molecular cloning and expression analysis. Yeast 21: 173-182.

Motoyama,A.B., Venancio,E.J., Brandao,G.O., Petrofeza-Silva,S., Pereira,I.S., Soares,C.M., *et al*. (2000) Molecular identification of *Paracoccidioides brasiliensis* by PCR amplification of ribosomal DNA. J Clin Microbiol 38: 3106-3109.

Mumberg,D., Muller,R., and Funk,M. (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res 22: 5767-5768.

Naiff,R.D., Ferreira,L.C., Barrett,T.V., Naiff,M.F., and Arias,J.R. (1986) Enzootic paracoccidioidomycosis in armadillos (*Dasypus novemcinctus*) in the State of Para. Rev Inst Med Trop Sao Paulo 28: 19-27.

Nascimento,E., Martinez,R., Lopes,A.R., de Souza Bernardes,L.A., Barco,C.P., Goldman,M.H. *et al*. (2004) Detection and selection of microsatellites in the genome of *Paracoccidioides brasiliensis* as molecular markers for clinical and epidemiological studies. J Clin Microbiol 42: 5007-5014.

Negroni,P. (1966) The *Paracoccidioides brasiliensis* lives saprophytically in the soil of Argentina. Prensa Med Argent 53: 2381-2382.

Nelson,W.J. (2003) Mum, this bud's for you: where do you want it? Roles for Cdc42 in controlling bud site selection in *Saccharomyces cerevisiae*. Bioessays 25: 833-836.

Nunes,L.R., Costa de Oliveira,R., Leite,D.B., da Silva,V.S., dos Reis,M.E., da Silva Ferreira,M.E. *et al*. (2005) Transcriptome analysis of *Paracoccidioides brasiliensis* cells undergoing mycelium-to-yeast transition. Eukaryot Cell 4: 2115-2128.

Ohkusu,M., Hata,K., and Takeo,K. (2001). Bud emergence is gradually delayed from S to G2 with progression of growth phase in *Cryptococcus neoformans*. FEMS Microbiol. Lett. 194, 251-255.

Ortiz,B.L., Diez,S., Uran,M.E., Rivas,J.M., Romero,M., Caicedo,V. *et al*. (1998) Use of the 27-kilodalton recombinant protein from *Paracoccidioides brasiliensis* in serodiagnosis of paracoccidioidomycosis. Clin Diagn Lab Immunol 5: 826-830.

Pan,S. and Cole,G.T. (1992) Electrophoretic karyotypes of clinical isolates of *Coccidioides immitis*. Infect Immun 60: 4872-4880.

Paris,S., Duran-Gonzalez,S., and Mariat,F. (1985) Nutritional studies on *Paracoccidioides brasiliensis*: the role of organic sulfur in dimorphism. Sabouraudia 23: 85-92.

Perfect,J.R., Magee,B.B., and Magee,P.T. (1989) Separation of chromosomes of *Cryptococcus neoformans* by pulsed field gel electrophoresis. Infect Immun 57: 2624-2627.

Pinto,A.R., Puccia,R., Diniz,S.N., Franco,M.F., and Travassos,L.R. (2000) DNA-based vaccination against murine paracoccidioidomycosis using the gp43 gene from *Paracoccidioides brasiliensis*. Vaccine 18: 3050-3058.

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.

Puccia,R., Carmona,A.K., Gesztesi,J.L., Juliano,L., and Travassos,L.R. (1998) Exocellular proteolytic activity of *Paracoccidioides brasiliensis*: cleavage of components associated with the basement membrane. Med Mycol 36: 345-348.

Queiroz-Telles,F. (1994). *Paracoccidioides brasiliensis* Ultrastructural Findings. In Paracoccidioidomycosis, Franco,M., Lacaz,C.S., Restrepo-Moreno,A. and del Negro,G.B. eds. (London: CRC Press), pp. 27-44.

Rappleye,C.A., Engle,J.T., and Goldman,W.E. (2004) RNA interference in *Histoplasma capsulatum* demonstrates a role for alpha-(1,3)-glucan in virulence. Mol Microbiol 53: 153-165.

Reinoso,C., Nino-Vega,G., San-Blas,G., and Dominguez,A. (2003) The genome of *Paracoccidioides brasiliensis*: an overview. IV Congreso Virtual de Micologia de Hongos Patógenos en América Latina.

Reinoso,C., Nino-Vega,G., San-Blast,G., and Dominguez,A. (2005) Random sequencing of *Paracoccidioides brasiliensis* genes. Med Mycol 43: 681-689.

Restrepo,A. (2000) Morphological aspects of *Paracoccidioides brasiliensis* in lymph nodes: implications for the prolonged latency of paracoccidioidomycosis? Med Mycol 38: 317-322.

Restrepo,A. and Jimenez,B.E. (1980) Growth of *Paracoccidioides brasiliensis* yeast phase in a chemically defined culture medium. J Clin Microbiol 12: 279-281.

Restrepo,A. and Tobón,A. (2005) *Paracoccidioides brasiliensis*. In Principles and Practice of Infectious Diseases. Mandell,G.L., Bennett,J.E., and Dollin,R. (eds). Philadelphia: pp. 3062-3068.

Restrepo,A., Cano,L.E., Brummer,E., and Stevens,D.A. (1982) Comparison of various techniques for determining viability of *Paracoccidioides brasiliensis* yeast-form cells. J Clin Microbiol 16: 209-211.

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

Restrepo,A., Salazar,M.E., Cano,L.E., and Patino,M.M. (1986) A technique to collect and dislodge conidia produced by *Paracoccidioides brasiliensis* mycelial form. J Med Vet Mycol 24: 247-250.

Restrepo,A., Salazar,M.E., Cano,L.E., Stover,E.P., Feldman,D., and Stevens,D.A. (1984) Estrogens inhibit mycelium-to-yeast transformation in the fungus *Paracoccidioides brasiliensis*: implications for resistance of females to paracoccidioidomycosis. Infect Immun 46: 346-353.

Restrepo,A., Trujillo,M., and Gomez,I. (1989) Inapparent lung involvement in patients with the subacute juvenile type of paracoccidioidomycosis. Rev Inst Med Trop Sao Paulo 31: 18-22.

Restrepo,B.I., McEwen,J.G., Salazar,M.E., and Restrepo,A. (1986) Morphological development of the conidia produced by *Paracoccidioides brasiliensis* mycelial form. J Med Vet Mycol 24: 337-339.

Restrepo-Moreno,A. (1994) Ecology of *Paracoccidioides brasiliensis*. In Paracoccidioidomycosis. Franco,M., Lacaz,C.S., Restrepo-Moreno,A., and del Negro,G.B. (eds). London: CRC Press, pp. 121-128.

Restrepo-Moreno,A. (2003) Paracoccidioidomycosis. In Clinical Mycology. Dismukes,W.E., Pappas,P.G., and Sobel,J.D. (eds). New York: Oxford University Press, pp. 328-345.

Restrepo-Moreno,A. and Schneidau,J.D., Jr. (1967) Nature of the skin-reactive principle in culture filtrates prepared from *Paracoccidioides brasiliensis*. J Bacteriol 93: 1741-1748.

Ricci,G., Mota,F.T., Wakamatsu,A., Serafim,R.C., Borra,R.C., and Franco,M. (2004) Canine paracoccidioidomycosis. Med Mycol 42: 379-383.

Richman,T.J. and Johnson,D.I. (2000) *Saccharomyces cerevisiae* cdc42p GTPase is involved in preventing the recurrence of bud emergence during the cell cycle. Mol Cell Biol 20: 8548-8559.

Richman,T.J., Sawyer,M.M., and Johnson,D.I. (1999) The Cdc42p GTPase is involved in a G2/M morphogenetic checkpoint regulating the apical-isotropic switch and nuclear division in yeast. J Biol Chem 274: 16861-16870.

Richman,T.J., Sawyer,M.M., and Johnson,D.I. (2002) *Saccharomyces cerevisiae* Cdc42p localizes to cellular membranes and clusters at sites of polarized growth. Eukaryot Cell 1: 458-468.

Roberts,R.L., Metz,M., Monks,D.E., Mullaney,M.L., Hall,T., and Nester,E.W. (2003) Purine synthesis and increased *Agrobacterium tumefaciens* transformation of yeast and plants. Proc Natl Acad Sci USA 100: 6634-6639.

Rodrigues,F., Ludovico,P., Sousa,M.J., Steensma,H.Y., Corte-Real,M., and Leao,C. (2003). The spoilage yeast *Zygosaccharomyces bailii* forms mitotic spores: a screening method for haploidization. Appl Environ Microbiol 69: 649-653.

Rodrigues,F., van Hemert,M., Steensma,H.Y., Corte-Real,M., and Leao,C. (2001) Red fluorescent protein (DsRed) as a reporter in *Saccharomyces cerevisiae*. J Bacteriol 183: 3791-3794.

Ruiz-Diez,B. (2002) Strategies for the transformation of filamentous fungi. J Appl Microbiol 92: 189-195.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1998) Molecular Cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Samsonoff,W.A., Salazar,M.E., McKee,M.L., Restrepo,A., Cano,L.E., and Edwards,M.R. (1991) Scanning electron microscopy of the conidia produced by the mycelial form of *Paracoccidioides brasiliensis*. Mycopathologia 114: 9-15.

San Blas,F. (1986). Ultrastructure of spore formation in *Paracoccidioides brasiliensis*. J Med Vet Mycol 24: 203-210.

San-Blas,F. and Cova,L.J. (1975) Growth curves of the yeast-like form of *Paracoccidioides brasiliensis*. Sabouraudia 1: 22-29.

San-Blas,F., San-Blas,G., and Cova,L.J. (1976) A morphological mutant of *Paracoccidioides brasiliensis* strain IVIC Pb9. Isolation and wall characterization. J Gen Microbiol 93: 209-218.

San-Blas,G. and San-Blas,F. (1977) *Paracoccidioides brasiliensis*: cell wall structure and virulence. A review. Mycopathologia 62: 77-86.

San-Blas,G. and San-Blas,F. (1994) Biochemistry of *Paracoccidioides brasiliensis* dimorphism. In Paracoccidioidomycosis. Franco,M., Lacaz,C.S., Restrepo-Moreno,A., and del Negro,G.B. (eds). London: CRC Press, pp. 49-63.

San-Blas,G., Nino-Vega,G., and Iturriaga,T. (2002) *Paracoccidioides brasiliensis* and paracoccidioidomycosis: molecular approaches to morphogenesis, diagnosis, epidemiology, taxonomy and genetics. Med Mycol 40: 225-242.

San-Blas,G., Nino-Vega,G., Barreto,L., Hebeler-Barbosa,F., Bagagli,E., Olivero de,B.R., *et al*. (2005) Primers for clinical detection of *Paracoccidioides brasiliensis*. J Clin Microbiol 43: 4255-4257.

San-Blas,G., San-Blas,F., Ordaz,D., Centeno,S., and Albornoz,M.C. (1984) Chemical changes in cell wall structure of five strains of *Paracoccidioides brasiliensis*. Sabouraudia 22: 255-257.

Sandhu,G.S., Aleff,R.A., Kline,B.C., and da Silva,L.C. (1997) Molecular detection and identification of *Paracoccidioides brasiliensis*. J Clin Microbiol 35: 1894-1896.

Sano,A. (1998) Pathogenicities and GP43kDa gene of three *Paracoccidioides brasiliensis* isolates originated from a nine-banded armadillo (Dasypus novemcinctus). Mycopathologia 144: 61-66.

Sano,A., Tanaka,R., Yokoyama,K., Franco,M., Bagagli,E., Montenegro,M.R. *et al*. (1998) Comparison between human and armadillo *Paracoccidioides brasiliensis* isolates by random amplified polymorphic DNA analysis. Mycopathologia 143: 165-169.

Semighini,C.P., de Camargo,Z.P., Puccia,R., Goldman,M.H., and Goldman,G.H. (2002) Molecular identification of *Paracoccidioides brasiliensis* by 5' nuclease assay. Diagn Microbiol Infect Dis 44: 383-386.

Severo,L.C., Geyer,G.R., Londero,A.T., Porto,N.S., and Rizzon,C.F. (1979) The primary pulmonary lymph node complex in paracoccidioidomycosis. Mycopathologia 67: 115-118.

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

Silva-Vergara,M.L. and Martinez,R. (1998) Role of the armadillo *Dasypus novemcinctus* in the epidemiology of paracoccidioidomycosis. Mycopathologia 144: 131-133.

Silva-Vergara,M.L., Martinez,R., Camargo,Z.P., Malta,M.H., Maffei,C.M., and Chadu,J.B. (2000) Isolation of *Paracoccidioides brasiliensis* from armadillos (*Dasypus novemcinctus*) in an area where the fungus was recently isolated from soil. Med Mycol 38: 193-199.

Silva-Vergara,M.L., Martinez,R., Chadu,A., Madeira,M., Freitas-Silva,G., and Leite Maffei,C.M. (1998) Isolation of a *Paracoccidioides brasiliensis* strain from the soil of a coffee plantation in Ibiá, State of Minas Gerais, Brazil. Med Mycol 36: 37-42.

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

Stover,E.P., Schar,G., Clemons,K.V., Stevens,D.A., and Feldman,D. (1986) Estradiol-binding proteins from mycelial and yeast-form cultures of *Paracoccidioides brasiliensis*. Infect Immun 51: 199-203.

Sudbery,P., Gow,N., and Berman,J. (2004). The distinct morphogenic states of *Candida albicans*. Trends Microbiol 12: 317-324.

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

Svidzinski,T.I., Miranda Neto,M.H., Santana,R.G., Fischman,O., and Colombo,A.L. (1999) *Paracoccidioides brasiliensis* isolates obtained from patients with acute and chronic disease exhibit morphological differences after animal passage. Rev Inst Med Trop Sao Paulo 41: 279-283.

Takeo,K., Ogura,Y., Virtudazo,E., Raclavsky,V., and Kawamoto,S. (2004). Isolation of a *CDC28* homologue from *Cryptococcus neoformans* that is able to complement *cdc28* temperature-sensitive mutants of *Saccharomyces cerevisiae*. FEMS Yeast Res 4: 737-744.

Teixeira,H.C., Calich,V.L., Singer-Vermes,L.M., Imperio-Lima,M.R., and Russo,M. (1987) Experimental paracoccidioidomycosis: early immunosuppression occurs in susceptible mice after infection with pathogenic fungi. Braz J Med Biol Res 20: 587-589.

Theodoro,R.C., Candeias,J.M., Araujo,J.P., Jr., Bosco,S.M., Macoris,S.A., Padula,L.O. *et al.* (2005) Molecular detection of *Paracoccidioides brasiliensis* in soil. Med Mycol 43: 725-729.

Thrash-Bingham,C. and Gorman,J.A. (1992) DNA translocations contribute to chromosome length polymorphisms in *Candida albicans*. Curr Genet 22: 93-100.

Tobón,A.M., Agudelo,C.A., Osorio,M.L., Alvarez,D.M., Arango,M., Cano,L.E., *et al.* (2003) Residual pulmonary abnormalities in adult patients with chronic paracoccidioidomycosis: prolonged follow-up after itraconazole therapy. Clin Infect Dis 37: 898-904.

Toledo,M.S., Suzuki,E., Straus,A.H., and Takahashi,H.K. (1995) Glycolipids from *Paracoccidioides brasiliensis*. Isolation of a galactofuranose-containing glycolipid reactive with sera of patients with paracoccidioidomycosis. J Med Vet Mycol 33: 247-251.

Torres-Guerrero,H. (1999) Ploidy study in *Sporothrix schenkii*. Fungal Genet Biol 27: 49-54.

van Burik,J.A., Schreckhise,R.W., White,T.C., Bowden,R.A., and Myerson,D. (1998) Comparison of six extraction techniques for isolation of DNA from filamentous fungi. Med Mycol 36: 299-303.

Van Damme,P.A., Bierenbroodspot,F., Telgtt,D.S., Kwakman,J.M., De Wilde,P.C., and Meis,J.F. (2006) A case of imported paracoccidioidomycosis: an awkward infection in The Netherlands. Med Mycol 44: 13-18.

Van den Berg,A.L., Ibrahim,A.S., Edwards,J.E., Jr., Toenjes,K.A., and Johnson,D.I. (2004) Cdc42p GTPase regulates the budded-to-hyphal-form transition and expression of hypha-specific transcripts in *Candida albicans*. Eukaryot Cell 3: 724-734.

Vicentini,A.P., Gesztesi,J.L., Franco,M.F., de,S.W., de Moraes,J.Z., Travassos,L.R., *et al*. (1994) Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. Infect Immun 62: 1465-1469.

Villar,L.A. and Restrepo,A. (1989) Virulence of a variant of *Paracoccidioides brasiliensis* that exists in the yeast form at room temperature. J Med Vet Mycol 27: 141-148.

Villemont,E., Dubois,F., Sangwan,R.S., Vasseur,G., Bourgeois,Y., and Sangwan Norreel,B.S. (1997) Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: Evidence of an S-phase control mechanism for T-DNA transfer. Planta 201: 160-172.

Vogt,K., Bhabhra,R., Rhodes,J.C., and Askew,D.S. (2005) Doxycycline-regulated gene expression in the opportunistic fungal pathogen *Aspergillus fumigatus*. BMC Microbiology 5[1].

Wanke,B. and Londero,A.T. (1994) Epidemiology and Paracoccidioidomycosis infection. In Paracoccidioidomycosis. Franco,M., Lacaz,C.S., Restrepo-Moreno,A., and del Negro,G.B. (eds). London: CRC Press, pp. 109-117.

Winson,M.K. and Davey,H.M. (2000) Flow cytometric analysis of microorganisms. Methods 21: 231-240.

Wood,J.S. (1982a). Genetic effects of methyl benzimidazole-2-yl-carbamate on *Saccharomyces cerevisiae*. Mol Cell Biol 2: 1064-1079.

Wood,J.S. (1982b). Mitotic chromosome loss induced by methyl benzimidazole-2-yl-carbamate as a rapid mapping method in *Saccharomyces cerevisiae*. Mol Cell Biol 2: 1080-1087.

Zeyl,C. (2004) Experimental studies on ploidy evolution in yeast. FEMS Microbiol Lett 233: 187-192.