Differential gene expression analysis of *Paracoccidioides brasiliensis* during keratinocyte infection

Roberta Peres da Silva, Marcelo Teruyuki Matsumoto, Jaqueline Derissi Braz, Aline Raquel Voltan, Haroldo Cesar de Oliveira, Christiane Pienna Soares and Maria José Soares Mendes Giannini

Correspondence

Maria José Soares Mendes Giannini
giannini@fcar.unesp.br

Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, UNESP, Araraquara CEP 14801-902, São Paulo, Brazil

Paracoccidioides brasiliensis is the agent of paracoccidioidomycosis, one of the most important systemic fungal diseases in Latin America. This initiates in lung tissue and can subsequently disseminate to other tissues. Clinical manifestations range from localized forms to disseminated disease that can progress to lethality, probably depending on the relationships among the virulence of the fungus, the immune response and the ability to interact with the surface structures and invade epithelial cells and mononuclear cells of the host. It is generally regarded as a multifocal disease, with oral lesions as the prominent feature. The aim of this study was to evaluate *P. brasiliensis* yeast infection in normal oral keratinocytes (NOKs). The differential expression of mRNAs and proteins was also determined when the fungus was placed in contact with the cell in order to characterize differentially expressed genes and proteins during *P. brasiliensis* infection. After contact with NOKs, the fungus appeared to induce alterations in the cells, which showed cellular extensions and cavitations, probably resulting from changes in the actin cytoskeleton seen at 5 and 8 h after infection. Levels of protein expression were higher after reisolation of the fungus from infected NOK culture compared with culture of the fungus in medium. The analysis identified transcripts related to 19 proteins involved in different biological processes. Transcripts were found with multiple functions including induction of cytokines, protein metabolism, alternative carbon metabolism, zinc transport and the stress response during contact with NOKs. The proteins found suggested that the yeast was in a stress situation, as indicated by the presence of RDS1. Nevertheless, the yeast seemed to be proliferating and metabolically active, as shown by the presence of a proteasome, short-chain acetylator, glucosamine-6-phosphate isomerase and ADP/ATP carrier transcripts. Additionally, metabolic pathways may have been activated in order to eliminate toxic substances from the cell as a zinc transporter was detected, which is a potential target for the development of future drugs.

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INTRODUCTION

Paracoccidioides brasiliensis is a thermomorphogenic pathogenic fungus, the aetiological agent of paracoccidioidomycosis (PCM), an endemic disease geographically limited to Latin America and one of the most prevalent human deep systemic mycoses found in Brazil, Colombia and Venezuela (San-Blas *et al.*, 2002). The transition from mycelium at 25 °C to yeast at 37 °C is essential for *P. brasiliensis* to establish the disease (Franco *et al.*, 1997). *P. brasiliensis* may be inhaled and, once in the lungs, the fungus initiates a process of morphological transition into the pathogenic yeast form (San-Blas *et al.*, 2002).

PCM has a multiplicity of clinical presentations, from cutaneous to systemic forms, and can attack various tissues, especially the lungs (Benard & Franco, 2007). Oral mucosal lesions are commonly multiple, with a granular, mulberry-like surface and micro-ulcerations, and microscopically are observed as a granulomatous inflammatory reaction and pseudoepitheliomatous hyperplasia that can be mistaken as neoplastic (Kaminagakura *et al.*, 2006; Moscardi-Bacchi *et al.*, 1989; Scully & de Almeida, 1992), which can be a problem clinically as the oral lesions of PCM closely resemble carcinoma (Kaminagakura *et al.*, 2006). The fungus may use a sequence of different mechanisms to become established in the host, from the first contact with host cells until the later stages of the disease. In particular, the mechanisms involved in

Abbreviations: NOK, normal oral keratinocyte; PCM, paracoccidioidomycosis; RDA, representational difference analysis.

dissemination and the steps by which the fungus crosses to the intravascular compartments of various organs are not at all clear (Franco, 1987; Kurokawa *et al.*, 2005). During human infection with *P. brasiliensis*, the first cells to encounter the conidia/mycelial fragments of the fungus may be alveolar macrophages and alveolar epithelial cells. Following this, migration of pathogenic yeast cells to other cells is considered a prerequisite for multiple organ invasion and dissemination of the fungus.

Pathogens have developed a diversity of strategies to interact with host cells, manipulate their behaviour and thus survive and propagate (Lengeler *et al.*, 2000; Mendes-Giannini *et al.*, 2008). Although, after spread, the fungus can cause injury throughout the oral cavity, so far it has not been reported to interact with keratinocytes. Keratinocytes are the major cell type in the skin and oral mucosa, and serve as a primary barrier between the external environment and the internal tissues. They also provide a barrier to micro-organisms, toxins and various antigens. Infection of host mucous cells may occur through the spread of *P. brasiliensis* yeast cells in this type of tissue, unlike in the primary infection in which conidia/mycelial fragments are the infectious particles.

The ability of *P. brasiliensis* to adhere to and invade non-professional phagocytes or epithelial cells has been recognized in previous studies (Hanna *et al.*, 2000; Lenzi *et al.*, 2000; Mendes-Giannini *et al.*, 1994, 2004). To establish infection in these cells, it is necessary for *P. brasiliensis* to be able to modify its expressed genes to adapt to changes in the host environment, so the identification of differentially expressed genes during the parasite–host interaction may help to elucidate the mechanisms used for the survival, resistance and growth of this fungus.

Many *P. brasiliensis* genes have been described as probably encoding proteins indispensable to this interaction. The proteins found to date have been determined by searching for differentially expressed genes participating in various metabolic pathways, the uptake and transport of metals such as iron, copper and zinc and the synthesis of virulence factors such as melanin and its precursors (Bailão *et al.*, 2006; Costa *et al.*, 2007; da Silva *et al.*, 2006; Hamilton & Gomez, 2002; Ratledge & Dover, 2000; Taborda *et al.*, 2008).

In this work, our goal was to evaluate the *P. brasiliensis* infection process in normal oral keratinocytes (NOKs), as well as to investigate candidate genes that may contribute to fungal adaptation during NOK infection. In this study, we showed that these cells may play an important role in disease pathogenesis, as *P. brasiliensis* was able to adhere to and invade the cells and modify their actin cytoskeleton, as also occurs in other cell types. The technique of representational difference analysis (RDA) was used to assess the transcription profile of *P. brasiliensis* in contact with keratinocytes.

METHODS

Micro-organism. *P. brasiliensis* strain Pb18 was isolated from a clinical case of PCM and maintained at the Faculty of Medicine of the University of São Paulo, Brazil. Pb18 was grown on Fava–Netto solid medium at 36 °C and subcultured every 3–4 days.

Cell culture. NOK cells immortalized with the *h-tert* gene were obtained from Dr Karl Hunger (Department of Medicine, Harvard Medical School, MA, USA). The cells were grown in keratinocyte serum-free medium (Gibco) at 36.5 °C and with 5% CO₂.

Assay of *P. brasiliensis*–NOK interaction. NOKs were cultured at 36.5 °C in six-well plates for 24 h and adjusted to 1×10^6 cells per well. *P. brasiliensis* was cultured on Fava–Netto solid medium for 3–4 days at 36 °C. A suspension was prepared in sterilized PBS, incubated for 10 min at 36.5 °C in the dark with 10 µM 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (BioChemika) and then washed with 5 vols 0.1% BSA in PBS and incubated on ice for 5 min. The sample was centrifuged at 2500 g for 5 min, washed twice in PBS and the fungus was resuspended in PBS at 1.0×10^6 yeast cells ml⁻¹. This suspension was added to the NOK cells and incubated for 2, 5 and 8 h. After each incubation period, the medium was discarded and the cells were washed with PBS, trypsinized, washed in medium with fetal calf serum, centrifuged and then fixed in 4% paraformaldehyde.

Flow cytometry. A FACSCanto flow cytometer (Becton Dickinson), pre-calibrated for immunofluorescence analysis, was used to determine the fluorescence intensities of the yeast and epithelial cells. Following adherence of the yeast to the epithelial cells, 100 µl cell suspension was mixed with 500 µl FACSFlow Sheath Fluid (Becton Dickinson) and applied to the instrument without further treatment. To determine the extent of adherence, uninfected cells and unlabelled yeast were used as internal controls to define the gate and self-fluorescence evaluation. The excitation wavelength was 488 nm, and emitted light was collected via a 530/30 nm band-pass filter. Data were processed and analysed using the FACSDiva software program. Assays were carried out in triplicate and the median result was expressed as the percentage of fluorescent cells per 10 000 events.

Scanning electron microscopy. The methodology was based on the technique used by Hanna *et al.* (2000). Monolayers of NOKs were infected with a standard suspension of Pb18. After infection, the cells were fixed for 18 h with 2% glutaraldehyde in 0.1 M cacodylate buffer, washed three times in cacodylate buffer for 1 h and post-fixed in 1% osmium tetroxide in 0.3 M cacodylate buffer for 2 h. The cells were then washed in cacodylate buffer and dehydrated in ascending grades of ethanol (30–100%). They were dried in liquid CO₂ using the critical point method, then used for scanning electron microscopy with a field emission gun.

Immunofluorescence assays for determination of cytoskeleton assembly. To study tubulin and actin changes that occur upon *P. brasiliensis* infection, NOK monolayers (1×10^6 cells per well) were cultured at 36.5 °C in 24-well plates for 24 h. The cells were infected with Pb18 suspension (1×10^6 cells ml⁻¹) and after 5 h of infection the monolayers were fixed as described above, washed in PBS and permeabilized in 0.5% Triton X-100 for 30 min. The cells were stained with anti-β-tubulin antibody (Sigma) and FITC-conjugated phalloidin (for detection of actin; Sigma) and anti-*P. brasiliensis* (cell-free) antibody for 1 h. Unbound antibodies were removed by washing in PBS and secondary Alexa Fluor 488- and Alexa Fluor-594-conjugated anti-rabbit antibodies (Molecular Probe) were added for 1 h in dark. The presence of tubulin, actin and Pb18 was observed simultaneously by confocal microscopy.

RNA isolation, cDNA synthesis and subtractive hybridization. A suspension adjusted to 1×10^6 NOKs ml^{-1} was cultured at 36.5°C in bottles for 72 h. A suspension of 1×10^6 Pb18 cells ml^{-1} was prepared in sterile PBS, added to the cells and incubated for 5 h at 36.5°C . After incubation, the medium was discarded and the cells were washed three times with PBS, treated with lysis buffer (0.25 M NaCl, 0.005 M EDTA and 0.0001% NP-40) and centrifuged at 4340 g for 5 min for recovery of the fungus. Isolation of total RNA from control Pb18 yeast (cultured in Fava-Netto medium) and from yeast incubated with NOK cells was performed with Trizol reagent (Gibco), following the manufacturer's instructions. The pellet was diluted in 30 μl DEPC-treated water and submitted to DNase I treatment (Fermentas) according to the manufacturer's instructions. The quality of RNA was assessed by determining the A_{260}/A_{280} ratio and by visualization of the RNA on a 1.2% agarose gel. First-strand cDNA synthesis was performed with Superscript III reverse transcriptase (Invitrogen), and the first strand was used as template to synthesize the second strand of cDNA. The cDNA RDA method described by Hubank & Schatz (1994) was used, as modified by Dutra *et al.* (2004). The resulting cDNAs were digested with the restriction enzyme *Sau3AI* and purified with a QIAquick purification kit (Qiagen). The products of digestion were bound to the first pair of RBam12/24 adapters, amplified and after new digestion and purification were used to form subtracted cDNA libraries using driver cDNA from 3/4-day-old-cultures of yeast cells and tester cDNAs synthesized from RNAs extracted from Pb18 obtained from yeast cells after incubation with NOK cells. The NBam12/24 adapters were ligated to the tester and the first differential product was obtained with a hybridization step (20 h at 67°C) of driver and tester cDNAs mixed at a 10:1 ratio, followed by PCR amplification with the NBam24 primer. To generate the second differential products, the JBam12/24 adapters were bound to the tester in a round of subtractive hybridization, and the driver and tester ratio was increased to 100:1; each round was purified and submitted to electrophoresis in 1.2% agarose gels. The oligonucleotides used for RDA are listed in Table 1.

Cloning and sequence analysis of the RDA products. The products of the second subtractive reaction were cloned directly into the pGEM-T Easy vector (Promega). *Escherichia coli* XL1 Blue competent cells were transformed with the ligation products. The recombinant clones were selected and plasmid DNA was prepared from the clones. Sequencing was performed by the method of Sanger *et al.* (1977) using a MegaBACE 1000 automatic sequencer (Amersham Biosciences). The reactions were performed with a DYEnamic ET Dye Terminator Cycle Sequencing kit (Amersham Biosciences) following the protocols recommended by the manufacturer, using the T7 forward oligonucleotide. Initially, the expressed sequence tags were processed using the program Phred (Ewing *et al.*, 1998). Only sequences with more than 100 nt and a Phred quality of ≥ 20 were selected. The removal of contaminants of vector sequences was performed using the program Crossmatch (Green, 2009) and the CAP3 program was used for the assembly of contigs (Huang & Madan, 1999). Resulting expressed sequence tags were compared with non-redundant sequences in GenBank using the program BLASTX (Altschul *et al.*, 1997). Only sequences with an e-value of $\leq 1e^{-4}$ were annotated and classified based on their putative molecular function and/or biological process using the Gene Ontology classification system.

Gene expression analysis. Genes known to be related to the adaptation of yeast to stress situations and also to virulence were chosen. Semi-quantitative RT-PCR and real-time PCR experiments were also performed to confirm the RDA results. For RT-PCR analysis, a OneStep RT-PCR kit (Qiagen) was used according to the manufacturer's protocol, and each PCR was performed using RNA as template in a 25 μl reaction mixture containing specific sense and antisense primers for glucosamine-6-phosphate isomerase, ubiquitin-conjugating enzyme variant MMS2 or the control housekeeping gene L34 (Table 1). The reaction mixture was incubated initially at 50°C for 30 min for reverse transcription, followed by 15 min at 95°C to degrade the transcriptase, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. The annealing temperature and number of PCR cycles were optimized for each gene to ensure that the analysis of each

Table 1. List of primers and adapters used in this study

Name	Sequence (5'→3')	Purpose
NBam24	AGGCAACTGTGCTATCCGAGGGAG	First-round RDA primer/adaptor
NBam12	GATCCTCCCTCG	First-round RDA adaptor
JBam24	ACCGACGTCGACTATCCATGAACG	Second-round RDA primer/adaptor
JBam12	GATCCGTTTCATG	Second-round RDA adaptor
Glucosamine-6-phosphate isomerase	CCCAGCCAACATCAATATCC	RT-PCR primer pair for amplification of glucosamine-6-phosphate isomerase transcript
	GATGGTGTCTGATGCGAGTG	
Ubiquitin-conjugating enzyme variant MMS2	TGGCCTAGCTAACGGAGATG	RT-PCR and quantitative real-time RT-PCR primer pair for amplification of ubiquitin-conjugating enzyme transcript
	ATTTTCACCGCATTGGATGT	
L34	ATGTCCTGTCTACACTGCGCAAG	Amplification of L34 ribosomal protein
	TGCTAACAGGGATACAGATGGAGAG	
RDS1 protein	CTATCACACACAATCGAACT	Quantitative real-time RT-PCR primer pair for amplification of RDS1 transcript
	AGGTAGTGTTAAGCTGTGAA	
PENR2	CCCTAGTTTCGAGAGCAACG	Quantitative real-time RT-PCR primer pair for amplification of PENR2 transcript
	GACCATCTTGGCGATTTCAT	

product fell within the exponential phase of amplification. The results were analysed on a 1.5% agarose gel. An analysis of relative differences was performed using Image J 1.38x software (<http://rsb.info.nih.gov/ij/>).

In the real-time PCR, in addition to the genes evaluated by RT-PCR, we also amplified the genes for RDS1 and PENR2 as follows. Reactions were performed with 12.5 µl master mix and 2 µl reverse transcriptase mix from a QuantiTect SYBR Green RT-PCR kit (Qiagen); each primer was added to a concentration of 400 nM and the final volume was adjusted to 25 µl. Reverse transcription was carried out at 50 °C for 30 min, followed by denaturation of the enzyme at 95 °C for 15 min. The PCR was performed with 45 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s, with a final cycle at 72 °C for 10 min. To confirm that each peak corresponded to a single fragment, electrophoresis was performed in 1.5% agarose gels in 1× TBE buffer for 2 h at 100 V. The gels were stained with GelRed and observed under UV light. Two independent RNA samples were prepared for use in experiments. The reactions were performed twice in triplicate, using an Applied Biosystems 7500 thermal cycler. The data were analysed using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). The primers for RT-PCR and quantitative real-time RT-PCR are listed in Table 1.

RESULTS

Infection of NOKs by *P. brasiliensis*

The infection index was determined by interactions between *P. brasiliensis* yeast cells and NOKs, as shown in Fig. 1. Control non-treated cells were used to calculate the percentage of total infection. The interaction was analysed by flow cytometry. Ten thousand events were collected for analysis as monoparametric histograms of log fluorescence and list mode data files. By setting up appropriate windows of light scatter intensities (forward and perpendicular light scatters) and adjusting the electronic gating option of the flow cytometer, analysis of fluorescence was restricted to cells in the P1 region (Fig. 1). We compared interactions at 2, 5 and 8 h p.i., analysing all yeast cells that were interacting with NOKs. Almost 30% of cells were infected by the yeast at 5 h and this value decreased at 8 h (Fig. 1). Statistically significance differences ($P < 0.05$) were observed at all times analysed.

The same pattern was observed following infection of A549 cells (data not shown).

Immunofluorescence assay for changes in the actin cytoskeleton

Microbial pathogens that invade non-professional phagocytic cells with rigid cytoskeletons can induce alterations in the underlying host cytoskeletal structure. We investigated whether internalization of *P. brasiliensis* would lead to actin and microtubule rearrangement in NOKs. The interaction of fungus with the NOKs after immunostaining for β -tubulin showed grouping of these filaments in the binding region of the fungus (Fig. 2b, arrow). Infected NOK

cells showed actin filaments condensed in the margins and projections in the contact areas with yeast cells. Recruitment of actin filaments was also observed to follow the binding of *P. brasiliensis* to NOKs. In infection with *P. brasiliensis*, the microfilament redistribution appeared as the formation of aggregates of F-actin that were co-located with some of the fungus adherence sites (Fig. 2d). The filaments were thin and were not arranged in parallel. After 5 h of contact with the fungus, there was an apparent loss of filaments around the cell periphery, showing an increased intensity of fluorescence within the cell.

Electron microscopy

P. brasiliensis yeast cells adhered to and invaded NOK cells. After the initial contact, the fungus appeared to induce alterations in the cells, which showed cellular extensions resulting from probable changes in the actin cytoskeleton, mainly at 5 and 8 h p.i. *P. brasiliensis* adheres to cells by a tubular structure, and the appearance of depressions on the cell surface suggests an active cavitation process affecting the cells. Several images revealed an extension of the cell membrane, which appeared to grow around the *P. brasiliensis* adherent form. In contact areas between the fungus and the cells, a material appeared to be released by the fungus, presumably to facilitate entry into the cell (Fig. 3).

RDA

To investigate gene expression during *P. brasiliensis* infection of NOK cells, we generated subtracted cDNA libraries from fungal cells harvested by cell lysis after 5 h of contact and compared them with those from *P. brasiliensis* grown in medium culture. Two reactions were carried out for each cDNA library, generating different electrophoretic patterns as shown in Fig. 4. Products that were differentially expressed were subjected to automatic sequencing, generating a total of 143 high-quality sequences of ten contigs and nine singlets.

The sequences were compared with sequences in the GenBank and Broad Institute databases using BLASTX and are listed in Table 2. They were related to different biological processes, such as metabolism proteins (elongation factor 2, PENR2 and PRE6), the stress response (RDS1), induction of the immune response (cytokine-inducing glycoprotein), sugar metabolism (glucosamine-6-phosphate isomerase), zinc transport (low-affinity zinc ion transporter) and fatty acid metabolism (short-chain fatty acid CoA ligase).

RDA validation

To validate the expression pattern, we used two methods, RT-PCR and real-time PCR. RT-PCR was used to evaluate the genes for the metabolism protein glucosamine-6-phosphate isomerase and ubiquitin-conjugating enzyme

Fig. 1. *P. brasiliensis* infection of NOKs evaluated by flow cytometry at 2, 5 and 8 h post-infection (p.i.). (a) Dot and histogram plots displaying the parameters of forward and perpendicular light scatter (upper panels) and fluorescence intensity at 2, 5 and 8 h p.i. (lower panels) for uninfected (NOKs) and infected (NOKs + Pb18) cells. (b) Graph showing the percentage of infected (adhesion plus internalization) cells at 2, 5 and 8 h p.i. *, $P < 0.05$.

variant MMS2 (Fig. 5). The gene for RDS1 was also analysed by real-time PCR (Fig. 6). The L34 housekeeping gene was used as a control in all experiments. It was found

that there was increased transcription of the tested genes in *P. brasiliensis* following infection of NOK cells compared with cultured *P. brasiliensis*.

Fig. 2. Confocal microscopy showing immunofluorescence of β -tubulin (a, b) and phalloidin (c, d) in uninfected NOK cells (a, c) and cells infected by *P. brasiliensis* (b, d) at 5 h p.i. β -Tubulin and actin filaments were labelled with Alexa Fluor 488-conjugated antibody and phalloidin-FITC, respectively (green). *P. brasiliensis* was detected with Alexa Fluor 594-conjugated antibody (red). The arrow in (b) indicates grouping of β -tubulin filaments in the binding region of the fungus, whilst the arrow in (d) indicates an aggregate of F-actin. Nuclei were stained with DAPI (blue). Original magnification, $\times 1024$ (a–c), $\times 4608$ (d).

DISCUSSION

PCM is a systemic infection. There is pulmonary involvement initially, but extrapulmonary sites such as the skin, oral mucosa (moriform stomatitis), pharynx or larynx (or a combination of both) and the apical region of teeth may also be affected (Benard & Mendes-Giannini, 2009). How and

why this fungus affects the oral cavity as a result of spread of the fungus, causing serious consequences, is not known. Additionally, the expression of genes during interaction with the oral mucosal cells has not yet been explored.

In this study, the ability of *P. brasiliensis* strain Pb18 to infect oral keratinocytes in culture was observed for what

Fig. 3. Infection of NOKs by *P. brasiliensis* at 2, 5 and 8 h p.i., demonstrated by scanning electron microscopy. The fungal cells indicated by an arrow in the upper images are shown at higher magnification in the corresponding lower images.

Fig. 4. Analysis of RDA products of NOK infection by gel electrophoresis. (a) First round of subtraction. (b) Second round of subtraction. The positions of molecular size markers (bp) are indicated.

we believe to be the first time. The same infection index was observed with A549 cell infection (data not shown). Previous studies have demonstrated the ability of *P.*

brasiliensis to adhere to and invade epithelial cells (Mendes-Giannini *et al.*, 2008), and the adhesion phenomenon is variable depending on the isolate (Andreotti *et al.*, 2005; Hanna *et al.*, 2000). Adhesion of *P. brasiliensis* to host tissues is a crucial step in the establishment of PCM. Infection of host mucous cells can occur through spread of *P. brasiliensis* yeast cells, unlike in the primary infection in which conidia/mycelial fragments are the infectious particles.

Pathogens with the capacity to invade cells often alter the components of the cytoskeleton to promote entry into the cell and this is done by rearrangement of actin; several virulence factors are key regulators of actin reorganization (Finlay & Falkow, 1988; Rosenshine *et al.*, 1992; Swanson & Baer, 1995). Our data confirmed the results of a previous study regarding the behaviour of actin in *P. brasiliensis* Pb18 infection of pneumocytes (Mendes-Giannini *et al.*, 2008). *P. brasiliensis* infection of keratinocytes showed a similar phenomenon to infection of pneumocytes, with apparent loss of peripheral filaments, suggesting that the binding of *P. brasiliensis* to NOKs

Table 2. Summary of the computational analysis of genes expressed by *P. brasiliensis* during infection in NOK cells

Protein	<i>e</i> value*	NCBI protein accession no.	Frequency (no. clones)	Putative molecular function and/or biological process
40S ribosomal protein S9 of <i>P. brasiliensis</i>	3e-27	EEH50187.1	5	Structural constituent of ribosome
60S ribosomal protein L27-A of <i>P. brasiliensis</i>	2e-22	EEH44323.1	1	Structural constituent of ribosome
ADP/ATP carrier protein of <i>P. brasiliensis</i>	9e-11	EEH45290.1	1	Transport of ATP
Conserved hypothetical protein of <i>P. brasiliensis</i>	3e-59	EEH46428.1	14	Unknown
Cytokine-inducing glycoprotein of <i>Cryptococcus neoformans</i>	2e-56	CAC78984.1	1	Induction of cytokines
Glucosamine-6-phosphate isomerase of <i>P. brasiliensis</i>	3e-19	EEH44112.1	14	Sugar metabolism
Golgi to plasma membrane transport-related protein of <i>Cryptococcus neoformans</i>	1e-10	XP_568859.1	1	Movement from Golgi to plasma membrane
Hypothetical protein CNBD4690 of <i>Cryptococcus neoformans</i>	4e-10	XP_775740.1	9	Unknown
Hypothetical protein CNBM0020 of <i>Cryptococcus neoformans</i>	2e-11	XP_772265.1	1	Unknown
Hypothetical protein PAAG_02259 of <i>P. brasiliensis</i>	5e-11	EEH40204.1	1	Unknown
Hypothetical protein PABG_06807 of <i>P. brasiliensis</i>	4e-42	EEH16720.1	1	Unknown
Low-affinity zinc ion transporter of <i>Cryptococcus neoformans</i>	1e-8	XP_570392.1	1	Transporter of zinc
PENR2 protein of <i>P. brasiliensis</i>	8e-44	EEH49739.1	5	Regulation of transcription
Predicted protein of <i>P. brasiliensis</i>	9e-12	EEH47615.1	1	Unknown
Proteasome component PRE6 of <i>P. brasiliensis</i>	3e-25	EEH46637.1	2	Hydrolysis of internal peptide bonds
RDS1 protein of <i>Cryptococcus neoformans</i>	4e-17	XP_568514	32	Stress response
RNA-binding protein of <i>P. brasiliensis</i>	2e-26	EEH48229.1	6	Interacts with RNA molecule
Short-chain fatty acid CoA ligase of <i>P. brasiliensis</i>	3e-16	EEH42484.1	43	Formation of short-chain fatty acids
Ubiquitin-conjugating enzyme variant MMS2 of <i>P. brasiliensis</i>	3e-52	EEH45652.1	4	Ubiquitin-conjugating enzyme

**e* value according to information from BLASTX searches at NCBI or the Broad Institute.

Fig. 5. RT-PCR results (left panels) to validate the transcript levels of glucosamine-6-phosphate isomerase (GPI; NCBI protein accession no. EEH44112.1) and ubiquitin-conjugating enzyme variant MMS2 (EEH45652.1) obtained by RDA in *P. brasiliensis* infection of NOK cells (lane 2), compared with growth of the fungus in Fava-Netto medium (lane 1). The values in the graph on the right represent the expression levels of the genes expressed in infected NOK cells normalized to the level of the control gene L34, which was given a value of 1.

triggers a signal that induces cytoskeletal changes such as the dissipation and redistribution of F-actin. Furthermore, aggregation of β -tubulin at the attachment sites of the yeast cells was also observed. The uptake of *P. brasiliensis* in these normally non-phagocytic cells involved the recruitment of microtubules and microfilaments, after adherence of the yeast to the cell surface, as observed by Mendes-Giannini *et al.* (2004).

The interaction of *P. brasiliensis* with keratinocytes was also analysed by scanning electron microscopy, in which we observed that the fungus adhered to and invaded NOK cells, signalling changes probably linked to cytoskeletal actin. The mechanism of internalization in oral keratinocytes, described here for the first time to our knowledge, suggests that this ability may be important in disease development. *P. brasiliensis* is not essentially an intracellular parasite (Tuder *et al.*, 1985), but epithelial and endothelial cells can be used as a place to escape defence

cells, as demonstrated in other diseases (Kumamoto & Vences, 2005; Qazi *et al.*, 2001; Vázquez-Boland *et al.*, 2001).

The capacity for survival and reproduction is essential for the establishment of infection, so the fungus must have developed a strategy to adapt to the host environment. Several studies have shown changes in the transcription profile of *P. brasiliensis* when grown under conditions that mimic infection in the host (Bailão *et al.*, 2006, 2007). In this study, we employed RDA to compare the *P. brasiliensis* transcriptome after infection of NOKs compared with that of *P. brasiliensis* cultured in Fava-Netto medium. This methodology is an alternative approach to identify genes related to host-pathogen interactions.

During infection, host nutrients are limited in this environment, so the metabolic pathways need other options to replace the glucose required for the synthesis of many macromolecules and also necessary for energy production for proliferation and growth. Infection of NOKs resulted in elevated expression of short-chain fatty acid CoA ligase (43 sequences) and glucosamine-6-phosphate isomerase (14 sequences) in the fungus, the high number of clones for these genes indicating that the main limitation to infection by *P. brasiliensis* is the acquisition of nutrients. Short-chain fatty acid CoA ligase, which catalyses the formation of acyl-CoA from a short-chain fatty acid, has a central role in the metabolism, development and pathogenicity of many fungi (Hynes *et al.*, 2006). Fatty acids can serve as sole sources of carbon and energy, whilst acyl-CoAs serve as important intermediates in diverse metabolic functions such as fatty acid transport, oxidative degradation of fatty acids and phospholipid biosynthesis, as well as enzyme activation, cell signalling and transcriptional regulation (Morgan-Kiss & Cronan, 2004). *Candida albicans* differentially regulates carbon assimilation pathways depending on the stage of infection (Barelle *et al.*, 2006; Lorenz & Fink, 2001). This is similar to the situation in *Cryptococcus neoformans*, where patterns of gene expression and adaptation were tissue specific for lung infection in mice (Hu *et al.*, 2008) or in a rabbit model of experimental meningitis (Lee *et al.*, 2010).

Fig. 6. Quantification of transcripts differentially expressed during *P. brasiliensis* infection of NOK cells compared with growth of the fungus in Fava-Netto medium. The values represent expression levels (normalized against the L34 gene) for the genes for glucosamine-6-phosphate isomerase (GPI; NCBI protein accession no. EEH44112.1), ubiquitin-conjugating enzyme variant MMS2 (EEH45652.1), RDS1 (XP_568514) and PENR2 (EEH49739.1).

Several studies have focused on the pathways of gluconeogenesis, the glyoxylate cycle and β -oxidation of fatty acids, as these pathways are specifically upregulated during contact with host immune cells in a variety of human fungal and bacterial pathogens, such as *P. brasiliensis* (Derengowski *et al.*, 2008), *Cryptococcus neoformans* (Fan *et al.*, 2005; Hu *et al.*, 2008; Rude *et al.*, 2002), *Candida albicans* (Barelle *et al.*, 2006; Fradin *et al.*, 2005; Ramírez & Lorenz, 2009), *Aspergillus fumigatus* (Ebel *et al.*, 2006; Olivas *et al.*, 2008) and *Mycobacterium tuberculosis* (McKinney *et al.*, 2000; Muñoz-Elías & McKinney, 2005).

N-Acetylglucosamine (GlcNAc) is the basic unit of chitin, the main component of cell walls of fungi and other microorganisms. In *P. brasiliensis*, the metabolism of GlcNAc during the parasitic phase may be essential for its survival during a change of environment, as the mucosal membrane is rich in amino sugars. Utilizing GlcNAc as an energy source may be an important adaptation for establishment of infection in NOKs. *Candida* species are able to use amino sugars such as GlcNAc and glucosamine as alternative carbon sources (Singh & Datta, 1979). *Candida albicans* has genes that encode GlcNAc kinase, GlcNAc-6-phosphate deacetylase and glucosamine-6-phosphate deaminase, which are present in a cluster as the Nag regulon (Sengupta & Datta, 2003), leading to the conclusion that there is a correlation between such specific adaptation and virulence of the organism, as *C. albicans* requires a functional GlcNAc catabolic pathway to become successfully established in systemic infections (Singh *et al.*, 2001). Due to the large number of sequences found for glucosamine-6-phosphate isomerase in this study, it is possible that the same metabolic pathway is necessary for *P. brasiliensis* to cause infection in NOKs.

The gene sequence for the RDS1 protein was found in 32 clones. This finding is similar to that observed by Goulart *et al.* (2010) during infection of macrophages by *Cryptococcus neoformans* and *Cryptococcus gattii*, indicating that *P. brasiliensis* is under high stress during the process of contact with NOKs. RDS1 has been identified in several fungi such as *Neurospora crassa*, *Magnaporthe grisea*, *Aspergillus nidulans*, *Fusarium graminearum* and *Ustilago maydis* and appears to be related to the fungal response to stress. In *Schizosaccharomyces pombe*, alterations in expression of the *rds1* gene were observed when the fungus was subjected to various conditions such as glucose, ammonia and phosphate deprivation and changes in CO₂ concentration and temperature (Ludin *et al.*, 1995). Kraus *et al.* (2004), using a microarray technique, found increased expression of RDS1 in *Cryptococcus neoformans* maintained at 37 °C and, similarly, Rosa e Silva *et al.* (2008), comparing the yeast at 25 and 37 °C, verified an increase in expression of this gene using RDA.

The cell is equipped with complex enzyme systems that signal and precisely control the cellular response to a medium-specific condition. Proteins related to cell control were also found in our study, as two clones of a proteasome

component, proteolytic complexes responsible for the degradation of many cellular proteins (Demasi *et al.*, 2003) that play an important role in regulating the cell cycle and signalling, including apoptosis and the elimination of abnormal proteins generated by mutation and oxidative damage (Berlett & Stadtman, 1997; Bochtler *et al.*, 1999; Coux *et al.*, 1996; Demasi *et al.*, 2003; Giulivi *et al.*, 1994; Ullrich *et al.*, 1999). Moreover, proteasomes are controlled by glutathione S-transferase and the related control of oxidation–reduction reactions and production of transcription factors (Demasi *et al.*, 2003; Silva *et al.*, 2008). Finally, it has been observed that the proteolytic activity controlled by proteasomes is increased in the proliferative (logarithmic) phase and gradually decreases on passing into the stationary phase (Bajorek *et al.*, 2003; Laporte *et al.*, 2008). These results suggest that, under the conditions tested in this study, the yeast form of the fungus is under stress but is metabolically active.

Although only one sequence related to the transport of zinc was found, this nutrient may have a key role in the infection process because it is an essential nutrient for the cell, comprising structural motifs for many transcriptional co-factors, as well as acting as a co-factor for several enzymes, and may also stabilize protein structure and facilitate oxidation–reduction reactions (Bird *et al.*, 2000; Kim *et al.*, 2008; Lieu *et al.*, 2006; Radisky & Kaplan, 1999; Rutherford & Bird, 2004). As a response to zinc deprivation, homologous genes for the transcription factor Zap1 (Eide, 2009; Zhao & Eide, 1997) lead to the process of filamentation in *Candida albicans* and *Aspergillus fumigatus*, and have been considered as virulence factors and promising candidates for elucidating the mechanisms of pathogenicity (Bignell *et al.*, 2005; Krappmann *et al.*, 2004; Moreno *et al.*, 2007). In this sense, the presence in our study of a low-affinity zinc ion transporter may indicate, indirectly, the need to eliminate reactive oxygen species through proteins such as cytosolic superoxide dismutase, a copper/zinc-dependent enzyme important for cell detoxification during oxidative metabolism, which is also regarded as a potential virulence factor (Cox *et al.*, 2003; Dias *et al.*, 2006; Narasipura *et al.*, 2003, 2005).

Another protein with increased expression in this study was the ADP/ATP carrier. This protein appears to have a critical role in energy metabolism in the transport and supply of ATP generated in mitochondria and transported to the cytosol (Traba *et al.*, 2009). More recently, studies have suggested that the participation of ADP/ATP carriers is important for the occurrence of apoptosis. In this case, the carriers would be one of the constituents of permeability transition pores that would control the release of apoptogenic factors such as cytochrome *c* (Lawen, 2007; Pereira *et al.*, 2007; Traba *et al.*, 2009).

The fungus–host interaction covers a complex network of signalling and metabolic pathways that are interconnected to respond to a given situation or medium conditions. In this sense, the technique of RDA was extremely helpful,

giving us an overview of the cell situation at that time. Thus, proteins that were found suggested that the yeast was in a stress situation (RDS1), but was proliferating and was metabolically active (proteasomes, short-chain acetylators, glucosamine-6-phosphate isomerase and ADP/ATP carrier). Additionally, metabolic pathways were being activated in order to eliminate toxic substances from the cell (zinc transporter), the latter, together with glucosamine-6-phosphate isomerase and other proteins of this route, being potential targets for the development of future drugs.

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