Overview and perspectives on the transcriptome of *Paracoccidioides brasiliensis*

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Summary

Paracoccidioides brasiliensis is a dimorphic and thermo-regulated fungus which is the causative agent of paracoccidioidomycosis, an endemic disease widespread in Latin America that affects 10 million individuals. Pathogenicity is assumed to be a consequence of the dimorphic transition from mycelium to yeast cells during human infection. This review shows the results of the *P. brasiliensis* transcriptome project which generated 6,022 assembled groups from mycelium and yeast phases. Computer analysis using the tools of bioinformatics revealed several aspects from the transcriptome of this pathogen such as: general and differential metabolism in mycelium and yeast cells; cell cycle, DNA replication, repair and recombination; RNA biogenesis apparatus; translation and protein fate machineries; cell wall; hydrolytic enzymes; proteases; GPI-anchored proteins; molecular chaperones; insights into drug resistance and transporters; oxidative stress response and virulence. The present analysis has provided a more comprehensive view of some specific features considered relevant for the understanding of basic and applied knowledge of *P. brasiliensis.*

Key words

Transcriptome, Expressed Sequence Tags, Dimorphic and thermo-regulated fungus, *Paracoccidioides brasiliensis*

Panorama y perspectivas del transcriptoma de *Paracoccidioides brasiliensis*

Resumen

Paracoccidioides brasiliensis es un hongo de dimorfismo termorregulado que es el agente causal de la paracoccidioidomicosis, una enfermedad endémica muy extendida en América Latina que afecta a 10 millones de individuos. Se asume que la patogenicidad es una consecuencia de la transición dimorfa de micelio a levadura durante la infección humana. Esta revisión muestra los resultados del proyecto de transcriptoma de *P. brasiliensis,* que generó 6.022 grupos ensamblados de las fases miceliar y levaduriforme. El análisis por ordenador utilizando herramientas bioinformáticas reveló varios aspectos del transcriptoma de este patógeno, como el metabolismo general y diferencial en el micelio y las levaduras, el ciclo celular, la replicación, reparación y recombinación del ADN, el aparato para la biogénesis del ARN, las maquinarias de traslación y destino de las proteínas, la pared celular, las enzimas hidrolíticas, las proteasas, las proteínas con anclaje GPI, los chaperones moleculares, el entendimiento de la resistencia a fármacos y los transportadores, la respuesta al estrés oxidativo y la virulencia. Este análisis ha proporcionado una visión más amplia de algunos aspectos específicos considerados relevantes para entender el conocimiento básico y aplicado de *P. brasiliensis.*

Palabras clave

Transcriptoma, Etiquetas de secuencia, Hongo dimorfo y termorregulado, *Paracoccidioides brasiliensis*

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1130-1406/01/10.00 Euros

Paracoccidioidomycosis (PCM) is the most important systemic mycosis in Brazil and widespread in Latin America with about 10 million individuals infected being ~2% developing the disease [88]. Since 85% of PCM cases occur in Brazil, this disease represents a major health problem, being classified as the first cause of death among systemic mycoses, and the eighth, with respect to infectious and parasitic diseases [33,88]. PCM represents a serious public health challenge, with social and economical importance. Infected individuals comprise rural workers living where forests and agriculture abound [16] and immunocompromised patients [94] including individuals with AIDS. PCM is characterised by granulomatous inflammation, suppression of cellular immunity and high antibody titres [40,90]. The disease may develop as a benign and localized form to severe and disseminated [49] affecting the skin, lymph nodes and various internal organs, including the lungs and the central nervous system [89]. All patients from whom the fungus is isolated should be treated, and pulmonary fibrosis is still the major sequel despite the availability of new antifungal drugs for therapy.

The etiological agent of PCM is the fungus *Paracoccidioides brasiliensis,* an ascomycete closely related to other pathogenic fungi such as *Blastomyces dermatitidis* and *Histoplasma capsulatum* [7]. *P. brasiliensis* exists as mycelium in the soil environment and as yeast in the host. The unicellular hyphae and fungal propagules or conidia are uninucleate, while yeasts are multinucleate. Infection typically occurs by inhalation of dry airborne spores, fungal propagules or mycelium fragments, which settle in the airways following the thermally regulated transition to the parasitic yeast phase [67,96]. The yeast form has a well-established habitat as a parasite of animals, including humans, armadillos and penguins [6,25]. Many aspects of *P. brasiliensis* ecology remain unclear, especially those that concern its environmental niche [68,88]. The morphological switch from mycelium to yeast is the most important biological feature that enables *P. brasiliensis* to colonize, invade and survive in the warm-blooded host [96]; strains unable to differentiate into yeast do not cause disease [14]. In vitro, this process can be reversibly driven mainly by a temperature switch from 26 °C to 36 °C. The morphogenetic changes are directly associated to the life cycle of this fungus; it undergoes molecular alterations during the morphogenetic switch from hypha to the yeast phase [36].

The degree of pathogenesis varies according with host features and infecting lineage virulence. The immune response of the hosts against *P. brasiliensis* depends on factors such as, sex, age, nutritional state and genetic inheritance. PCM can be restricted to respiratory tract or disseminated all over the organism, and so, being lethal [49]. Thus, the forms of PCM can be divided into two groups: PCM infection, which is generally self-limited and restricted to the site of contact with fungi fragments or to a single organ, affecting both sexes indistinctly; and PCM disease, which preferentially attacks males and can evolve benignly to a PCM infection or disseminate systemically imputing severe damage to the host.

The fungus-host interaction will depend mainly on the host immunological response and fungal virulence. Clinical and experimental data indicate that cell-mediated immune response is the main mechanism of defense against *P. brasiliensis* infection, whereas specific antibodies produced in large amounts do not confer protection [11,24,47,60,66]. Protective cell-mediated immune response in PCM is characterized by the production of cytokines (TNF-α, IL-12 and IFN-γ) that are required for the activation of macrophages, the major defensive cell against *P. brasiliensis* [5,27,102]. In the absence of such cytokines, such as in susceptible hosts, macrophages serve as a protected environment in which fungus can undergo intracellular replication and disseminates from the lungs to other organs [17,18,19,20,50,75] as suggested in histoplasmosis [113]. All patients from whom the fungus is isolated must be treated, and despite new antifungal drugs, pulmonary fibrosis is still the most frequent sequel. The outcome of infection depends on several factors, including host responses and the virulence of the infecting isolate. PCM treatment lasts up to five years and uses basically sulphonamides, azoles and amphotericin B, with successful rates [52].

Information on the genetic composition of *P. brasiliensis* is scarce. The nuclear genome size estimated by PFGE (Pulsed Field Gel Eletrophoresis) is around 30 Mb and confocal fluorescence microscopy studies suggest that *P. brasiliensis* is haploid/diploid or even aneuploid [42]. The electrophoretic pattern revealed chromosomal polymorphism in the fungus, which presented 4-5 chromosomal DNA molecules according to the analyzed isolate, showing molecular sizes ranging from 2-10 Mb [42,71]. Based on the DNA sequencing of ~50 Kb of a *P. brasiliensis* genome fragment, it is estimated that is a total of 7,500-9,000 genes [86].

Our group has been working for the last 10 years in order to identify the differentially expressed genes involved in the dimorphism of *P. brasiliensis* and also genes encoding proteins which are immunogenic in humans during infection. Several genes up-regulated in mycelium and yeast cells have been previously identified by DDRT-PCR (Diferencial Display Reverse Transcriptase PCR), proteome analysis and immunoscreening of cDNA libraries from mycelium and yeast cells using patient's sera [1,8,34-37,43,46,58,73,82,92,93,109].

An important and efficient approach for the comparative study of various genomes is the sequencing of expressed sequence tags (ESTs) which reflects the expression profile of different tissues, cell types or developmental stages [84]. EST data is useful for the discovery of homologous genes, detection of polymorphism and alternative splicing of mRNAs; identification of vaccine candidate molecules, new drug targets, gene prediction and expression studies. This review will focus on some biological aspects derived from *P. brasiliensis* transcriptome project which was carried out by the PbGenome Network, a consortium of 11 public and private institutions from the Midwest region of Brazil [43,44].

The transcriptome project of *P. brasiliensis* – an overview

The Pb Genome project aimed to identify the transcribed genes of mycelium and yeast cell-types of *P. brasiliensis* separately, being of particular interest those related to the dimorphic process, which could be key to a better comprehension of the pathobiology of this fungus. Ultimately, we sought the understanding of the cellular differentiation process at the molecular level, by means of the massive identification and annotation of genes.

The strategy employed to study the *P. brasiliensis* (Pb01 isolate) transcriptome was to sequence ESTs derived from non-normalized cDNA libraries from mycelium and yeast cells cultivated at 23 °C and 36 °C, respectively (Figure). A total of 25,511 clones were sequenced corresponding to 6,022 groups which represents the repertoire of *P. brasiliensis* expressed genes [15]. This covers about

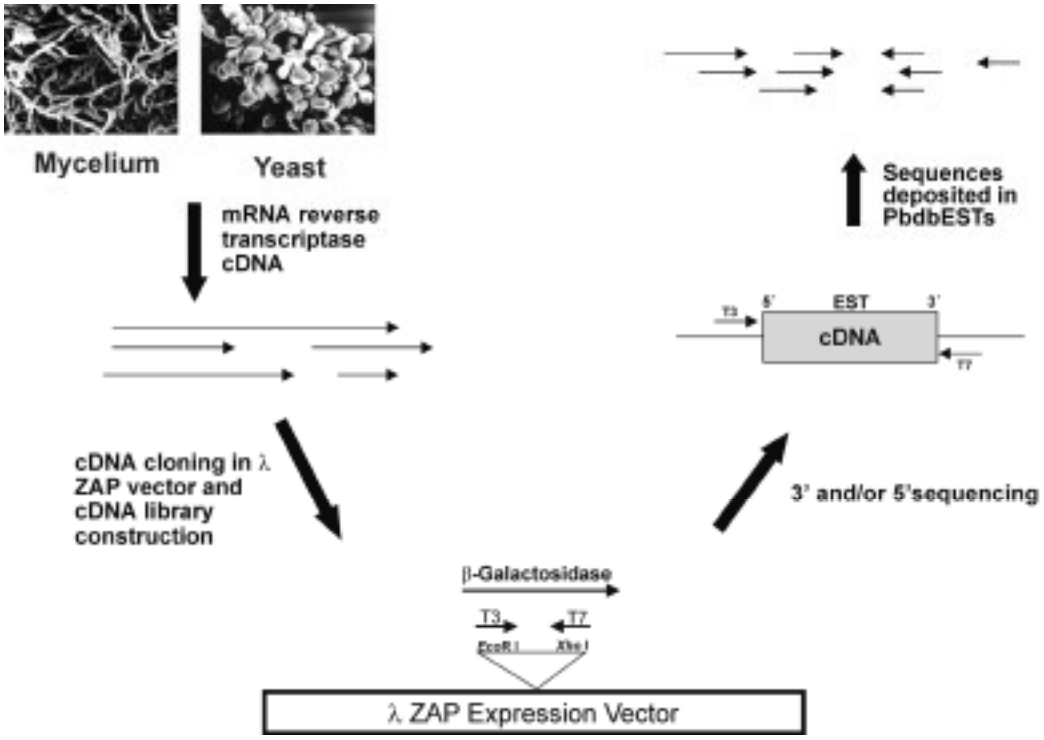

Figure. Strategy for EST generation employed in the *P. brasiliensis* transcriptome project. Briefly, mRNA purified from mycelium and yeast cells were used as template for cDNA synthesis and then cloned into the multiple cloning site (MCS) of the λZAP expression vector (Stratagene). cDNA were sequenced from the 5´-end generating an EST (expressed sequence tags) collection which was deposited in the *P. brasiliensis* ESTs data bank (PbdbESTs) accessed through https://www.biomol.unb.br/Pb.

80% of the total number of predicted genes since it is estimated that *P. brasiliensis* contains ~8,000 genes. The 6,022 clusters were categorized as follows: 2,129 successfully annotated, 1,604 non-conclusively annotated and 2,289 not identified. The PbAESTs *(P. brasiliensis* Assembled ESTs) were classified in 18 functional COG categories: cellular metabolism (29%), transcription (12%), protein synthesis (10%), energy production (9%), control of cellular organization (4%) and other categories.

Metabolic features. Annotation of the of *P. brasiliensis* transcriptome has set the grounds for a global understanding of its metabolism in both mycelia and yeast forms [4,43]. This fungus is able to use several carbohydrate sources including starch; and it can store reduced carbons in the form of glycogen and trehalose; these provide energy reserves that are relevant for metabolic adaptation, protection against stress and infectivity mechanisms. The glyoxylate cycle, which is also involved in pathogenicity, is present in this fungus. Classical pathways of lipid biosynthesis and degradation, including those of ketone body and sterol production, are well represented in the database of *P. brasiliensis.* It is able to synthesize *de novo* all nucleotides and amino acids, with the sole exception of asparagine, which was confirmed by the fungus growth in minimal medium. Sulphur metabolism, as well as the accessory synthetic pathways of vitamins and cofactors, is likely to exist in this fungus

We have identified up-regulated genes in mycelium and yeast cells encoding enzymes of central metabolic pathways (glycolysis, citrate cycle, sulphur and amino acid metabolism, alcohol fermentation and glyoxylate cycle), most of them experimentally confirmed by cDNA microarray and confirmed by Northern blot analysis [43]. The overall analysis indicates that alcohol fermentation occurs preferentially in yeast cells while mycelium saprophytic cells possess an aerobic metabolism. The putative capacity of the pathogen to also grow in anaerobiosis was evidenced by the alternative conversion of pyruvate to ethanol. Lastly, it may be able to utilize two-carbon sources in the form of acetate and ethanol through the glyoxylate cycle, since several pathways that provide substrates for the glyoxylate cycle are up-regulated in the yeast cells.

Cell cycle, DNA replication, repair and recombination. Data derived from transcriptome analysis revealed that cell cycle, DNA replication, DNA repair, and the recombination machineries are highly similar to that of the yeast *Saccharomyces cerevisiae* [87]. Among orthologs detected in both species there are genes related to cytoskeleton structure and assembly, chromosome segregation and cell cycle control genes (CDCs). We identified at least one representative gene from each step of initiation of DNA replication.

We also identified the major players in DNA damage repair in *P. brasiliensis,* with the exception of photo-reactivation repair mechanism. Oxidative DNA damage is mainly repaired by BER (base excision repair), so this kind of repair may play an important role in host defense resistance. *P. brasiliensis* had shown a large resistance to high concentrations of H_2O_2 (Dantas, personal communication) that can induce oxidative DNA damage. So, maybe BER could be an important mechanism in the

response of *P. brasiliensis* against oxidative stress caused by H_2O_2. In a growth-restricting environment, genetic adaptation of a microbial population involves mechanisms (e.g. error-prone polymerases) that lead to an elevated mutation rate. Evidence for the presence of mutagenic pathways in *P. brasiliensis* may account for the high variability observed in karyotypes of different isolates and the difficult to determine if *P. brasiliensis* isolates are one single species.

RNA biogenesis apparatus. The *P. brasiliensis* RNA biogenesis apparatus is very similar to those of other eukaryotes, such as *S. cerevisiae* [2]. PbAESTs related to almost all categories of *S. cerevisiae* RNA biogenesis were found. Two out of 12 *S. cerevisiae* RNA pol II core subunits, Rbp3 and Rpb7, were found, probably reflecting the growth phase from where the cDNA libraries, used in ESTs generation, were constructed. We have also found orthologs to TBP, and at least one subunit of each TFII in *P. brasiliensis* transcriptome, except TFIIB.

With respect to the pre-mRNA processing, 65 PbAESTs orthologs to *S. cerevisiae* basal splicing machinery and 21 orthologs of 5'and 3'-ends formation processes were found. Components involved in RNA interference were detected, suggesting that this gene expression regulation mechanism is probably used by *P. brasiliensis.* Twelve PbAESTs related to Pol I and Pol III machineries were assigned as *S. cerevisiae* orthologs. Finally, 25 and 10 PbAESTs associated to rRNA and tRNA processing were detected. Altogether, our results enable us to depict, for the first time, a global view of transcription and RNA processing in *P. brasiliensis.*

Even though *P. brasiliensis* is classified as an ascomycete, no sexual cycle has ever been observed. However we found transcripts encoding transcription factors possibly related to sexual development such as MCM1, a MAD box protein, which interacts with cofactors that regulate mating in yeast, homologues to the mating type MAT-1 and NsdD, from *Aspergillus nidulans,* and a putative MAT-2 encoding sequence. In addition to the genes described above, the description of other transcription factors regulated by signal transduction pathways strongly suggest, a not yet experimentally observed, sexual cycle in *P. brasiliensis.*

Translation and protein fate machineries. *P. brasiliensis* translational and post-translational machineries are very similar to those of other eukaryotes, such as *S. cerevisiae* [103]. In the *P. brasiliensis* transcriptome we were able to find 78 of the 79 predicted cytosolic ribosomal proteins, corresponding to the whole small subunit and almost complete large subunit set of proteins at the exception of that encoding L23. Transcripts encoding proteins of the large subunit (L24, P1 and P2) and of the small subunit (S2, S3, S10 and S14) are highly expressed. Also, the L19 cytosolic ribosomal protein of *P. brasiliensis* revealed to be differentially expressed in yeast cells.

From the analysis of 27 genes related to translation initiation in *S. cerevisiae,* 19 orthologs were identified in the *P. brasiliensis* transcriptome. All eukaryotic elongation factors were detected being eEF1A one of the most expressed genes. A transcript for the putative initiation factor eIF5A was found in our analysis, which is not essential to the general protein synthesis but is essential to yeast viability. We also found eEF3 which is required for ribosome translocation in fungi and the translation termination factor eRF3, but not eRF1. Sixteen PbAESTs, showing aminoacyl-tRNA synthetases predicted activities were found in our analyses.

Of the mitochondrial ribosomal proteins, we found orthologs to *S. cerevisiae* large (20) and small (18) ribosomal subunit proteins. Although orthologs to *S. cerevisiae* mitochondrial EF-Tu, EF-G and RF1 were detected, no sequences corresponding to functional EF-Ts were identified.

As for the post-translational apparatus, 64 transcripts associated to protein modifications and 28 to protein degradation were identified. These results suggest that in *P. brasiliensis* these machineries are well conserved, when compared to other organisms.

Cell wall. Besides being the first barrier against host defences, the cell wall of human pathogenic fungus displays important antigens, thus presenting an active role during infection [64]. Cell wall is a dynamic structure, whose components are continuously modified and rearranged during fungal life cycle [83]. A striking difference in the composition of the cell wall of *P. brasiliensis* is that α–glucan is the main yeast cell wall polysaccharide, while β–glucan and galactomannan predominate in mycelium.

Morphogenetic shift from hypha to the yeast form is essential to *P. brasiliensis* pathogenicity and cell wall α-1,3-glucan and β-1,3-glucan seem to contribute to this process [95]. In this view, studies on the enzymes involved in cell wall biosynthesis and recycling, as well as on the cell-wall-associated molecules, should provide important information for the design of drugs that affect selectively the pathogen, and for new preventive or therapeutic approaches. The *P. brasiliensis* transcriptome project has revealed several data concerning the gene products that could be involved in this fungus cell wall metabolism [107].

Chitosan was not yet identified in *P. brasiliensis* cell wall; nevertheless, the gene encoding chitin deacetylase *(cda),* the enzyme that converts chitin to chitosan, is over expressed in the yeast phase, according to transcriptome and cDNA microarray analyses [43,44]. Hydrophobins are small proteins that form an amphipathic film at hydrophilic/hydrophobic interfaces covering fungal structures [114]. Two hydrophobin single-copy genes were detected in *P. brasiliensis* genome; Northern blot analysis revealed that both mRNAs are mycelium-specific and highly expressed during the onset of the mycelium-to-yeast transition [1].

Although yeast cells present a higher chitin content than mycelium, the chitin synthase genes Pbr*chs*1, Pbr*chs*2, Pbr*chs*3, Pbr*chs*4 and Pbr*chs*5 transcripts levels were higher in the former [79], suggesting an involvement of these multigenic family in morphogenesis. Our group identified a new *P. brasiliensis* chitin synthase gene (Pbr*chs*6) whose expression is restrict to the mycelium phase.

Genes whose products are involved in the synthesis of N-linked outer chain mannans, such as *dpm*1, *pmt*1, *mnn*2 and *mnn*9 were represented in *P. brasiliensis* transcriptome. Their transcripts were exclusive of the yeast phase, except for the *mnn*2 mRNA which was present in both phases.

Some transglycosidase genes *(bgl*2, *gas*1, *crh*1, *gel*1, *gel*2 and *gel*3*),* possibly related to the cell wall synthesis and fungal morphogenesis [10] were identified in the *P. brasiliensis* transcriptome. In this context, mycelium and yeast cells were tested for several glycohydrolases activities: high levels of β-glucanases, low amounts of α-glucanase, chitinase and maltase were detected. β–1,3–glucanase increasing levels correlated to the shift to the mycelial phase (unpublished data).

Taken together, these data can shed some light on the *P. brasiliensis* poorly understood cell wall metabo-

lism, whose comprehension is essential to the development of more effective prophylactic and therapeutic procedures against this pathogen.

Hydrolytic enzymes. Saprophytic fungi display a broad spectrum of protein- and polysaccharide-hydrolysing enzymes which allow the utilization of important available nutrients. This versatility is required since in the natural environment a large diversity of substrates, including animal, vegetal and fungal sources is expected to be found. Since the mycelium form of *P. brasiliensis* is believed to be a saprobe, it should also be expected to have the enzymatic machinery necessary for the conversion of complex substrates [104]. Analysis of the *P. brasiliensis* transcriptome has revealed this to be true and these data was also further confirmed by enzymatic tests with the supernatants of *P. brasiliensis* cultures grown with different carbon sources [12].

The most important polysaccharides available in the natural habitats of *P. brasiliensis* are hemicellulose, cellulose, and starch which can be hydrolysed to simple sugar for fungal metabolism. Xylanases are key enzymes in the hydrolysis of hemicellulose. Although xylanase activity was detected in both fungal forms, it is six times higher in mycelium than in yeast, which is expected since hemicellulose is an abundant carbon source in the environment. *P. brasiliensis* also secretes cellulases confirming the transcriptome data which indicated the presence of β-1,4-glycosidase and endoglucanase homologues.

Another important plant polysaccharide is starch. As expected, amylolitic activity was detected in *P. brasiliensis* mycelium cells and an α-amylase ortholog was found in *P. brasiliensis* transcriptome. Likewise, chitinolytic activity was only observed in mycelium cells. A chitinase ortholog gene that contains motifs for extracellular localization was found.

Proteases. Proteases can be classified as aspartyl proteases, cysteine proteases, metalloproteases, serine proteases and threonine proteases, depending on the nature of the active site. Many of these enzymes are of medical importance, including exogenous proteases encoded in the genomes of disease-causing organisms. A significant number (53) of protease genes were identified in the *P. brasiliensis* transcriptome database [80]. A total of 15 cDNAs, which encode energy independent protease homologs in the fungus transcriptome were annotated (3 aspartyl, 2 cysteine, 8 metallo and 2 serine proteases). Among those, 2 are exopeptidases and the remaining 13 are endopeptidases.

A gene coding an aspartyl protease of the A1 family (clan AA) is present in *P. brasiliensis* transcriptome. Zinc-containing metalloproteases are widely distributed in prokaryotic and eukaryotic organisms and are classified into four groups comprehending DD-carboxypeptidases, carboxypeptidases, zincins and inverzincins [70]. From the eight identified energy independent zinc metalloproteases in the *P. brasiliensis* transcriptome, four present the consensus motif HEXXH which define those proteases as members of the zincins family. Among the cysteine proteases, a caspase homolog was detected, suggesting that the programmed cell death in *P. brasiliensis* could be proteolytically regulated by this class of molecules [98]. In addition, a Kex2 endoprotease was identified among the serine proteases. The Kex2 endoprotease presented the highest identity to the previously described *kex2* gene of *P. brasiliensis* [108].

As for genes coding energy dependent proteases, 12 ESTs were detected. Among these, one corresponds to a Lon protease, a multi-functional enzyme that is conserved from *Archaea* to mammalian mitochondria, which not only degrades protein substrates but also binds DNA. Lon protease was previously described in *P. brasiliensis* [9].

The endopeptidase Clp/Hsp100 is a cytoplasmic protease that plays an important role in many cellular processes, including regulation of stress responses and protein quality control. Clp/Hsp100 proteins, such as ClpA and ClpX, associate with the ClpP protease and direct the degradation of substrate proteins bearing specific sequences. The first Clp protein described in the *P. brasiliensis* was the ClpB [58]. ClpB is also a heat shock protein which is induced upon the mycelium to yeast transition in *P. brasiliensis.* Other members of the Clp protease family were identified by analysis of the *P. brasiliensis* transcriptome: ClpA and ClpP2

The 26S proteasome is a member of a family of 'chambered proteases' and consists of the 20S proteasome, which forms the proteolytically active core and a regulatory 19S complex. The 20S proteasome of higher eukaryotes is composed of seven distinct α and β subunits [63]. ESTs encoding all the α subunits (1 to 7) and six homologues to the 20S b subunit (1 to 6) are present in *P. brasiliensis* transcriptome. Of special note is the presence of the β subunits 1, 2 and 5 suggesting that the complex is proteolytically active in *P. brasiliensis.* The eukaryotic 26S proteasome recognizes substrates that are tagged with a “polyubiquitin chain” - a polymer assembled from the small (8 kDa), conserved protein ubiquitin. ESTs coding deubiquitinating enzymes are present in this transcriptome. In addition, components of the regulatory 19S complex were found in the *P. brasiliensis* transcriptome in a total of nine different subunits.

GPI-anchored proteins. Detection of glycosylphosphatidylinositol (GPI) membrane anchors in proteins of *P. brasiliensis* was described for the first time in 1995 [56]. The *P. brasiliensis* transcriptome analysis allowed an efficient retrieval of novel parasite GPI proteins [30]. Several studies have now established that GPI-anchored proteins represent a large class of functionally diverse proteins. They can be enzymes, surface antigens, adhesion molecules, and surface receptors [31]. GPI-anchored proteins are leading vaccine candidates, though to be of major importance for infection [39]. These proteins have certain characteristics which allow their recognition, mainly N-terminal signal peptides and C-terminal features that mediate GPI anchor addition at amino acid residue designated the omega (ω) site [54]; a serine-threonine rich sequence which provides sites for glycosylation; and the presence of basic or hydrophobic residues in the ω-region that partially determine cellular localization [28,111,54,55]. By searching some of these characteristics in the translated sequences derived from *P. brasiliensis* ESTs, 20 predicted GPI-anchored proteins were identified: nine enzymes (α-amylase, aspartic proteinase, Cu- and Zn-containing superoxide dismutase 1, ECM33 and DFG5-like, β-1,3-glucanosyltransferases 1, 2 and 3, and phospholipase B); two structural proteins (Crh-like and GPI-anchored cell wall protein); one adhesion molecule (Extracellular Matrix Protein, EMP); three surface antigens (expression library immunization antigen 1, proline-rich antigen, β-1,3-glucanosyltransferase 1); and six hypothetical proteins. Predicted localization in plasma membrane or cell wall was reported for most of them.

Metabolism of GPI-proteins involves different processes: GPI biosynthesis, attachment of the GPI anchor to the protein carboxyl-terminus in the endoplasmic reticulum, GPI protein trafficking and sorting, and protein release by the action of phospholipases.

In mammals, around 20 genes participate in the GPI biosynthesis pathway and are called PIG (phosphatidylinositol-glycan) genes. The glycoslyltransferase complex composed by the proteins Pig-A, Pig-C, Pig-H, GPI1, Pig-P and DPM2 (Dolichol-Phosphate-Mannose 2) catalyses the first step in the GPI synthesis [112]. Genes encoding Pig-A, Pig-C, Pig-P and DPM2 of the glycoslyltransferase complex were detected in *P. brasiliensis* transcriptome. A gene homolog to *PIG-L,* which codes an enzyme catalyzing the second step of GPI synthesis, was present in the transcriptome. In addition, *PIG-O,* which codes an enzyme involved in the EtNP transfer to the first and third mannose residues of the GIP anchor, was identified. Therefore, most of the genes involved in GPI biosynthesis were identified in *P. brasiliensis,* six remaining to be identified or to be described as being inexistent in this parasite.

Attachment of the GPI to a protein involves cleavage of the pre-protein at a hydrophobic aminoacid sequence, followed by the attachment of the cleaved sequence to the fully assembled GPI via a transamidase reaction. Human GPI transamidase is a protein complex, containing five homologous subunits (GPI8, PIG-S, PIG-T, and PIG-U) [57]. All of those encoding transamidases ESTs were detected in the analysis of the *P. brasiliensis* transcriptome with the exception of ESTs encoding PIG-U.

Cleavage of GIP anchor by phospholipases (PL) is a mean of selective protein release. In *P. brasiliensis,* a potent PLC capable of selectively hydrolyze GPI anchor was detected [56]. Two open reading frames with high sequence homology to phosphatidylinositol specific phospholipase C (*PI-PLC*) and phospholipase D (*PLD*) of *Aspergillus nidulans* and *Aspergillus oryzae* respectively, were revealed in the *P. brasiliensis* transcriptome.

Molecular chaperones. Molecular chaperones or stress proteins were first described as heat shock proteins (HSPs), since they are over-expressed in response to heat shock. Other environmental parameters, such as oxidative [72], osmolarity [99] and cold shock [59] stresses, also induce the expression of molecular chaperones. Another type of chaperone is the carbohydrate trehalose, which is produced at high levels in response to several stresses in *S. cerevisiae* and other organisms. This molecule stabilizes proteins and biological membranes, being thus considered a chemical chaperone. Its synthesis and degradation is tightly controlled in response to heat stress (for review see [110]) and it is also produced by *P. brasiliensis,* according to our transcriptome data analysis [77].

Molecular chaperones are involved in protein folding and renaturing and are also implicated in other biological processes, including the dimorphic transition of fungi and immunopathogenicity of infectious diseases. The *P. brasiliensis* HSP70 is preferentially expressed in the yeast phase [37]. Recombinant *P. brasiliensis* HSP60 was recognized by 75 serum samples of infected patients, which suggests the usefulness of HSP60 singly or in association with other recombinants antigens in the PCM diagnosis [35].

In the *P. brasiliensis* transcriptome we have identified 438 ESTs (184 in mycelium and 253 in yeast) which were clustered in 48 distinct chaperones or co-chaperones transcripts [43]. These genes were classified in families, corresponding to three small chaperones, nine HSP40s, ten HSP60s, seven HSP70s, five HSP90s, four HSP100s and ten other chaperones. Calnexin, the cytoplasmic *hsp*60 (*cct7*) and the *sba*1 co-chaperone genes were over expressed in mycelium cells, while the co-chaperone *cpr*1, *hsp*42, *hsp*60, *hsp*70 and *hsp*90 sequences were up regulated in the yeast form. The increased expression (about 38% higher) of heat shock genes observed in the yeast phase was expected, since the differentiation of *P. brasiliensis* cells relies on a thermal shift from the environmental temperature to around 37 °C both in vitro as in vivo. Transcriptome data greatly improved our knowledge of *P. brasiliensis* molecular chaperones, since only eight HSPs had been previously reported in this pathogen.

Transporters and insights on drug resistance. In the analysis of microbial genome, specially those pathogenic ones, the identification of transmembrane protein transporters are of special interest. This is an important class of proteins once they can play a pivotal role in microbial survival and pathogenesis, including, drug resistance. In the *P. brasiliensis* trancriptome we found 26 groups (including singlets and contigs) that show a high similarity degree with transporters proteins, 22 that probably coding for ABC (ATP Binding Cassette) transporters and four that probably code for MFS - Major Facilitator Superfamily [32]. Among ABC transporters we found, for example, *Candida albicans CDR 1* [85] and *CDR 2* [97], *S. cerevisae PDR5* [45] and *A. nidulans ATRF* [3] orthologs. Regarding MSF we were also able to identify a *C. albicans MDR1* [23] ortholog, also pointed out as an important virulence gene. All of those genes are related in these fungi to azole resistance. These findings along with the recent reported ketoconazole resistant *P. brasiliensis* isolation from PCM patients [53] advice us to this pathogen resistance potential emergence.

Oxidative stress response. Oxidative stress is an imbalance between oxidants and antioxidants in favor of the former [100]. It can be caused by reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and peroxynitrite ($ONOO^-$), a product of the reaction between superoxide with nitric oxide (NO). Enzymatic and non-enzymatic defense systems function to avoid the biological damage caused by ROS. Intracellular parasites should have antioxidants to protect against endogenously formed ROS and against ROS produced by the host cells. The enzymatic defense system present in *P. brasiliensis* include catalase, superoxide dismutase, cytochrome c peroxidase and peroxiredoxin [26].

Glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) is an abundant thiol tripeptide that takes part in the metabolism of ROS. It protects against oxidative stress by maintaining the cytosol of the cells more reduced. GSH may react with the hydroxyl radical directly, producing water [101]. Its synthesis occurs by the consecutive action of γ-glutamyl-cysteine synthetase and glutathione synthetase. Genes coding both enzymes were identified in the *P. brasiliensis* transcriptome, as well as that coding for glutathione reductase (GR), responsible for reducing oxidized glutathione (GSSG) back to GSH using NADPH as an electron donor. However, a glutathione peroxidase (GPx) gene is probably absent in the parasite's transcriptome. A substitute for this enzyme may be a 1-Cys peroxiredoxin with GPx activity [26].

Glutathione S-transferases (GST) conjugate GSH to DNA and lipid hydroperoxides, as well as mutagens, carcinogens, and other toxic chemical substances [61]. These enzymes interact with kinases and have GSH-dependent peroxidase and isomerase activities [74]. *P. brasiliensis* has a GST homolog belonging to the class Omega [13] and a microsomal counterpart of this enzyme.

The Cu^{+2} and Fe^{+2} ions play an important role in ROS production. Organisms need these them for transport, protection against oxidative stress, cell growth and development. However, these ions can catalyze hydroxyl radical formation by the Fenton reaction: $Fe^{+2} + H_2O_2 + H^+ \rightarrow Fe^{+3} + H_2O + HO^{\bullet}$ [51]. Therefore, the cellular levels of Fe^{+2} and Cu^{+2} should be carefully controlled. Noteworthy is the presence of a frataxin gene homolog in *P. brasiliensis.* In *S. cerevisiae,* frataxin functions both as a chaperone for Fe^{2+} when mitochondrial iron is limiting, and as a storage molecule for Fe^{3+} when iron is in excess [81]. In humans, defects in frataxin causes Friedreich ataxia (FRDA), a severe neuro- and cardio-degenerative disease in humans, in which mitochondria are unable to handle iron properly [62,91]. Studies on *P. brasiliensis* frataxin may help to clarify the function of this protein in humans.

The transcripton factors Yap1 and Skn7 function in signaling pathways used by fungal cells to control the expression of genes involved in the oxidative-stress response [76]. The presence of ESTs coding both factors in *P. brasiliensis* is important to preserve cellular viability when ROS are produced endogenously or as a result of host immune attack.

Virulence. The search for PbAESTs related to virulence genes was based on those orthologs that fitted molecular Koch´s postulate: Falkow's postulate [29,41] and made possible the annotation of 28 virulence PbAESTs [43,105]. Among those groups are metabolism gene orthologs (eight different groups), two PbAESTs that probably code for ICL (isocitrate lyase) and MLS1 (malate synthase) *C. albicans* glyoxylate cycle orthologs, that is upregulated in *P. brasiliensis* yeast form [43]. This phenomenon was also described for *C. albicans* during its intracellular phase [65], where it is thought to play a crucial function in nutritional furnish. A similar mechanism can be postulated for *P. brasiliensis* into the macrophage, after phagocytosis. Those genes related to cell wall biosynthesis and stabilization is also reported as critical to pathogenesis. We were able to find six PbAESTs that shares high similarity degree with fungal cell wall biosynthesis that were previously reported as important virulence genes [21,22,69,106]. The disruption of these genes affects *C. albicans* survival and attenuates its virulence, so these can be potential drug targets, also because cell wall is the key difference between fungal and human cell. During the infection, *P. brasiliensis* is submitted to oxidative stresses caused by reactive oxygen (ROI) and nitrogen (RNI) intermediates generated during immune response to infection. In our transcriptome we found at least four groups that probably codes for enzymes that are able to ROI and RNI detoxification, whose other fungi orthologs also fit Falkow´s postulate, decreasing their virulence when disrupted, and reversing this phenotype when the null mutants were supplied with the disrupted gene. The other ten PbAests assigned as potential virulence genes have miscellaneous fungi orthologs [43], as for example: phospholipase, urease, multi drug resistance transporter, signal transduction genes and other diverse functions.

Perspectives

Functional analysis of the genes described in the *P. brasiliensis* transcriptome will yield relevant information about cellular differentiation, pathogenicity and/or virulence as genetic tools become available for this pathogen. However, functional analysis by gene disruption in *P. brasiliensis* is hampered by the evidence that isolates have different number of chromosomes [42] and cells are normally multinucleated.

Peptide analogues to human proteins have been reported as potent anti-fungal agents [48]. The high potency and low toxicity make these peptides strong candidates for the treatment of several mycoses. These diseases are traditionally treated with amphotericin B and azoles (fluconazole or itraconazole, among others) but these drugs are becoming less effective as drug resistance arise among several pathogenic fungi. However, therapeutic peptides are less likely to induce drug resistance; for example, peptides derived from MSH (melanocite stimulating hormone) have shown no toxicity in pre-clinical and in vitro studies [48].

Among the perspectives that we foresee as a consequence of the transcriptome project we would like to highlight the following:

1) In vivo expression analysis will be extended to the genes proposed to be involved in host-pathogen interaction, including the differentially expressed genes in mycelium and yeast cells, heat shock, essential genes, putative drug targets and virulence factors. cDNA microarray experiments are currently being carried out in order to evaluate the in vivo expression profile of these genes in macrophages and human pulmonary epithelial cells infected by *P. brasiliensis.*
2) *S. cerevisiae* can be used as a tester model for gene functional analysis by trans-complementation as recently demonstrated [78].
3) RNA interference (RNAi), a natural process which relies in the ability of the cell to target and destroy foreign genetic material, may represent an attractive alternative for gene analysis in *P. brasiliensis.* This approach has been successfully used in other fungi such as *H. capsulatum, Cryptococcus neoformans* and *Neurospora crassa* [38]. The identification of five *N. crassa* orthologs (*rrp-3, qde-2, sms-2, dcl-2 and recQ-2*) in the *P. brasiliensis* transcriptome is an evidence that RNAi may also occur in this fungus thus encouraging the development of an efficient strategy for functional gene analysis.
4) *In silico* design of synthetic peptides based on ESTs sequences may generate new antifungal molecules for the treatment of PCM.

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