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Yeast Primer

New insights into a complex fungal pathogen: the case of *Paracoccidioides* spp.

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Abstract

Paracoccidioidomycosis is a systemic mycosis endemic to Latin America, with *Paracoccidioides brasiliensis* and *P. lutzii* being the causal agents of this disorder. Several issues have been raised in the 100 years since its discovery and in this article we discuss features of this fascinating fungal pathogen, including its biology, eco-epidemiology and aspects of its pathogenicity. We also consider some of its virulence determinants, the most recent advances in the study of its metabolic pathways and the molecular and genetic research tools developed for this research. We also review the animal models used to study host–fungal interactions and how the host defence mechanisms against this pathogen work. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: ***Paracoccidioides brasiliensis*; *Paracoccidioides lutzii*; paracoccidioidomycosis; virulence factors; metabolic pathways; host defense mechanisms**

Introduction

The fungus *Paracoccidioides* spp. is the causal agent of paracoccidioidomycosis (PCM), a systemic mycosis endemic to Latin America with Brazil, Colombia, Argentina and Venezuela being the countries with the highest numbers of reported cases. An estimated 10 million people have been infected with this fungus to date (Brummer *et al*., 1993; Restrepo *et al*., 2015).

Paracoccidioides is a thermally dimorphic fungal pathogen that exhibits two morphotypes, i.e. a mould occurring at temperatures $< 28\,^{\circ}C$, composed of thin septated hyphae that in turn produce conidia (considered as the infectious propagules) (Restrepo *et al*., 2011, 2015) and a yeast found in cultures or in the host at $37\,^{\circ}C$ composed of variably-sized oval to round cells (blastoconidia), characterized by the formation of larger mother cells surrounded by multiple daughter cells, resembling a pilot wheel (Figure 1) (Restrepo *et al*., 2011, 2015; Marques, 2013).

The genus *Paracoccidioides* belongs to the Phylum Ascomycota, Class Euromycetes, Order Onygenales and Family Ajellomycetaceae (Onygenaceae) (Bagagli *et al*., 2008). It comprises two species: *P. brasiliensis*, considered to be a complex of four distinct phylogenetic lineages (S1, PS2, PS3 and PS4), and the recently described *P. lutzii* (formerly 'Pb01-like') (Matute *et al*., 2006; Teixeira *et al*., 2009; Salgado-Salazar *et al*., 2010; Theodoro *et al*., 2012; Teixeira *et al*., 2013).

Clinical presentations of PCM include subclinical or asymptomatic infection and the symptomatic or clinical manifested disease which cause an acute/subacute or a chronic form, the latter involving the lungs as well as other organs (Restrepo *et al*., 2015; Bocca *et al*., 2013; Restrepo *et al*., 2012).

Various functional (*in vitro* and *in vivo*) and molecular studies have been performed in order to evaluate and identify the role of several genes and proteins involved in fungus–host interactions; more recently, new strategies using anti-sense RNA technology have allowed the presence of virulence factors to be demonstrated, as well as that of molecules involved in both the oxidative and metabolic pathways (Menino *et al*., 2012). Several animal models have led to an understanding of the

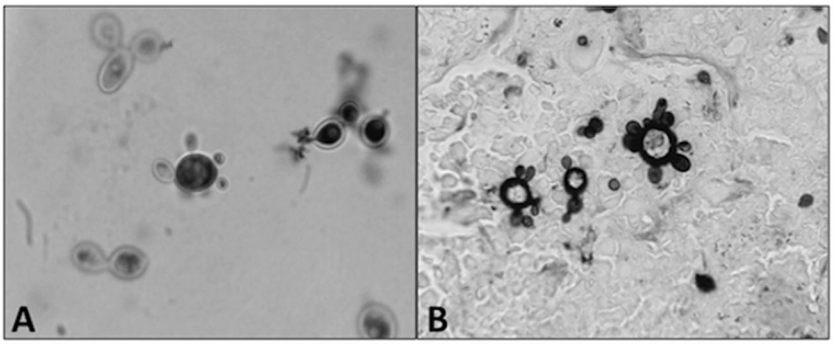

Figure 1. (A) *Paracoccidioides brasiliensis* yeast surrounded by budding daughter cells; wet mount from culture. (B) *P. brasiliensis* in mouse tissue; GMS stain; magnification ≈ ×1000

pathogenesis of this fungal infection, from the initial infection process through the chronic stages, using both the conidia and yeast cells (González *et al*., 2008b; Calich *et al*., 1985; Singer-Vermes *et al*., 1993a).

Here we present an overview of some of the scientific breakthroughs achieved in studies of *Paracoccidioides* spp. and briefly touch on some key aspects of its biology, including its eco-epidemiology, pathogenesis, host defence mechanisms, genome, genetics, metabolism and the use of new molecular tools to understand the biology of this fungal pathogen.

Paracoccidioidomycosis: the disease

PCM is acquired after inhalation of infectious propagules in the environment, leading to a primary pulmonary infection. If the infection is not controlled by the immune response, patients may develop clinical manifestations, mainly as an acute/subacute or a chronic form (Restrepo *et al*., 2012, 2015).

Although subclinical or asymptomatic infection is not associated with any specific clinical characteristics, in these patients the fungus may remain latent in the tissue for years without any manifestation of disease. The acute/subacute or juvenile-type disease evolves rapidly and mainly affects children or young adults, representing about 10% of all cases. This clinical form is characterized by involvement of the reticulo-endothelial organs (lymph nodes, liver, spleen) as well as skin and bone; digestive tract manifestations are also common (Restrepo *et al*., 2012, 2015). The chronic or adult-type form is the most common clinical presentation and accounts for approximately 80% of cases. This clinical form is characterized by infection of the lungs and extrapulmonary organs (mainly skin and mucous membranes), accompanied by the development of ulcerative, granulomatous and infiltrated lesions. The adrenal glands and CNS may also be involved. Pulmonary fibrosis, the main sequela of this disease, may develop in 50% of patients with the chronic form (Restrepo *et al*., 2012, 2015).

Eco-epidemiology

As described above, PCM is restricted to certain countries of Latin America. This mycosis has an estimated incidence of 1–3 cases/100 000 inhabitants (Bocca *et al*., 2013; Bellissimo-Rodrigues *et al*., 2011) and a mortality rate of 1.4/million, the highest value for any systemic mycosis (Coutinho *et al*., 2002). One of its most peculiar characteristics is that it is more often diagnosed in males than females (ratio 13:1), and occurs in individuals engaged in agricultural activities (Restrepo *et al*., 2012, 2015; Colombo *et al*., 2011; Bellissimo-Rodrigues *et al*., 2013). It is noteworthy that no outbreaks of this mycosis have ever been reported.

Regarding its phylogenetic classification, *P. brasiliensis* S1 is widely distributed throughout South America, PS2 has been identified only in Brazil and Venezuela, PS3 is apparently restricted to regions of Colombia and PS4 has to date been

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reported only from Venezuela (Matute *et al*., 2006; Salgado-Salazar *et al*., 2010; Theodoro *et al*., 2012). The other species, *P. lutzii*, is found mainly in central, south-western and northern Brazil, as well as Ecuador (Teixeira *et al*., 2009, 2013; Theodoro *et al*., 2012).

Several environmental and ecological factors have been associated with the diagnosis of PCM, including the presence of tropical and subtropical forests, moderate-to-high precipitation rates (2000–2999 mm/year), watercourses, mild temperatures (<27 °C), fertile soils and elevation < 800 m (Calle *et al*., 2001; Bagagli *et al*., 2003; Restrepo *et al*., 2012, 2015). In addition, the El Niño southern oscillation (ENSO) climatic anomaly has been linked with a cluster of 10 cases of acute PCM (Barrozo *et al*., 2010).

This fungal pathogen has been isolated in culture, as well as being detected by molecular and serological methods, from several wild and domestic animals; these include armadillos (Bagagli *et al*., 2003; Corredor *et al*., 2005), raccoons, Brazilian guinea pigs, spiny tree porcupines, ferrets and tayras (Richini-Pereira *et al*., 2008; Albano *et al*., 2014), dogs (Ricci *et al*., 2004; de Farias *et al*., 2011; Fontana *et al*., 2010), pigs (Belitardo *et al*., 2014a), rabbits (Belitardo *et al*., 2014b), cats (Oliveira *et al*., 2013), dairy goats (Ferreira *et al*., 2013), sheep (Oliveira *et al*., 2012) and chickens (Oliveira *et al*., 2011). *Paracoccidioides* has also been detected in soil samples from around or inside armadillo burrows (Theodoro *et al*., 2005).

Pathogenesis

Different *in vivo* and *in vitro* models have been devised to understand the pathogenesis of PCM. *In vivo*, it has been observed that once conidia or yeast cells of *P. brasiliensis* reach the lungs, these propagules interact initially with the extracellular matrix (ECM) proteins, epithelial cells, alveolar macrophages and pulmonary dendritic cells. Apparently, these interactions are mediated by adhesin-type molecules present on the fungal surface that instead recognize ECM proteins, also present on the epithelial cell surface (Caro *et al*., 2008; González *et al*., 2005a, 2008a). In addition, pulmonary cells (mainly macrophages) are activated and an inflammatory process occurs involving the production of pro-inflammatory cytokines and chemokines; this process is characterized by expression of adhesion molecules in the host and subsequent recruitment of neutrophils and macrophages to the infection site (González *et al*., 2003, 2005b). After interaction of the fungal propagules with neutrophils and macrophages, these phagocytic cells become activated and express molecules, such as nitric oxide, lysozyme and reactive oxygen intermediates, that may exert a fungicidal effect against *P. brasiliensis*; however, if the fungus is able to overcome these mechanisms, dissemination to other organs and systems will occur (Gonzalez *et al*., 2000, 2008b; Moreira *et al*., 2008a, 2008b).

The initial interaction of the phagocytic cells with *P. brasiliensis* has been described as being partly mediated by several molecules including complement receptor-3 (CR3), mannose receptor, dectin-1, Toll-like receptors (TLR)-2 and TLR-4, among others (Jiménez *et al*., 2006; Loures *et al*., 2009, 2010; Bonfim *et al*., 2009).

Moreover, in PCM patients B lymphocytes are activated at polyclonal level with specific antibodies (mainly IgA, IgG and IgE) produced against the fungus (Chequer-Bou-Habib *et al*., 1989; Baida *et al*., 1999).

Regarding the immune response developed in PCM patients, it has been found that those with the severe form show increased production of Th2 cytokines (mainly IL-10, IL-4 and IL-5) whereas the Th1 cytokine levels (mainly IFNγ and IL-2) are low (Kashino *et al*., 2000; Benard, 2008). In addition, the different T cell subsets accomplish different functions in this mycosis, as evaluated in animal models; thus, T $CD8^+$ cells participate in the control of fungal burden, whereas T $CD4^+$ cells are involved in the delayed-type hypersensibility response and production of specific protective antibodies (Chiarella *et al*., 2007). Tγ/δ lymphocytes appear to participate in the polyclonal activation of B cells, while T-reg ($CD4^+$ $CD25^+$ $FoxP3^+$) cells are involved in the development of a local and systemic immune response in PCM (Munk *et al*., 1995; Moreira *et al*., 2008a, 2008b).

Life cycle

An important aspect of the life cycle of *Paracoccidioides* is its thermo-dimorphism, in

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which the mycelial morphotype occurs at ambient temperature, whereas the yeast morphotype grows at 37 °C, equivalent to that of the mammalian host (Queiroz-Telles, 1994).

This is an important issue, since during the life cycle the fungus has to adapt to different conditions in the outside environment and within the host cell; in addition, cell cycle control is vital to maintaining correct cellular function and the transfer of genetic information to new daughter cells. Computational studies searching for genes involved in the cell cycle of *P. brasiliensis* have permitted the discovery of genes involved in cell cycle control, cytoskeleton structure and chromosome segregation, similar to those reported in *Saccharomyces cerevisiae* (Reis *et al*., 2005). Few studies on this topic have been carried out for *Paracoccidioides*, however. Analysis of *P. brasiliensis* nuclear DNA by flow cytometry enabled four different cellular subpopulations (R1, R2, R4 and R5) to be identified, indicating a 1–4-fold increase in genomic DNA content, characteristic of multiple budding and/or polynucleated cells, as expected in *Paracoccidioides* (Almeida *et al*., 2006). Approximately 90% of the overall population consisted of two main subpopulations, having 1n and 2n DNA content, R1 and R2 respectively; of note, the R2 subpopulation consisted of mononucleate and binucleate cells that may correspond to cells at different phases of the cycle. Alternatively, these two nuclei may correspond to multinucleate cells; treatment with antifungal drugs induced arrest in the cell cycle profile, suggesting an alteration in nuclear division of *P. brasiliensis* yeast (Rodrigues *et al*., 2003; Almeida *et al*., 2006).

On the other hand, several pieces of evidence suggest that this fungal pathogen has a sexual stage. These evidences include the capacity of recombination and the presence of two mating type idiomorphs; thus, each *Paracoccidioides* spp. isolate contains the **a**-box (MAT1-1) or HMG (MAT-12) mating type idiomorph (Matute *et al*., 2006; Torres *et al*., 2009).

Genome and genetics

The first studies in this area were conducted by San-Blas (1986), who demonstrated that both mycelia and yeast morphotypes contain multiple nuclei, whereas conidia present only a single nucleus. Pulsed-field gel electrophoresis (PFGE) assays were later used to obtain two clinical isolates of *P. brasiliensis*, for which four megabase-sized (2.0–10.0 Mb) bands were identified. A genome size of 45.7–60.9 Mb was estimated for these isolates, based on ranging microfluorometric analysis (M. I. Cano *et al*., 1998). The same group subsequently employed the contour-clamped homogeneous electric field gel electrophoresis (CHEF) technique and reported a molecular size in the range 3.2–10 Mb for *P. brasiliensis*, with a genome size of approximately 29.7 Mb (Montoya *et al*., 1999). The differences in genome size suggest that the nuclei of *P. brasiliensis* yeast cells could be diploid, without discarding the possibility of the isolates being haploid or aneuploid. In a later, well-conducted study, Almeida *et al*. (2007a) used flow cytometry (FCM) protocols to evaluate the nucleic content of 10 *P. brasiliensis* isolates from four different PCM-endemic areas (Brazil, Colombia, Uruguay and Venezuela; these isolates represented three different identified cryptic species (S1, PS2 and PS3) (Matute *et al*., 2006). Results of this study indicated that *P. brasiliensis* were mononucleate yeast cells with a genome size in the range 23–31 Mb, similar to that seen in the previous study (Montoya *et al*., 1999). Furthermore, the ploidy ratio of 1.0:1.1 observed in the isolates evaluated indicates that this fungus has a haploid DNA content (Almeida *et al*., 2007a).

In a collaborative study between the Broad Institute and *Paracoccidioides* research community, three referenced isolates (Pb18, Pb03 and Pb01) were selected, representing the S1, PS2 and *P. lutzii* lineages, respectively, for sequencing and genomic analysis. The assembly size for these strains varied in the range 29.1–32.9 Mb and 7875–9132 genes were identified (Desjardins *et al*., 2011). Even with the methods available at that time, the results produced high-quality draft assemblies, although these included many gaps and nucleotides of uncertain or low quality in the final consensus sequences. More recently, this limitation was overcome by employing high sequencing depth to improve the original assemblies, generated from Sanger sequence reads of the same isolates, allowing more complete and accurate reference assemblies to be obtained (Muñoz *et al*., 2014). *Paracoccidioides* spp. genome information is

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available at: http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis

These results could be used in future for diverse molecular and biochemical projects as well as in functional studies, not only of *Paracoccidioides* but also in other fields of medical mycology.

Metabolic pathways

Several studies have been carried out with a view to understanding the metabolic pathways in *Paracoccidioides* spp. that are involved both in cell growth and adaptation to host conditions. These have demonstrated that fungal pathogens have sufficient metabolic plasticity to allow them to exploit different carbon sources (Brock, 2009; Fleck *et al*., 2011). Arraes *et al*. (2005) found that *P. brasiliensis* is able to develop the most important pathways of central metabolism, involving carbohydrates, lipids, amino acids and nucleotides (Arraes *et al*., 2005). The major metabolic pathways described so far for *P. brasiliensis* are shown in Figure 2.

Carbohydrates

With regard to metabolism of carbohydrates, *Paracoccidioides* spp. has both glycolytic and gluconeogenic pathways, the former to obtain pyruvate and the latter to synthesize glucose from non-carbohydrate carbon substrates. The enzymes required for these processes are present in both the mycelium and yeast morphotypes, suggesting that this fungus has sufficient metabolic plasticity to employ different carbon sources and produce glucose, as has been described for other fungi (Arraes *et al*., 2005; Brock, 2009; Fleck *et al*., 2011; Ene *et al*., 2012; Price *et al*., 2011). Tavares *et al*. (2015) demonstrated that *Paracoccidioides* spp. employ a fermentation process to obtain energy; this strategy could thus facilitate fungal adaptation to glucose-poor microenvironments, such as the phagosomes inside leukocytes. In addition, this pathogen could also produce ATP under low oxygen conditions, in turn reducing the levels of reactive oxygen species produced by the host (Tavares *et al*., 2015). This was confirmed by Felipe *et al*. (2005), who reported that the fermentation process was mainly developed in *Paracoccidioides* yeast cells; meanwhile aerobic-dependent ATP production

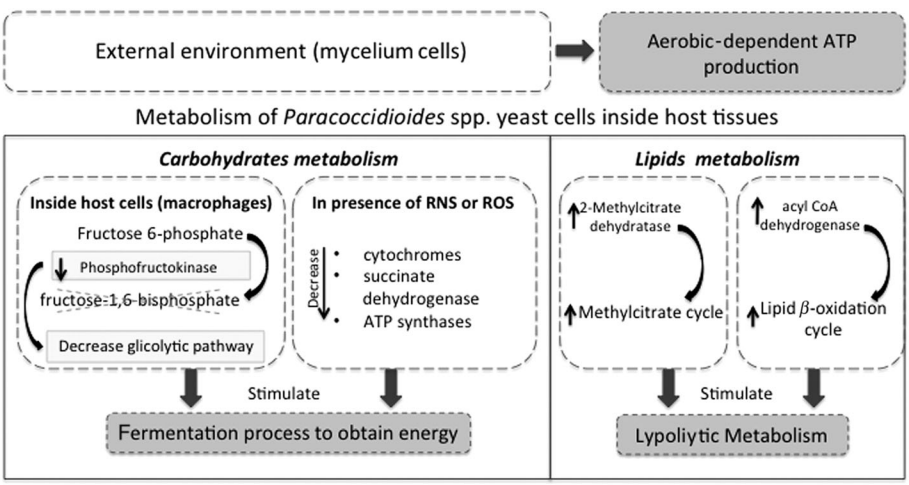

Figure 2. A scheme representing the major metabolic pathways described in *Paracoccidioides*. Inside the host tissues, this fungal pathogen reduces the levels of phosphofructokinase, the enzyme which catalyses phosphorylation of fructose-6 phosphate to fructose 1,6-bisphosphate. In the same way, reactive oxygen species (ROS) or reactive nitrogen species (RNS) decrease the levels of enzymes involved in aerobic respiration. Alterations to any of these pathways generate decreases in the glycolytic pathway. To compensate for the decrease in ATP obtained by carbohydrate metabolism, the fungus possibly increases lipolytic metabolism, this being supported by increases in the methyl citrate and lipid β-oxidation cycles

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occurs in the mycelium morphotype. Tavares *et al.* (2007) demonstrated that *Paracoccidioides* spp. co-cultured with murine macrophages showed reduced expression of the mRNA levels of the gene coding for phosphofructokinase, an enzyme that catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate in the glycolytic pathway, which would provide an alternative way to obtain energy. To confirm this, proteomic studies were performed using *Paracoccidioides* yeast cells after exposition to reactive nitrogen species (RNS) by adding of *S*-nitrosoglutathione (GSNO; a stress generator). In these studies, reduced levels of the enzymes involved in aerobic respiration were observed, such as cytochromes, succinate dehydrogenase and ATP synthases (Parente *et al.*, 2015). These results reinforce the hypothesis that this fungal pathogen uses a fermentation process to obtain energy inside host cells.

On the other hand, a better understanding of the metabolism of the fungus during its adaptation to the host tissues is important to elucidate the alternative metabolic pathway it might adopt under conditions of carbon starvation. Lima *et al.* (2014) reported that the major changes in the transcriptional profile of *Paracoccidioides* during carbon starvation were related to gluconeogenesis and ethanol production. These findings were supported by modulation of the glyoxylate and tricarboxylic cycles; degradation of amino acids and fatty acids was also observed.

Lipids

Regarding the metabolism of lipids, 16 enzymes have been identified in *Paracoccidioides* as responsible for ergosterol biosynthesis, and many others are involved in different cellular processes. An important characteristic of this fungus is its ability to disseminate from the lungs to other organ systems (Mendes-Giannini *et al.*, 2008). During this process, the fungus must adapt to lipolitic metabolism; Bailão *et al.* (2006) demonstrated that this process is characterized by the expression of 2-methylcitrate dehydratase and acyl CoA dehydrogenase genes which are involved in the methyl citrate and lipid β-oxidation cycles, respectively. In addition, high concentrations of proteins involved in lipid synthesis (fatty acid synthase subunit β-dehydratase, fatty acid synthase subunit α-reductase and trans-2-enoyl CoA reductase) have been observed during the interaction of *Paracoccidioides* and RNS (Parente *et al.*, 2015).

Amino acids and nucleotides

Paracoccidioides can synthesize all amino acids except asparagine (Asp). The complete pathway for DOPA–melanin biosynthesis has also been found in this fungus (Arraes *et al.*, 2005). It has been demonstrated that during nitrosative stress in *Paracoccidioides*, four enzymes involved in amino acid metabolism (branched chain amino acid aminotransferase, acetolactate synthase, isovaleryl-CoA dehydrogenase and a subunit of methylcrotonoyl-CoA carboxylase) showed increased activity (Parente *et al.*, 2015); these results are in agreement with those of Missall *et al.* (2006), who observed that amino acid metabolism is affected by nitrosative stress in *C. neoformans*. Further studies are required to characterize the role of RNS in both the synthesis and catabolism of these molecules in *Paracoccidioides*.

To date, seven enzymes involved in *de novo* purine synthesis and four in pyrimidine biosynthesis have been described in *Paracoccidioides*. This fungus probably also produces GMP and AMP *de novo*; however, this hypothesis needs to be tested further, since these previous findings were based on transcriptome analysis (Arraes *et al.*, 2005).

'Moonlighting' proteins are exceptional multifunctional molecules that can accomplish several functions pivotal to cellular metabolism (Huberts and van der Klei, 2010). Several moonlighting proteins are expressed in *Paracoccidioides*, including aconitase, aldolase, glyceraldehyde 3-phosphate dehydrogenase, isocitrate lyase, malate synthase, triose phosphate isomerase, fumarase and enolase. In addition to their metabolic functions, these proteins are involved in cell wall biosynthesis/remodelling, adaptation to different environmental conditions and pathogenesis (Marcos *et al.*, 2014). However, more studies are needed in order to understand their specific and global functions in *Paracoccidioides*.

Metals (iron, zinc, copper) and haemoglobin

Various metals, such as iron, zinc and copper are essential to the metabolism of different microorganisms, including fungal pathogens such as *Paracoccidioides* These metals participate in

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different metabolic pathways as cofactors and are associated with proteins to form metalloproteins, which play an important role in cell membranes and are involved in cellular respiration (Nevitt, 2011).

Various studies regarding the role of iron in *Paracoccidioides* have demonstrated that this metal is required for conidia-to-yeast transition (an essential event for the development of the disease), as well as yeast replication inside macrophages and monocytes (Cano *et al*., 1994; Gonzalez *et al*., 2007). Parente *et al*. (2011) subsequently demonstrated that, under iron-depleted conditions, *P. brasiliensis* undergoes a glycolytic process instead of oxidative pathways, allowing it to survive inside the host cell. Other studies have demonstrated iron to be an important micronutrient required for the growth of several pathogens, including *Paracoccidioides*. Expression of genes involved in the production of siderophores (important iron chelators) under iron-limited conditions has been demonstrated; these siderophores are present at both the extracellular and intracellular levels as coprogens, ferrichromes and fusarinines, and their expression is associated with survival of *Paracoccidioides* after co-culture with IFNγ-activated macrophages (Silva-Bailão *et al*., 2014). Moreover, siderophore production and iron uptake have been described as important factors in cellular metabolism, as well as acting as virulence factors for fungal pathogens (Schrettl *et al*., 2004; Hwang *et al*., 2008).

In another related study, it was also demonstrated that *Paracoccidioides* may use haemoglobin as an iron source, most probably through receptor-mediated pathways, since iron-related genes that encode haemoglobin receptors were identified in the genome of this fungal pathogen. In this study, the authors observed that expression of these related genes, including those encoding for proteins involved in amino acid assembly, as well as nitrogen, sulphur and iron–sulphur metabolism, were induced under low-inorganic iron conditions or in the presence of haemoglobin (Bailão *et al*., 2014).

Although copper and zinc homeostasis have not been extensively studied in *Paracoccidioides*, increased copper transportation has been observed in the tissues of mice infected with *P. brasiliensis* (Bailão *et al*., 2006). Increased levels of mitochondrial copper transporter and Cu/Zn superoxide dismutase were subsequently observed in *P. brasiliensis* yeast cells infecting macrophages (Tavares *et al*., 2007). Metalloproteins are known to be involved in many cellular processes, including general metabolism and virulence. Several metalloproteins, such as Cu-, Fe- and Zn-binding proteins, have been identified using bioinformatic tools, representing 7% of the total proteins encoded by the genome of *Paracoccidioides*. Zinc proteins are the most abundant, representing 5.7% of the fungus proteome, with copper and iron proteins accounting for 0.3% and 1.2%, respectively (Tristão *et al*., 2015).

All the above studies clearly demonstrate the importance of metals (particularly iron) in the cellular metabolism of *Paracoccidioides* as well as its interaction with host cells. However, the exact mechanism by which these metals promote or participate in the metabolism of this fungus is not yet fully understood. Further studies should be carried out on this important topic.

Oxidative and nitrosative stress response

It has recently been demonstrated that the alternative oxidase (PbAOX) plays an important role in intracellular redox balancing in *P. brasiliensis*; this enzyme is also involved with other components in the mitochondrial respiratory process (Ruiz *et al*., 2011; Martins *et al*., 2011). Moreover, additional studies have shown that PbAOX is important not only for maintaining cellular homeostasis, by assisting redox balancing during cell growth and morphological changes, but also in fungal defence against the oxidative stress imposed by immune cells to combat *P. brasiliensis*. This demonstrates that the oxidative stress response is essential to both fungal survival and adaptation to host conditions (Hernández *et al*., 2015; Ruiz *et al*., 2011).

Hydrogen peroxide is another molecule that participates in oxidative stress. After the addition of H_2O_2 to *Paracoccidioides* cultures, it was observed that this pathogen expressed anti-oxidant enzymes, such as catalase, superoxide dismutase, cytochrome *c* peroxidase and thioredoxin, with substantial changes in its metabolism, as observed by activation of pathways to provide NAD(P)H (de Arruda Grossklaus *et al*., 2013). These studies also showed that an appropriate response to oxidative stress of *Paracoccidioides* is an essential event for its dimorphism process and adaptation to host conditions, both requiring metabolic adaptation by the fungus.

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On the other hand, RNS has been reported to reduce the activity of the mitochondrial transport chain in *Paracoccidioides*, possibly through inhibition of aconitase, cytochrome oxidase and other mitochondrial proteins involved in this process (Parente *et al*., 2015; Mason *et al*., 2006). The inhibition exerted by RNS compounds is apparently a non-selective but irreversible process, whereas nitric oxide induces a rapid, selective but reversible inhibition of cytochrome oxidase (Brown, 1999). These results clearly indicate that these reactive species not only alter the mitochondrial transport chain in *Paracoccidioides* but also affect important metabolic processes related to ATP production, dimorphism and host adaptation, among others.

Molecular and research tools

Among the molecular tools that have been employed for studying *Paracoccidioides* spp., amplification of nucleic acids (mainly DNA) has been useful for diagnosing PCM. Development of PCR protocols using primers or probes and targeting the *GP43* gene or the ITS1 ribosomal DNA regions have been the most common assays reported. These have proved to be highly sensitive and specific, able to detect as few as 10 copies or 1.1 pg/ml DNA (Semighini *et al*., 2002; Dias *et al*., 2012). Other protocols using conventional, nested or semi-nested PCR are useful for detecting fungal DNA in clinical samples, with the exception of those from serum, which do not appear to be effective (Pitz Ade *et al*., 2013; Dias *et al*., 2012; Teles and Martins, 2011; Motoyama *et al*., 2000).

On the other hand, taking into account that homologous recombination is unusual in *Paracoccidioides*, the development of knockout isolates is not currently feasible (Sturme *et al*., 2011). Thus, one of the most important advances for functional studies of *Paracoccidioides* has been the implementation of antisense technology. Initially, Leal *et al*. (2004) reported transformation of *P. brasiliensis* yeast cells using *Agrobacterium tumefaciens* GV3101 and the vector pAD1625, although transformation efficiency was not satisfactory (Leal *et al*., 2004). Afterwards, an efficient strategy based in *Agrobacterium tumefaciens*-mediated transformation (ATMT) was used to obtain *P. brasiliensis* isolates, with downregulation of specific genes; in this study the authors presented evidence for the occurrence of random single-gene copy integration per haploid nucleus and the generation of homokaryon progeny, a finding that was key to subsequent studies of the biology of this fungus (Almeida *et al*., 2007b). This approach quickly began to be used for functional studies in *P. brasiliensis*. The first target was CDC42; decreased expression of this molecule in *P. brasiliensis* yeast was associated with reduced cell size and more homogeneous cell growth, alteration in the typical polymorphism of wild-type cells. Increased phagocytosis and decreased virulence in the mutant cells in a mouse model of infection were also observed (Almeida *et al*., 2009). Several subsequent studies using this technology have allowed the role of various molecules during host–fungus interactions to be defined; these molecules include adhesins (PbHAD32 and GP43), stress proteins (PbAOX), heat shock proteins (HSP90) and other important antigens, such as Pb27 (Hernández *et al*., 2010, 2012; Ruiz *et al*., 2011; Tamayo *et al*., 2013; Torres *et al*., 2013, 2014; Torres-Gómez *et al*., 2013; Bailão *et al*., 2014). It is noteworthy that using these mutants (silenced isolates) in both *in vitro* and *in vivo* (animal) models allowed the involvement of these molecules in several processes, including conidia-to-yeast transition, adhesion and cell homeostasis to be confirmed. The mutant strains showed decreased virulence in all cases. One of the most important results was that the targeted genes remained silenced for long periods, even up to a year after several cultures, or after recovery from animal tissues several months after infection. The expression levels of the majority of genes knocked down using the antisense technology are shown in Figure 3.

An additional strategy to silence genes has been implemented for *Paracoccidioides*; use of RNAi technology to decrease the expression levels of Pb*GP*43 and Pb*P*27 genes indicated reduced expression of these genes in mutant strains, albeit only during the first 20 days after selection, indicating that gene silencing by this methodology was not stable over time (Torres *et al*., 2013). In a later attempt to improve RNAi technology in *Paracoccidioides*, successful knockdown of GP43 was achieved through the expression of intron-containing hairpin RNA (ihpRNA). Employing this strategy reduced Gp43 transcript levels by 73.1% (Goes *et al*., 2014).

The implementation of molecular tools for both diagnosis and research has allowed significant

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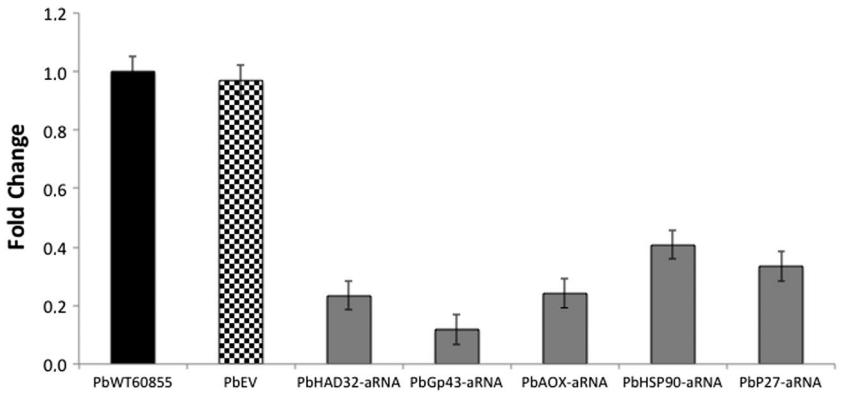

Figure 3. Generation of knocked-down strains of *P. brasiliensis*. Gene expression levels of various in wild-type (PbWT), WT transformed with the empty vector (PbEV) and *P. brasiliensis* yeast transformed with the antisense RNA (Pb-X-aRNA) after subculture for 120 days. Gene expression levels obtained by RT–PCR were normalized to the internal reference, Tubulin-2 (TUB2); $*p < 0.05$ compared with PbWT and PbEV

advances to be made in both fields. However, new tools will need to be developed to evaluate several genes at the same time at functional levels, since host–parasite interactions generate complex responses. Further studies are needed to elucidate the role of genes involved in various metabolic processes, as well as those involved in the response to the host attacks and others encoding for virulence factors.

Virulence factors

As with other dimorphic fungal pathogens, *Paracoccidioides* spp. exhibits several virulence mechanisms that allow it to combat and overcome host defences (Rappleye and Goldman, 2006). Two main virulence mechanisms are critical for the establishment of disease, the first being the ability of the fungus to adhere to both extracellular matrix (ECM) proteins (mainly laminin, fibronectin, fibrinogen, collagen) and epithelial pulmonary cells. This adherence mechanism is mediated by molecules, named adhesins, present on the fungal surface that allow it not only to adhere but also to invade the host tissues, contributing to development of the mycosis. Thus, several adhesins have been reported, including the 14–3–3 protein enolase, triose phosphate isomerase (TPI), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the glycoprotein Gp43 (the main immunodominant antigen) and a hydrolase (HAD32) (de Oliveira *et al*., 2015; Pereira *et al*., 2007; Barbosa *et al*., 2006; Torres *et al*., 2013; Mendes-Giannini *et al*., 2006; Hernández *et al*., 2010). Moreover, the 14–3–3 protein and Gp43 induce apoptosis in human pneumocytes (Silva *et al*., 2015). The second virulence mechanism is the conidia/mycelium-to-yeast transition, a morphological process due not only to the temperature shift to 37 °C but also to the availability of organic sulphur compounds (Menino *et al*., 2013b). A negative regulator of the inorganic sulphur assimilation pathway has also been described. This molecule (SconCp) is considered to be a novel virulence determinant in *P. brasiliensis* (Menino *et al*., 2013b).

Other virulence mechanisms have also been described. Thus, α1,3-glucan, present in the yeast cell wall, has been suggested to form a 'protective shield' against host defence mechanisms (San-Blas *et al*., 1977; Rappleye and Goldman, 2006). Melanin, a pigment produced by different pathways, is present in *P. brasiliensis* and is considered to be an important virulence factor that reduces susceptibility to host defence mechanisms and antifungal drugs (Taborda *et al*., 2008).

Biofilm formation is generally associated with antimicrobial resistance and avoidance of host defences. More recently, it has been reported that *P. brasiliensis* yeasts are able to form biofilms and that their presence is associated with overexpression of adhesins and enzymes (Sardi *et al*., 2015).

Other virulence determinants include metalloproteinases, phospholipases, an alternative oxidase (AOX; an enzyme involved in intracellular redox

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balancing), p27 and heat-shock proteins (Tristão *et al*., 2015; Soares *et al*., 2011; Ruiz *et al*., 2011; Tamayo *et al*., 2013; Torres e*t al*., 2014). In addition, *P. brasiliensis* is capable of producing an exocellular serine–thiol proteinase which can degrade the basement membrane, allowing the fungus to invade and disseminate to other organ systems (Puccia *et al*., 1998).

Animal models

While taking into account that the exact moment when the host acquires the infection remains unknown, animal models have provided important tools to allow host–fungus interactions to be studied, from the beginning of infection to the development of chronic stages that mimic development in humans. Several animal models have been devised and various fungal strains of different morphotypes (conidia or yeast) and virulence have also been employed. Thus, animal models for this mycosis include mice (Calich *et al*., 1985; Cano *et al*., 2000; Defaveri *et al*., 1982; McEwen *et al*., 1987; Moscardi and Franco, 1980), hamsters (Essayag *et al*., 2002; Iabuki and Montenegro, 1979; Peraçoli *et al*., 1982), guinea pigs (Fava-Netto *et al*., 1961) and rats (Iovannitti *et al*., 1999). The mouse model is the one that has been the most used. Different inbred strains of mice inoculated intraperitoneally with yeast cells of *P. brasiliensis* showed significantly varying patterns of susceptibility. The A/SN strain was found to be the most resistant, while BIOD2/nSn, BIO.A and BIOD2/oSn were the most susceptible strains. These susceptibility differences were independent of the size of challenge inocula and histocompatibility complexes. However, gender is a factor, since male BALB/c and BIOD2/nSn mouse strains were more susceptible to infection than those of females (Calich *et al*., 1985).

The same group subsequently established a genetically controlled murine model of PCM using isogenic mice, which allowed several parameters of the host–parasite interactions to be investigated. In this model, resistant mice inoculated intraperitoneally or intratracheally with *P. brasiliensis* yeast showed efficient macrophage activation, Delayed-type Hypersensitivity (DTH) response, low levels of specific antibodies and a tendency towards resolution of the infection process, suggesting the development of a Th1 immune response. By contrast, susceptible mice developed a predominantly Th2 immune response with inefficient macrophage activation, depressed DTH reactions, high levels of antibodies, greater dissemination of the fungus and the development of chronic progressive disease (Singer-Vermes *et al*., 1993a, 1993b; Cano *et al*., 1995). Furthermore, athymic or nude (nu/nu) and euthymic (nu/+) mice have been evaluated in order to evaluate the involvement of T cells. Similar participation of inflammatory cells (neutrophils and macrophages) as well as levels of ECM protein production were observed in the two mouse types. The only difference was that the euthymic animals showed lesions with a more pronounced tendency to encapsulate, increased ECM proteins and higher titres of specific antibodies, indicating that T cells could participate in the containment and control of infection (Lenzi *et al*., 1994; Burger *et al*., 1996a, 1996b). In addition to humoral and cellular studies, other investigations have been carried out to evaluate the innate immune response in PCM, in order to understand the initial host–fungus interactions. These studies have allowed the study of mechanisms such as complement protein activation, the microbicidal activity of natural killer cells and phagocytes, and production of inflammatory eicosanoids, cytokines and chemokines (Calich *et al*., 2008).

More recently, knockout mice have allowed the role of several molecules that participate in host–*Paracoccidiioides* interactions to be elucidated. These include interleukin (IL)-10, IL-12, IL-18 (Costa *et al*., 2013; Livonesi *et al*., 2008; Ketelut-Carneiro *et al*., 2015), nitric oxide (Bernardino *et al*., 2013), dectin-1 (Loures *et al*., 2014); MyD88, Toll-like receptor (TLR)-2, TLR-4, TLR-9 (González *et al*., 2008c; Loures *et al*., 2009, 2015; Menino *et al*, 2013a), CCR-5 (Moreira *et al*., 2008a, 2008b) and caspase-1 (Ketelut-Carneiro *et al*., 2015).

The observation that PCM is more frequent in males than females was attributed to the protection conferred by the female hormone 17-β-oestradiol, an elegant animal study being performed to confirm this hypothesis. The course of infection in male and female mice in relation to their hormonal status indicated that, after infection with *P. brasiliensis* conidia, normal males showed progressive infection, whereas normal females restricted

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proliferation and progressive disease. Castrated animals exhibited a lesser capacity to restrict disease progression. In addition, castrated male mice reconstituted with 17-β-oestradiol initially restricted proliferation, whereas castrated female mice reconstituted with testosterone were unable to restrict disease. These results confirmed that 17-β-oestradiol confers protection during initial infection in females (Aristizábal *et al*., 2002).

Host defence mechanisms against *Paracoccidioides* spp.

Most knowledge concerning the host defence mechanism against *Paracoccidioides* spp. comes from the infected host (particularly animal models) and from *in vitro* studies using both primary cell or cell line cultures. Different cell types are involved in the defence mechanism against this fungal pathogen, including macrophages, neutrophils, dendritic cells (DCs), T lymphocytes and natural killer (NK) cells. Macrophages, neutrophils and DCs are classified as 'professional phagocytes' and participate in the innate and acquired phases of immunity. Their activation is fundamental to the control of pathogen growth. Both normal macrophages and neutrophils are permissive to *P. brasiliensis* growth, while phagocytes activated by cytokines are able to restrain fungal multiplication (Pina *et al*., 2008; González *et al*., 2003, 2000; Rodrigues *et al*., 2007; Tavian *et al*., 2008; Kurita *et al*., 2005). The fungicidal activity of these phagocytes is mediated by nitric oxide or reactive oxygen species (ROS) production, these being molecules that deplete fundamental metabolites for fungal cell cycling and subsequent growth (Pina *et al*., 2008; González *et al*., 2003, 2000; Rodrigues *et al*., 2007; Tavian *et al*., 2008). In addition to NO and ROS production, the indoleamine 2,3-dioxygenase (IDO; a enzyme that catalyses tryptophan metabolism and is produced mainly by macrophages and DCs) participates in the control of fungal infection (Araújo *et al*., 2014).

The development and activation of dendritic cells are associated with secretion of IFNγ, IL-4 and IL-17 and increased development of T-reg cells, with all of them exerting a positive effect in controlling fungal infection (Pina *et al*., 2013).

Besides the phagocytosis mechanism, neutrophils release structures called extracellular traps (NETs), which are composed of nuclear (decondensed DNA and histones) and granular material such as elastase; more recently, we have demonstrated that although *P. brasiliensis* is able to induce NETs, this mechanism is ineffective in killing the fungus (Mejía *et al*., 2015).

T cells comprise different subpopulations that include mainly $CD4^+$, $CD8^+$ and T-reg ($CD4^+CD25^+PhoxP3^+$) cells. Both $CD4^+$ and $CD8^+$ T cells are necessary for controlling paracoccidioidal infection; in this case, $CD8^+$ T cells control fungal load, while $CD4^+$ T cells regulate antibody production and DTH reactions (Cano *et al*., 2000; Chiarella *et al*., 2007). Depletion of T-reg cells led to a less severe infection in mice, indicating that these cells have a suppressive effect on the immune response, preventing tissue pathology by limiting the inflammatory reaction (Felonato *et al*., 2012). Nonetheless, the host susceptibility pattern appears to account for functionality of these cells; thus, $CD4^+$ T cells play a protective role in the resistant and intermediate mouse strains, whereas in susceptible mice they are deleted or anergic. These facts indicate that genetic resistance to PCM is associated with concomitant $CD4^+$ and $CD8^+$ T cell immunity, secreting type 1 and type 2 cytokines (Cano *et al*., 2000; Chiarella *et al*., 2007). Resistant mice develop higher numbers of more potent T-reg cells than susceptible individuals (Felonato *et al*., 2012).

Moreover, NK cells accomplish two fundamental functions: (a) they are able to kill *P. brasiliensis* cells directly or recognize and kill infected cells through a cytotoxic mechanism; (b) they produce pro-inflammatory cytokines (IFNγ and TNFα) that in turn influence the acquired immune response against this fungus (Longhi *et al*., 2012).

Interactions between immune host cells (mainly phagocytic cells) and *Paracoccidioides* spp. are mediated by recognition of conserved fungal structures, known as pathogen-associated molecular patterns (PAMPs), by means of germline-encoded pattern recognition receptors (PRRs) present on the host cell surface (Leibundgut-Landmann *et al*., 2012; Brown, 2011). PRRs comprise several molecules, including Toll-like receptors (TLR), the C-type lectin receptor (CRL) dectin-1, mannose receptors (MR) and complement receptors (CR), among others. This interaction is critical for

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priming an appropriated immune response with synthesis of pro-inflammatory cytokines and chemokines, ROS production and phagocytosis activation; nevertheless, PPR signalling could be beneficial or detrimental to the host, depending on its background. Thus, dectin-1, MR, TLR-2 and TLR-4 control lymphocyte proliferation in *P. brasiliensis* infection, or could exert negative effects by inducing a more severe infection, leading to tissue damage for an uncontrolled or enhanced pro-inflammatory immune response (Loures *et al*., 2009, 2010, 2015).

As described above, protective immunity to *Paracoccidioides* infection was shown to be mediated by IFNγ, IL-12 and TNFα, using gene knockout or cytokine-depleted mice (L. E. Cano *et al*., 1998; Arruda *et al*., 2002; Bernardino *et al*., 2013); while IL-4 and IL-10 apparently both show detrimental effects (Pina *et al*., 2004; Costa *et al*., 2013). In addition to cytokines, lipid mediators such as leukotrienes participate in host defence against *Paracoccidioides*; thus, mice deficient in 5-lipoxygenase, a key enzyme that catabolizes the arachidonid acid present in the cell membrane, show increased fungal burdens and mortality (Santos *et al*., 2013).

Concluding remarks and future directions

New advances in *Paracoccidioides* biology have been described. These include the description of *P. lutzii* as a new species and annotation of the genome which is already available. Thus, the implementation of novel genetic and molecular tools and the availability of the entire genomic sequence could lead to remarkable progress in the study of virulence determinants and the search for targets in the design of new drugs and diagnostic assays, with a view to improving management of this mycosis.

Nevertheless, several questions remain to be answered regarding the pathogenesis of this dimorphic fungus, its adaptation mechanisms to the human host and how the host defence mechanisms work, as well as its diverse metabolic pathways.

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