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Mestrado em Genética Molecular

Trabalho efectuado sob a orientação de:
Orientador: Professor Doutor Fernando Rodrigues
Co-orientador: Professor Doutor Agostinho Almeida

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ABSTRACT

Paracoccidioides brasiliensis, a thermal dimorphic fungus, is the etiological agent of paracoccidioidomycosis, the most common systemic mycosis affecting the rural population of Latin America. During the last decade, molecular approaches have allowed a broader insight into *P. brasiliensis*. However, knowledge regarding fundamental biology of this fungus has been greatly hampered by the absence of appropriate genetic tools. We present results concerning the development of a strategy for gene targeted mutagenesis that could increase the probability of homologous recombination (HR) in *P. brasiliensis*, using antisense RNA (aRNA) technology to down-regulate the *KU80* gene, a key component of the nonhomologous end joining (NHEJ) pathway. However, the aRNAs developed within this work were not able to downregulate *PbKU80*'s expression, thus other targeted sequences should be employed. The present work also focuses on the analysis of different morphological features (mother cell size and form; bud number, size and form) and possible correlations that might exist among them, and between the expression of the Rho-like GTPase Cdc42 (Pbcdc42p), a pivotal molecule in cellular division and shape. We used real-time PCR (RT-PCR) to determine *PbCDC42* transcript levels and stereomicroscopy (by contour measurements of 150 mother cells and buds) for morphological analysis of exponentially growing batch culture yeast cells from the 3 described cryptic species (S1, PS2, and PS3), using both clinical and environmental isolates. We show that cell size and cell form of both the mother and bud cells are very heterogeneous both within the same strain and among the strains. Regarding bud number per mother cell, most strains showed no predominance or budding pattern. Moreover, no associations were detected between mother cell and bud cell size and form, suggesting that *P. brasiliensis* yeast cells do not obey the critical mass rule between mother and daughter cells conversely to other yeast cells like *Saccharomyces cerevisiae*. Interestingly, expression of *PbCDC42*, although heterogeneous among the groups of the 3 cryptic species, seems to show a negative correlation with bud number in the tested strains, but not with any of the other analyzed morphological features. Altogether, this study provides a quantitative assessment of morphological traits of *P. brasiliensis* yeast cells, supporting the hypothesis that *P. brasiliensis* does not follow standard rules of cell growth.

RESUMO

Paracoccidioides brasiliensis, um fungo termo-dimórfico, é o agente etiológico da paracoccidioidomicose, a micose sistémica mais comum que afecta a população rural da América Latina. Durante a última década, abordagens moleculares têm permitido uma visão mais ampla sobre *P. brasiliensis*. No entanto, o conhecimento acerca da biologia fundamental deste fungo tem sido dificultado pela ausência de ferramentas moleculares adequadas. Neste trabalho apresentamos resultados relativos ao desenvolvimento de uma estratégia para mutagénese dirigida que poderia aumentar a probabilidade de recombinação homóloga (RH) em *P. brasiliensis*, aplicando a tecnologia de RNA antisense (aRNA) para diminuir a expressão do gene *KU80*, um interveniente chave na via nonhomologous end joining (NHEJ). No entanto, as moléculas antisense desenvolvidas no âmbito deste trabalho não foram capazes de reduzir a expressão de *PbKU80*, assim, outras sequências antisense deverão ser desenvolvidas. O presente trabalho também se debruçou na análise de diferentes características morfológicas (tamanho e forma da célula mãe; número, tamanho e forma das gémulas) e possível correlação que possa existir entre elas, e entre a expressão da Rho-like GTPase CDC42 (Pbcdc42p), uma molécula crucial na forma e divisão celulares. Usámos a técnica de PCR em tempo real para determinar os níveis de expressão de *PbCDC42* e a estereomicroscopia (por medição do contorno de 150 células mãe e suas gémulas) para a análise morfológica de células de levedura em crescimento exponencial pertencentes a 3 espécies crípticas descritas (S1, PS2 e PS3), usando tanto isolados clínicos como ambientais. Mostrámos que tanto o tamanho como a forma de ambas as células mãe e respectivas gémulas são muito heterogéneos, tanto para a mesma estirpe, como entre estirpes diferentes. Quanto ao número de gémulas por célula mãe, na maioria das estirpes não se verificou qualquer predominância ou padrão de gemulação. Além disso, não foram encontradas associações entre o tamanho e a forma das células mãe e respectivas gémulas, sugerindo que as células leveduriformes de *P. brasiliensis* não obedecem ao modelo ideal de gemulação exibido entre célula mãe e gémula, ao contrário de outras leveduras como *Saccharomyces cerevisiae*. Curiosamente, a expressão de *PbCDC42*, embora heterogénea entre as 3 espécies crípticas, parece apresentar uma correlação negativa com o número de gémulas nas estirpes testadas, mas não com qualquer das outras características morfológicas analisadas. Em suma, este estudo faculta uma avaliação quantitativa das características morfológicas das células leveduriformes de *P. brasiliensis*, apoiando a hipótese de que este fungo dimórfico não segue as regras standard do crescimento celular.

ÍNDICE

Chapter 1

General Introduction

1.1 *Paracoccidioides brasiliensis*: A Brief Overview

1.1.1 Paracoccidioidomycosis: Biological Agent and Disease

Fungi are ubiquitous organisms occurring in almost all habitats. While more than one hundred thousand fungal species are known to this date, only a minority is responsible for causing disease in mammals and, particularly, in humans. When inside the host, morphological transitions are frequently required to express a certain degree of virulence in particular fungi. Notable among these important dimorphic fungal pathogens are *Candida albicans*, *Histoplasma capsulatum*, *Cryptococcus neoformans* and *Paracoccidioides brasiliensis* whose morphogenetic processes are the subjects of intense studies (Momany 2005, Berman 2006).

P. brasiliensis grows as a saprophytic mycelium at environmental temperatures while at the temperature of the infected host it switches to the pathogenic yeast form. Adolfo Lutz was the first to isolate this fungus in 1908, officially recognized in 1971 as the causative agent of paracoccidioidomycosis (PCM), one of the most frequent systemic mycoses affecting the rural population of Latin America (Lutz 1908, Restrepo and Tobón 2005).

To understand the pathophysiology of any infectious agent, one key piece of knowledge is the determination of its habitat. In the case of *P. brasiliensis*, its precise ecological micro-niche remains undefined, mainly due to the difficulty to isolate *P. brasiliensis* mycelia form (Montenegro and Franco 1994). It is important to emphasize that the PCM "reservarea", i.e., the location where the pathogen habitat and infection coincide, is influenced by several abiotic factors such as water availability (Simões *et al.* 2004). Epidemiologic studies suggest that PCM's endemic area is located in regions with a certain ecological characteristics which the following factors favor the presence of *P. brasiliensis*: temperatures between 17 to 24 °C, high humidity with annual pluviosities ranging from 500 to 2,500 mm^3, abundant forests and watercourses, short winters and rainy summers (Brummer *et al.* 1993, Restrepo-Moreno 2003). Therefore, *P. brasiliensis'* microenvironment is normally defined as exogenous to humans with several pieces of evidence pointing out the soil of humid tropical and subtropical forests of Central and South America as its most probable habitat (Hogan *et al.* 1996). Consistently, *P. brasiliensis* has frequently been isolated from the nine-banded armadillo *Dasypus novemcinctus* in Brazil (Bagagli *et al.* 1998, Bagagli *et al.* 2003; Silva-Vergara and Martinez 1999, Silva-Vergara *et al.* 2000) and from the naked-tailed armadillo *Cabassous centralis* in Colombia (Corredor *et al.* 1999, Restrepo *et al.* 2000). It was found in 75-100 % of armadillos from hyperendemic PCM areas (Bagagli *et al.* 1998,

Bagagli *et al.* 2003, Restrepo *et al.* 2000), and was recovered from young and older adult animals with no sign of disease (Bagagli *et al.* 1998, Bagagli *et al.* 2003). By having a low body temperature, ranging from 32.7 to 35.3ºC (Boily 2002), a weak immunological response system (Purtilo at al. 1975) and constant putative contact with *P. brasiliensis* in the soil, armadillos may acquire the pathogen repeatedly. The demonstration of a high incidence of *P. brasiliensis* in these animals has opened new perspectives for comprehending this pathogen's ecology and evolution. It is becoming clear that this group of fungal pathogens has evolved to a lifestyle associated with animal hosts, with the maintenance of the saprobic phase in the environment. This reinforces the hypothesis that parasitism could not be considered a blind alley, but rather a two-way street for dimorphic pathogenic fungi, with the preservation of the saprobic form-coupled with a parasitic form in animals (Bagagli *et al.* 2006).

Although for many years classic systematic has classified *P. brasiliensis* as an imperfect fungus within the phylum Deuteromycota in the class of Hyphomycetes mainly due to lack of identification of sexual structures (San-Blas *et al.* 2002) recent advance of molecular methodologies (including caryotyping, multilocus enzyme electrophoresis, RAPD, RFLP among others; Bowman *et al.* 1996) based on epidemiological typing and population genetics have proved helpful in the resolution of taxonomic ambiguity. Phylogenetic comparisons based on the rDNA 28S ribosomal subunit, has more precisely placed *P. brasiliensis* as belonging in the order Onygenales, family Onygenaceae (phylum Ascomycota), together with other human pathogenic fungi such as *Blastomyces dermatitidis, Histoplasma capsulatum, and Coccidioides immitis* (Leclerc *et al.* 1994, Bagagli *et al.* 2006). Recently, based on phylogenetic analysis of 65 isolates, three different phylogenetic species (S1, PS2, and PS3) of *P. brasiliensis* were recognized using a combined data set of eight regions in five nuclear loci (Matute *et al.* 2006) (Figure 1).

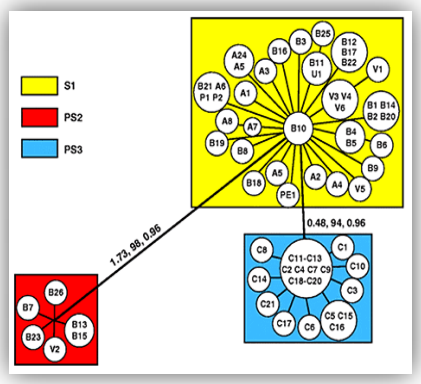

Figure 1. Unrooted tree showing the partitions found in *P. brasiliensis*, based on combined data of five nuclear loci obtained with weighted maximum parsimony. S1 consists of a major group of isolates from Brazil, Argentina, Venezuela, Peru and Paraguay, PS2 represents a cryptic phylogenetic species of *P. brasiliensis* (so far, six isolates from Brazil and Venezuela) and PS3 forms a separate group of Colombian isolates. The values above the branches represent their individual support: the first is the tree length, the second is the weighted high bootstrap, and the third is the posterior probabilities (adapted from Matute *et al.* 2006).

In addition, a clade of 17 genotypically similar isolates, including Pb01, a *P. brasiliensis* isolate that has been the subject of intense molecular studies for many years, was shown to exhibit great genomic and morphological divergence from the S1/PS2/PS3 species clade. This "Pb01-like" group was even recommended to be considered a new phylogenetic species, since it is strongly supported by all independent and concatenated genealogies, increasing enormously the genomic variation known in the *Paracoccidioides* genus (Carrero *et al.* 2008, Teixeira *et al.* 2009).

PCM has been found in 14 Latin American countries, with the highest incidence in Brazil (80%) followed by Venezuela and Colombia (Figure 2). Over 10 million people are estimated to be infected by this fungus but only up to 2% of them develop the disease (McEwen *et al.* 1995). Notheless, mainly due to world globalization and intense migratory connections, PCM has also been reported in other areas such as North America, Asia and Europe (Gushulak and MacPherson 2000).

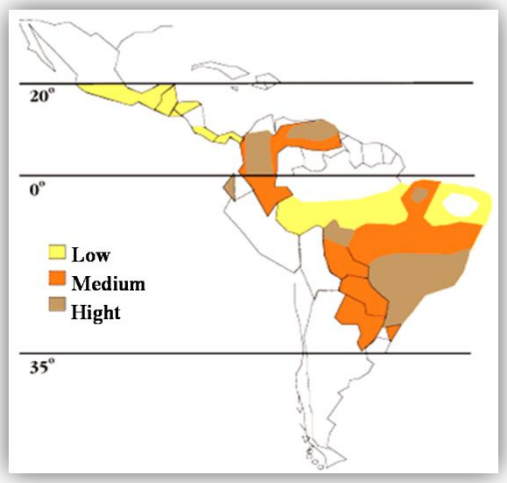

Figure 2. Geographic distribution of PCM in Central and South America. Incidence (low, medium and high) is labeled by different colours (adapted from Shikanai-Yasuda *et al.* 2006).

P. brasiliensis belongs to the Group 3 Biological Agents characterized by those organisms that: i) can cause severe human disease and present a serious hazard to workers, ii) may present a risk of spreading to the community, but iii) there is usually efective prophylaxis or treatment available (DIRECTIVE 2000/54/EC. L 262-21-L 262/45. 18-9-2000). For these reasons, research developed on *P. brasiliensis* has to be carried out at a Biosafety Level 3 Facility characterized by special safety practices, equipment and facilities.

1.1.2 Pathogenesis and Clinical Manifestations

The long latency period of the disease and the lack of epidemic outbreaks also create difficulties in determining under which circumstances the primary infection occurs (Franco *et al.* 2000). However, with the accumulating epidemiological, clinical and experimental evidence, the pathogenic process is thought to occur via inhalation of airborne fungal propagules (e.g., mycelial fragments or conidia). Once inside the host, the propagules produced reach the pulmonary alveolar epithelium and transform into the parasitic yeast form (Franco 1987, McEwan *et al.* 1987, Brummer *et al.* 1993) that may lead to diverse clinical manifestations (Montenegro and Franco 1994).

Depending on host factors, strain-level virulence differences, and environmental conditions, a wide spectrum of clinical and pathological manifestations can be observed in these patients (Montenegro and Franco 1994), where the vast majority (up to 60 % in areas of endemicity) develop only asymptomatic or subclinical PCM (Souza *et al.* 2000), which sometimes progresses into a disease with a diversity of clinical forms. The disease presents two main clinical forms: the acute or sub-acute form (juvenile type), and the chronic form (adult type) (Franco *et al.* 1987, Lacaz 1994) (Figure 3). The acute or sub-acute clinical form affects mainly children and young adults of both sexes, representing only 3 to 5% of all cases. It is characterized by a rapid and severe evolution (weeks to months) with dissemination to the reticulo-endothelial system leading to a marked involvement and hypertrophy of spleen, liver, lymph nodes, and bone marrow. On the other hand, the chronic clinical form occurs mainly in adult males (approximately 80 to 90%) and can be restricted to only one organ or disseminated to several organs and tissues, occurring more frequently in the lungs, oral and laryngeal mucous membranes, skin, lymph nodes, and adrenal glands with a slower course of infection (months to weeks). Furthermore, whereas the acute or sub-acute clinical form of PCM results in a significant rate of mortality, the chronic clinical form leads to a considerable patient morbidity (Brummer *et al.* 1993). Nevertheless, other clinical settings have been recently more frequently detected. For example, the central nervous system has also been identified as a target for PCM (neuroparacoccidioidomycosis, NPCM) (Tristano *et al.* 2004, Fagundes-Pereyra *et al.* 2006, Pedroso *et al.* 2009).

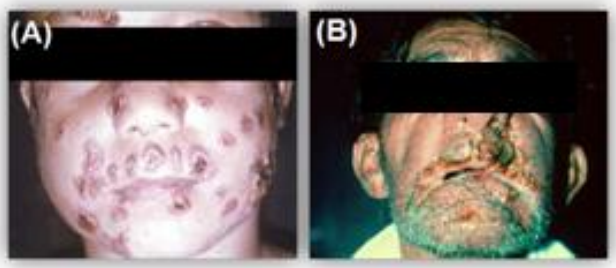

Figure 3. Clinical forms of Paracoccidioidomycosis. (A) Acute or juvenile form of PCM (patient with mucocutaneous lesions). (B) Chronic or adult form of PCM (patient with extensive skin involvement and ulcerated lesions) (Corporación para Investigaciones Biológicas', CIB, archives).

One important feature of this systemic mycosis is its marked incidence in males, with an estimated ratio of male:female of approximately 48:1 (Restrepo and Greer 1983). Several studies have addressed this sex bias, suggesting that the mechanism underlying this process involves hormonal regulation. Exposure of *P. brasiliensis* to female hormones, such as estrogen, blocks the conidia- or mycelium-to-yeast transition (Restrepo *et al.* 1984, Salazar *et al.* 1988, Clemons *et al.* 1989), probably via a cytosolic steroid-binding protein (Loose *et al.* 1983), and thus contributing to a lower percentage of infected female (Aristizabal *et al.* 1998). In spite of these evidences, the molecular events that rule this particular phenomenon are still unknown.

1.1.3 Striking Characteristics of Thermodimorphism

An important feature of several fungal pathogens is their inherent ability to assume either a filamentous or a unicellular morphology in response to changes in environmental conditions when they infect host tissues (San-Blas at al. 2002). This process, broadly referred to as dimorphism, is an intrinsic genetic property of certain fungi, and appears to be linked to pathogenicity, since strains unable to undergo the morphologic transition are mostly avirulent (Maresca and Kobayashi 2000).

Temperature, nutritional factors, or both, are usually the agents that activate this change in morphology (San-Blas *et al.* 2002). However, in *P. brasiliensis* the ability to conduct a transition between the yeast and mycelial forms is only dependent on the temperature, making it amenable to study the molecular and biochemical events that regulate this phenomenon (Szaniszlo *et al.* 1983).

P. brasiliensis' morphological shift is accompanied by extensive modifications in the cell membrane and cell wall composition (San-Blas and San-Blas 1977, San-Blas and San-Blas 1994). Regarding cell wall composition, the mycelia-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). Moreover the cell membrane lipid composition changes, particularly glycosphingolipids (Toledo *et al.* 1995). 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.

P. brasiliensis grows as a yeast form at 36-37 ºC in host tissues and culture media such as Brain Heart Infusion agar or Sabouraud agar. Yeast colonies become visible within 3 to 7 days of incubation at 36 ºC (depending on the isolate) appearing soft, wrinkled and tan to cream (Figure 4A). Microscopically, yeast cells are multinucleated and of varying sizes (4-35 μm), usually oval to elongated, and have a thick refractile cell wall and a cytoplasm that contains prominent lipid droplets (Restrepo and Jimenez 1980) (Figure 4C).

P. brasiliensis mycelia is usually cultured in modified synthetic McVeigh Morton agar at temperatures ranging from 19 ºC to 28 ºC, producing within 15 to 30 days small, irregular, and white to tan colonies with short hairy looking mycelia (Figure 4B). Microscopically, hyphae are thin (1 to 3 μm), multinucleated and septated structures (Figure 4D) from which arthroconidia and pedunculated and single-celled conidia are produced when specific isolates are cultured under conditions of nutritional deprivation. These conidia are uninucleated and round shaped structures measuring less than 5 μm in diameter (Figure 4E-F), and respond to temperature changes, transforming either into yeast cells at 36 ºC or producing hyphae at lower temperatures.

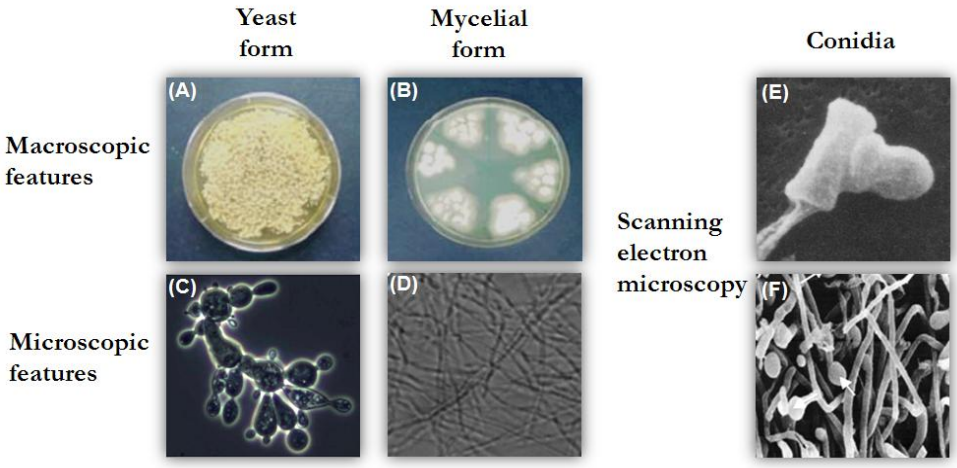

Figure 4. *P. brasiliensis* yeast (36 ºC), mycelial (18 ºC) and conidial forms. Macroscopic features of colonies from yeast (A) and mycelial form (B). Microscopic features of cells grown in batch culture from yeast (C) and mycelial form (D). Scanning electron microscopy analysis of conidia cells (E) and (F)(white arrows indicate intercalary conidia) (adapted from Wanke and Londero 1994 and Almeida, A.J *et al.* unpublished data).

P. brasiliensis yeast form is generally characterized by a multiple budding phenotype and a polymorphic cell growth, leading to the formation of cells with extreme variations in shape and size, as well as in the number of buds that are produced depending on the environmental nutritional media (Figure 4C). In compliance with these cellular features, conclusive histological diagnosis of PCM has traditionally relied on the identification of the most characteristic feature of the yeast form of *P. brasiliensis*, i.e., the pilot's wheel appearance of the mother cell surrounded by multiple peripheral daughter cells (Restrepo-Moreno 2003). More recently, it has been suggested that *P. brasiliensis* may follow an alternative control mechanism during cell growth and possess an unusual scenario for polarity establishment and maintenance. Consequently, there is the question of whether or not an accurate regulation of the cellular division exists.

According with *P. brasiliensis* features and the role of Ccd42p, a pivotal molecule in numerous cellular events, such as polarity signaling during growth and morphogenesis (Cotteret and Chernoff 2002), Almeida *et al.* (2009) evaluated the role of this Rho-like GTPase in the polymorphic morphology and virulence of this pathogenic fungus. In fact, they showed that a lower expression of *PbCDC42* results in a decrease in the size of buds and mother cells, characterized by being more spherical, less elongated and polymorphic than wild-type cells, suggesting a differential control of the apical-isotropic switch during growth. This study also showed that silencing *PbCDC42* facilitates *P. brasiliensis* phagocytosis and decreases virulence. Although Cdc42p has been implicated in a wide variety of cellular processes, there is still little insight into the mechanisms of action or the conservation of function for Cdc42p within these processes. While interacting with multiple regulators and effectors, Cdc42p seem to be conserved in most cell types, however it is dangerous to extrapolate precise Cdc42p functions or mutational phenotypes from one organism to another, given, for instance, the differences in phenotypes seen between analogous *cdc42* mutants in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Adams *et al.* 1990, Ziman *et al.* 1991, Miller *et al.* 1994). It is unfortunate that despite all the detailed information about Cdc42 functions and interacting proteins, there little experimental data addressing the specific mechanism(s) of action for Cdc42p in these different cellular processes (Johnson 1999).

1.1.4 Trends in Genome and Genetic Tools

Research on PCM and its etiological agent, *P. brasiliensis*, has promoted a fast growth with the introduction of molecular and chemical tools, opening new roads for a better understanding of this health problem. Research fields have been mostly directed towards the study of host-pathogen interactions and morphogenesis (San-Blas and Nino-Vega 2008), although the knowledge in other areas such as molecular diagnosis, epidemiology, immunology, taxonomy and genetics has also been enhanced. As in other pathogenic fungi, advances in pulsed-field gel electrophoresis (PFGE) methodologies and confocal fluorescence microscopy have allowed the genomic characterization and chromosomal mapping of *P. brasiliensis* (Montoya *et al.* 1997, Cano *et al.* 1998, Feitosa *et al.* 2003). The nuclear genome size was estimated by PFGE at around 30 Mb, and confocal fluorescence microscopy provided strong evidence to the haploid/diploid or even aneuploid nature of *P. brasiliensis* (Feitosa *et al.* 2003). DNA sequencing of ~50 Kb showed a density of one gene per 3.0-3.7 Kb, suggesting that 7500-9000 genes were present in its genome (Reinoso *et al.* 2005). More recently, new insights regarding *P. brasiliensis* genome size and ploidy were obtained by Almeida *et al.* (2007a). They used a flow cytometry (FCM) protocol to characterize cellular morphology and nuclei content of the various subpopulations discriminated during cell cycle profile analysis, a technique not only further informative concerning genome size and ploidy but also complementary to other DNA content quantification methods. Cell cycle analysis revealed a genome size ranging from 26.3 to 35.5 Mb per uninucleated yeast cell. The ploidy, assessed by comparing genome size by FCM with the average haploid size obtained from electrophoretic karryotyping, showed that *P. brasiliensis* present a haploid or at least an aneuploid DNA content. Cell cycle progression of *P. brasiliensis* yeast cells analyzed by flow cytometry, under different environmental conditions, seem to indicate that *P. brasiliensis* yeast cells may possess alternative control mechanisms during cell growth to manage multiple budding and its multinucleate nature (Almeida *et al.* 2006). Moreover, it is also imperative to mention the recent advances made by the Broad Institute, specifically the Fungal Genome Initiative (FGI) Project. This project, has already sequenced the genome of 3 *P. brasiliensis*'s species, namely Pb01 (a clinical isolate from an acute form of PCM in an adult male), Pb03 (one of the better-studied isolates of PS2) and Pb18 (the representative of the major phylogenetic group S1).

Regarding genetic manipulation, 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 and protoplasting among others have proved inefficient in *P. brasiliensis*. In the light of this, *Agrobacterium tumefaciens*-mediated transformation system (ATMT) with transformation efficiency of 78 ± 9 transformants/co-cultivation (5 ± 1 transformants/10^6 target cells), was developed and used as molecular toolbox for this dimorphic fungus (Almeida *et al.* 2007b).

One specific approach to study gene function and molecular genetics in eukaryotic organisms has been direct gene targeting. For some time now, genetic manipulation of prokaryotes and eukaryotes and the technologies involved have been the focus of many research labs, promoting ongoing development of new methodologies ranging from gene knock-out/in, point mutations, and RNA silencing (Bertling 1995). Gene targeting is a method for modifying the structure of a specific gene without removing it from its genomic environment. This process involves the construction of a piece of DNA which is then introduced into the cell where it replaces or modifies the normal *locus*, and for several model organisms, such as *S. cerevisiae*, *Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana* and mice, the generation of knock-out/in mutants is already standardized (Bertling 1995). The method of disrupting, modifying or replacing a target gene is supported by the cellular machinery that requires the action of a branch pathway of DNA double-strand break (DSB) repair mechanism (Bertling 1995, Ninomiya *et al.* 2004). Eukaryotes have developed two main pathways to deal with this type of DNA damage: nonhomologous end-joining (NHEJ) and homologous recombination (HR), often described as "error-prone" and "error-free" respectively. The first, NHEJ, involves direct ligation of the strand ends independent of DNA homology, whereas the second, HR, involves interaction between homologous sequences (Shrivastav *et al.* 2008, Christmann *et al.* 2003).

The usage of NHEJ and HR depends on the phase of the cell cycle. NHEJ occurs mainly in G0/G1, whereas HR occurs during the late S and G2 phases (Takata *et al.* 1998, Johnson and Jasin, 2000). The NHEJ system involves the recognition of and binding to damaged DNA occurs by the Ku70–Ku80 complex. Thereafter, the Ku heterodimer binds to DNA–PKcs, forming the DNA–PK holoenzyme. DNA–PK activates XRCC4–ligase IV, which links the broken DNA ends together. Before re-ligation by XRCC4–ligase IV, the DNA ends are processed by the MRE11–Rad50–NBS1 complex, presumably involving FEN1 and Artemis. On the other hand, HR starts with nucleolytic resection of the DSB in the 5' → 3' direction by the MRE11–Rad50–NBS1 complex, forming a 3' single-stranded DNA fragment to which Rad52 binds. Rad52 interacts with Rad51, provoking a DNA strand exchange with the undamaged, homologous DNA molecule. Assembly of the Rad51

nucleoprotein filament is facilitated by different Rad51 paralogues (such as Rad51B, Rad51C and Rad51D, XRCC2 and XRCC3). After DNA synthesis, ligation and branch migration, the resulting structure is resolved (Christmann *et al.* 2003).

These mechanisms are conserved in evolution, but have different contribution to overall DSBs repair within different eukaryotic cells (Pastwa and Błasiak 2003). For example, the budding yeast *S. cerevisiae* repairs DNA DSB mainly by the HR system. However, it seems that most fungi and higher eukaryotes, including plants and mammals, have an "inefficient homologous integration system" using the NHEJ pathway to repair DNA DSB (Kooistra *et al.* 2004). Due to either high activity of NHEJ or poor efficiency of HR, targeting of genes at a desired *locus* in the dimorphic fungus *P. brasiliensis* is difficult being a rate-limiting step for further progress in this area.

The NHEJ has been blocked/downregulated in order to increase the rate of homologous integration of exogenous DNA in several fungal models including *Neurospora crassa*, different *Aspergillus* spp., *Cryptococcus neoformans*, *Claviceps purpurea*, *Magnaporthe grisea* and *Sordaria macrospora* among many others (Ninomiya *et al.* 2004, Goins *et al.* 2006, Pöggeler and Kück 2006, da Silva Ferreira *et al.* 2006, Haarmann *et al.* 2008, Villalba *et al.* 2008, Guangtao *et al.* 2009).

Very recently, another technique has been used in *P. brasiliensis*, namely antisense aRNA technology (Almeida *et al.* 2009, Hernández *et al.* 2010). Antisense refers to short DNA or RNA sequences, termed oligonucleotides, which are designed to be complementary to a specific gene sequence. The goal is to alter specific gene expression resulting from the binding of the antisense oligonucleotide to a unique gene sequence. Antisense technology was first effectively used in plants to alter the levels of various degradative enzymes or plant pigments, but was rapidly applied to mammalian cells. The exact mechanism by which antisense technology prevent protein production from a targeted gene remains uncertain. Proposed mechanisms include triplex formation, blocking RNA splicing, preventing transport of the mRNA antisense complex into the cytoplasm, increasing RNA degradation, or blocking the initiation of translation. Delivery of antisense oligonucleotides into target cells or the cell nucleus has been problematic. Currently, the most problematic aspect associated with antisense technology revolves around the specificity of their action. In some cases, non-specific antisense sequences, in other words, sequences which do not bind to the targeted gene or RNA, have prevented gene expression to the same degree as their sequence-specific antisense counterparts. This has led to considerable complication in data interpretation and requires detailed and careful data

analysis. Since antisense technology focuses on preventing gene expression, it has been most widely applied to cancer gene therapy (Lichtenstein and Nellen 1997, Morcos 2007).

1.1.5 Laboratory Diagnosis and Molecular Identification

Histopathologic diagnosis is typically made through morphological criteria, mainly upon the identification of the characteristic multiple-budding feature of the yeast form (San-Blas *et al.* 2002). The isolation of the causative agent from clinical samples is essential to confirm accurate identification but is time-consuming since microbiological procedures may take over 20 days (Brummer *et al.* 1993). Alternatively, the diagnosis of PCM by indirect serological methods (e.g., complement fixation, agar-gel immunodiffusion and immunoenzymatic assays) that rely on antibody detection and is of considerable value due to the wide range of clinical presentations and the time-consuming procedures for the isolation of *P. brasiliensis* from clinical specimens (de Camargo *et al.* 1984, Cano and Restrepo 1987, Taborda and Camargo 1994, Ortiz *et al.* 1998). However, antibody levels may be absent in immunocompromised patients, or may remain present months after successful therapy (San-Blas *et al.* 2002). Nevertheless, serological diagnosis has sometimes shown altered sensitivity and specificity (may vary from 65 to 100 %) due to cross-reactivity with other fungi (e.g., *H. capsulatum*), or due to the use of undefined antigenic proteins derived from diverse cellular components of both yeast and mycelia forms of more than one *P. brasiliensis* strain (Lacaz *et al.* 1991, Freitas-Silva and Roque-Barreira 1992). However, advances in molecular biology have allowed the production of more reproducible and defined antigenic proteins through gene cloning and sequencing.

Molecular diagnosis of PCM has also been under development, with several primers being proposed as specific probes for clinical and field uses. For example, Goldani *et al.* (1995) reported the cloning and sequencing of a species-specific 110 bp DNA fragment generated by PCR from *P. brasiliensis.* Additionally, the specific DNA fragment from three different isolates of *P. brasiliensis* was amplified by PCR with primers mostly complementary to non-actin sequences of the 110 bp DNA fragments (Goldani *et al.* 1995). The literature shows that *P. brasiliensis* DNA samples have not only been applied in standard polymerase chain reactions (sPCR), but also in other amplification methods such as PCR-enzyme immunoassay (PCR-EIA), real time PCR (RT-PCR) and nested PCR (nPCR) in an attempt to improve test specificity/sensitivity (Goldani *et al.* 1995, Sandhu *et al.* 1995, Sandhu *et al.* 1997, Diez *et al.* 1999, Bialek *et al.* 2000, Gomes *et al.* 2000, Motoyama *et al.* 2000, Lindsley *et al.* 2001, Semighini *et al.* 2002).

1.1.6 Aim and outline of the thesis

The work presented during this thesis, was developed within the research line of the Microbiology and Infection Research Domain of the Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho.

Despite recent advances, knowledge regarding the fundamental biology of *P. brasiliensis* has been hampered by the absence of appropriate genetic tools. Therefore to further contribute to elucidate biological phenomena of this pathogen, we aimed to develop a strategy for gene targeted mutagenesis. Moreover, to better understand the relationship between the mechanisms that regulate cellular division of *P. brasiliensis* and its distinctive multiple budding phenotype and particular morphological trait, we analyzed different biological parameters of yeast cell morphology of several isolates and expression of the Rho-like GTPase Cdc42.

Chapter 1 provides an overview on the current knowledge concerning the etiological agent *P. brasiliensis* and the systemic mycosis it causes, paracoccidioidomycosis. The methodologies used to study this dimorphic pathogenic fungus as well as molecular developments on *P. brasiliensis* are also addressed, leading the reader to Chapters 2 and 3.

In Chapter 2, the development of an efficient methodology for gene disruption in *P. brasiliensis* was attempted. In summary, work developed throughout this chapter proposes to down-regulate the non-homologous end joining pathway, by decreasing the expression levels of the *Ku80* gene, a component of the DNA-dependent protein kinase catalytic subunit (the Ku70-Ku80 heterodimer), would increase homologous recombination, an essential tool to produce strains suitable to HR allowing future functional genomics studies.

Chapter 3 focuses on the quantification of different morphologic aspects that the yeast form of this dimorphic fungus presents, both inter- and intra-strain. Moreover, the hypothetical role of *PbCDC42* in the heterogeneous cell size and form of several *P. brasiliensis* strains from the described cryptic species was addressed.

Finally, Chapter 4 presents the main conclusions bringing together Chapter 2 and 3 in the context of the initially proposed objectives, in order to provide a global perspective of the work and possible lines for future research.

Chapter 2

Development of Targeted Gene Disruption in *Paracoccidioides brasiliensis*: Downregulation of the Non-Homologous End Joining Pathway

2.1 Introduction

The incidence of fungal infections has been increasing, particularly in patients who are immunocompromised by human immunodeficiency virus infection. Systemic fungal infections in such patients are often life threatening. *Paracoccidioides brasiliensis*, the causative agent of PCM is among this group of pathogenic fungi that are of clinical significance and scientific interest (Guarro *et al.* 1999, Restrepo-Moreno 2003, Chakrabarti and Shivaprakash 2005). Even though during the last decade molecular approaches have allowed a broader insight into *P. brasiliensis*, knowledge regarding the fundamental biology of this pathogenic fungus has been hampered by the absence of appropriate genetic tools (San-Blas *et al.* 2002, Felipe *et al.* 2005, Almeida *et al.* 2007). In particular, the development of an efficient gene targeted mutagenesis for gene disruption, vital to understand the role of specific genes in several biological processes such as dimorphism, pathogenesis, among others. In order to accomplish disruption, modification or replacement of a target gene investigators usually take advantage of the cellular machinery that requires the action of a DNA double-strand break (DSB) repair mechanism (Haarmann *et al.* 2008 Villalba *et al.* 2008). DSBs are the most severe form of DNA damage, mostly induced by ionizing radiation, some chemicals, like anticancer drugs, or arise spontaneously during DNA replication, and can result in cell death or a wide variety of genetic alterations including large- or small-scale deletions, loss of heterozygosity, translocations, and chromosome loss (Pastwa and Błasiak 2003, Shrivastav *et al.* 2008).

Eukaryotes have two main pathways to deal with this type of DNA damage: nonhomologous end-joining (NHEJ) and homologous recombination (HR) (Christmann *et al.* 2003, da Silva Ferreira *et al.* 2006). In *Saccharomyces cerevisiae* the most important DSB repair pathway is HR, while other organisms, such as humans, preferentially use NHEJ (Ninomiya *et al.* 2004). The NHEJ machinery for DSBs repair is mediated by the essential DNA binding Ku70-Ku80 heterodimer, which directs the DNA-PK to the end of a DSB, thus stabilizing its DNA binding, that along with other components execute crucial steps in NHEJ repair (Pöggeler and Kück 2006). Recently, da Silva Ferreira *et al.* (2006) disrupted *Aspergillus fumigatus* gene homologous to *Neurospora crassa KU80*. Transformation of the *A. fumigatus KU80* disruption yielded 80% transformants exhibiting integration at the homologous site, compared to 3 to 5% for a wild-type recipient. To our knowledge, the rate-limiting step for further progress in the dimorphic fungus *P. brasiliensis* is the low percentage of homologous integration.

Thus, we decided to downregulate the *P. brasiliensis KU80* gene in order to increase the integration of introduced exogenous DNA fragments by homologous recombination. Specifically, we amplified a DNA fragment from the cDNA library using degenerated primers designed based on Ku80p sequence alignment from *Ajellomyces capsulatus*, *Coccidioides immitis*, *Penicillium marneffei* and *Microsporum canis* to isolate *PbKU80* gene and applied antisense RNA (aRNA) technology using oligonucleotides targeting the 5' untranslated region of *PbKU80* to knockdown its expression in *P. brasiliensis* yeast cells.

2.2 Materials and Methods

2.2.1 Microorganisms and culture media

Escherichia coli XL-1-Blue

E. coli XL-1-Blue strain (Bullock *et al.* 1987) was used as host for plasmid amplification and cloning. *E.coli* was grown on Luria Bertani (LB) medium (Sambrook *et al.* 1998) at 37 ºC. For bacterial selection, kanamycin, 50 mg/L, or ampicillin, 100 mg/ml were used as supplements.

Agrobacterium tumefaciens LBA1100

A. tumefaciens LBA1100 (C58C1 with a disarmed octopine-type pTiB6 plasmid) (Beijersbergen *et al.* 1992) was used as recipient for binary vectors. *A. tumefaciens* was maintained at 28 °C on LB medium containing spectinomycin, 250 mg/L and rifampicin, 20 mg/L. For selective purposes, kanamycin, 100 mg/L was employed.

During Agrobacterium tumefaciens-mediated transformation (ATMT) procedures, *A. tumefaciens* LBA1100 containing the binary vector pUR5750 (conferring kanamycin resistance in *A. tumefaciens* and *E. coli*) was applied (de Groot *et al.* 1998).

Paracoccidioides brasiliensis

P. brasiliensis yeast cells, strain ATCC 60855, Pb18 and Garcia, were maintained at 36 ºC by periodic subculturing in brain heart infusion (BHI) solid medium (1.5% wt/vol agar). For the assays carried out in this study, yeast cells were grown in both BHI and modified synthetic McVeigh Morton (MMcM) liquid media (Restrepo and Jimenez 1980), at 36 ºC with aeration on a mechanical shaker (200 rpm).

2.2.2 Cloning vectors

pCR35

The plasmid pCR35, kindly provided by C. A. Rappleye (Department of Microbiology, Ohio State University, Columbus, Ohio, USA) (Rappleye *et al.* 2004) is a 6823 bp vector that contains the *GFP* gene downstream from *Histoplasma capsulatum* promoter region calcium-binding protein (*CBP1*). Additionally it contains the neomycin phosphotransferase II gene (*nptII*), the *E. coli* selectable kanamycin resistance marker, the *Podospora anserina* URA5 gene (*PaURA5*) which encodes orotidine-5'-monophosphate pyrophosphorylase that leads to uracil auxotrophy in *Histoplasma capsulatum* and one Ori.

pUR5750

The plasmid pUR5750 (de Groot *et al.* 1998) is a 15731 bp binary vector. This vector contains a transferred DNA (T-DNA) harbouring, between left and right border sequences, an *E. coli* hygromycin B phosphotransferase (*HPH*) gene driven by the *Aspergillus nidulans* glyceraldehydes 3-phosphate (*GPD*) promoter and transcriptional terminator (*TRPC*) from pAN7-1 and the *A. tumefaciens* selectable kanamycin resistance marker, neomycin phosphotransferase II gene (*nptII*), flanked by the nopaline synthase promoter (Pnos) and terminator (Tnos). Additionally it contains the neomycin phosphotransferase III gene (*nptIII*), the *E. coli* selectable kanamycin resistance marker.

2.2.3 Nucleotide and protein sequence analysis

Sequence similarity searches were performed using BLAST (**http://blast.ncbi.nlm.nih.gov/Blast.cgi**). Both nucleotide and protein sequence alignments were carried out with the DNAMAN Version 4.0 program (Lynnon BioSoft).

2.2.4 Cloning of *P. brasiliensis* aRNA PbKU80 oligonucleotides and binary vector construction

DNA and total RNA from exponentially growing *P. brasiliensis* yeast cultures were extracted using Trizol standard procedures (van Burik *et al.* 1998).

Recombinant DNA manipulations, agarose gel electrophoresis and polymerase chain reaction (PCR) were conducted according to Sambrook *et al.* (1998). Plasmid purification was carried out using the Quiagen® plasmid purification procedure.

E. coli transformation by heat shock was performed as described elsewhere (Sambrook *et al.* 1998).

Binary vector was mobilized to *A. tumefaciens* LBA1100 by electroporation as described by Dulk-Ras and Hooykaas (Dulk-Ras and Hooykaas 1995). Briefly, after preparation of *A. tumefaciens* cells for electroporation, bacterium cells were thawed on ice. 2 to 3 μL of plasmid DNA were added to 40 μL of cell suspension and transferred to an electroporation cuvet. Electroporation was done applying an electric pulse at 2.5 kV, 25 μF and 200 Ω with a time constant of approximately 4.7 ms. After *A. tumefaciens* cells recovery in 1 mL of SOC medium at 29 ºC for 2 h with shaking (180 rpm), aliquots of cell mixtures were inoculated onto LB selection plates and incubated at 28 ºC for 3 days.

The parent plasmids used in this study are summarized in Table 2. The parent plasmid for pCR35::A1ku80 and pCR35::A2ku80 was pCR35, and the parent plasmids for pUR5750::A1 and pUR5750::A2ku80 were pCR35::A1, pCR35::A2ku80 and pUR5750.

PCR amplification was performed using degenerate primers (Deg1 and Deg2, Table 1) designed based on protein sequence alignment of the Ku80p from *Ajellomyces capsulatus* (GenBank accession number GG663365), *Coccidioides immitis* (ac. no. XP_001248033), *Penicillium marneffei* (ac. no. XP_002151653) and *Microsporum canis* (ac. no. EEQ38520) using the cDNA library of *P. brasiliensis* produced in our laboratory. The sequence of the 1315-bp PCR product (STAB VIDA, Oeiras, Portugal) was used to design 4 primers to obtain 2 antisense molecules (A1ku80 and A2ku80) from the 5' UTR region using the primers in Table 1 (F1/R1; F2/R2).

The obtained antisense fragments were digested with *Asc*I and *Xho*I and cloned into the *Asc*I/*Xho*I site of pCR35. The ligated DNA was introduced in *E. coli* by heat shock and positive clones selected on LB kanamycin plates. To identify pCR35::A1ku80 and pCR35::A2ku80 colony PCR was performed using the external primers (P3/P4) for A1/A2ku80 cassette. 200 ng of DNA were added to 18 μL of reaction mixture defined by reaction buffer 1×, 2 mM $MgCl_2$, 200 μM dNTP, 200 μM of each primer and 1 unit of Taq polymerase. The PCR reaction cycling was as follows: 1 cycle at 94 ºC for 10 min, 35 cycles at 94 ºC for 15 s, 58 ºC for 30 s, 72 ºC for 2 min, and 1 final cycle at 72 ºC for 10 min. Some pCR35::A1/A2ku80 positive clones were then selected for a second screening of colony minipreps by digesting the DNA with *Kpn*I, which originated one fragment with 1823 bp for pCR35::A1ku80 and 1809 bp for pCR35::A2ku80, confirming the insertion of the A1/A2ku80 fragments on pCR35.

Binary vectors were constructed by cloning the *Kpn*I fragments from pCR35::A1/A2ku80 into the *Kpn*I site of the binary vector pUR5750. The ligated DNA was introduced in *E. coli*. Selection was performed on LB kanamycin plates. In order

identify positive clones, colony PCR was performed using the external primers (P5/P6) for A1/A2ku80 cassette as described above. Positive pUR5750::A1/A2ku80 clones were selected for a colony miniprep sceening digesting the DNA with *Kpn*I, which provided a digestion pattern of 2 fragments of 15731 and ≈1800 bp, respectively. The obtained binary vector was mobilized to *A. tumefaciens* LBA1100 ultracompetent cells by electroporation as described above and transformants were isolated by kanamycin selection at 50 mg/L.

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

Primer sequences (5'-3')[a]
Deg1-AARGTNCCNCCNAARGCNAAR
Deg2-GGNTTYAARGARGARGAYAAR
F1-ccgctcgagTTATGATCAATTTTTCCTCC
R1-ggcgcgccTACCATGGTGACAATTGGCC
F2-ccgctcgagAGGAAAACCGCGAATCTCGG
R2-ggcgcgccATTAATCTGGCTTATGTCTT
P3-ggggtaccCCGCGGATCACGGTATCGATGA
P4-ggggtaccCCGGTACCTAGGTGGATCCAAT
P5-ggggtaccGATCGGTGCGGGCCTCTTCG
P6-ggggtaccCATGACGGCCATCATGCCAA
Hyg1-ATGCCTGAACTCACCGCGAC
Hyg2-TTCTACACAGCCATCGGTCC
RT-PCR
P1 Pbku80-GGAGCTACAAGCTCAAAGCAA
P2 Pbku80-GCCTGAAAGTGGCTTTTCTG
P1 Pbtub2-AGCCTTGCGTCGGAACATAG
P2 Pbtub2-ACCTCCATCCAGGAACTCTTCA

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

Table 2. Features of the parent plasmids used in this study.

Plasmid	Features between RB and LB	Resistance gene for selection in *E. coli*	Resistance gene for selection in *A. tumefaciens*	Resistance gene for selection in *P. brasiliensis*
pCR35	n.a.	Kanamycin	n.a.	n.a.
pUR5750	Pnos::*ntpII*::Tnos Ttef::*hph*::Ptef	Kanamycin	Kanamycin	Hygromycin B

Hph, hygromycin B phosphotransferase gene; LB, left border; n.a., not applicable; *nptII*, neomycin phosphotransferase II gene; Pnos, nopaline synthase promoter; Ptef, translation elongation factor-1 α promoter; RB, right border; Tnos, nopaline synthase terminator, Ttef, translation elongation factor-1 α terminator.

2.2.5 *Paracoccidioides 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 (AS), 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 growth phase (48 to 60 h).

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). 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 ºC 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 2 mL non-selective BHI liquid medium containing cefotaxime, 200 mg/L, 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 ºC, 200 rpm, before plating in selective media (HygB 50 μg/ml). Selection plates were incubated at 36 ºC 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

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2.2.6 Analysis of transformants by 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 unit of *Taq* polymerase. The PCR reaction cycling was as follows: 1 cycle at 94 ºC for 10 min, 40 cycles at 94 ºC for 15 s, 56 ºC for 30 s, 65 ºC for 60 s, and 1 final cycle at 65 ºC for 10 min. A fragment of 982 bp was amplified using the

primers indicated in Table 3 (Hyg1/Hyg2). Two different control samples were applied during PCR procedures: pUR5750 plasmid DNA and *P. brasiliensis* ATCC60855 wild type DNA.

2.2.7 Mitotic stability

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. Previously tested for mitotic stability, transformants were then selected for posterior assays.

2.2.8 Real-time polymerase chain reaction (RT-PCR)

Total RNA 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 ºC followed by 60 min at -80 ºC) 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 for the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Nutley, NJ, USA) to amplify the *P. brasiliensis Ku80* gene (184 bp) and the *TUB2* gene (64 bp) using the primers indicated in Table 4. 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 ºC for 20 s, a melting step of 55º-95 ºC (0.5 ºC/s), and a final cooling at 40 ºC for 30 s. Real-time quantification was performed on a LightCycler System (Roche) using threshold cycle (Ct) values for *TUB2* transcripts as the endogenous reference. mRNA differential *Ku80* expression levels were evaluated by normalizing *Ku80* Ct values with the reference and comparing the ratio amongst the tested samples.

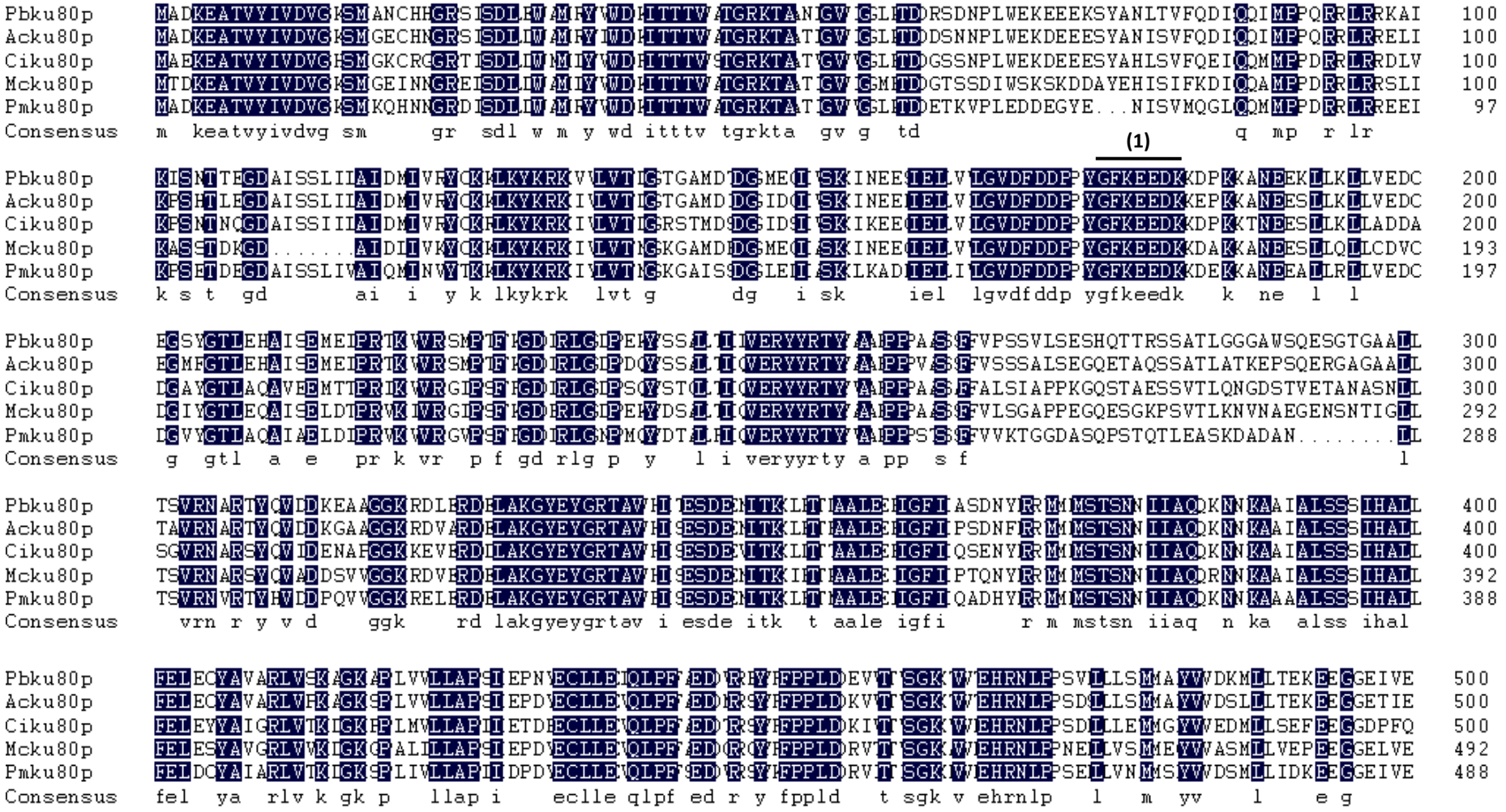
2.3 Results and Discussion

2.3.1 Isolation of *PbKU80* and construction of Aku80

Despite new advances on molecular manipulation of *P. brasiliensis*, specifically the recent development of an efficient transformation and a gene expression and interference system developed by our group (Almeida *et al.* 2007b, 2009), functional genetic studies in *P. brasiliensis* has been impaired due to the absence of tractable molecular techniques, namely an efficient procedure allowing the generation of gene-knockout strains by HR.

As formerly stated, the rate of homologous integration of exogenous DNA can be increased by blocking/downregulating NHEJ key players's function such as Ku80 (Ninomiya *et al.* 2004, Goins *et al.* 2006). In this sense, we established an antisense RNA (aRNA) system to downregulate the expression of *PbKU80*. Thus, to isolate *P. brasiliensis* KU80 (*PbKU80*) homolog, degenerate primers were designed from the highly conserved amino acid sequences GFKEEDK and KVPPKAK (positions 175-181 and 565-571 respectively) based on the alignment of counterparts from different organisms (Figure 1). Two DNA fragments of 888 and 675 bp were obtained by PCR on *P. brasiliensis* cDNA library. The DNA sequence analysis of these fragments revealed an incomplete open reading frame (ORF) with high degree of similarity to *KU80* ORFs from other organisms (data not shown). The sequences of the isolated cDNA fragments revealed a 769-bp ORF flanked by a 342-bp 5' untranslated region (5'UTR) and by a 186-bp 3' untranslated region (3'UTR) together with a poly(A) tail (Figure 2). Then, with two sets of primers targeting the *PbKU80* 5'UTR (Figure 2A), two different aRNA molecules (A1KU80 and A2KU80) of 140 and 126-bp were amplified for independent downregulation of *PbKU80* gene. To allow the construction of antisense molecules, specific restriction sites, namely *XhoI* and *AscI* were respectively added in the 5' and 3' ends of both sequences.

The repressing oligonucleotides were afterward individually cloned in pCR35 under the control of the Calcium-Binding Protein Promoter (*CBP1*) region from *H. capsulatum* (Rapleye *et al.* 2004).


(1)

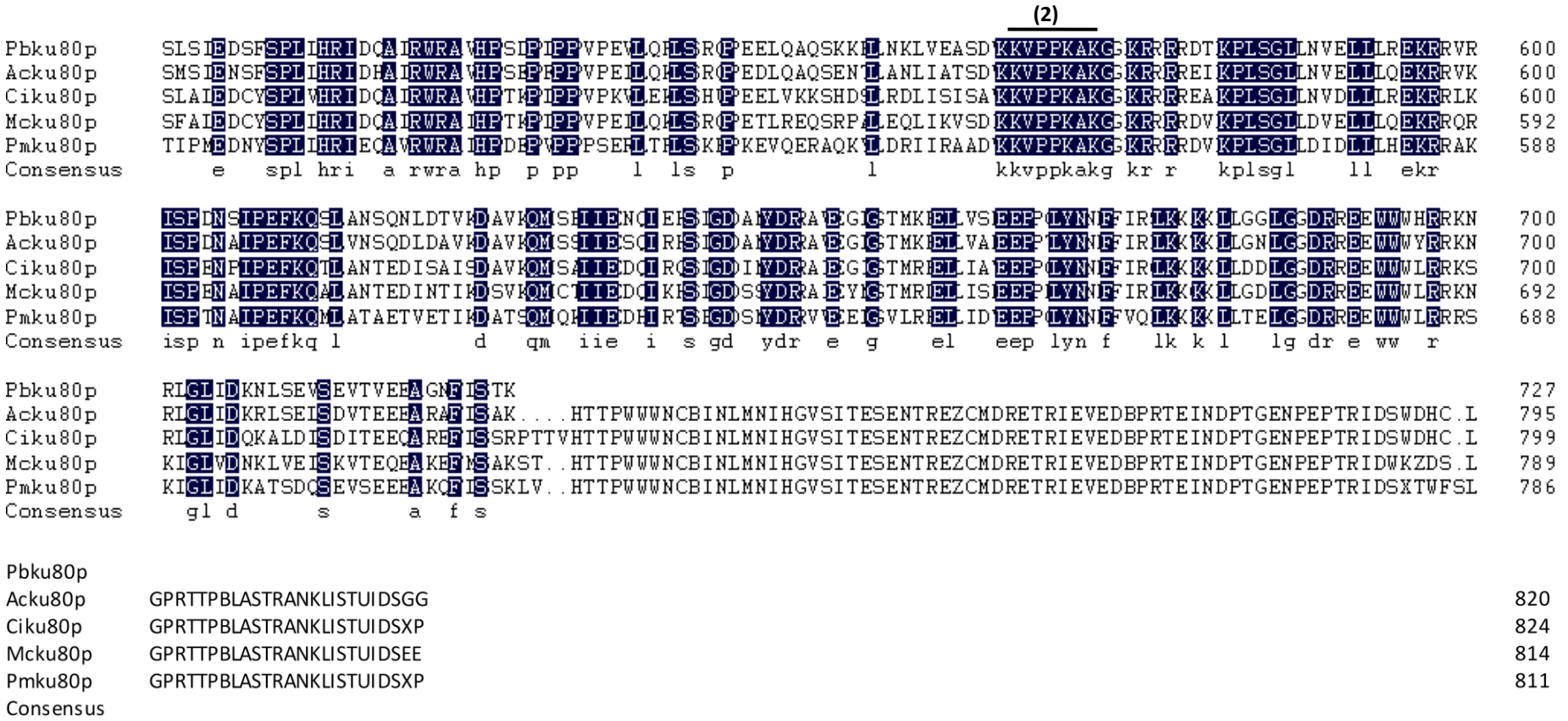


Figure 1. Multiple sequence alignment of the deduced amino acid sequence of *P. brasiliensis KU80* (Pbku80p), *Ajellomyces capsulatus KU80* (Acku80p), *Coccidioides immitis KU80* (Ciku80p), *Microsporum canis KU80* (Mcku80p) and *Penicillium marneffei KU80* (Pmku80p). In black are underlined the degenerated primers (1) Deg2 and (2) Deg1.

A

F1

-342 TCAGG***TTATG ATCAATTTTT CCTCC***TCTTC TCCCTCTTCA TCTTTCCATG TCCAAGTGCA

-282 GTACTAAGAA GAAAGATGGC GGACAAGGAA GCCACTGTGT ATATTGTGGA TGTCGGAAAA

R1

-222 TCTAT*GGCCA ATTGTCACCA TGGTA*GATCT ATATCAGACC TCGAGTGGGC GATGCGCTAT

F2

-162 GTCTGGGACA AGATCACAAC CACGGTTGCC ACTGGC*AGGA AAACCGCGAA TCTCGG*AGTA

-102 ATTGGTCTCA AAACAGATCG CTCTGACAAT CCATTATGGG AGAAAGAGGA AGAGAAAAGC

R2

-42 TATGCCAATT TGACCGTTTT TC*AAGACATA AGCCAGATTA AT*

```
1       ATG GCG GAC AAG GAA GCC ACT GTG TAT ATT GTG GAT GTC GGA AAA TCT
        Met Ala Asp Lys Glu Ala Thr Val Tyr Ile Val Asp Val Gly Lys Ser

49      ATG GCC AAT TGT CAC CAT GGT AGA TCT ATA TCA GAC CTC GAG TGG GCG
        Met Ala Asn Cys His His Gly Arg Ser Ile Ser Asp Leu Glu Trp Ala

97      ATG CGC TAT GTC TGG GAC AAG ATC ACA ACC ACG GTT GCC ACT GGC AGG
        Met Arg Tyr Val Trp Asp Lys Ile Thr Thr Thr Val Ala Thr Gly Arg

145     AAA ACC GCG AAT CTC GGA GTA ATT GGT CTC AAA ACA GAT CGC TCT GAC
        Lys Thr Ala Asn Leu Gly Val Ile Gly Leu Lys Thr Asp Arg Ser Asp

193     AAT CCA TTA TGG GAG AAA GAG GAA GAG AAA AGC TAT GCC AAT TTG ACC
        Asn Pro Leu Trp Glu Lys Glu Glu Glu Lys Ser Tyr Ala Asn Leu Thr

241     GTT TTT CAA GAC ATA AGC CAG ATT AAT ATG CCT CAA ATC CGC GAA CTG
        Val Phe Gln Asp Ile Ser Gln Ile Asn Met Pro Gln Ile Arg Glu Leu

289     CGT AAA GCG ATC AAA ATC AGC AAT ACA ACT GAA GGA GAC GCA ATA TCA
        Arg Lys Ala Ile Lys Ile Ser Asn Thr Thr Glu Gly Asp Ala Ile Ser

337     TCC CTT ATC TTG GCG ATT GAT ATG ATT GTA CGA TAC TGC AAG AAA TTG
        Ser Leu Ile Leu Ala Ile Asp Met Ile Val Arg Tyr Cys Lys Lys Leu

385     AAA TAC AAA AGG AAA GTC GTC CTT GTT ACG GAT GGA ACA GGT GCT ATG
        Lys Tyr Lys Arg Lys Val Val Leu Val Thr Asp Gly Thr Gly Ala Met

433     GAT ACG GAT GGG ATG GAG GGA ATT GTA TCC AAA ATA AAC GAG GAA TCG
        Asp Thr Asp Gly MET Glu Gly Ile Val Ser Lys Ile Asn Glu Glu Ser

481     ATT GAA CTT GTA GTC CTG GGT GTA GAT TTC GAT GAC CCA GAG TAC GGA
        Ile Glu Leu Val Val Leu Gly Val Asp Phe Asp Asp Pro Glu Tyr Gly

529     TTC AAG GAA GAG GAC AAG
        Phe Lys Glu Glu Asp Lys
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B

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1       AAG GTA CCA CCC AAA GCC AAA GGT CTC AAA CGA GTC AGA GAC ACA GAA
        Lys Val Pro Pro Lys Ala Lys Gly Leu Lys Arg Val Arg Asp Thr Glu

49      AAG CCA CTT TCA GGC TTA AAC GTC GAA GAG CTT CTC CGG ACG GAG AAA
        Lys Pro Leu Ser Gly Leu Asn Val Glu Glu Leu Leu Arg Thr Glu Lys

97      CGT GTG AGG ATA TCC CCG GAC AAC TCC ATA CCC GAA TTC AAG CAG TCC
        Arg Val Arg Ile Ser Pro Asp Asn Ser Ile Pro Glu Phe Lys Gln Ser

145     CTG GCG AAT TCT CAG AAC CTT GAT ACA GTC AAG GAT GCC GTT AAG CAG
        Leu Ala Asn Ser Gln Asn Leu Asp Thr Val Lys Asp Ala Val Lys Gln

193     ATG TCC CCC ATC ATA GAA AAC CAA ATA GAG CAT AGC TTG GGC GAT GCC
        Met Ser Pro Ile Ile Glu Asn Gln Ile Glu His Ser Leu Gly Asp Ala

241     AAC TAT GAT CGG GCC GTT GAA GGC TTG GGC ACC ATG AAA GAA GAG CTG
        Asn Tyr Asp Arg Ala Val Glu Gly Leu Gly Thr Met Lys Glu Glu Leu
```

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289      GTT TCC TTT GAG GAG CCT GGT CTA TAT AAC GAT TTC ATC CGT AGC TTG
         Val Ser Phe Glu Glu Pro Gly Leu Tyr Asn Asp Phe Ile Arg Ser Leu

337      AAA GCG AAA CTG CTT GGG GGT GAA CTC GGT GGA GAT AGG CGC GAG ATG
         Lys Ala Lys Leu Leu Gly Gly Glu Leu Gly Gly Asp Arg Arg Glu Met

385      TGG TGG CAT GTG AGG AAG AAT AGA CTG GGG TTG ATT GAT AAG AAT CTC
         Trp Trp His Val Arg Lys Asn Arg Leu Gly Leu Ile Asp Lys Asn Leu

433      TCA GAA GTC TCT GAA GTG ACG GTG GAA GAA GCT GGG AAT TTT TTG TCC
         Ser Glu Val Ser Glu Val Thr Val Glu Glu Ala Gly Asn Phe Leu Ser

481      ACA AAA TGA
         Thr Lys ***

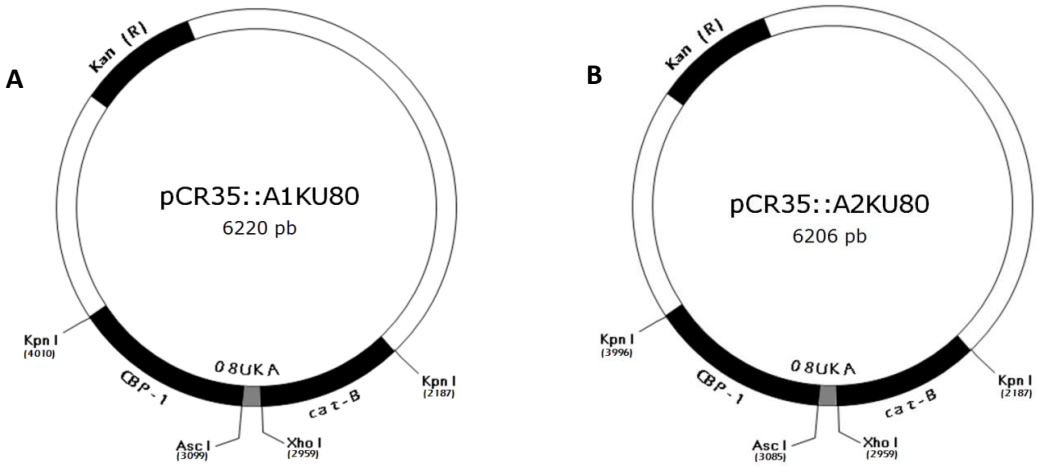

+1       GTACACTAGT TTCGGTGAGA ATATTCCTGC CATGGTTTTG GAGTGTTCGA CATTATGAGC
+61      GAATCCGTAT CCATACACGC CCCCTCAATG GATGATTGAT CATCAGGGTA GATTCCTTGT
+121     CTTAAGTCTA ATAAAATTGG ATTATTCATA TTATCGCGAA AAAAAAAAAA AAAAAAAAAA
+181     AAAAAA
```

Figure 2. Nucleotide and deduced amino acid sequence of the incomplete ORF of *P. brasiliensis* cDNA encoding the *KU80* homolog. The *PbKU80* cDNA sequence contains an incomplete 1035-bp open reading frame flanked upstream by a 342-bp 5' untranslated region (A) and downstream by a 186-bp 3' untranslated region (B). In grey are highlighted the sequences used for the construction of A1KU80 aRNA and A2KU80 aRNA.

A1KU80 and A2KU80 aRNA constructs were amplified together with the flanking CBP-1 promoter and cat-B terminator, with *Kpn*I restriction sites, and cloned into the binary vector pUR5750. This is a crucial and helpful tool for *A. tumefaciens*-mediated transformation, for the single copy insertion of the cassette (T-DNA) with the antisense oligonucleotide into *P. brasiliensis* genome.

2.3.2 Cloning strategy

The strategy pursued in this study resulted in the construction of four plasmids, pCR35::A1KU80, pCR35::A2KU80, pUR5750::A1KU80 and pUR5750::A2KU80 (Figure 3).



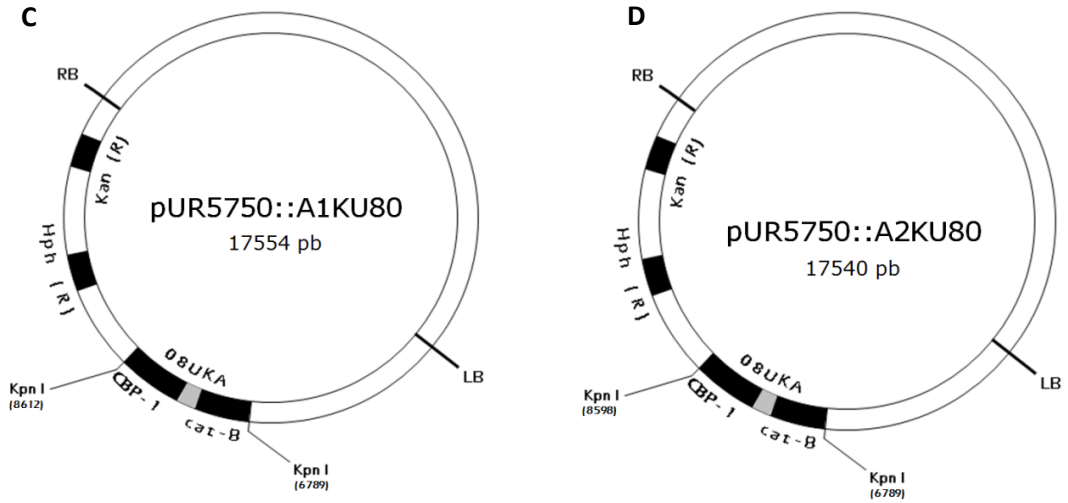


Figure 3. (A) pCR35::A1KU80, (B) pCR35::A2KU80, (C) pUR5750::A1KU80 and (D) pUR5750::A2KU80 vectors. For vector properties see Table 2, and for construction strategies see Material and Methods. Hph (R), hygromycin resistance selectable marker; Kan (R), kanamycin resistance selectable marker; CBP-1, calcium-binding protein promoter; 08UKA, antisense oligonucleotides of *PbKU80* gene; cat-B, intergenic region downstream of the CATB gene as transcriptional termination signal; LB, left border; LR, right border.

In Figure 4 is represented the strategy used to clone the antisense oligonucleotides into the vectors to carry out gene transfer into *P. brasiliensis*.

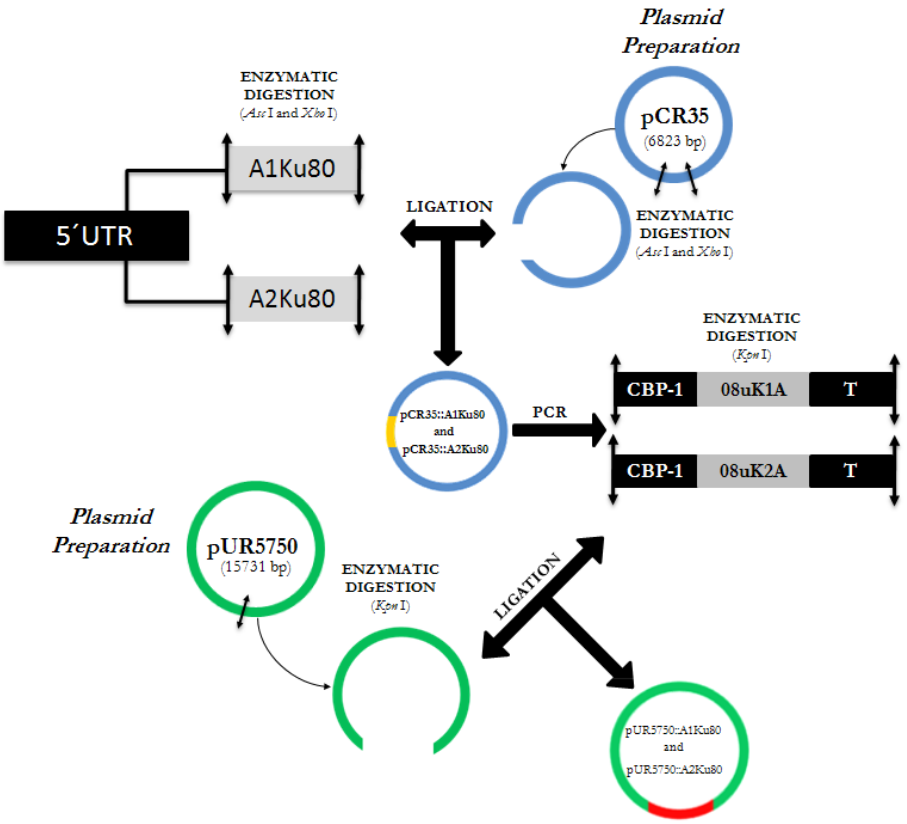


Figure 4. Scheme of the strategy used for cloning the antisense molecules, as described in Materials and Methods.

It is important to emphasize the fact that the final cloning was performed with a very large plasmid (over 15 kb), which becomes an obstacle during the cloning of the antisense oligonucleotides, forcing numerous attempts, as well as the variation of several parameters involved, that could be affecting the process.

A DNA molecule needs to display several features to be able to act as a vector for gene cloning. Apart from having to be able to replicate within the host cell, a cloning vector also needs to be relatively small, ideally less than 10 kb, as large molecules tend to break down during purification, and are also difficult to manipulate. Beyond the size, the copy number of a plasmid (number of molecules of an individual plasmid that are normally found in a single bacterial cell) is also an aspect particularly important as far as cloning is concerned, since a useful cloning vector needs to be present in the cell in multiple copies so that large quantities of the recombinant DNA molecule can be obtained (Brown 2006). First reported by de Groot *et al.* (1998), binary vector pUR5750 besides being a low copy plasmid is approximately 16 kb, being a critical factor with respect to transformation efficiency (Brown 2006). Moreover, adds to the fact that in practice, a key factor to success in a gene cloning is the ability to thoroughly select positive clones (Brown 2006).

2.3.3 *A. tumefaciens*-mediated transformation of *P. brasiliensis*

The development of efficient genetic transformation systems for fungi has been crucial for establishing a link between *in vitro* DNA analysis and its i*n 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).

The recently optimized ATMT protocol of *P. brasiliensis* is represented in Figure 5 (Almeida *et al.* 2007b). ATMT systems take advantage of a natural plant transformation process brought about by *A. tumefaciens*, a bacterial plant pathogen that carries a ~200-kbp tumor-inducing (Ti) plasmid containing the transferred-DNA (T-DNA), a DNA segment that is randomly inserted into the plant genome during infection (Hoekema *et al.* 1984).

After construction of pUR5750::A1KU80 and pUR5750::A2KU80, binary vectors were electroporated into *A. tumefaciens* strain LBA1100 which carries the virulence (*vir*) genes that code for the T-DNA transfer system. The initial transformation was performed by co-cultivating *P. brasiliensis* yeast cells with *A. tumefaciens* on IM with acetosyringone (AS)

which is an inducer of expression of the *vir* genes in *A. tumefaciens* being considered essential for fungal transformation.

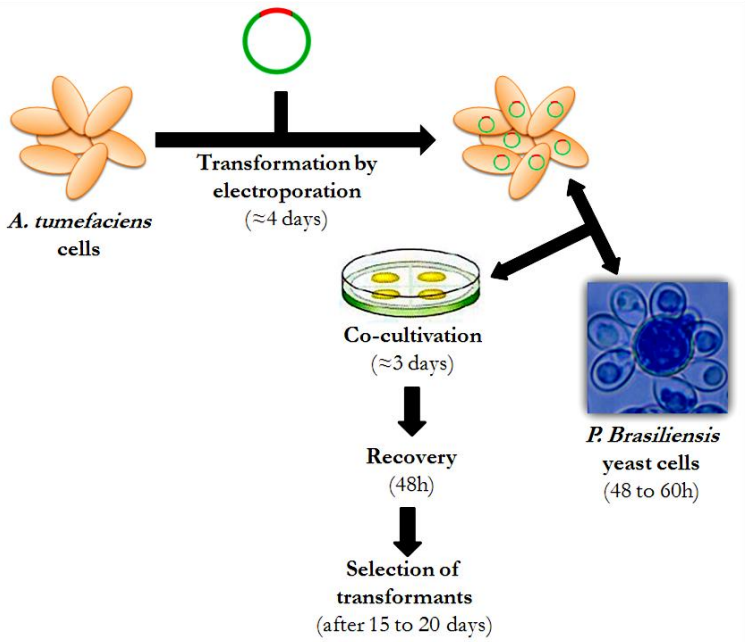


Figure 5. Schematic representation of ATMT of *P. brasiliensis* yeast cells (adapted from Martins 2005).

2.3.4 Downregulation of *PbKU80* using antisense technology

As stated above two different aRNA oligonucleotides designed from the 5'UTR region of *PbKU80* were cloned and inserted into the *P. brasiliensis* genome using ATMT (Figure 6).

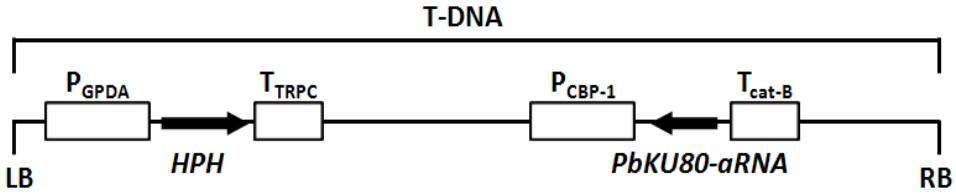


Figure 6. Transfer-DNA (T-DNA) constructs for antisense RNA (aRNA) silencing of *PbKU80* in *P. brasiliensis* via *Agrobacterium tumefaciens*-mediated transformation. T-DNA harboring the hygromycin B phosphotranseferase (*HPH*) gene driven by the *Aspergillus nidulans* glyceraldehyde 3-phosphate (*GPD*) promoter and transcriptional terminator (*TRPC*) with a *PbKU80*-aRNA oligonucleotide under the control of the calcium-binding protein (*CBP1*) promoter from *Histoplasma capsulatum*.

The transformation of *P. brasiliensis* with pUR5750::A1KU80 and pUR5750::A2KU80 was carried out in 3 different strains (Pb18, Pb60855 and Pb Garcia). After transforming *P. brasiliensis* with these binary vectors, transformants were selected using hygromycin B as a selective agent. 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.

Furthermore, genomic DNA from 3 *P. brasiliensis* 60855 transformants of each antisense was extracted and used as template for PCR analysis as described in Material and Methods. The presence of *hph* gene was confirmed by the visualization of a 982 bp fragment in all transformants assayed (Figure 7).

Figure 7. PCR amplification from *P. brasiliensis* 60855 using primers Hyg1 and Hyg2 (as described in Material and Methods). Lanes: m, 100 pb molecular marker; 1-6, *P. brasiliensis* transformants; 7, pUR5750 plasmid DNA; 8, *P. brasiliensis* wild type; 9, negative control. Positive reactions yielded a 982 bp fragment from *hph* gene amplification.

Quantitative real-time polymerase chain reaction (RT-PCR) was conducted both in wild-type and aRNA selected isolates transformed with T-DNA harboring A1KU80 and A2KU80, in order to evaluate *PbKU80* expression levels. Threshold cycle (Ct) values of *Pb*KU80 were compared with the single copy endogenous reference, the *TUB2* gene.

Independent of the aRNA oligonucleotide inserted into *P. brasiliensis* genome among randomly selected clones, *PbKU80*'s expression was not decreased (Figure 8). Transcript levels of *PbKU80* did not vary among the studied clones, both with and without the aRNA molecules (data not shown).

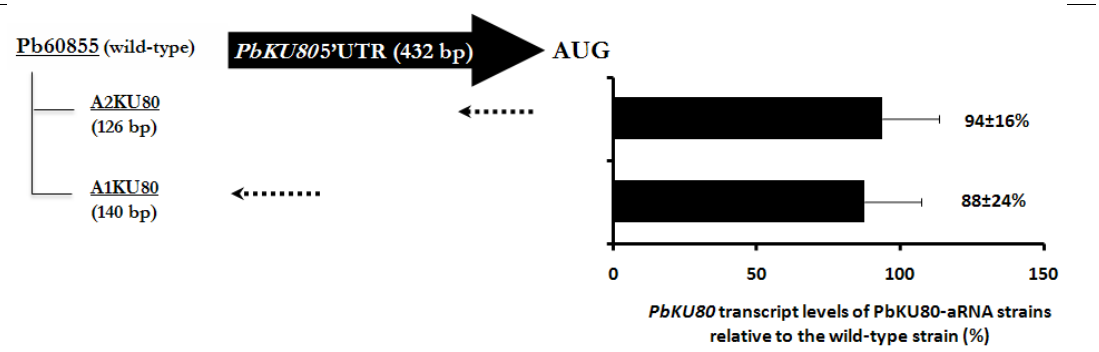


Figure 8. Silencing of *PbKU80* from *P. brasiliensis* 60855 by targeted antisense RNA (aRNA) expression in *P. brasiliensis* wild-type cells decreases mRNA levels. Gene expression levels of *PbKU80* in *P. brasiliensis PbKU80*-aRNA cells harboring distinct repressing oligonucleotides designed in the 5'UTR compared to the respective wild-type strain.

The hypothesis tested in this study was that down-regulation of a specific component required for the NHEJ pathway, in particular the *PbKU80* gene, may result in the HR machinery taking over the integration of exogenous targeting vectors in *P. brasiliensis* genome.

The applied strategy for "silencing," or perhaps more appropriately, perturbing *PbKU80*'s expression was not effective. The notion that gene expression could be modified through use of exogenous nucleic acids derives from studies by Paterson *et al.* (1977), who first used single-stranded DNA to inhibit translation of a complementary RNA in a cell-free system. Since then, technologies using nucleic acids to manipulate gene expression evolved, and problems associated with the use of this technology for modifying gene expression are well known.

One such problems is the presence of a variety of repair/editing enzymes such as helicase and RNA unwindase that can unwound RNA-RNA duplexes and consequently, the ribosomal complex may bind to the mRNA decoding it to produce the corresponding protein (Nellen and Lichtenstein 1993). In addition, the ribosomal complex itself has unwindase activity that likely permits "reading" of the complexly fold mRNA. In these cases, an antisense effect might not occur (Shakin and Liebhaber 1986). Alternatively, the dsRNA may serve as a substrate for editing enzymes such as double-stranded RNA adenosine deaminase (DRADA) (Kim and Nishikura 1993, Kim *et al.* 1994, Kim *et al.* 1994), which as its name implies converts adenosine to inosine by a mechanism called hydrolytic deamination (Polson *et al.* 1991). This enzyme's activity, firstly termed as dsRNA unwinding/modifying activity, is completely specific to dsRNA, although there is no strict sequence requirement in the substrate dsRNA (Bass and Weintraub 1988, Wagner *et al.* 1989). On the other hand, a relatively long double-helical structure of at least 100 bp of duplex seems to be required for efficient modification (Nishikura *et al.* 1991).

The nature of the antisense molecules used for mRNA targeting is also very important in these events that are triggered as a result of duplex formation (Gewirtz *et al.* 1998). In practice, only a few complementary oligonucleotides can successfully hybridize to a targeted mRNA, largely because of problems of target accessibility, which in turn may be because of the secondary or tertiary mRNA structure (Tu *et al.* 1998). Whether being used as an experimental reagent or pharmaceutical, antisense molecules need to meet certain physical requirements to make them useful (Gewirtz *et al.* 1998). After being transcribed, antisense molecules need to be able to hybridize with their intended target. The ability of an antisense molecule to form a stable hybrid is minimally a function of the antisense

molecule's binding affinity and sequence specificity. Binding affinity, in turn, is a function of the number of hydrogen bonds formed between the antisense molecule and the sequence to which it is targeted, having direct influence on the melting temperature or T_M. The T_M, temperature at which 50% of the double-stranded material is dissociated into single strands, depends also on the size and concentration of the antisense oligonucleotides, the nature of the base pairs, and the ionic strength of the solvent in which hybridization occurs (Gewirtz *et al.* 1998).

In summary, the developed strategy for gene targeted mutagenesis did not efficiently modulate the expression of *PbKU80*. Thus, to achieve the proposed goals, other antisense molecules need to be designed, perhaps with a different length or from another location without being the 5' UTR.

Chapter 3

Yeast Cellular Morphology of *P. brasiliensis* and *CDC42* Expression

The results presented over this chapter were published in a conference proceeding,

Almeida A.J., Barros D., Hernández O., Oliveira P., McEwen J., Ludovico P. and Rodrigues F. (2009) *Paracoccidioides brasiliensis* yeast cells do not obey standard rules during cell growth, at the National Congress of Microbiology, "MICROBIOTEC 2009", in Vilamoura, Portugal (poster presentation).

3.1 Introduction

Generally, yeasts life cycle have three phases: cell division, mating and sporulation (Chant 1999). So, given sufficient nutrients, a cell in preparation for cell division increases in size, copies its DNA and prepares to divide (Herskowitz 1988). In what concerns cell division, an alteration of cell shape is made via the generation of asymmetry in the organization of cytoskeletal elements, usually leading to actin localization at sites of growth (Perez and Ricón 2010). On the other hand, in all eukaryotic cells, from yeast to mammals, signaling pathways that control actin organization and morphogenetic processes are regulated by the Rho family of GTPases like CDC42 (Perez and Ricón 2010).

However, the process by which each mother cell gives rise to one ellipsoidal daughter cell made of entirely new cell surface material seems not to occur in *Paracoccidioides brasiliensis*. Moreover, the absence of an active size-sensing mechanism that control the cell cycle ensuring the maintenance of a proper balance between growth and proliferation rates in this pathogenic fungus appears to be evident (Almeida *et al.* 2006, 2009).

Besides its distinctive budding pattern, the etiological agent of PCM also exhibits a polymorphic cell growth, defined by the existence of extreme variations in cell size and shape in both mother and bud cells during growth within the same cellular population. This aspect suggests once more that *P. brasiliensis* yeast cells may follow, if not a different set of rules, at least a lax control of the establishment and maintenance of polarity during growth (Almeida *et al.* 2009). Therefore, and since there are very few studies on these issues as relevant factors (Brummer *et al.* 1990, Svidzinski *et al.* 1999, Kurokawa *et al.* 2005), one of the main goals of the work developed herein was to analyze different morphological features, such as mother cell size and form, bud number, size and form of *P. brasiliensis* wild-type yeast cells from the 3 recently classified distinct lineages (S1, PS2, and PS3) (Matute *et al.* 2006) and possible correlations that might exist among them, using both clinical and environmental isolates. Moreover, we additionally aimed to evaluate a possible correlation between the expression of Cdc42 (Pbcdc42p) with the analyzed morphological features. This pleiotropic Rho GTPase signaling molecule is a critical modulator within a variety of biological processes, namely, as a polarity cue molecule during growth and morphogenesis, presenting overlapping functions in various cell types and both unicellular and multicellular organisms (Cotteret and Chernoff 2002, Harris and Momany 2004). For example, in the budding yeast *Saccharomyces cerevisiae* Cdc42 is involved in the coordination of bud emergence and growth, cell cycle progression, cytokinesis, actin cytoskeleton remodeling, pseudohyphal growth, vesicle dynamics, and mating (Johnson 1999, Nelson

2003). In addition, a class of *CDC42* mutants in *S. cerevisiae* displayed a multiple budding phenotype and a disturbed control of polarized growth (Richman and Johnson 2000, Caviston *et al.* 2002, Richman *et al.* 2002, 2004). However, in dimorphic and filamentous fungi, this pivotal molecule was shown to intervene in phenomena such as the morphological transition, correct polarized growth, pathogenesis and cell separation (Ye and Szaniszlo 2000, Boyce *et al.* 2001, Michel *et al.* 2002, Ushinsky *et al.* 2002, Bassilana *et al.* 2003, Van den Berg *et al.* 2004, Mahlert *et al.* 2006). Moreover, Almeida *et al.* (2009) evaluated the role of this pivotal molecule during cell growth and virulence trhough antisense aRNA technology. By knocking-down *PbCDC42's* expression in *P. brasiliensis* yeast cells, a decrease in cell size and more homogenous cell shape was promoted. Also, by altering the typical polymorphism of wild-type cells, due to the *PbCDC42's* reduced expression levels, an increased phagocytosis and decreased virulence in a mouse model of infection was revealed.

3.2 Materials and Methods

3.2.1 Microorganisms and culture media

For the assays carried out in this study *P. brasiliensis* yeast cells were maintained at 36ºC by periodic subculturing to fresh media every 4 days in brain heart infusion supplemented with 1% glucose (BHI) liquid media (Restrepo and Jimenez 1980) with aeration on a mechanical shaker (200 rpm). Ten isolates of *P. brasiliensis* were employed, six obtained from patients with PCM and four isolated from natural sources (Table 1).

Table 1. List of *Paracoccidioides brasiliensis* strains analyzed during this study.

Strain identification	Phylogenetic species	Country of isolation	Source	Citation
18	S1	Brazil	Chronic PCM	(Teixeira *et al.* 1987)
T15LN1	S1	Brazil	Natural*	(Hebeler-Barbosa *et al.* 2003)
Gonchart	S1	Colombia	Chronic PCM	(Matute *et al.* 2006)
T10B1	PS2	Brazil	Natural*	(Hebeler-Barbosa *et al.* 2003)
2	PS2	Venezuela	Chronic PCM	(Morais *et al.* 2000)
BT84	PS2	Brazil	PCM	(Hebeler-Barbosa *et al.* 2003)
Cabasus	PS3	Brazil	Natural*	(Matute *et al.* 2006)
60855	PS3	Colombia	Chronic PCM	(Gomez *et al.* 2001)
Garcia	PS3	Colombia	Chronic PCM	(Matute *et al.* 2006)
Tapias	PS3	Brazil	Natural*	(Matute *et al.* 2006)

***** Environmental and non-human host sources

3.2.2 Real-time polymerase chain reaction (RT-PCR)

Total RNA from exponentially growing *P. brasiliensis* yeast cultures was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) standard procedures and heat shock treatment (20 min at 65ºC followed by 60 min at -80ºC) 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 for the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Nutley, NJ, USA) to amplify the *P. brasiliensis CDC42* gene (152 bp) and the *TUB2* gene (64 bp) using the primers indicated in Table 2. 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 ºC for 20 s, a melting step of 55º-95 ºC (0.5 ºC/s), and a final cooling at 40 ºC for 30 s. Real-time quantification was performed on a LightCycler System (Roche) using threshold cycle (Ct) values for *TUB2* transcripts as the endogenous reference. mRNA differential *CDC42* expression levels were evaluated by normalizing CDC42 Ct values with the reference and comparing the ratio amongst the tested samples.

Table 2. Primers used in this study for Real-Time Polymerase Chain Reaction (RT-PCR).

Primer sequences (5'-3')
P1Pbcdc42-CGTTACATCCCCAGCATCTT
P2Pbcdc42-TCTTCTGCTTGGCCAACTTT
P1Pbtub2-AGCCTTGCGTCGGAACATAG
P2Pbtub2-ACCTCCATCCAGGAACTCTTCA

3.2.3 Microscopic count methods

The quantification of morphological parameters, namely number of mother and bud cells, cell size and form of *P. brasiliensis* exponentially growing yeast cells was performed using Stereo Investigator software (MicroBrightField) and a camera (DXC390; Sony, Japan) attached to a motorized microscope (Axioplan 2; Zeiss, Germany). A minimum of 150 mother cells and respective buds were analyzed.

Stereo Investigator is a stereology system that allows us to obtain the most efficient, precise and unbiased estimates of cell populations, as well as morphometric properties of biological structures. This software has been cited in over one thousand peer-reviewed journal articles and it has contributed to recent advances in disease research, including Alzheimer's, Parkinson's, and Huntington's.

Bright field microscopy was performed with a Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany) epifluorescence microscope fitted with 10x eyepieces and 100x (oil immersion) objective and equipped with a Carl Zeiss AxioCam (HR/MR) and Axio Vision 3.1 software.

3.2.4 Reproducibility of the results and statistical analysis

Data are reported as 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 for Windows (San Diego, CA, USA). The One-way ANOVA analysis of variance was applied to assays in Figure 2 ($p<0.05$) and a linear regression for Figure 4A and 6.

3.3 Results and Discussion

3.3.1 Evaluation of morphological parameters reveals high heterogeneity among *P. brasiliensis* strains

P. brasiliensis has been the subject of numerous investigations concerning its ecology (Restrepo 1985, Bagagli *et al.* 2008), thermal dimorphism (Patiño *et al.* 1984, Theodoro *et al.* 2008), ultrastructure (Carbonell 1967, Carbonell and Gill 1982), growth characteristics (Arango and Restrepo 1976, Kashino *et al.* 1985) and biochemistry (San-Blas *et al.* 1984, Campo-Aasen 1985), but attempts to correlate physiological patterns have failed (Guarro *et al.* 1999). This dimorphic pathogenic fungus presents as its most distinctive feature a multiple budding phenotype; however, the molecular bases underlying this particular morphological trait are still unknown (Restrepo-Moreno 2003), although some significant progresses have recently been made (Almeida *et al.* 2006, 2007 and 2009).

One of the main goals of the work developed in this chapter was to characterize different morphological features of *P. brasiliensis* wild-type cells using both clinical and natural strains (Table 1). To achieve our purpose we used a stereomicroscope which allowed the contour measurement of exponentially growing batch culture yeast cells from the recently classified 3 different phylogenetic species (S1, PS2, and PS3). Overall, 150 mother cells and respective buds were analyzed from 10 different strains (Table 1).

A preliminary analysis, based on microscopic observation of some strains, revealed a high heterogeneity among *P. brasiliensis* strains (Figure 1). It is a well-known fact that fungi

are extremely variable organisms with strains of the same species showing different morphological features (Kashino *et al.* 1985). As shown in Figure 1, there is large heterogeneity regarding the morphological features of the studied strains: there are spherical mother cells with similarly-sized buds all over its surface; single budded cells; abnormal mother cells with elongated or thin-necked buds; mother cells with buds of different sizes; and large unbudded cells.

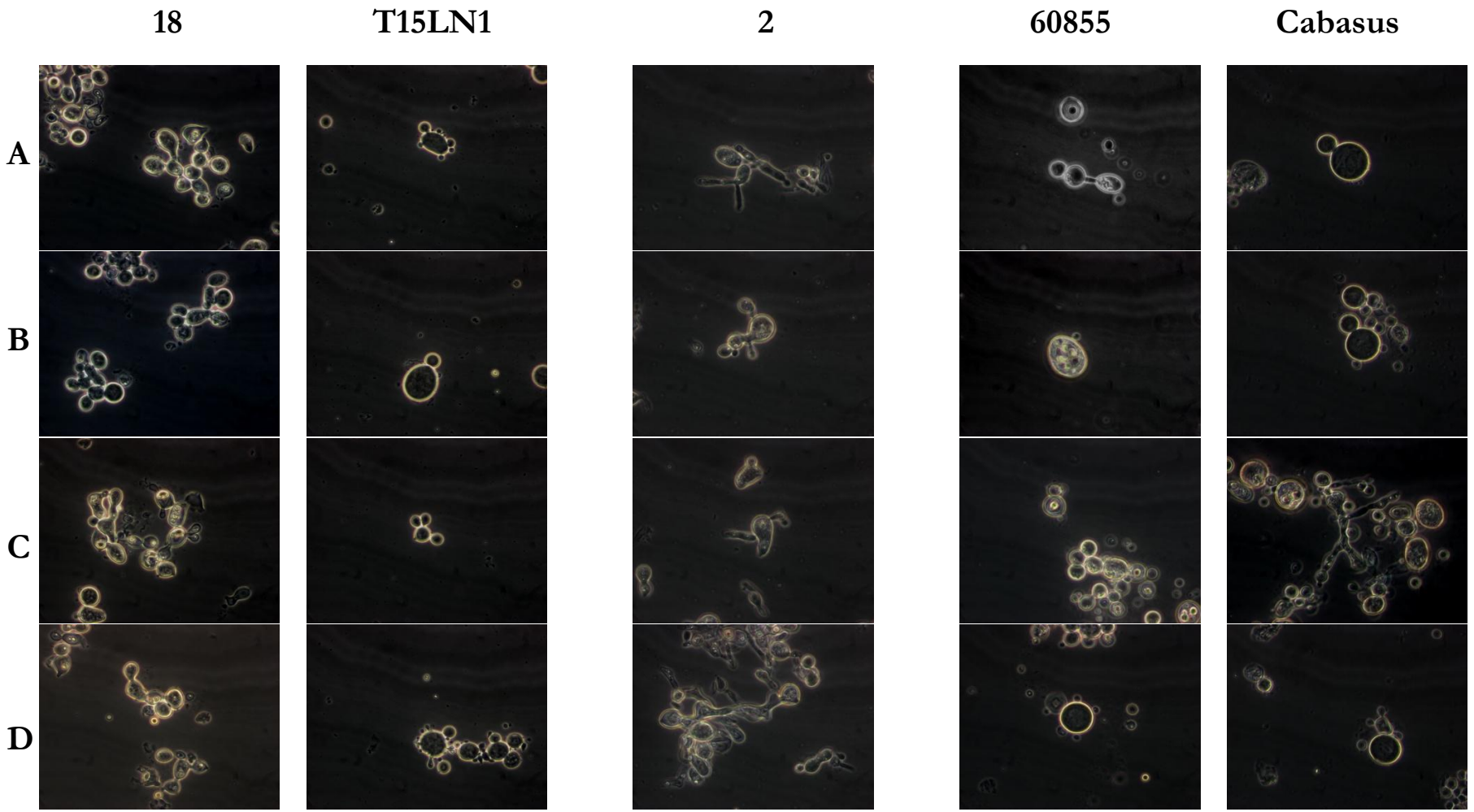


Figure 1. Bright field microscopy analysis of *P. brasiliensis* wild-type yeast cells. Representative images of different strains (18, T15LN1, 2, 60855 and Cabasus) reveal high heterogeneity both within and among strains. Magnification, ×1000. A, B, C and D stand for replicas of the same strain.

Morphological parameters were also quantitatively evaluated to allow a more detailed analysis (Figure 2).

Starting with the evaluation of the cell size, both the mother (Figure 2AI) and bud cell (Figure 2AII) are very heterogeneous both within the same strain and among different strains. Even more interesting is the elevated variation of bud cell size per mother cell that is also very high (Figure 2AIII). In fact, mother and bud cell size may vary, depending on the strain, from 8 to 710 μm^2 and from 3 to 260 μm^2, respectively. However, heterogeneity is such that the highest and the lowest measured averages do not correspond to the same strains that present these extreme values. Although all strains present high variation in mother and bud cell size, the strains with greater variation are T10B1, 60855 and Tapias for mother cell size and 2, 60855 and Tapias for bud size. The strains with the highest variation of bud cell size per mother cell are strains 2 and 18.

Regarding cell form, a high heterogeneity is also patent (Figure 2B). Cell form is measured by the complexity of the drawn contour; a perfect circle corresponds to 1.0, but when it flattens out, this value approaches 0.0. Both the mother (Figure 2BI) and bud cell (Figure 2BII) are very heterogeneous either within the same strain or among different strains. Also, the variation of bud cell form per mother cell is very high (Figure 2BIII). The mother cells of strain Tapias are the most circular, while the mother cells of strains 2 and BT84 are characterized by a more apical-type growth. Tapias also presents the rounder bud cells, while strains 2 and BT84 are on the other extreme. With respect to average bud form per mother cell, T10B1 is the strain with smaller variation and strains 2 and BT84 with the highest variation.

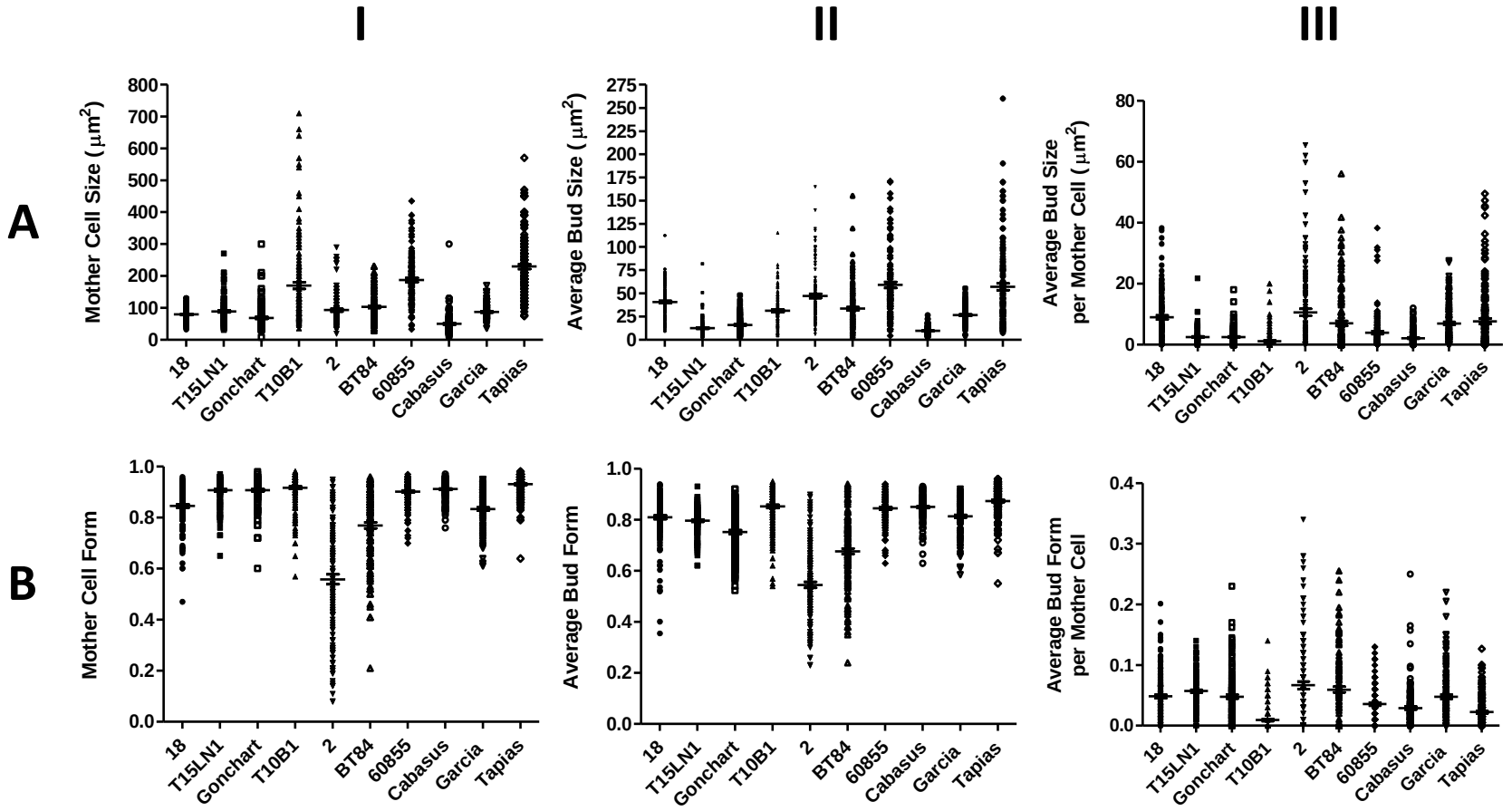


Figure 2. Morphological analysis of *P. brasiliensis* wild-type yeast cells using a stereoscope microscope. Results show high heterogeneity in cell size (A) and form (B) in both mother (I) and bud (II) cells. As the contour shape approaches of a perfect circle this value tends to 1.0. As the contour shape flattens out, this value approaches to 0.0. The average bud size and form per mother cell (III) represented by the standard error of the mean (SEM) of IIA and IIB is also very high, meaning that one mother cell has buds with very different sizes and forms.

Besides cell size and form, bud number per mother cell was also quantified (Figure 3). Regarding this aspect, while most strains showed no predominance, strains T10B1 and T15LN1 presented a higher frequency of cells with 1 and more than 5 buds, respectively. Strains 18, Garcia, Gonchart and Cabasus presented a higher percentage of mother cells with 2 and 3 buds, while strains 2 and Tapias were mainly characterized by presenting mother cells with 1 and 2 buds.

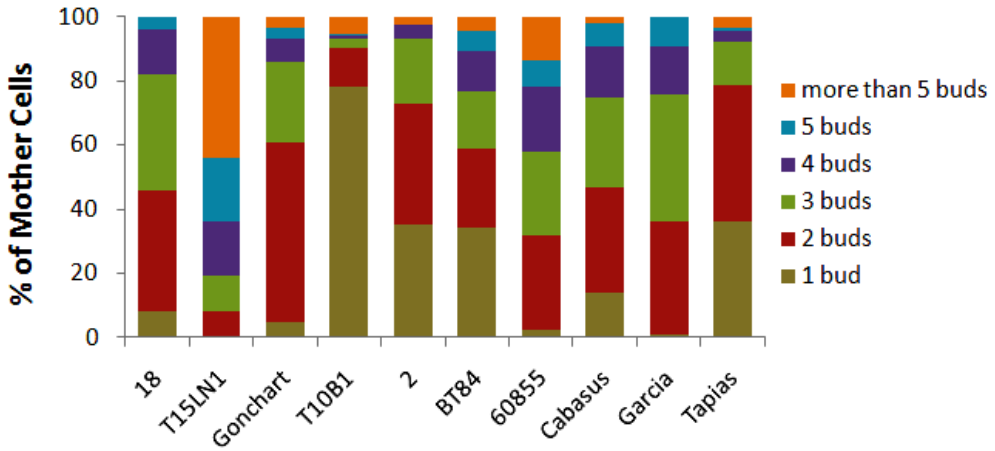


Figure 3. Budding pattern of 10 *P. brasiliensis* strains (18, T15LN1, Gonchart, T10B1, 2, BT84, 60855, Cabasus, Garcia and Tapias).

These data reinforce the polymorphic nature of *P. brasiliensis* wild-type yeast cells among all the studied strains. However, taking into account the extensive variability in *P. brasiliensis*, no association is detectable between the phenotypic differences in the strains herein analyzed and the cryptic species of *P. brasiliensis*.

To further analyze the collected data, we have inquired on possible correlations between the studied biological variables. Figure 4A shows the correlation between mother cell size and average bud size and Figure 4B illustrates the correlation among mother cell size and bud number per mother cell. No association or trend between the analyzed parameters was found; however, one cannot rule out the possibility that the reduced sample size may be masking an important relationship between the biological parameters.

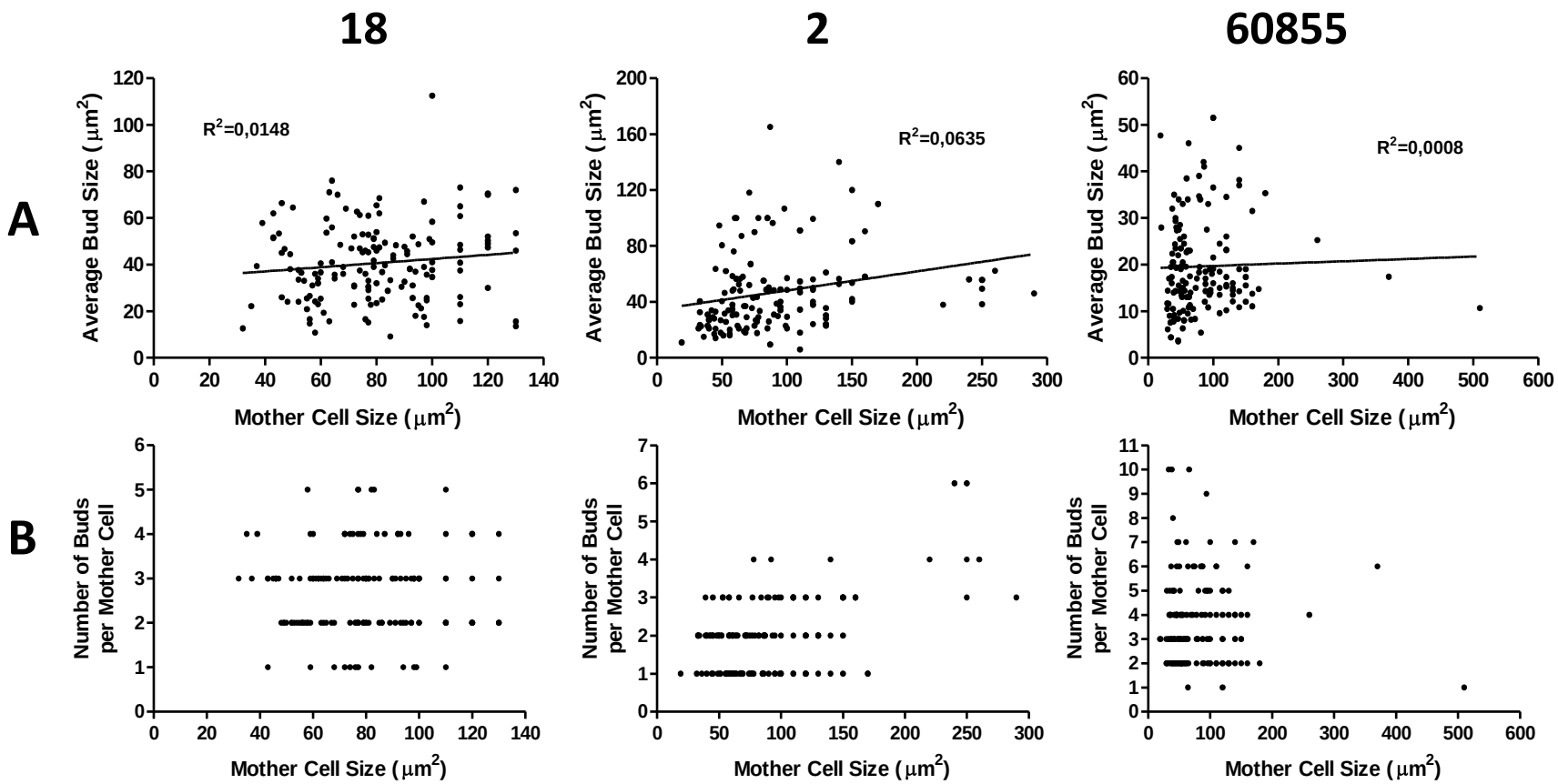


Figure 4. Relation between analyzed morphological parameters for strains 18, 2 and 60855. (A) Correlation between mother cell size and average bud size. (B) Correlation between mother cell size and number of buds per mother cell. R^2, coefficient of determination.

As reported earlier, the morphological data here obtained varied among the studied strains, and it is clear that the heterogeneity revealed by *P. brasiliensis* wild-type yeast cells is significant even within the same strain.

The most physiologically relevant cell cycle control stands for the coordination between cell growth and cell division. This coordination makes it possible to maintain cell size homeostasis, thus preventing cells from becoming too small or too large (Porro *et al.* 2009). In the budding yeast *Saccharomyces cerevisiae*, a model for understanding the cell cycle progression of eukaryotic cells, cells have to grow to a critical cell size before start budding (Porro *et al.* 2009). In contrast, *P. brasiliensis* does not seem to follow this rule since mother cells of various sizes were observed within the same strain, but we also must take into account the age of mother cells (i.e., the number of cycles it has passed through). Regarding bud cells, once again in *S. cerevisiae* all daughters have the same critical cell size requirement (Porro *et al.* 2009) while in *P. brasiliensis* this does not happen since mother cells with buds from different sizes can be observed.

3.3.2 Effect of *PbCDC42* expression on the cellular morphology of *P. brasiliensis*

The process of budding in *Saccharomyces cerevisiae* is initiated once per cell cycle at a specific time of the cell cycle and at a specific location on the cell surface; however, even though research around the molecular nature of the temporal and spatial regulatory mechanisms has been strengthen little is known. Moreover, it is also unclear what factors, if any, among the numerous proteins required to make a bud are involved in the determination of budding frequency (Caviston *et al.* 2002), although, a recent study has shown that to ensure the formation of only one bud, cells must polarize toward one, and only one, site. Howell and co-workers (2009), in order to assess whether singularity is linked to the Rho family GTPase Cdc42, which concentrates at polarization sites establishing polarity, modified the Cdc42p amplification mechanism by creating a novel fusion protein designed to "rewire" the endogenous yeast polarization pathway. The rewired cells polarized and successfully proliferated, but often polarized to two sites simultaneously and sometimes made two buds, violating singularity. Even cells that made only one bud sometimes initiated two clusters of Cdc42, but then one cluster became dominant. Combined experimental and theoretical analysis of both wild-type and rewired cells suggests that when more than one Cdc42p cluster forms, the amplification mechanisms engender competition between the clusters, eventually producing a single bud.

The process by which the three-dimensional organization of subcellular constituents, which ultimately determines an organism's characteristic growth and shape, is generated and maintained is called morphogenesis. During this complex process, the establishment of cell polarity is critical, thereby leading to asymmetrical growth, resulting in formation of buds (Johnson 1999). Also, in this context it is essential to distinguish the concept of polarity establishment, defined as an asymmetric distribution of specific proteins and organelles near a defined spatial site, allowing the cell to increase its surface in an asymmetrical trend, from the concept polarized growth which requires secretion and surface extension (Bähler and Peter 2000). Both events require the recruitment of proteins such as the essential small Rho-like GTPase Cdc42p and those involved in Cdc42p-mediated pathways (Cabib *et al.* 1998, Johnson 1999, Pruyne and Bretscher 2000).

In this study, the expression levels of this central molecule were accessed in seven *P. brasiliensis* strains, two from groups S1 and PS2 and 3 from group PS3 (Figure 5).

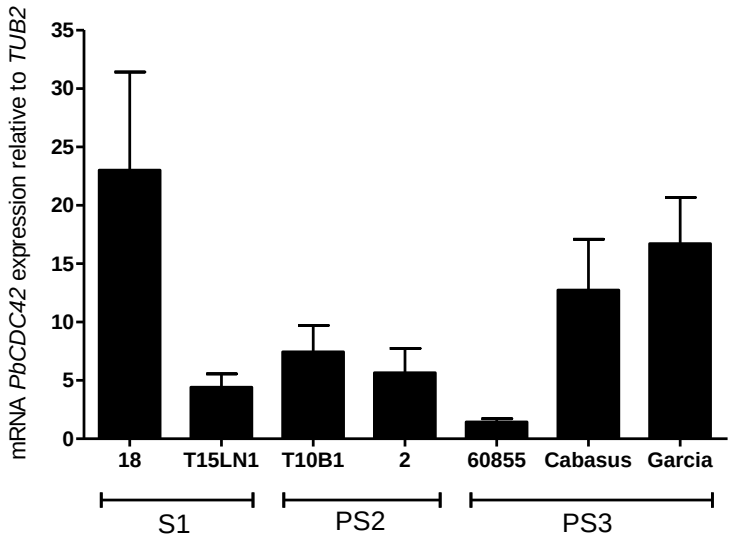


Figure 5. Gene expression levels obtained by RT-PCR analysis of *PbCDC42* in *P. brasiliensis* wild-type yeast cells (*PbCDC42* expression levels were normalized using the internal reference *TUB2*).

PbCDC42 expression levels seem heterogeneous within the 3 cryptic species groups, although with less variability in group PS2. Nonetheless, more strains must be analyzed before making any definite conclusions.

Variations in gene expression of *P. brasiliensis* have previously been found between strains belonging to S1 and PS2 species (Hebeler- Barbosa *et al.* 2003; Carvalho *et al.* 2005), and their phenotypic differences were not associated with cryptic species. Nevertheless, the two species share host species, humans and armadillos, and both are capable of inducing disease (Carvalho *et al.* 2005). For example, *PbGP43*, the first gene fully characterized in *P.*

brasiliensis, which encodes an immune dominant diagnostic antigen (gp43) has also been reported to show heterogeneity between different strains (Carvalho *et al.* 2005, Costa *et al.* 2010). However, a recent study by Matute and his colleagues have revealed that *PbCDC42* show very little variation during evolution (Matute *et al.* 2008).

We also evaluated a possible correlation between *PbCDC42*'s expression and the morphological features previously quantified.

Interestingly, expression of *PbCDC42*, although heterogeneous among the 3 cryptic species, shows a certain level of correlation with bud number in the tested strains (Figure 6), but not with any of the other analyzed morphological features (data not shown). The results showed that increasing *PbCDC42* expression, the number of mother cells with 2 and 3 buds (Figure 6A) and the number of mother cells with more than 5 buds (Figure 6B) decrease.

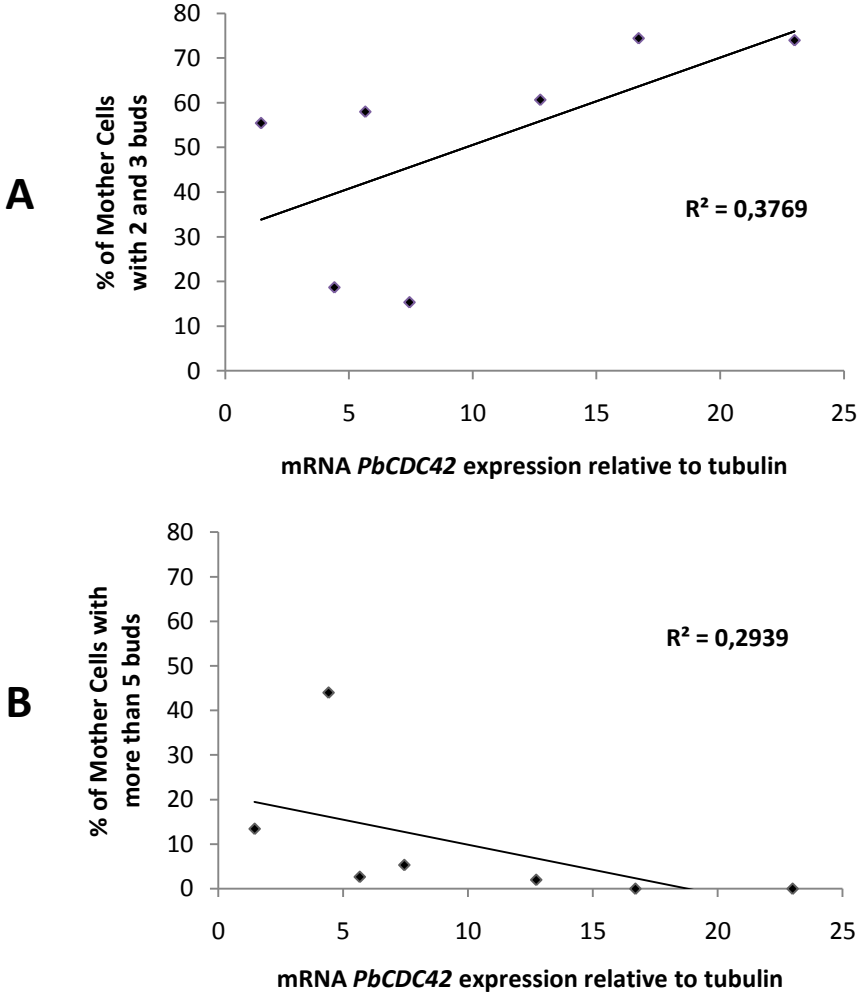


Figure 6. Relation between the percentage of mother cells with 2 and 3 buds (A) and with more than 5 buds (B) and the *PbCDC42* expression levels for strains 18, T15LN1, T10B1, 2, 60855, Cabasus and Garcia.

Over the years, diversity among *P. brasiliensis* strains has been reported at several levels, namely in terms of microscopic (Svidzinski *et al.* 1999, Restrepo 2000), macroscopic (Macoris *et al.* 2006), growth and transition characteristics (San-Blas *et al.* 1997), lipid composition (Hamdan *et al.* 1992), cell wall components (Crott *et al.* 1997), isoenzyme profiles (Svidzinsky *et al.* 1995), antigen production (Mendes-Giannini *et al.* 1995, Moura-Campos *et al.* 1995), protein contents (Salem-Izacc *et al.* 1997), and virulence degree (Hebeler-Barbosa and Bagagli 2003). Comparative studies of *P. brasiliensis* strains have been made i*n vivo* and i*n vitro* by several authors. Some studies employed to analyze growth curves patterns, colony morphology, ultrastructure of yeasts have shown variability between *P. brasiliensis* strains, but no association with pathogenicity was observed (Kashino *et al.* 1985, 1987). The different morphological and physiological characteristics observed in those studies were ascribed not only to the heterogeneity of *P. brasiliensis* strains, but also to the various experimental conditions employed as well as to the different sources at geographically distinct places from which the dimorphic fungus was isolated and how long ago it was (Kashino *et al.* 1985, 1987, Calcagno *et al.* 1998, Sano *et al.* 1998, Kurokawa *et al.* 2005). Genetic changes or microevolution on standard strains have also been suggested for other fungi such as *C. neoformans*, *B. dermatitidis* and *H. capsulatum*, being associated with phenotypic changes and loss of virulence (Kurokawa *et al.* 2005). Even when maintained and submitted to strictly the same procedures *P. brasiliensis* heterogeneity within the strains was demonstrated. This fact indicates that morphological and physiological characteristics of *P. brasiliensis*'s strains, under controlled conditions, are dependent on intrinsic biological properties of these fungi (Kashino *et al.* 1987). On the other hand, Carvalho and co-workers (2004) believe that features like animal model, route of infection and type of inoculums (degree of *in vitro* or *in vivo* adaptation, culture and growth conditions, etc.) might considerably affect morphological and physiological characteristics of *P. brasiliensis*'s strains. Although these studies elucidated some biological aspects of different *P. brasiliensis* strains, further investigations were and still are necessary to better characterize the causal agent of PCM.

P. brasiliensis's genetic heterogeneity has also been assessed by several genetic tools, such as random amplified polymorphic DNA (RAPD) analysis (Molinari-Madlum *et al.* 1999, Kurokawa *et al.* 2005), restriction fragment length polymorphism (RFLP) (Niño-Vega *et al.* 2000) and polymorphic microsatellites located on noncoding and coding sequences (Nascimento *et al.* 2004, Matute *et al.* 2006). Multiple factors are likely to contribute to the high variability in the genome and regulation of transcriptome of *P. brasiliensis* and other

fungi (e.g., the residence of the fungus in many soil types or microclimates). Thus, genetic drift and unique selection pressures impacting on the organism in various environmental niches may also influence its genetic structure (Calcagno *et al.* 1998).

In the case of *P. brasiliensis*, it is particularly important to classify its strains because this fungus is the causative agent of PCM, one of the most prevalent systemic mycoses (Restrepo-Moreno 2003). PCM infection has several pathologies which have been classified into categories according to type of lesion and patient characteristics, leading to the occurrence of different clinical forms of the disease (Restrepo-Moreno 2003). This diversity has been reported has been due to not only host-related factors (e.g. gender, age, immunological status) but also to characteristics of the infecting agent, mainly its virulence., suggesting that strain variability plays an important role in host-parasite relationships (Kashino *et al.* 1985, 1987, Franco 1987). This fact was already been reported by Franco *et al.* (1996) when *P. brasiliensis* antigen batches from the same strain showed immunological and biochemical differences under controlled incubation conditions. On the other hand, previous studies by Almeida and co-workers (2006) reported a differential cell cycle progression of *P. brasiliensis* yeast form during batch growth cultures in defined or complex nutritional environments.

Taking in account Almeida *et al.* (2009) previous work, where *PbCDC42* was reported for the first time as being responsible for the yeast cell growth regulation, a detailed analysis of its pathway's underlying molecular machinery may provide further insight into the regulation of cell growth and pathogenesis, not only of *P. brasiliensis*, but also other human fungal pathogens.

With this work, we have proven in a quantified and structured way the highly heterogenic morphology of the dimorphic fungus *P. brasiliensis*, showing variation both inter- and intra-strain.

Altogether, the present investigation provides a quantitative assessment of morphological traits of *P. brasiliensis* yeast cells, suggesting that this pathogenic fungus does not follow standard rules during growth. The true relevance for its adaptation and survival throughout its life cycle will require more detailed analysis in different environmental conditions.

Chapter 4

Concluding Remarks and Future Prospects

The increased incidence of systemic fungal infections in the past two decades has been overwhelming, constituting a major public health problem in many parts of the world, both in developed and developing countries. Fungi are extremely adapted for survival as evidenced by their ubiquity in nature, however, of the estimated several hundred thousand species of fungi, less than 150-200 were considered to be pathogens of humans (Chakrabarti 2005). One such fungal pathogen is *Paracoccidioides brasiliensis*, the etiological agent of paracoccidioidomycosis (PCM), the most important systemic mycosis in Central and South America (Restrepo-Moreno 2003).

With this thesis, we intended to further contribute to elucidate biological phenomena of *P. brasiliensis*, following two main directives: on one hand to continue the development of molecular tools for *P. brasiliensis* and on the other to characterize cellular morphology of *P. brasiliensis* wild type yeast cells. In compliance with the primary objectives of this thesis, a gene targeted mutagenesis methodology, applying antisense RNA (aRNA) technology, was developed that allows gene disruption through homologous recombination, by down-regulating *PbKU80's* expression, a key player of the non-homologous end joining (NHEJ) pathway (Chapter 2). For that, repressing oligonucleotides targeting the 5'UTR with single copy insertion of the cassette (T-DNA) into *P. brasiliensis* genome were used. The application of this method proved unsuccessful with very low reduction of *PbKU80*'s expression levels, suggesting that the characteristics of the designed aRNA oligonucleotides hampered the success of this technique. Whether because of the fact that *P. brasiliensis* is relatively unamenable in what refers to tractable molecular techniques or because of the reasons previously discussed, ongoing work on our laboratory is exploiting other hypotheses.

Specifically, work is being developed on:

i. Down-regulation of other key players involved in the NHEJ pathway such as *KU70* or a gene involved in the ligation step of DNA in DSBs repair, for instance the DNA ligase IV gene (*Dnl4*);

ii. Development of a tetracycline operator system that allows the modulation of gene expression based on prokaryotic regulatory elements that respond to minute tetracycline concentrations without compromising the eukaryotic host.

In addition, since *P. brasiliensis* yeast form is generally characterized by a multiple budding phenotype and a polymorphic growth suggesting that its yeast cells do not obey the critical mass rule between mother and daughter cells like other yeasts (e.g., *S. cerevisiae*), a great effort was conducted in order to characterize cellular morphology of *P. brasiliensis* wild-type yeast cells and a possible involvement of the Rho-like GTPase Cdc42p in the process. The morphological assessment by quantitative analysis confirmed the polymorphic nature and growth of *P. brasiliensis* wild-type yeast cells among all existing cryptic species complex. Furthermore, no associations were detected between the various parameters analyzed with exception to *PbCDC42* expression levels which, although heterogeneous among the 3 cryptic species, showed correlation with bud number, but not with any of the other analyzed morphological features.

These results represent only the beginning of multiple future purposes, prospecting the morphological assessment of *P. brasiliensis* yeast cells as an area under intense discussion. In line with the objective to characterize cellular morphology of *P. brasiliensis* wild type yeast cells, several integrative studies are underway, or, at least, under evaluation:

i. Investigate the particularities that the pleiotropic Pbcdc42p may have and underlie its specific function in *P. brasiliensis*;

ii. Specific mutations in the Rho-like GTPase gene *CDC42* from *Saccharomyces cerevisiae*:
    a. site-directed mutagenesis of *ScCDC42*;
    b. evaluation of the phenotype of *S. cerevisiae* Δ*cdc42* cells harboring the mutagenic proteins;

In addition, ongoing work in our lab is being addressed to investigate host factors in acquiring PCM. Following this line, the identification of host-genetic variants that may enhance predisposition to infection by *P. brasiliensis* and the detection of defined virulence factors of this dimorphic fungal pathogen are in sight.

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