PANEL SESSION 1
BIOCHEMISTRY; MOLECULAR AND CELLULAR BIOLOGY; GENETICS

01-01

INTERNALIZATION OF Paracoccidioides brasiliensis AND CYTOSKELETAL ORGANIZATION BY EPITHELIAL CELLS

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Paracoccidioidomycosis presents a variety of clinical manifestations, and the fungus *Paracoccidioides brasiliensis* can reach many tissues, most importantly the lungs. Understanding the mechanisms of dissemination is based on indirect evidence, and the polymorphic aspects of disease suggest that several virulence mechanisms are involved. The ability of the pathogen to interact with the host superficial structures is essential to its virulence, but little is known about interactions between cells and *P. brasiliensis*. For this reason, interactions between *P. brasiliensis* and epithelial Vero and A549 cells were evaluated, with the emphasis on the induction of cytoskeletal alterations during the interaction process fungus-cells, by indirect immunofluorescence. Cytoskeleton components like actin, tubulin and cytokeratin were involved in *P. brasiliensis* invasion process. Cytochalasin D and colchicine, substantially reduced invasion, indicating the participation of microfilaments and microtubules in this mechanism. Through an immunoblot assay, gp 43 was also recognized by anti-actin and anti-cytokeratin sera, but not by anti-tubulin sera. Thus, it seems that this antigen could be a ligand for some components and may be a candidate a invasin. Using the TUNEL with fluorescent probe technique to label cells undergoing DNA fragmentation, it was shown that *P. brasiliensis* inducess apoptosis in infected cells. The adhesion and invasion of epithelial cells by *P. brasiliensis* may represent strategies employed to thwart the host immune response, and may help in the dissemination of the pathogen.

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01-02

IMMUNOCYTOCHEMISTRY KINETIC STUDY OF THE PRESENCE OF JBE (JACALIN BINDING EXOANTIGEN) IN Paracoccidioides brasiliensis YEAST FORMS

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Paracoccidioides brasiliensis is responsible for the most widespread human systemic mycosis in South America. This fungus synthesizes and releases different exoantigens, many of which are glycoproteins, that participate in the pathogenesis of the disease. In a previous evaluation of exoantigen preparations, the most readily recognized components by serum IgG from patients with paracoccidioidomycosis was gp 43, a well characterized *P. brasiliensis* antigen. In order to characterized others exoantigens, we isolated a jacalin binding exoantigen, denominated JBE, of the supernatant of the liquid culture yeast of *P. brasiliensis* through the affinity chromatography in column of immobilized jacalin. Such preparation presents, in SDS-PAGE, band of 190 to 70 kDa and, in the presence of 2 ME, only the 70 kDa band. Study by Immunoblot, the bands developed with policlonal antibodies anti-gp 190 and anti-gp 70 showed similarities among them. The band of 70 kDa, has also been studied due to its lectin character and was denominated paracoccin. In an attempt of immunolocalization such components in the yeast, we developed immunocytochemistry assay, through electronic microscopy, with rabbit IgG anti-gp 190 and goat anti-IgG rabbit conjugated with colloidal gold. Samples of yeast cuts of 7, 14, 21 and 28 days of culture from Modified Negroni were assayed. The immunolocalization revealed JBE, mostly in the yeast cellular walls and, in most quantity in the cultures of 7 and 21 days. It suggests that such localization may influence the interaction of yeast and host cells.

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01-03

CHARACTERIZATION OF A GENE WHICH ENCODES A MANNOSYLTRANSFERASE HOMOLOG OF Paracoccidioides brasilienses

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We screened an expression library of the yeast form of *Paracoccidioides brasiliensis* with a pool of human sera that was pre-adsorbed with mycelium, from patients with paracocidioidomycosis (PCM). A sequence (Pbymnt) encoding an antigenic protein was obtained and characterized. A genomic clone was obtained by PCR of *P. brasiliensis* total DNA. The cloned genomic sequence identified a single open reading frame coding for a protein containing 357 amino acid residues, 39.78 kDa. The deduced amino acid sequence exhibits identity to mannosyl and glycosyl transferases from several sources. The deduced amino acid sequence contains a DXD motif, characteristic of the glycosyltransferases. Hidropathy analysis revealed that the deduced protein presents a single transmembrane region near the amino terminus of the molecule suggesting a type II membrane protein. The Pbymnt was expressed preferentially in the yeast fungus parasitic phase. The nucleotide sequence of Pbymnt and its flanking regions had been submitted to GenBank under accession number AF374353. A recombinant protein reactive to sera of patients with paracoccidioidomycosis was obtained in *Escherichia coli* after expression of the Pbymnt in pBluescript SK+. Overall, our data, suggest that Pbymnt encodes one member of a glycosyltransferase family of proteins and that our strategy ws useful in the isolation of differentially expressed genes. A cDNA was obtained by RT-PCR and cloned into expression vector. The purification of the recombiannt protein is under progress.

Suported by CNPq, FUNAPE-UFG

01-04

FUNCTIONAL AND GENOMIC ANALYSIS OF A CALMODULIN FROM THE FUNGUS Paracoccidioide brasiliensis

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Calmodulin (CaM) belongs to a family of calcium binding proteins modulating intracellular calcium signaling and acts on several metabolism pathway and gene expression regulation. *P. brasiliensis* (*Pb*) is a dimorphic and human pathogen fungus, which causes the Paracoccidioidomycosis in the yeast form. The differentiation from mycelium to yeast forms is dependent of temperature. Studies of drugs effect in the Ca^{+2}/CaM signal transduction pathway were performed inducing *in vitro* the differentiation process in the presence of EGTA, a calcium chelator, TMB-8, which blocks calcium entry, and the CaM antagonists 48/80, R24571 (calmidazolium), TFP (trifluoperazine), and W7. All drugs were able to inhibit the mycelium to yeast differentiation process. The more efficient effect was observed for 24 μM R24571, followed by 50 μM TFP, 100 μM W7, and 10 mM EGTA. The gene encoding CaM was isolated screening the *Pb* genomic library using a homologue probe obtained by PCR. The isolated genomic clone contains the CaM gene lacking the 3' region, which was obtained by 3'RACE. The amplified sequence presents 924 nucleotides and 5 conserved introns after the ATG. The transcriptions factor motifs and a polyadenylation site were identified by several computational programs. The primary sequence similarity and multiple alignments were done using the FASTA and Pileup programs. The deduced amino acid sequence exhibits four typical calcium-binding EF-hand motifs and is identical to CaM from *H. capsulatum*, *A. oryzae*, and *A. nidulans*; similar to *N. crassa* (99.3%), human CaM (84%), and to *P. tetraurelia* (81.2%). In conclusion, the drug effects results mainly indicate the role of CaM_Pb in *Pb* differentiation process. Indeed, the experimental conditions not affected the cellular viability, which reinforces the drug specificity on the differentiation process.

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01-05

STRUCTURAL MODEL FOR CALMODULIN FROM Paracoccidiodes brasiliensis BY MOLECULAR MODELING

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Calmodulin (CaM) modulates intracellular calcium signaling acting on metabolism process and gene expression regulation. The cellular differentiation of some dimorphic fungi is dependent of the Ca^{+2}/CaM signal transduction pathway. *Paracoccidioides brasiliensis* (*Pb*) is the human pathogen dimorphic fungus. It switches between mycelium and yeast forms to infection establishment. Here, we present the three-dimensional model of CaM from *Pb* (CaMPb) by molecular modeling. According to primary sequence several conserved structures of CaM were found on PDB. The Ca^{+2}/CaM from *P. tetraurelia* was retained as CaMPb template and its coordinates were assigned using InsightII program. The minimized model was submitted to molecular dynamics (MD) simulations of 100 ps in a water box of $90^3 Å^3$. The MD was performed in a supercomputer available at Université Henry-Poincaré, France, using NAMD Software. CaMPb consists of two domains each one comprised of two EF-hand motifs. Ca^{+2}-bound motifs are in open conformation with hydrophobic clefts solvent exposed corresponding to the probably CaMPb site for CaM target proteins and pharmacological agents. After MD simulation the secondary structures feature of the EF-hand motifs were preserved with all Ca^{+2} correctly coordinated. The structural overview of CaMPb model has no significant changes (rmsd 1.3Å). 90.1% and 9.9% of residues are in most favorable and in allowed regions. Ca^{+2}/CaM inhibitor drugs were effectives to block the *Pb* mycelium to yeast transition. These results indicate that Ca^{+2}/CaM signal transduction pathway is important in the differentiation process. Based on data that a chromofungin inhibits CaM action in fungi and considering the structural properties of CaMPb model, we proposed further studies to test this peptide as a potential CaMPb antagonist.

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01-06

GENETIC POLYMORPHISM OF Paracoccidioides brasiliensis BY PFGE AND RAPD

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Paracoccidioides brasiliensis, the causative agent of paracoccidioidomycosis (PCM), is refractory to conventional genetic analysis. Previous work with RAPD or PFGE revealed *P. brasiliensis* genetic polymorphism. We worked concomitantly on PFGE and RAPD to evidence genetic polymorphism among 13 isolates, from different sources (8 isolates from chronic clinical forms, 4 from acute forms, and 1 from soil) and geographic regions (11 from Brazil, 1 from Venezuela and 1 from Peru). For PFGE, yeast cells immobilized into agarose plugs ($3x10^7$ cells/plug), had their cell walls removed by enzymatic digestion, followed by lysis. Stable and reproducible karyotypes were observed. Preliminary analysis showed polymorphism of number (4 to 5) and sizes of chromosomal bands (2 to 10 Mb). Polymorphism makes it difficult to correlate the banding pattern among isolates so gels were transferred to membranes and available genetic markers will be used to hybridize Southern blots. We standardized experimental protocols for extracting from agarose plugs the high quality DNAs obtained to use it into RAPD. Six random primers were tested for discriminative capacity at RAPD-PCR. All primers generated polymorph banding patterns. Results on each primer and combination of the results for all six primers, by the UPGMA method, were used to build individual and a general phenogram. This is the first time that RAPD and PFGE, together, compare genetic patterns of a set of *P. brasiliensis* isolates. No obvious correlation could be established so far, however, two isolates which present basic Gp43 isoforms, previously studied by sequence alignment of the gp43 gene, associated with chronic pulmonary PCM (Morais *et al.*, 2000), grouped together and also shared the same geographic origin and identical karyotype profile.

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01-07

NEW ASPECTS ABOUT THE EXOCELLULAR THIOL-DEPENDENT SERINE PROTEINASE FROM *Paracoccidioides brasiliensis*

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In paracoccidioidomycosis, an exocellular thiol-dependent serine proteinase from *Paracoccidioides brasiliensis*, previously characterized by our group, has a possible role in fungal dissemination. It is capable of selective degradation, *in vitro*, of proteins associated with the basal membrane, like laminin, fibronectin, type IV collagen and proteoglycans. The proteinase shows strong hydrolytic activity against quenched fluorescent peptides derived from Abz (O-aminobenzoyl)-MKRLTL-EDDnp(ethylenediaminedinitrophenyl), which are specifically cleaved at the L/T bond. Attempts to purify the proteinase have been hampered by self degradation and/or aggregation with highly glycosylated fungal components. However, its relative migration could be localized in SDS-PAGE gels by substrate cleavage detected with an agarose overlay containing Abz-MKALTLQ-EDDnp or by zymograms, where the gel is impregnated with gelatin. Hydrolysis by the serine-thiol proteinase was seen as a diffusely migrating component between 68 and 43 kDa. The activity was enriched in fractions obtained after chromatography in Phenyl Superose hydrophobic columns followed by affinity separation in concanavalin A. A broad SDS-PAGE gel band well stained with silver, but poorly with Coomassie blue, was observed co-migrating with the hydrolytic activity. Analysis of enzymatically obtained internal peptides contained in the gel-excised band indicated the presence of a mixture of four or more molecules, but none was analogous to proteinases. Recently, a new purification step, namely reverse phase chromatography in C4 columns, has been introduced after fractionation of culture supernatants in Phenyl Superose. Preliminary results showed that activity resisted treatment with organic solvents and was eluted in two distant peaks. Further analyses are being carried out and will be presented.

01-08

IDENTIFICATION AND CHARACTERIZATION OF A GENE THAT ENCODES THE STAGE-SPECIFIC PROTEIN PbY20 FROM THE DIMORFHIC FUNGUS *Paracoccidioides brasiliensis*

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Paracoccidioides brasiliensis is a dimorphic fungus that occurs as mycelium (26°C) and yeast form (36°C). Cunha *et al.* (1999) identified by two dimensional electrophoresis an yeast-phase specific protein denominated PbY20. The 34 N-terminal amino acids residues shown high similarity with specific allergens, Alt a7 (*Alternaria alternata*) and Cla h5 (*Cladosporium herbarum*). To amplify a DNA fragment, relative to PbY20 by PCR, we designed three primers by alignment of cDNAs relatives to proteins Alt a7; Cla h5 and the obr1 gene from *Schizosaccharomyces pombe*. The complete sequence has 1154 pb and a deduced open reading frame of 203 amino acids showing a high identity with the proteins 1,4-benzoquinone reductase from *Phanerochaete chrysosporium* (72%), Alt a7 (70%), NADH:quinone oxidoreductase from *Gloeophyllum trabeum* (67%), Cla h5 (68%), Pst2p and YCP4 from *Saccharomyces cerevisae* (65%) and a protein of brefeldin A resistance from *S. pombe* (59%). Brefeldin A is a fungal metabolite which causes disassembly of the Golgi apparatus. The 1,4-benzoquinone reductase may transform brefeldin A in an inactive form. The northern blot analysis using total RNAs obtained from different time intervals of temperature-induced mycelium tó yeast transition (0h, 1/2h, 2h 6h 12h, 24h and Y) showed that there is an increase in pby20 mRNA levels in the yeast form. The western blot using the extract of mycelium and yeast total protein confirmed the diferential expression of the PbY20 protein in the yeast form..

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01-09

CHARACTERIZATION OF A MALATE DEHYDROGENASE FROM Paracoccidioides brasiliensis

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The human pathogen *Paracoccidioides brasiliensis* is the ethiological agent of paracoccidioidomycosis (PCM) the most frequent deep mycosis in Latin America. The fungus transits between the mycelia to the yeast form during the infection pathway. The fungus presents a complex antigenic structure and some antigens had been characterized. In this work we present the characterization of a new antigen from *P. brasiliensis*, Malate Dehydrogenase (MDH), molecular mass of 34 kDa, pI 6.5, highly expressed in the fungus parasitic phase. On basis of aminoacid sequences of MDH, as well as on nucleotide sequences of MDH from other organisms, it was designed oligonucleotides in order to obtain, by PCR, the related genomic fragment in *P. brasiliensis*. The obtained MDH fragment demonstrated homology to MDH from other organisms, like *Malassezia furfur*. The fragment was used in the screening of cDNA library.

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01-10

THE kex GENE FROM THE DIMORPHIC AND HUMAN PATHOGENIC FUNGUS Paracoccidioides brasiliensis

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Kexin-like protein is a component of the subtilase family of proteinases involved in the processing of proproteins to their active forms. The members of the kexin subfamily are synthesized with a signal peptide followed by a propeptide at the amino-terminus, a subtilisin-like catalytic domain and a P domain, which is essential for the catalytic activity and stability. The carboxy-terminal region may or may not have an S/T-rich sequence, a C-rich sequence, an amphipathic region, a single transmembrane domain and a cytoplasmatic domain. Disruption of the kex gene in *C. albicans* and *Y. lipolytica* affects hyphae production and induces morphological cell defects, strongly suggesting a possible role of Kexin-like proteins in dimorphism of human pathogenic fungi. In this work, we report the nucleotide sequence of kex gene cloned from the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis* (Pbkex). An open reading frame (ORF) of 2622 bp was identified in the complete sequence, interrupted by only one intron of 93 bp. The 5'-non coding region contains consensus sequences such as canonical TATA, CAAT boxes and putative motifs for transcriptional factors binding sites. In the 3'non coding region were observed motifs for efficient and positioning elements, as well as for poli (A) site. The deduced protein sequence consists of 842 amino acids residues showing identity to Kexin-like proteinases from *A. niger* (55%), *E. nidulans* (53%) and *C. albicans* (48%). Comparative sequence analysis of *P. brasiliensis* Kexin-like protein reveals the presence of homologous regions related to a signal peptide, a propeptide, a subtilisin-like catalytic domain, a P domain, a S/T rich region and a transmembrane domain.

01-11

IDENTIFICATION OF AN EARLY EXPRESSED GENE IN DIMORPHIC FUNGI Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis is the etiologic agent of paracoccidioidomycosis (PCM). The fungus is thermally dimorphic, growing as a multicellular mycelial form at environmental temperatures (26°C) and as an unicellular large globose yeast cells at 37°C. Using Differential Display Reverse Transcriptase-PCR (DDRT-PCR) to compare multiple mRNA populations to screening early expressed genes during the dimorphic process of *P. brasiliensis*, we were able to identify the M-Y2 fragment, which recognized a mRNA of approximate 3.2 kb and is not expressed in mycelium, but shows a significant expression after 2 hours with maximum expression at 24 hours the temperature shift. Both strands of the M-Y2 fragment were sequenced and deposited in dbEST under accession n° BE758603. Databank searches for homology were performed and no significant homology was found. The M-Y2 fragment was used as probe to screening a genomic library of *P. brasiliensis*. Several clones were found and six of them were isolated and, following total DNA extraction, were analyzed by single (*Hind*III) and double digestion (*Hind*III/*Xba*I). The digested DNA from all clones analyzed by Southern blotting, using the M-Y2 fragment as a probe. Three distinct hybridization patterns were obtained by single (HindIII or XbaI) and double (*Hind*III/*Xba*I) digestions. A 5.0 kb fragment, product of HindIII digestion from G3 clone was chosen for subcloning into pUC18 vectors. The pH1 clone was obtained and sequencing is under way, in order to obtain the complete sequence of differentially expressed M-Y2 gene.

01-12

EXPRESSION OF CATALASE IN THE PATHOGENIC FUNGUS Paracoccidioides brasiliensis

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The pathogenic fungus *Paracoccidioides brasiliensis* is the ethiological agent of Paracoccidioidomycosis (PCM), the most prevalent deep mycosis in Latin America. The fungus grows as mycelia or yeast on dependence of the temperature. The mycelia constitute the infective phase that differentiate to the yeast form establishing the infection in the human lungs. An important aspect in the disease establishment is the fungus potential in modulating the host immune system. Catalases, metaloenzymes that catalyse the conversion of hydrogen peroxide to H_2O and O_2 are imunodominant antigens and putative virulence factors in several pathogens. The expression of a *P. brasiliensis* catalase, molecular mass of 61kDa, pI 6.2 was analysed by one and two-dimensional western blotting of cellular extracts probed to a monoclonal antibody raised against catalase of *Toxoplasma gondii*. It was shown that the catalase from *P. brasiliensis* is strongly induced during the transition from mycelia to yeast, the infective pathway of *P. brasiliensis*. In addition, during the reverse process, the catalase expression is repressed. Clinical isolates of *P. brasiliensis* analysed in yeast and mycelia forms present the enzyme expression. The expression of catalase was also analysed in yeast and mycelia in the presence of hydrogen peroxide. It was not found alterations in the pattern of catalase in the presence of H_2O_2 suggesting that the enzyme could not be regulated by the substrate concentration.

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01-13

PHENOTYPIC CHARACTERISTICS OF Paracoccidioides brasiliensis STRAINS

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The dimorfhic fungus *Paracoccidioides brasilensis* (Pb) is one of the most prevalent invasive fungal pathogenes in Latin America. In this study, we investigate the following proofs for phenotypic characteristics of Pb isolates: urease production, behavior in canavanine-glycine-bromothymol blue (CGB) and tetrazolium chloride (TTC) media and in Odds & Abbott system. Twenty different isolates of Pb were grown in Bacto-Peptone, Dextrose-Agar, pH 5.5 at 27°C, and one loopful of each of these cultures were transfered onto Petri dishes of basal media. All isolates were plated in duplicates at 27°C and were examined daily for 30 days in order to observe the colony growth and proteinase production for Odds & Abbott system, growth with pigmentred to TTC, Growth color change from yellow to blue to CGB and yellow to red urease. All isolates of Pb were positive in all proofs and their Odds & Abbott biotype was found to be 777. They all showed the same profile when cultured on urease, CGB and TTC media.

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01-14

PURIFICATION AND CHARACTERIZATION OF 200 kDa ANTIGEN, A PUTATIVE FORMAMIDASE HOMOLOG OF Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis, the ethiological agent of Paracoccidioidomycosis (PCM) presents a growing medical importance. The fungus grows as yeast or mycelium and the dimorphic transition is the main event in the infection establishment. An important aspect of the host-pathogen interaction with direct role in pathogenesis, is the fungus potentially in modulating the host immune response. In this vein, our group is engaged in a program to characterize antigens of *P. brasiliensis* that could contribute to the generation of the immune response. A 200kDa protein, acid isoeletric point, preferentially expressed in the yeast cells and reactive to sera of PCM patients was purified through isoeletric focusing using the Rotofor System. Amino acid sequences from three internal peptides were obtained after digestion of the protein with endoproteinase Lys-C. Comparative analysis with sequences found at database showed the protein to be a formamidase (EC 3.5.1.49) of *P. brasiliensis*. Interestingly, it has been described in pathogenic fungi of plants that some nitrogen catabolic enzymes, such as amidases and formamidases play a role in the pathogenicity. A genomic fragment coding for formamidase was obtained by DNA amplification using degenerated primers. The cloning of the entire gene/cDNA of this serum reactive protein of *P. brasiliensis* will allow future studies on the molecule role in the fungus.

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01-15

HETEROLOGOUS EXPRESSION, PURIFICATION AND IMMUNOLOGICAL REACTIVITY OF A RECOMBINANT HSP60 FROM Paracoccidioides brasiliensis

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Paracoccidioidomycosis (PCM) is a fungal disease caused by *Paracoccidioides brasiliensis*, a thermal dimorphic fungus which is geographically confined to Latin America. The fungus presents thermal dimorphism, growing as mycelium (22°C-28°C) and as yeast *in vitro* and at 37°C, in the host tissues. The complete cDNA of HSP60 from *P. brasiliensis* was overexpressed in *Escherichia coli* host to produce high levels of recombinant protein. The protein was purified by affinity chromatography. A total of 169 human serum samples were tested for reactivity by Western blot analysis with the purified HSP60 recombinant protein. Immunoblots with serum samples from PCM patients were performed and reactivity was observed in 73 of the 75 serum samples analyzed, showing 97.3% sensitivity. The recombinant HSP60 did not react with sera from patients with aspergillosis, cryptococcosis, sporotrichosis, or tuberculosis. Reactivity to HSP60 was observed in sera from 7% normal subjects and 11.5% patients with histoplasmosis. The high sensitivity and specificity (93.3%) for HSP60 suggested that the recombinant protein can be used singly or in association with other recombinant antigens to detect antibody responses in *P. brasiliensis* infected patients.

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01-16

EFFECT OF Eugenia uniflora LEAVES EXTRACT ON DIMORPHISM OF Paracoccidioides brasiliensis

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Eugenia uniflora L. (Myrtaceae) is a shrubby tree of Brazilian origin with edible cherry-like fruits. The leaves has been used in folk medicine as antidiarrheic, diuretic, antirheumatic and antifebrile. Ethanol extract and its aqueous fraction from leaves have been investigated for their growth inhibitions on the yeast cells and during the mycelial-to yeast-phase transitions of *P. brasiliensis* (Pb01 strain) induced by a temperature shift from 25 to 36°C in Negrone medium. When the concentration of the ethanol extract and aqueous fraction was 0.5 mg/ml, the inhibition of yeast grown, after 15 days at 36°;C, were 79 and 53%, respectively. In addiction, the aqueous fraction was able to inhibit the dimorphism in a dose-dependent manner. The highest dose tested (1.0 mg/ml) produced a 90% of the inhibition of transition phases, while 0.5 mg/ml gave an inhibition of 53%. On the other hand, this fraction exhibited little citotoxicity after 15 days exposure. The cell viability was decreased by 21.9% at the concentration of 1.0 mg/ml, indicating that it did not cause injury to cells. HPLC fractionation of the aqueous fraction has led to the identification of macrocyclic dimeric ellagitannins, oenothein B (retention time of 12.4 min), eugeniflorin D1 (r.t. 13.6 min) and eugeniflorin D2 (r.t. 8.9 min) as principal chemical components.

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01-17

STRUCTURAL ANALYSIS OF GENES RELATED TO CELL WALL METABOLISM IN ISOLATES OF *Paracoccidioides brasiliensis*

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Paracoccidioides brasiliensis is a dimorphic organism that grow as budding yeast or mycelium of filamentous hyphae. In *P. brasiliensis* both the molecular architecture and the functional components of the cell wall vary between yeast and mycelia forms of the organism. The cell wall of *P. brasiliensis* is composed of chitin, glucan and mannan. Genes that code for enzymes involved in metabolism of the cell wall are of great interest, since the understanding of this process could help to elucidate aspects of morphogenesis, invasion, infection and virulence of this fungus. We previously characterized in Pb 01 (ATCC MYA, 826) the PbFKS1, PbNAG1 and PbYmnt genes coding for b-D-1,3 glucan synthase, N-acetil-b-D-glucosaminidase and manosyltransferase, respectively. In order to compare the cited sequences in isolates of *P. brasiliensis* we selected the Pb 4940 that transits between mycelia and yeast on dependence of serum. The isolate Pb 01 requests a temperature upshift to perform the dimorphic transition. Oligonucleotides of the described genes were used in PCR reactions of the Pb 4940 DNA. PCR and Southern blot analysis showed the presence of those genes in Pb 4940 isolate. The fragments amplified from DNA of Pb 4940 isolate were cloned and sequenced. Interestingly, the PbFKS1 gene was deleted at the 5´ intron. This finding could be a start for studies of phylogenetic aspects of *P. brasiliensis*.

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01-18

MOLECULAR CHARACTERIZATION OF ARMADILLOS' *Paracoccidioides brasiliensis* ISOLATES: CORRELATION WITH VIRULENCE AND GEOGRAPHIC ORIGIN

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The armadillo *Dasypus novemcinctus* has been confirmed as the first natural reservoir of *P. brasiliensis* and its geographic distribution is similar to the distribution of human Paracoccidioidomycosis (PCM). This work analysed the virulence profiles and genetic diversity of *P. brasiliensis* isolates from different armadillos and clinical isolates. The animal isolates were obtained from the Botucatu endemic area of PCM, with exact information of the collection sites. The clinical isolates, except Pb265, were obtained from patients that live in the same endemic area. Virulence was evaluated in an experimental hamster model by colony-forming units (CFU) and histopathological analysis in the testis, liver, spleen, lung and circulating specific antibodies measured by ELISA. Molecular characterisation was evaluated by RAPD methods and sequencing analyses of the internal transcribed spacer regions (ITS 1 and ITS 2) and 5.8S ribosomal gene. All isolates from armadillos were virulent, with dissemination to many organs. The clinical isolate Pb265 was less virulent. The isolates were classified into four categories according to number of CFU per gram of tissue. Molecular analysis showed large genetic similarity between all isolates and did not separate armadillos from clinical isolates. One strain (T10B1) presented distinct genetic patter in both analysis (RAPD and sequencing). This strain can represent a particular genotype of the *P. brasiliensis*. Thus, the nine-banded armadillos that have already been considered as an important animal sentinel for PCM, can also be very useful for mapping the biological variations of this fungus, in its ability of causing disease in animal and human hosts.

01-19

THE GP43 GENE (PbGP43) FROM Paracoccidioides brasiliensis: ANALYSIS OF THE TRANSCRIPT

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Paracoccidioidomycosis (PCM) is a granulomatous disease endemic in many countries of Latin America. It is caused by *Paracoccidioides brasiliensis*, a temperature-dependent dimorphic fungus. Gp43 is the main diagnostic fungal antigen, besides being protective against murine PCM by eliciting specific cellular immunity. Our previous work [Morais et al. 2000, J. Clin. Microbiol. 38: 3960] showed the existence of polymorphism in the PbGP43 gene when 17 fungal isolates from pulmonary and lymphatic PCM were analyzed. The phylogenetic distribution of the sequences separated, in a distant group from the others, 3 isolates from patients with pulmonary PCM. In the present work we show the polymorphism in 306 bp of the 5'non-translated region of the PbGP43 ORF. Among the 17 fungal samples analyzed we found 0-4 informative sites, but none was within putative transcription motifs. The phylogenetic distribution of the sequences was similar to that of the PbGP43 coding region. In order to determine the PbGP43 transcription start and end sites, RNA from some isolates were submitted to primer extension and 3´RACE reactions. Three transcription initiation sites were detected in 4 isolates. The most abundant site was 25 bp upstream (-25) to the translation initiation codon. The other two sites were at positions –33 and –35. Fifty-six 3´RACE clones from 10 isolates were sequenced and 13 different polyadenylation cleavage sites were detected in the PbGP43 transcript between positions 91 and 128 downstream to the stop codon. All isolates, except one, showed cleavage site heterogeneity, but the 3'non-translated region of the PbGP43 transcript was fairly conserved. We have not been able to establish any correlation between polyadenylation cleavage site preference and other characteristics of the isolates.

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01-20

INHIBITORS OF THE DELTA$^{24(25)}$STEROL METHYLTRANSFERASE AS POSSIBLE ANTIFUNGAL AGENTS AGAINST Paracoccidioides brasiliensis

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The enzymes delta$^{24(24')}$ and delta$^{24(25)}$sterol methyltransferases (SMTs) catalyze an essential step in the biosynthesis of sterols in fungi, protozoa and plants, being absent in vertebrates. Therefore, they are potential specific targets for antifungal therapy. In *Pneumocystis carinii*, the effectivity of an SMT inhibitor, 20-piperidyl-2-il-5alpha-pregnan-3beta,20(R)-diol (22,26-azasterol), as antiproliferative drug *in vitro*, has been proven (1). We have studied growth inhibition in *Paracoccidioides brasiliensis*, by means of 22,26-azasterol and analogs. *P. brasiliensis*, strain Pb73 (ATCC 32071), yeast phase, was plated in PYG agar, and incubated at 37°C. As inhibitors we tested: 22,26-azasterol (AZA-1), 22-piperidin-2-il-pregnan-22(S),3beta-diol (AZA-2) and 22-piperidin-3-il-pregnan-22(S),3beta-diol (AZA-3) between 10^{-5} and 10^{-10} M. As positive growth control, the fungus grown in the absence of inhibitors was used. As negative control, the fungus was plated with ketoconazole 10^{-8} M. AZA-1 and AZA-2 were 100% inhibitory at concentrations of $5x10^{-6}$ M, while AZA-3 was so at $1x10^{-6}$ M. MIC calculations were done in liquid media (2). Neutral lipid analysis by means of GC-MS showed that the main free sterol present in control cells was ergosta-5,22-dien-3beta-ol (68%), followed by lower amounts of ergosterol (21%) and lanosterol (11%). On exposure to either AZA 1, 2 or 3, there was an appreciable accumulation of lanosterol (50-60%), providing evidence that the delta$^{24\ (25)}$ SMT step of sterol biosynthesis was being inhibited by the azasterols. These results should be extended in order to explore the potential clinical use of these drugs.

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01-21

STRUCTURES OF POLYSACCHARIDES F1SS FROM Paracoccidiodes brasiliensis POINT TO A CLOSE TAXONOMIC RELATIONSHIP OF THIS FUNGUS TO CERTAIN ONYGENALES

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Paracoccidiodes brasiliensis is considered a member of the order Onygenales, by means of molecular evidence (a). Lacking sexual reproduction, its systematic position and phylogenetic relationship with other members of that order is uncertain. Currently, the structural analysis of cell wall polysaccharides has become a powerful weapon in the definition of genera and determination of phylogenetic relationships in fungi (b). Therefore, we have included *P. brasiliensis* in a study aimed to analyze F1SS polysaccharides (the cell wall water-soluble fraction from alkaline extracts) from Onygenales. Structures of *P. brasiliensis* F1SS polysaccharides from both morphological (mycelial and yeastlike) phases were determined. Mannose and galactose were components of both polymers. Methylation and NMR studies revealed that F1SS from both phases contained [→2,6)-α-D-Man*p*-(1→;→6)-α-D-Man*p*-(1→yα-D-Gal*f*-(1→] residues, while F1SS from the yeastlike phase additionally contained [α-D-Man*p*-(1→;→2)-α-D-Man*p*-(1→] and [β-D-Gal*f*-(1→] residues. These moieties are closely related to those of F1SS from species of *Ascocalvatia*, *Onygena* and some *Aphanoascus*, confirming the positioning of *P. brasiliensis* as a member of the Onygenales.

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01-22

A PRELIMINARY ANALYSIS OF THE GENOME OF Paracoccidiodes brasiliensis

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Fungal genomes are variable in size and it has been described that the size of *Paracoccidiodes brasiliensis* genome is about 30 Mb. By digestion of the genomic DNA from strain IVICPb 73 (ATCC 32071), we have constructed a gene bank of 50,000 colonies with a size average of the insert of 8 kb (10x coverage) in *E. coli* DH5α;. We have fully sequenced 7 clones comprising 50,655 bp and an 8th clone (after isolation of the *URA3* gene) comprising additional 3322 bp. The sequence has a 44.36% GC content and presents 22 putative ORFs (nine of them, partial) and one tRNA, giving a density of one gene per 3.5-4.5 kb. Thus we may suggest that the genome of *P. brasiliensis* contains between 7,500 to 9,000 genes in agreement with the number of genes estimated in ascomycetous fungal genomes (between 7,000 to 10,000). The 22 coding sequences cover 46% of the total sequence. Introns are present in 12 out of the 22 sequences. While 15 sequences have a corresponding fungal homologue, four lack homology with any ORF described so far in databanks, one shows homology with a protein of unknown function, and two sequences have homologues in *Bacillus halodurans* and *Pseudomonas aeruginosa* proteins.

01-23

ACTIN GENE IN *Paracoccidioides brasiliensis*

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Actin is a pivotal structure in the cytoskeleton of many cells. In dimorphic fungi, actin plays an important role in morphogenesis. To clone the actin gene in *Paracoccidioides brasiliensis*, DNA was extracted as before (Niño-Vega *et al.*, 2000). By means of alignment of amino acid sequences from several fungi, high homology zones were determined, from which degenerate oligonucleotide primers were designed (Sambrook *et al.*, 1989). By PCR, a unique product (approx. 800 bp) was amplified, which was subsequently cloned and sequenced. A *P. brasiliensis* partial genomic DNA library was constructed; the PCR amplified product was used as primer to search for recombinant colonies carrying the fragment under study. To search the initiation codon and the promoter region of the gene, the method reported by Zhang and Gurr (2000) was used. A 1.7 kb fragment was obtained and sequenced, containing the promoter region. Northern analyses of the gen during both M to Y and Y to M transitions showed differential expression, with higher levels of expression during the yeast phase.

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01-24

Paracoccidioides brasiliensis ORNITHINE DECARBOXYLASE GENE

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In *Paracoccidioides brasiliensis*, the activity of ornithine decarboxylase (ODC) increases through yeast like (Y) growth and in transition from the mycelial (M) phase to the infective Y phase, being associated to yeast budding. *P. brasiliensis* ODC gene (PbrODC) was studied in strain Pb73. After DNA extraction (Niño-Vega *et al.*, 2000), a partial library was constructed from *Pst*I fragments searched by colony hybridization, with the use of a 273 bp ODC fragment as the primer (Torres-Guzmán *et al.*, 1996). The gene has an ORF of 1413 bp, with a 72 bp intron, coding for a 447 amino acid protein, molecular weight 50 kDa, isoelectric point 4.9, and high similarity to other fungal ODCs. Phylogenetic trees based on ODC genes suggest that *P. brasiliensis* groups with Ascomycetes, order Onygenales. PbrODC expression during Y growth and dimorphic transition was determined by RT-PCR. It was constant throughout both processes, suggesting a post-transcriptional regulation of the gene product.

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01-25

EFFECT OF Solanum ALKALOIDS ON DIMORPHISM OF Paracoccidioides brasiliensis

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Glycoalkaloidic fractions rich in alpha-solasonine and jurubine from *Solanum lycocarpum* A. St. Hil. (lobeira) and *S. paniculatum* L. (jurubeba), were tested as growth inhibitors on the yeast cells as well as during the mycelial-to yeast-phase transitions of *P. brasilensis* (Pb01 strain) induced by a temperature shift from 25 to 36°C in Negrone medium. The alkaloidic fractions from ripe fruits, at 0.5 and 1.0 mg/ml, moderately or considerably inhibited yeast growth. At the same concentration, the dimorphism process showed a reduction of 60 and 80% inhibition for alkaloidic fraction from *S. lycocarpum* and *S. paniculatum*, respectively, compared to the control. Both rate and the extent of alkaloidic activities were enhanced when the concentration was increase. The observed antifungal activity has been proposed by ability of these steroidal glycoalkaloids to complex with membrane 3-hydroxy sterols and disrupt membrane integrity and function.

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01-26

HYGROMYCIN B ACQUIRED PHENOTYPE IN Paracoccidioides brasiliensis VIA PLASMID DNA INTEGRATION

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In this report we describe a reproducible procedure for the transformation of yeast cells of the human pathogenic fungus *Paracoccidioides brasiliensis* by electroporation with the plasmid pAN7.1 (Punt *et al.*, 1987) harboring the *hph* gene, which confers hygromycin resistance to the transformed cells. This is the first report of transformation in *P. brasiliensis* and provides a means for the genetic manipulation of this fungus towards a better understanding of its biology and the mechanisms involved in gene functions, cell differentiation, host-pathogen interactions including virulence and pathogenicity. Transformation was achieved using electroporation protocol, with intact or linearized plasmid DNA. The fungus was transformed using 200 mM manitol, 5 and 7 KV/cm field strength, 25 μF of capacitance, 400Ω of resistance, 5 μg of plasmid DNA and 10^7 eight days old yeast cells in 400 μL, and selected in overlaid 30 μg/mL hygromycin B (hygB) containing BHI medium. The transformation efficiency obtained was 8-transformants/μg of DNA after the first selection in BHI containing 30 μg/mL hygB. Based on the final hygB resistance phenotype, putative transformants could be divided into two groups. The majority exhibited a high degree of instability losing the resistance phenotype after four passages on selective medium. The other group presented low rate of growth in selective medium. The degree of instability observed was probably due to nuclei number or genomic rearrangements of integrated copies of *hph* gene of *P. brasiliensis* yeast cells. Using hph1 and hph2 primers, it was possible to amplify an expected PCR product of 462 bp in length. All the selected clones, independent of the vector, presented the expected fragment demonstrating the presence of the *hph* sequence into the transformant genomes. Southern blotting analysis also demonstrated the transformation of Pb01 yeast cells. These data certainly represent a great advance on the molecular biology of *P. brasiliensis* since it opens the possibility to direct experiments to study *P. brasiliensis* gene functions involved in host-pathogen interactions, pathogenicity, virulence and/or multi-drug resistance, making possible the development of more specific diagnosis and new approaches to PCM treatment.

01-27

EFFECT OF Hyptis passerina ESSENTIAL OIL ON DIMORPHISM OF Paracoccidioides brasiliensis

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The essential oil from aerial parts of *Hyptis passerina* Mart. ex Benth. (Lamiaceae) were analysed by gas chromatography/quadrupole mass spectroscopy. The major constituents were valeric acid 4-pentenyl ester (25.8%), caryophyllene oxide (19.5%) and (E,E)-farnesyl acetate (10.3%). The oil was investigated for their antifungal activity against the yeast cells and during the mycelial-to yeast-phase transitions of *P. brasilensis* (Pb01 strain) induced by a temperature shift from 25 to 36°C in Negrone medium. In these experiments, the yeast growth was reduced to 32.6 and 48.4% of the control values by essential oil at 0.5 and 1.0 mg/ml, respectively. The dimorphic process was stronger blocked (>95% inhibition compared to the control) when mycelia were placed to transform to yeast at the same concentrations. In contrast, the essential oils from aerial parts of *H. velutina* Pohl ex Benth. and *H. recurvata* Poit. had no effect on yeast grown.

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01-28

EXPRESSION OF RECOMBINANT GP43 IN BACTERIA

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Gp43 is the major diagnostic antigen from *Paracoccidioides brasiliensis*. More importantly, it is capable of inducing protective cellular immunity in mice. It is reactive with most sera from patients with paracoccidioidomycosis (PCM), which preferentially recognize peptide epitopes. The antigen is specific for PCM when tested in its native conformation. In ELISA, cross-reactivity can eventually occur due specially to a galactose-containing epitope. The PbGP43 gene encodes a protein of 416 amino acids, from which 35 correspond to the leader sequence not found in the processed secreted molecule. *P. brasiliensis* isolates could be grouped according to the genetic polymorphism in the PbGP43. The most polymorphic sequences (3 among 17) were from isolates of pulmonary PCM and encoded basic isoforms of gp43 (previously shown to be less antigenic), when all the others encoded isoforms of neutral isoelectric points. The PbGP43 cDNA from *P. brasiliensis* B-339 has previously been expressed in fusion with gluthatione S-transferase, as inclusion bodies in *Escherichia coli*. Immunoblotting showed that all sera from patients with PCM, but not with other mycoses, reacted with the recombinant protein of the processed gp43. Reactivity with fusion proteins containing sub-fragments of the N-terminal, internal or C-terminal regions occurred eventually and the C-terminal was the most antigenic (S.N. Diniz, 1996, M.S. thesis). Presently, cDNA fragments of the PbGP43 encoding the processed antigen were obtained by reverse transcriptase polymerase chain reaction (RT-PCR) from total RNA of several *P. brasiliensis* samples. The isolates were chosen according to the PbGP43 sequence in order to obtain gp43 recombinants of different isoforms. The cloned cDNA was sequenced and sub-cloned into vectors for expression in bacteria of gp43 containing a histidine-tag. In Western blotting, all isoforms were positive with rabbit anti-gp43 immune-serum and with a couple of sera from PCM patients. The recombinants are currently being purified to be tested in ELISA with a number of patients' sera.

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01-29

MOLECULAR CLONING AND CHARACTERIZATION OF A CDNA ENCODING THE FRUCTOSE 1,6-BIPHOSPHATE ALDOLASE ANTIGEN OF Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis is a human pathogenic fungus that constitutes a major medical problem in Latin America. The microrganism is the ethiological agent of Paracoccidioidomycosis (PCM), a systemic disease with a high prevalence in the rural population of the region. Although it has been described a complex antigenic composition for *P. brasiliensis*, there is still a paucity of purified and characterized antigens. In this work we describe a new antigen of *P. brasiliensis*, a protein of 39 kDa, pI 6.2, reactive to serum of PCM patients. The protein was gel isolated and amino acid sequences from N-terminal and internal peptides were obtained. Homology search analysis revealed the protein to be a fructose1,6-biphosphate aldolase - FBP (aldolase) from *P. brasiliensis*. Degenerated primers were designed on basis of the amino acid sequences. A genomic fragment of 499 bp was obtained presenting homology to aldolases from several sources. The genomic fragment was used as a probe in the screening of a cDNA library of *P. brasiliensis*. Two cDNAs were obtained with different restriction patterns. The cDNAs were partially characterized. The nucleotide sequences and the deduced proteins presented identity to fructose biphosphate aldolase from several sources. An analysis at the Prosite database evidenced the consensus signature for fructose biphosphate aldolase classe II.1. The total characterization of the cDNAs is under progress.

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01-30

EXPRESSION OF PARACOCCIN, AN N-ACETYLGLUCOSAMINE-SPECIFIC LECTIN, IN DIFFERENT Paracoccidioides brasiliensis ISOLATES.

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Paracoccin is a lectin component with affinity for N-acetylglucosamine which has been previously identified in a jacalin-binding fraction obtained from the culture medium of BAT isolate of *P. brasiliensis*. It could be further purified in a GlcNAc-Agarose 6B column, and consists of a band of 70 kDa component in SDS-PAGE under reducing conditions. In the present work we obtained paracoccin expression in six isolates detected by Western blotting using rabbit anti-paracoccin antibodies. The lectin was then isolated from culture supernatants of two fungal isolates by affinity chromatography in GlcNAc-Agarose. Isolates Peru and BAT were good paracoccin producers under our culture conditions, and have been chosen for further studies. In both cases, the paracoccin preparations contained components of 70 and a 55 kDa under reducing conditions, but eventually a high molecular weight component with diffuse migration was also detected, suggesting that the lectin may be associated with glycoconjugates or polysaccharides. Paracoccin is apparently a minor exocellular component and could only be purified in small amounts either from supernatant fluids or from cells growing on agar medium. The presence of glycosylation was tested in paracoccin preparations by enzymatic treatment with N-glycosidases, which did not alter the electrophoretic mobility of the 70 and 55 kDa bands. This topic will be further addressed using alternative approaches. It has been previously shown that paracoccin localized to the cell surface of *P. brasiliensis* mainly on the budding region. Therefore, we investigated its interaction with chitin, a beta-1,4-homopolymer of GlcNAc, conspicuously present in fungi. Among the components bound to chitin flakes and eluted with GlcNAc, those of 70 and the 55 kDa were recognized by anti-paracoccin antibodies. The paracoccin-chitin interaction and the localization of the component on the budding regions suggest that paracoccin may have a role in *P. brasiliensis* growth and cell wall biogenesis.

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01-31

CHARACTERIZATION OF SEQUENCES ENCODING THE TRIOSE PHOSPHATE ISOMERASE ANTIGEN OF Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis (PCM), the most prevalent deep mycosis in Brazil. The fungus grows as mycelia at 22°C, and as yeast at 36°C *in vitro* and in the host infected tissues. *P. brasiliensis* presents a complex antigenic composition. Although some antigens had been identified there is still a paucity of purified and characterized antigens. In this work we identified and characterized a new antigen of *P. brasiliensis* by amino acid sequencing and homology search analysis. The protein is homologous to Triose Phosphate Isomerase (TPI) of several sources. On basis of the obtained aminoacid sequences it was designed oligonucleotides. Sense and antisense primers were able to amplify two distinct DNA fragments, 0.9 and 1.3 kb respectively (GenBank AF387658 and AY037936), both presenting homology to TPI genes from several organisms. Both sequences CDNAs are under characterization were used in the screening of a *P. brasiliensis* expression library.

01-32

GENETIC TRANSFORMATION OF Paracoccidioides brasiliensis MEDIATED BY Agrobacterium tumefaciens

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Background: Genetic transformation allows the understanding of gene function. Several fungi have been transformed successfully employing CaCl, electroporation, biolistic methods, and recently, transformation based on the cooperation of *Agrobacterium tumefaciens* used as a facilitator. At present no system of transformation is available for *P. brasiliensis*. **Methods:** *P. brasiliensis* ATCC-60855, ATCC-200273, *A. tumefaciens: GV-1040, LBA 4404,* and *EHA 101* strains were used, as well as plasmids pAD-1624, pAD-1625, containing into their T-DNA, the hygromycin phosphotransferase (hph) gene. *A. tumefaciens* strains were transformed by electroporation, then the bacteria vir genes of the bacteria were activated and co-cultivated with yeast phase cells of *P. brasiliensis* at 28°C per 8 days. After co-cultivation the recombinant cells of *P. brasiliensis* were selected by growing at 35°C in BHI media containing, cefotaxime and hygromycin. **Results:** Several colonies of *P. brasiliensis* transformants which contained the hph gene were obtained. The transformation was confirmed by growing the *P. brasiliensis* in selective media and by PCR amplification of the hph gene using specific primers, which gave an amplicon of 220 bp in the hygromycin resistant colonies. No bacterial contamination was detected in the culture, neither by PCR with specific ribosomal primers for *A. tumefaciens*. **Conclusions:** a transformation system for *P. brasiliensis* has been devised. We are planning to use these system to introduce DNA into the fungus in order to disrupt several genes by homologous recombination, thus gaining an understanding on their function.

01-33

CHARACTERIZATION OF A cDNA ENCODING THE GLYCERALDEHYDE 3-P-DEHYDROGENASE ANTIGEN (GAPDH) of Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis is the ethiological agent of paracoccidioidomycosis (PCM) a major medical problem in Latin America. The fungus mycelia propagules when inhaled differentiate to the yeast phase establishing the infection in the human lungs. The disease establishment depends, among several aspects, on the ability of the fungus to modulate the host immune system. In this work we describe the isolation of a cDNA encoding the Glyceraldehyde 3-P-Dehydrogenase (PbGAPDH1) antigen of *P. brasiliensis*. The cDNA (Pbgapdh1) presented 1717 nucleotides in length, with an open reading frame (ORF) of 1014 bp, coding for a protein with a predicted molecular mass of 36 kDa and pI of 6.8. It was observed in the deduced protein four potential N-glycosylation sites (N-X-S/T-X). Also, it was found the consensus active site region (ASCTTNCL), the conserved binding site for NAD and a consensus binding site for inorganic phosphate. These consensus regions suggest that the protein can present enzymatic activity in *P. brasiliensis*. The expression of Pbgapdh1 was analyzed by two-dimensional electrophoresis of proteins and northern-blot. It was detected a single mRNA species of 1.8 Kb, preferentially expressed in the yeast cells phase, in agreement to the high levels of the GAPDH detected in this parasitic phase of *P. brasiliensis*. The heterologus expression of GAPDH is under progress.

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01-34

MOLECULAR CLONING AND CHARACTERIZATION OF A cDNA ENCODING THE N-ACETYL-β-D-GLUCOSAMINIDASE HOMOLOG OF Paracoccidioides brasiliensis.

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A cDNA encoding the N-acetyl-β-D-glucosaminidase (NAG) enzyme of *Paracoccidioides* brasiliensis named Pb NAG1 was cloned and characterized. The nucleotide sequence of the cDNA with 2663 nucleotides, presented a single open reading frame encoding a protein with a predicted molecular mass of 64.73 kDa, isoeletric point of 6.35. The predicted protein is composed of a putative 30-amino-acid signal peptide. The deduced protein shares sequence similarity to classical NAGs. The primary sequence of Pb NAG1 was used to infer phylogenetic relationships. The amino acid sequence of Pb NAG1 has 45, 31 and 30% identity to the sequences of those from *Trichoderma harzianum, Emericella nidulans* and *Candida albicans*, respectively. In particular, it was observed striking homology with the active site regions of the glycosyl hydrolases, family 20 proteins. The active site consensus motif GXDE and the catalytic Asp and Glu residues described as characteristic of NAGs were found in the presently described protein, reinforcing that it belongs to the glycosyl hydrolases family 20. The nucleotide sequence of Pb nag1 and its flanking regions has been submitted to GenBank under accession number AF419158. The production of NAG was studied in yeast cells. The NAG activity was detected in the extra cellular media of cells grown in the presence of N-acetylglucosamine (GlcNAc) suggesting that the enzyme can be secreted in yeast cells of *P. brasiliensis*.

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01-35

RESTRICTION ENZYME ANALYSIS OF MITOCHONDRIAL DNA OF Paracoccidioides brasiliensis

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Paracoccidiodes brasiliensis (Pb) is a dimorphic fungus that causes a systemic granulomatous mycosis. This thermal fungus grows in a yeast and pathogenic phase at 36°C and in a mycelial and infectant phase at room temperature (25°C). The mitochondria seems to be involved in the transition of mycelial to yeast phase induced by temperature shift when ATP levels and respiration rates decline and growth is adapted to higher temperatures (Medoff et al. 1987). The genetic composition of the fungus and information about the mitochondrial genome size is virtually unknown. We have improved the method for extraction of mitochondrial DNA and we have done a restriction analysis in 7 different isolates of Pb (18, 339, AP, SS, 608, Peru, 470). Six different restriction endonucleaseses were used in order to analyse the pattern of restriction fragments in each isolate as follows: *Eco*RI, *Hind*III, *Bgl*II, *Xho*I, *Xba*I and *Sac*I (10U enzyme/mg DNA). The digestions were made for 2hs at 37°C and the fragments observed in 1% agarose gels. The digestion with each enzyme for each isolate resulted in different pattern of bands suggesting a restriction fragment length polymorphism and we estimated the genome size of mitochondrial DNA around (70 to 80Kb) in those isolates.

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01-36

HETEROLOGOUS EXPRESSION CONSTRUCTS AND PROTOCOLS FOR PURIFICATION OF A DIFFERENTIALLY EXPRESSED HYDROPHOBIN FROM Paracoccidiodes brasiliensis

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Paracoccidioides brasiliensis, the etiological agent of paracoccidioidomycosis (PCM), is a dimorphic fungus found as mycelia (the putative infective form) at 25°C and as yeast at 37°C. The mycelium to yeast transition seems be essential for the establishment of infection *in vivo*. The characterization of stage-specific genes may be of great importance in the understanding of its life cycle and in the future development of new strategies for PCM treatment. Using DDRT-PCR methodology we have isolated a mycelium especific cDNA fragment which had its full-length sequence obtained by 5´RACE and RT-PCR. This sequence (Pbhyd) has a 303 bp ORF similar to hydrophobins, small proteins secreted by filamentous fungi. These proteins are very diverse in amino-acid sequence but they all have a highly conserved arrangement of eight cysteine residues, a typical hydropathy pattern and the ability of self-assemble into an amphipathic film. These proteins are related to a variety of functions in fungal growth, development, infection and pathogenicity. Although a large number of sequences probably enconding such proteins have been described in various fungi, only in a few cases has the protein itself been isolated and characterized. In order to actually confirm that the protein encoded by PbHyd is an hydrophobin we report our strategy for cloning and express this cDNA both in bacterial and yeast cells. After production and subsequent purification of PbHyd encoded protein, we intend to proceed to the amino-terminal sequencing and antibodies production for later studies on hydrophobin function in *P. brasiliensis* infection and pathogenesis. Such experiments could help us to evaluate if this protein share same properties described for other pathogenic fungi hydrophobins and define its potential role in the PCM.

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01-37

SAPROBE GROWTH OF Paracoccidioides brasiliensis IN SOIL AND ITS DETECTION BY CULTURE, ANIMAL INOCULATION AND PCR

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The habitat of the fungus *P. brasiliensis*, the etiological agent of paracoccidiodomycosis, is still unknown. The soil seems to be the major source of infection. The present study aimed to standard the detection of its saprobe micelial phase in soil using three techniques: culture, animal inoculation and PCR. The saprobe growth was obtained by seeding micelial fragments or yeast cells in sterile soil (with and without poultry feces enrichment) and incubating it at 25°C until 3 months. The detection of the pathogen in soil was carried out by: i) direct culture of soil and its dilutions (1:5 and 1:25) in Sabouraud Dextrose Ágar at 25°C, ii) animal inoculation by intratestis route in hamsters, with plating of their organs in Mycosel® after 8 weeks and iii) molecular detection. For fungal DNA extraction four different methods were employed: i) phenolic with glass beads, ii) indirect according to Tsushima (1995) followed by extraction with QIAgen® Plant Kit, iii) indirect and direct according Tsushima (1995) and iv) FastDNA® Spin Kit for Soil. The primers used in PCR were ITS4/ITS5 and two pair of primers (specific for *P. brasiliensis*) in the Nested PCR: forward/Lo (outer primers) and Up/P300 (inner primers). The detection of the saprobe phase in soil was positive by direct culture and in 1:5 dilution, and negative in 1:25 dilution and by animal inoculation. It was obtained a high amount of DNA using the FastDNA® Spin Kit for Soil, but this DNA could not be amplified. The extraction by the procedures (ii) and (iii) showed a positive PCR reaction. We concluded that *P. brasiliensis* is a fastidious fungi to recover from its saprobe phase, and that molecular techniques should be adopted in parallel with traditional methods for the detection of the fungus in nature.

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01-38

YEAST MORFOLOGY AND TRANSITION TO THE MICELIAL PHASE IN ARMADILLOS AND CLINICAL P.brasiliensis ISOLATES, AT DIFFERENT TEMPERATURES

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P. brasiliensis, the etiological agent of Paracoccidioidomycosis, is a dimorphic fungus, which grows as a saprobe mycelium at 25°C and as unicellular yeast at 36°C, essential to its dissemination and pathogenic action. The present study aimed to characterize the yeast form of fifteen isolates (10 from armadillos and 5 from human patients) and to observe its morphological change according to the temperature decrease. The fungus was cultivated in GPY medium (Dextrose, Peptone and Yeast Extract) and firstly incubated at 36°C. Fifteen days later, the morphological aspects were analyzed in the microscope and a new culture was made, decreasing the temperature, in 1°C, gradually from 35 to 25°C. It was observed a morphological diversity among the isolates at 36°C (yeast phase). The characteristics analyzed to such comparison were: size and shape (round or stretched) of the cells, presence of budding and big cells (approximately 10X larger than the frequent small cells). Bt84 and Bt85, two clinical isolates have stretched yeasts with few budding, while for the most part of armadillo's isolates, the yeast was predominantly round and had a lot of budding. One important and very variable characteristic was the transition temperature of each isolate. Most isolates started the hypha production between 30 and 28°C, except isolates Bt85 and T13LN1, which started the transition at 33°C and 27°C respectively. In general, the transition to micelial form occurred gradually, except for the isolate T1F1, which changed abruptly to the micelial phase at 28°C. The improvement of the knowledge about the isolates biodiversity, particularly about the phenotypic variation of the dimorphism and its genetic base, will be helpful for a better elucidation of the pathogenic dissemination of *P. brasiliensis* and its parasite adaptation to the host tissues (human being and the armadillo, *Dasypus novemcinctus*).

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01-39

MOLECULAR CLONING AND CHARACTERIZATION OF A CDNA ENCODING THE Paracoccidioides brasiliensis L35 RIBOSOMAL PROTEIN

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Paracoccidioides brasiliensis is the causative agent of Paracoccidioidomycosis (PCM) the most prevalent systemic mycosis in Latin America. The disease, that presents multiple clinical forms, is considered a health problem in the endemic areas. The fungus occurs as two morphological stages, mycelia and yeast. It produces mycelial spores that are inhalated by the host and differentiate into yeast cells establishing the infection. We report the cloning of a cDNA (Pbl35) encoding for a ribosomal protein of *P. brasiliensis*, from a yeast expression library. The cloned Pbl35 was 630 bp in length encoding an open reading frame of 125 amino acids with predominant basic composition. Pbl35 presented pyrimidine sequences at the 5' untranslated region (UTR), as found in other eukaryotic ribosomal proteins and a putative polyadenylation sequence at the 3' UTR. The predicted molecular mass of PbL35 was 14.5 kDa, pI of 11.0. Motifs for phosphorylation and N-myristylation were present in PbL35. A characteristic nuclear target sequence (NLS) was also identified. Expression of a 700 bp transcript was observed by northern blot analysis. The PbL35 and the deduced amino acid sequence constitute the first description of a ribosomal protein in *P. brasiliensis*. The cDNA (Pbl35) and the deduced protein were deposited on GenBank under accession number AF416509.

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01-40

CHARACTERIZATION OF A CHAPERONE CLPB HOMOLOG OF Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis is the etiological agent of Paracoccidioidomycosis (PCM), an endemic disease in Latin America. The fungus grows as yeast or mycelia on dependence of the temperature. *P. brasiliensis*, as a dimorphic organism, is confronted to a heat stress as a regular feature of its life cycle. ClpB, a member of the ClpATPase family, is a heat shock protein preferentially induced in the parasitic stage of pathogenic organisms. We report the cloning and sequence analysis of a genomic clone encoding a *P. brasiliensis* ClpB chaperone homolog (PbClpB). The clpb gene was identified in a l Dash II library. Sequencing of Pbclpb revealed a long open reading frame capable of encoding a 792 amino acid, 87.9 kDa protein, pI of 5.34. The predicted polypeptide contains several consensus motifs of the ClpB proteins. Canonical sequences like two putative nucleotide-binding sites, chaperonins ClpA/B signatures and highly conserved casein kinase phosphorylation domains are present. ClpB is 69% to 49% identical to members of the ClpB family from several organisms from prokaryotes to eukaryotes. The clpb expression was analysed by RT-PCR and northern blot. RT-PCR assays provided a product of 138 bp, in yeast and mycelia phases of *P. brasiliensis*, showing 100% identity to the Pbclpb. The transcript of Pbclpb was detected as a mRNA species of 3.0 kb preferentially expressed in the yeast parasitic phase of the fungus. Also a 89 kDa protein was detected in yeast cells of *P. brasiliensis*. The PbClpB is the first described ClpB in a pathogenic fungus. The Pbclpb was deposited on GenBank under accession No. AF449501.

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01-41

ULTRASTRUCTURAL AND IMMUNOCYTOCHEMISTRY SEQUENCIAL STUDY OF HeLa CELLS INFECTION BY YEAST FORMS OF Paracoccidiodes brasiliensis

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The aim of the present study was to demonstrate sequencial pathogenic mechanisms of adhesion, invasion and intracellular parasitism of yeast cells of *Paracoccidiodes brasiliens*, after infection of HeLa culture cells. Ultrastructural observations after 3 and 5 h p.i. demonstrated details of simultaneous mechanisms of adhesion and invasion by the fungus. Adhesion showed intimate interation of the cell pathogen with HeLa membrane that shows a cytoplasmatic expansion, forming a bridge between interacting host-parasite. This interaction triggers rearrangement of host-actin cytosqueleton observed by dark areas in the micrographs represented by a mixed process of cavitation and pseudopodelike structures. The fungus can be internalized into host-phagossomes showing evidences of wall-less protoplasm of *P. brasiliensis*. Ultrastructural observations also revealed many morphologically different forms of this pathogen in intracellular parasitism.. Forms in apparent degeneration presenting a thin cell wall and few cytoplasmatic organells were observed, as the histological forms described in PCM lesions coded "ghost" forms of *P. brasiliensis*. Different and bizarre intracellular. forms of the pathogen as small forms could be identified by immunomicroscopy reactions. These results demonstrated for the first time that *P. brasiliensis* is *in vitro* a facultative parasite. Adhesions forms just reacted with anti-whole cells antiserum and anti-gp43 antiserum demonstrating that gp43 is secreted by the infective form of *P. brasiliensis* before adhesion. These reactions suggest that gp43 is probably involved in the HeLa cells infection by *P. brasiliensis*.

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01-42

MOLECULAR CLONING AND CHARACTERIZATION OF A cDNA ENCODING A CATALASE ANTIGEN FROM Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis is the ethiological agent of Paracoccidioidomycosis (PCM), a human disease that affects inhabitants of the Latin America areas. The fungus that presents thermal dimorphism, alternates between yeast and mycelium forms and the dimorphic transition is the main event in the infection establishment. Catalases (E.C. 1.11.1.6) are antioxidant metalloenzymes which are virtually ubiquitous among aerobic organisms and protect cells against oxidative damage caused by hydrogen peroxide produced by phagocytes cells of the host. We obtained amino acid sequences from five internal peptides of a catalase of *P. brasiliensis*, apparent molecular mass of 61 kDa, pI 6.2, reactive to serum from PCM patients. Based on those sequences, oligonucleotides were constructed in order to amplify, by PCR, a genomic fragment of catalase from *P. brasiliensis*. The fragment obtained had 690 bp and was used as a probe in the screening of a cDNA library. One cDNA clone was obtained and characterized. The deduced amino acid sequence showed homology to catalases from other fungus. Analysis of the cDNA sequence showed regions presumed to be essential to catalysis and to the heme ligation. In addition a specific region for peroxissomal catalase was observed. These findings suggest the protein to be a functional enzyme in *P. brasiliensis*. The expression of the antigen catalase was analyzed by northern blotting. It was observed a preferential expression in the yeast cells, suggesting a potential role of catalases in this fungus parasitic phase.

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01-43

IDENTIFICATION OF NOVEL POTENTIAL VACCINE CANDIDATES AGAINST Paracoccidioides brasiliensis BY AN ANTIGENIC SEQUENCE TAG STRATEGY

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Prophylactic vaccines have undoubtedly improved human health throughout the 20th century. However, an effective anti-paracoccidioidomycotic vaccine remains to be developed. This is a goal to be pursued, since paracoccidioidomycosis (PCM) is restricted to Latin America and Brazil is nowadays the major endemic country. In this context, another strategy to discover genes that encode novel potential vaccine candidates is the partial sequencing of cDNA clones previously selected with immunoglobulins (Igs) to generate Antigen Sequence Tags (ASTs). In the present work, 320 recombinant clones with an average insert length of 1006 bp from a *Paracoccidioides brasiliensis* yeast cell cDNA library were screened with affinity chromatography-purified IgG from anti-F0, anti-FII, anti-FIII rabbit and PCM patients sera by indirect ELISA. Of these, 119 were selected by the immunoscreening procedure and ASTs were acquired from the 5'-end of 68 cDNA clones. Comparison at protein level of ASTs to sequences in the nonredundant GenBank at NCBI revealed 10 significant homologies to fungal genes encoding proteins of known function. One of them matched calcineurin B of Neurospora crassa with 32% identity and 51% similarity. Calcineurin B is involved in a wide variety of responses to environmental cues essential for the survival of different fungi. This AST originated from a clone reactive to anti-F0 and PCM patients Igs. Intrinsic sequence features such as transmembrane regions were predicted by TMHMM2 in the Simple Modular Arquitecture Research Tool (SMART) in 28 ASTs and therefore could represent integral membrane protein targets.

01-44

MOLECULAR CHARACTERIZATION OF THE MDJ1 HOMOLOG FROM Paracoccidioides brasiliensis

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Temperature-dependent cellular differentiation in the lungs of *Paracoccidioides brasiliensis* from mycelium to yeast is an essential requirement for the occurrence of paracoccidioidomycosis (PCM). Stress proteins are probably involved in this process and deserve to be studied. We have recently characterized PbLON, a LON gene homologue encoding an ATP-dependent mitochondrial proteinase. In yeast, Lon is a heat shock molecule involved in the turnover of short-lived functional proteins and in the Mdj1-dependent hydrolysis of misfolded proteins. Mdj1 is a mitochondrial member of the DnaJ (Hsp 40) family, whose molecules are organized in modules consisting of a J domain followed by a glycine/phenylalanine-rich segment and four CXXCXGXG (zinc finger) domains. The C-terminus is not conserved. A partial sequence of the MDJ1 homologue was found in *P. brasiliensis*, in opposite direction to the PbLON gene and sharing the 5´ non-translated region. This sequence consists of 906 bp interrupted by a 110-bp intron, as confirmed by sequencing of a cDNA fragment obtained by reverse transcriptase polymerase chain reaction (RT-PCR). The ORF known so far includes all the domains mentioned above, except for one still missing zinc finger. Attempts to obtain the 3´ region of PbMDJ1 by 3´RACE have so far been unsuccessful. Expression of the PbLON and PbMDJ1 transcripts is under investigation in total RNA isolated from *P. brasiliensis* after heat shock and at different phases of the mycelium to yeast (M-Y) or yeast to mycelium (Y-M) transition, by using real time RT-PCR. Preliminary results obtained suggest that there was a decrease in PbMDJ1 expression during the Y-M differentiation, while the amount of PbLON was not changed. Additionally, there was a two-fold increase in both transcripts after heat shock at 42°C for 30 min. Further results will be presented.

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01-45

CLONING OF PARTIAL SEQUENCES OF *pac*C GENES FROM Paracoccidioides brasiliensis AND Aspergillus fumigatus

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In some yeasts and filamentous fungi, homologues to the *Aspergillus nidulans pac*C gene product have been implicated in the induction of genes which must be expressed exclusively at alkaline pH, and in the repression of those whose expression must occur at an acidic environment. In these microorganisms, ambient pH is related to the production of enzymes of industrial interest, metabolites such as penicillin and in the pathogenesis against humans, as it was demonstrated for the opportunistic dimorphic fungus *Candida albicans*. In view of this last datum, we are interested in investigating whether the pH of the host niche can affect the gene expression and virulence in *Paracoccidioides brasiliensis* and in *Aspergillus fumigatus*. *P. brasiliensis* is a dimorphic fungus which is endemic in the rural areas of Latin America and causes a severe systemic mycosis, while *A. fumigatus* is responsible for about 95% of the human aspergilloses, specially affecting immunodeficient patients. From the DNA sequence alignment of *pac*C genes of different fungi, we have designed a pair of degenerated primers spanning the region encoding the three zinc-finger domains of the PacC transcription factor. These primers were employed to amplify *P. brasiliensis* and *A. fumigatus* total DNA in a typical PCR. The cloned *A. nidulans pac*C gene was used as a positive control. For both fungi, we obtained amplification products of about 200 bp, which is compatible to the *A. nidulans* corresponding sequence. PCR products were sequenced and the *P. brasiliensis* amplicon displays 79% of similarity with the *A. nidulans pac*C zinc-finger encoding region, while for *A. fumigatus* this value raises to 82%. Southern blot analysis of *P. brasiliensis* total DNA suggests that the *pac*C gene occurs in a single copy. *P. brasiliensis* and *A. fumigatus pac*C partial sequences will be employed for the screening of genomic libraries in order to clone the complete genes. After molecular characterization, expression analyses and gene disruption, we believe we can evaluate a possible role of the *pac*C homologues in the regulation of the pathogenesis process of these fungi.

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01-46

SEQUENCING OF 40 Kb FROM THE MITOCHONDRIAL GENOME OF Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis mitochondrial DNA was partially sequenced and we present the results from a fragment of 41,174 bp that represents 2/3 of whole genome. We made comparisons against the non redundant protein database from NCBI and found the coding sequences for seven well known genes ordered as follow: nicotinamide adenine dinucleotide dehydrogenase chain VI (NADH6), cytochrome c oxidase polypeptide III (COX3), mitochondrial ribossomal protein (RMS5), cytochrome c oxidase polypeptide I (four exons of COX1), ATP synthase subunit 9 (ATP9), cytochrome c oxidase polypeptide II (COX2), nicotinamide adenine dinucleotide dehydrogenase chain 5 (NADH5). Between the exons of COX1 was found four intronic open reading frames. The position of these genes in the fragment appear as discribed above whose are very similar with Aspergillus nidulans. Using the tRNAscan we detected at least seventeen tRNA genes coding for alanine, arginine, asparagine, aspartic acid, glutamic acid, histidine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan and valine.

01-47

APPROACH TO EST SEQUENCING FOR Paracoccidioides brasiliensis FUNCTIONAL GENOME

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Nowadays, most of the genome projects dedicate a part of their effort to the sequencing of Expressed Sequence Tags (ESTs), wich are short DNA sequences (200-500 bp) generated from the 3' or the 5' ends of randomly selected cDNA clones. EST sequencing provides a rapid scan for expressed genes and a tag of each gene. The network of Centro-Oeste "Functional and Differential Genome of *P. brasiliensis*" has as general objective of mapping functional genes expressed in both forms of *P. brasiliensis*. *Paracoccidioides brasiliensis* is a dimorphic fungus, existing as mycelium at 26°C and yeast at 36°C and its transition was associated to infection. The mapped ESTs will be used to screen for genes expressed differentially or transiently during mycelium to yeast transition by means of microarray. This will allow the study of genes potencially involved in the host-fungus adaptation, in the maintenance of the differentiated state and in virulence and/or pathogenicity. The established strategy was to sequence the 5' extremity of cDNAs from mycelium and yeast libraries, constructed in λ ZAP II phages. The cDNA libraries were plated, isolated phages were eluted and amplified by PCR using the vector primers (T3 and T7); the products were then submitted directly to automatic sequencing using Pharmacia's MegaBace sequencer. After validation of sequences with PHRED and elimination of vector sequences with CROSS_MATCH, the sequences were submitted to database analysis using NCBI's BLASTx. Our initial results indicate a successful strategy a functional genome projet, that will be used for large scale screening of novel news.

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01-48

PARTIAL TRANSCRIPTOME CHARACTERIZATION OF THE DIMORPHIC AND PATHOGENIC FUNGUS *Paracoccidioides brasiliensis*

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Paracoccidioides brasiliensis is a pathogenic fungus with a dimorphic life cycle. The transition from mycelium to yeast is assumed to be critical for infection of human host. Our goal was the identification of expressed genes in mycelium and yeast forms by EST (Expressed Sequence Tag) sequencing and generate initial information on the fungus transcriptome. We have standardized the EST production protocol using cDNA libraries from mycelium and yeast cells culture. By means of bioinformatics tools, we had annotated potential functions for these genes. Individual EST sequences were clusterized by CAP3 and annotated by using blastx similarity analysis and INTERPRO scan. Three different databases (GenBank *nr*, COG - Cluster of Orthologs Groups and GO – Gene Onthology) where used to help annotation. A total of 3.938 (Y=1.654 and M= 2.274) ESTs were generated and clustered in 597 contigs and 1563 singlets. From these, 1337 clusters were successfully annotated from which 894 were classified in 18 functional COG categories, as following: 44% corresponded to genes involved in cellular metabolism; 25% in information storage and processing, 19% involved in cellular processes (cell division, post-translational modifications, etc) and 12% corresponded to genes with unknown functions. Bioinformatic analysis identified several interesting genes potentially involved in the dimorphic transition, drug resistance and virulence and pathogenesis determination. Further analysis of these genes may suggest new insights in pathology and treatment of paracoccidiodomycosis.

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01-49

MULTIDRUG RESISTANCE (MDR) GENE FROM P. brasiliensis: A PARTIAL CHARACTERIZATION

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Paracoccidioides brasiliensis is a dimorphic fungus which occurs either as mycelium (26°C) or yeast forms (36°C). It is the ethiological agent of paracoccidioidomycosis (PCM), a systemic mycosis wich is restricted to Latin America. It affects mostly rural workers and immunologically deficient patients. Multiple drugs resistance (MDR) is a phenotype observed in several pathogenic microorganisms, including the fungi *Candida albicans* and *Trichophyton rubrum*. Identification and characterization of genes related to this phenotype represents a new approaches to treat PCM and other diseases. Analysing a database of expressed sequences tags (EST) from the mycelium and yeast forms of *P. brasiliensis* we observed a sequence wich has shown high aminoacid identity (82%) and similarity (89%) with a MDR gene from *Emericella nidulans*. Conserved domains analyses done in this sequence have shown the presence of ABC (ATP-Binding Cassete) transport motifs from *Tricophyton rubrum*. Four other EST with similarity to MDR gene were found. These transporters are highly conserved ATPases, found from bacteria to man. The members of this protein family have many diferent functions, ranging from nutrients receptors to the transporting of citotoxic agents through the cell membrane. This later function can result in reduction of the intracellular accumulation of toxins and drugs. The perspectives of this work are: isolation and structural characterization of the complete MDR gene, performing resistance assays to different drugs and gene expression profiles . Even though drug resistance in *P. brasiliensis* has not been shown yet, it conciable that probably under selective pressure this microorganism will behave like other pathogenic fungus.

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01-50

HEAT SHOCK PROTEIN FAMILIES IDENTIFIED IN *Paracoccidioides brasiliensis* PARTIAL TRANSCRIPTOME BY DATABASE ANALYSIS

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Paracoccidioides brasiliensis is a Latin American endemic thermodimorphic pathogenic fungus. We are sequencing its functional and differential genomes, generating ESTs (Expressed Sequence Tags) wich are compared to databases. In this work, we screened this partial transcriptome searching for Heat Shock Proteins (HSPs), a family of ubiquous and highly conserved proteins involved in protein folding, aggregation prevention, signal transduction, protein degradation and antigenic response. They are divided in two major groups, the molecular chaperones and the chaperonins. Some HSPs have been described in *P. brasiliensis*, the most important of wich are HSP70, shown to be differentially expressed in the yeast form, and HSP60. These HSPs are involved in cellular differentiation and pathogenesis. We were able to identify 21 expressed genes possibly coding different HSPs; of these, 4 are co-chaperones, 7 are chaperones and 6 are chaperonins. The other four are two HSP100, one possible lysossomal membrane chaperone and prefoldin, novel proteins wich are yet to be fully described. In the chaperone group, we have isolated 3 HSP40 (DNAJ) genes, 3 HSP70 (DNAK) and 1 HSP90. HSP90 is a promising target for future studies, because it has been recently conected with protein degradation by the proteasome and with steroid hormone recptors. In the chaperonin group, we isolated 3 low-molecular weight HSPs and 3 proteins from the HSP60 sub-family, including mitochondrial homologs HSP10 (GroES) and HSP60 (GroEL), wich form a large multi-subunit machine. In the co-chaperone group, 3 sequences code for HSP90 co-chaperones and one codes for a poorly characterized one. This work suggests this approach can be useful in the identification of target genes in the study of *P. brasiliensis* pathogenesis and dimorphism.

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01-51

PARTIAL MutS HOMOLOG IDENTIFICATION IN *Paracoccidioides brasiliensis*

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Genomic integrity is maintained by DNA repair mechanisms. Replication errors, spontaneous or induced base modifications, and recombinational events can produce mispaired and unpaired bases in DNA. The major pathway for correction of mismatches arising during replication is the MutHLS pathway of *Escherichia coli* and related pathways in other organisms. MutS initiates repair by binding to the mismatch, and activates together with MutL the MutH endonuclease, which incises at hemimethylated dam sites and thereby mediates strand discrimination (Marti *et al.*, J. Cell Physiol., 2002). Multiple MutS and MutL homologues exist in eukaryotes, which play different functions in the mismatch repair (MMR) pathway or in recombination. In humans, at least six proteins are necessary for MMR. MMR appears to be one pathway associated with the repair of 8-oxo-guanine lesions, highly mutagenic products of the interaction between reactive oxygen species and DNA (Mazurek *et al.*, J. Biol. Chem., 2002). We identified a MutS homologue in the dimorphic fungi *Paracoccidioides brasiliensis*. In a partial DNA fragment, we found a continuous open reading frame of 720 nucleotides, encoding a deduced protein sequence of 240 amino acids. Protein–sequence analysis revealed that *P. brasiliensis* MutS contains one ATPase domain between amino acids 66-237 and a DNA-binding domain between amino acids 4-52. The complex transformation that *P. brasiliensis* suffers *in vivo* could favour oxidative stress. Repair of DNA oxidative damage by the MMR pathway may participate in *P. brasiliensis* temperature adaptation.

01-52

Cu-Zn AND Mn SUPEROXIDE DISMUTASES FROM *Paracoccidioides brasiliensis*

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The discovery and characterization of superoxide dismutase (SOD) enzymes in parasitic fungi can provide much of the basis for future understanding of antioxidant defense systems in these organisms. Most importantly, it can lead to the postulation of the oxidative stress participation in pathogenicity. Copper-zinc-containing superoxide dismutases (CuZnSODs) are present in all eukaryotic cells. In animal cells, most CuZnSOD is located in the cytosol, but some appears present in lysosomes, nucleus and the space between inner and outer mitochondrial membranes (Halliwell and Gutteridge, 1999). Manganese-containing SOD (Mn-SOD) are widespread in bacteria, plants and animals. In most animal tissues and yeast, MnSOD is located in the mitochondria. We identified CuZnSOD and MnSOD genes (partial sequence) in the parasitic fungi *Paracoccidioides brasiliensis*. We found continuous open reading frames of 1068 and 1074 nucleotides, encoding deduced proteins of 356 and 358 amino acids for CuZnSOD and MnSOD, respectively. According to pairwise alignments, *P. brasiliensis* CuZnSOD shares high degrees of amino acid identity (76-77%) with the *Neurospora crassa*, *Aspergillus fumigatus* and *Emericella nidulans* CuZnSOD proteins and lower degree of amino acid identity (67%) with CuZnSOD from *Saccharomyces cerevisiae*. *P. brasiliensis* MnSOD shares high degrees of amino acid identity (77%) *Emericella nidulans* MnZnSOD protein and lower degree of amino acid identity (57%) with MnSOD from *Arabidopsis thaliana*. Preliminar analysis indicate that MnSOD is predominantly expressed in yeast cell type of *P. brasiliensis*.

01-53

IDENTIFICATION OF RETROTRANSPONS ELEMENTS IN THE PARTIAL TRANSCRIPTOME OF THE PATHOGENIC FUNGUS Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis is the etiologic agent of paracoccidioidomycosis (PCM), a systemic human mycosis that affects predominantly rural communities from South and Central America. The dimorphic fungus *P. brasiliensis* is found as mycelium (M) or spore at environmental temperature or in the yeast form (Y) at 37°C. The dimorphic transition from mycelium to yeast seems to be a condition for the establishment of infection. The functional genome of these two forms is being sequenced. The chosen approach was the EST (Expressed Sequence Tags) generation from mycelium and yeast forms of the fungus. The genes were classified by similarity analysis of translated ESTs against the proteic databases GenBank (NR), COG and GO. Blastx algorithm was used for comparisons. Interpro scanning was also used as an accessory tool. Among other several genes, it was possible to identify many retrotransposons transcripts of *P. brasiliensis*. The occurrence of transpositions into coding sequences and their initiation of chromosome rearrangements have huge impact on gene expression and genome evolution. Eight different groups of retrotransposons were identified; three were contigs and five were singlets. Two contigs and three singlets presented strong similarity to various gypsy-class retrotransposons, especially with the elements GRH and MAGGY of *Magnaporthe grisea*. The two other singlets exhibited similarity with members of the LINE-1 class (L1). One of the singlets presented homology with L1 members of *M. grisea* and the another with TAD elements LINE-like of *Neurospora crassa*. The last contig showed similarity to TY3 elements of *Saccharomyces cerevisiae*. This strategy showed valid for the identification of retrotransposons and will help to understand the evolution of this pathogenic fungus.
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01-54

UBIQUITIN GENES IN THE PARTIAL TRANSCRIPTOME CHARACTERIZATION OF *Paracoccidioides brasiliensis*

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Paracoccidioides brasiliensis is the etiologic agent of paracoccidioidomycosis (PCM), a human systemic disease, endemic in Latin America. This multinuclear fungus is found in mycelium form at 26°C. At 37°C these cells differentiate in yeast, the infecting form. Due to large clinical importance its transcriptome is being characterized and in this work we focused the polyubiquitin ESTs. Polyubiquitin consist in a tandem arrangement of repeated ubiquitins genes. Its primordial function is to mark proteins for proteosomic degradation or endocitosis also involved in vital cell processes, like DNA repair, nuclear transport, signal transduction, transcriptional regulation, apoptosis and protein turnover. The ubiquitin can be encoded by three genes (*ubi1, ubi3* and *ubi4*). In *Aspergilus nidulans*, the expression of the *ubi1* and *ubi4* genes were induced upon heat shock and shows a complex expression model in stress conditions. In *Candida albicans*, the expression of the genes *ubi3* and *ubi4* was analysed in stress condicions (temperature and alimentary lack) and only *ubi3* gene was restrained. In the fungus *Gibberella pulicaris* the *ubi1* gene is induced during heat shock. Because of its highly conserved gene sequence and also due to its ubiquous distribution among the fungi, we choose two polyubiquitin ESTs for further analysis. When compared with database, one of these EST shows high aminoacid identity (98%) and similarity (98%) with *ubi4* sequence of *Saccharomyces cerevisiae*. The other EST shows high identity (77%) and similarity (79%) with *ubi1* sequence of *Emericella nidulans*. Since these genes are involved in protein turnover and seems to be regulated by heat shock and stress conditions they could have important role in transition process of *P. brasiliensis*.

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01-55

IDENTIFICATION AND PARTIAL CHARACTERIZATION OF RIBOSOMAL PROTEINS FROM THE SMALL AND LARGE SUBUNITS OF Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis is the etiological agent of Paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in Latin America. The fungus occurs as two morphological stages, mycelia and yeast. The eukaryotic ribosome consists of RNA, more than 70 ribosomal proteins and other accessory factors. These proteins are named in accordance to the subunit of the ribosome, which they belong to, as small (S1 to S31) and large (L1 to L44). In this work we performed comparative analyses of the deduced amino acid sequences from cDNAs enconding putative ribosomal proteins of *P. brasiliensis*. We identified up to now 26 S and 40 L ribosomal proteins in *P. brasiliensis*. The partial amino acid sequences obtained for all the ribosomal proteins present predominance of basic amino acids, one characteristic of those proteins. High scores of identity were obtained with distinct organisms, with a wide distribution in the evolutionary scale, from human to yeast. For example, identity for L14 r-protein of *P. brasiliensis* and L14 from mammalian cells and *Saccharomyces cerevisae* showed values of 57% and 53%, respectively. Also the L4 of *P. brasiliensis* and L4 p-like r-protein of *Schizosaccharomyces pombe* showed identity of 48%. Studies on the composition and structure of *P. brasiliensis* ribosomal proteins will be of importance to clear up the role of these molecules in the process of proteins synthesis and ribosomal assembly.

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01-56

PARTIAL SEQUENCES OF cDNAs RELATED TO BIOSYNTHESIS OF THE CELL WALL OF Paracoccidioides brasiliensis

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P. brasiliensis is an opportunistic fungal pathogen that causes life-threatening infections of the organism. Because of the paucity of current antifungal drugs and targets, the development of new agents directed at novel targets is welcome. Particularly appealing are agents directed at fungus-specific targets, such as the cell wall. At present, two classes of cell wall inhibitors, glucana and chitin synthase inhibitors, are in development. Hydrolytic enzymes inhibitors also can affect fungal wall metabolism synergistically. There are evidences that fungi may adapt to the inhibition on synthesis of one wall component by compensatory production of another; this again leads to the theoretical expectation that hits on two or more targets could produce an enhanced effect. Here we report partial sequences of cDNAs associated to cell wall biosynthesis of mycelium and yeast forms of *P. brasiliensis*. At the moment, we found 1 glucan synthase, 2 chitin synthases, 1 beta(1-3)endoglucanase, 1 chitinase, 4 beta-glucosidases, 1 chitin deacetylase, 3 mannosyltransferases and 4 mannosidases. The glucan synthase and chitin synthase 2 were decribed previously by *P. brasiliensis*. One mannosyltransferase has homology to a PbYMnt, previously decribed by our group. The new cDNAs present partial identity to sequences described in database and special motifs were found concerning to glycosyl hydrolases. Temporal expression of these genes are of particular interest because their products may participate in wall modification during both sporulation and growth, thereby, represent potential molecular targets for novel antifungal drugs. Furthermore, immunological studies of these and other isolated parasitic cell wall components could result in the identification of antigens with demonstrated impact on host response to paracoccidioidomycosis infection.

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01-57

PARTIAL cDNAs ENCODING AMINOPEPTIDASE-LIKE PROTEINS FROM Paracoccidioides brasiliensis

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Paracoccidioides brasiliensis, the etiological agent of Paracoccidioidomycosis (PCM) presents a growing medical importance. The fungus grows as yeast or mycelium and the dimorphic transition is the main event in the infection establishment. The fungus is an active participant of the infective process and putative virulence factors like proteases have been described. One of the major groups of peptidase degrading enzymes is constituted by proteins with an aminopeptidase (Ap) activity. Many of those peptidases are zinc metallopeptidases. In this work we performed comparative analyses of the deduced amino acid sequences from five cDNAs encoding putative Ap proteins in *P. brasiliensis*. The presence of typical zinc binding motifs found in zinc metallopeptidases were found. Of special note is the presence of consensus motifs that characterize the Ap family and also the zinc class. Identity for one Ap of *P. brasiliensis* and a related Ap from *Aspergillus niger* and *Saccharomyces cerevisae* showed, for example, values of 82% and 68%, respectively. Further studies will focus on those proteins in *P. brasiliensis*.

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01-58

COMPARATIVE STUDY OF CELL FREE ANTIGENS (CFA) EXPRESSED BY DIFFERENT FORMS OF P. brasiliensis DURING INFECTION OF HELA CELLS, DEMONSTRATING DIFFERENCES BETWEEN CFA EXTRACTION FROM CULTURES OF THE FUNGUS WITH CFA FROM INFECTIVE AND INTRACELLULAR CELLS

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Adhesion, invasion and intracellular parasitism of *P. brasiliensis* yeast forms were proved by ultrastructural and immunocytochemistry micrographs. The aim of this work was study CFA extracted from forms the fungus during HeLa infection. By this comparative study of CFA extracted from four Pb18 forms:1-Pb18 in Fava Netto's culture(PbFN); 2-Forms from fungus in pre-cultures containing D-galactose(PC); 3-Fungus named infecting forms of *P. brasiliensis* from infected culture medium(I.F);4-Intracellular forms (ICF)of the pathogen, obtained, after HeLa infected lysis cultures 2h post-infection. The CFAs from these four forms were then performed by SDS-PAGE and demonstrated different bands. CFA from PC and IF not expressed gp43, but CFA from ICF presented intense 43kDa band. Anti-FIC identified gp43 purified in CFAs from strains of *P. brasiliensis* and from ICF. Indirect ImmunoFluorescence showed that PC and IF expressed gp43 only in the cytoplasm of the fungus.

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01-59

LECTIN AND ENZYMATIC ACTIVITIES OF PARACOCCIN DERIVED FROM DIFFERENT *Paracoccidioides brasiliensis* ISOLATES

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We have shown that *P. brasiliensis* yeasts from the BAT isolate release paracoccin, an GlcNAc binding protein. This 70 kDa antigen is expressed on the surface of *P. brasiliensis* yeast and establishes sugar dependent interactions with laminin and murine macrophages. The latter is responsible for nitric oxide production. We also showed that paracoccin has GlcNAcase activity, apparently independent of the lectin activity. Paracoccin properties have encouraged us to extend its detection to Pb18, 339 and BOAS isolates, beyond BAT (standard isolate). By Western blot assay using anti-paracoccin antibody, a 70 kDa band was detected in all isolates tested. A semi-quantitative immunoenzimatic assay reaction of anti-paracoccin with all tested isolates was at least 50% lower than the reaction with BAT. Paracoccin was sugar affinity purified from the culture supernatant of each isolate and studied regarding lectin and enzymatic activities. The purification yields were 50% lower for the tested isolates than the yield provided by BAT. All paracoccin preparations (100 ng) have interacted with laminin (250 ng), coated to microplate wells. In comparison to paracoccin obtained from BAT, the binding of paracoccin from Pb18 and 339 were respectively 50 and 70% smaller, whereas the binding of paracoccin from BOAS was 20% higher. The specific enzymatic activity of paracoccin against the chromogenic pseudosubstrate p-nitrophenyl N-acetyl-β-D-glucosaminide was not detectable when derived from 339, whereas paracoccin from BOAS and Pb18 provided activities twice higher than BAT. Our results show that paracoccin expression is shared by several *P. brasiliensis* isolates. Nevertheless, paracoccin derived from distinct isolates had different extent of lectin and enzymatic activities.

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01-60

ADHERENCE OF *Paracoccidioides brasiliensis* CONIDIA TO EXTRACELLULAR MATRIX PROTEINS

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In *Paracoccidioides brasiliensis* (Pb) infection, the interaction between conidia and host tissues probably involves specific recognition of extracellular matrix proteins (ECMP) by the fungal propagules. *In vitro* immunofluorescence assays have shown that both conidia and mycelial fragments, bind to products such as laminin of mouse origin, as well as to human fibronectin and fibrinogen. It also was observed that antibodies against laminin and fibrinogen were capable of interacting with Pb propagules. Additionally, microplate adherence assays showed that the interaction between streptavidin-peroxidase labeled conidia and immobilized laminin, fibronectin and fibrinogen was dose dependent and could be saturated with increasing concentrations of ECMP. These results suggest differences on the binding ability of conidia to ECMP, apparently with lower affinity for fibronectin and indicate that both conidia and mycelial fragments, do interact with important ECM components. This ability could well be related to fungal virulence and pathogenesis of paracoccidioidomycosis.