Capítulo V

Artigos em colaboração
Transcriptional Profiles of the Human Pathogenic Fungus
Paracoccidioides brasiliensis in Mycelium and Yeast Cells*

Received for publication, January 18, 2005, and in revised form, March 15, 2005 Published, JBC Papers in Press, April 22, 2005, DOI 10.1074/jbc.M500625200


From the "Departamento de Bioquímica, Universidade Federal de Mato Grosso do Sul, 74001-970, Goiânia, GO, Brazil, iDepartamento de Genética, Universidade de São Paulo, 14040-900, Ribeirão Preto, SP, Brazil, and jFaculdade de Odontologia, Universidade de São Paulo, 14040-900, Ribeirão Preto, SP, Brazil.

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis, a disease that affects 10 million individuals in Latin America. This report depicts the results of the analysis of 6,022 assembled groups from mycelium and yeast phase expressed sequence tags, covering about 80% of the estimated genome of this dimorphic, thermo-regulated fungus. The data provide a comprehensive view of the fungal metabolism, including electron subtraction and cDNA microarrays. Also, a significant differential expression pattern in mycelium and yeast cells was detected, which was confirmed by Northern blot analysis, providing insights into differential metabolic adaptations. The overall transcriptome analysis provided information about sequences related to the cell cycle, stress response, drug resistance, and signal transduction pathways of the pathogen. Novel P. brasiliensis genes have been identified, probably corresponding to proteins that should be addressed as virulence factor candidates and potential new drug targets.

The dimorphic human pathogenic fungus Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis (PCM) (1), a major health problem in Latin America. High positive skin tests (75%) in the adult population reinforce the importance of the mycosis in endemic rural areas, where it has been estimated to affect around 10 million individuals, 2% of whom will develop the fatal acute or chronic disease (2). The acute form of PCM chiefly compromises the reticuloendothelial system; the chronic form mainly affects adult males with a high frequency of pulmonary and/or mucocutaneous involvement (1). Chronic severe multifocal PCM may also cause granulomatous lesions in the central nervous system (3). Regardless of the affected organ, PCM usually evolves to the formation of fibrotic sequelae, permanently hindering the patient’s health.

P. brasiliensis Undergoes a Dimorphic Process in Vivo—It is assumed that the fungus exists as a soil saprophyte, producing propagules that can infect humans and produce disease after transition to the pathogenic yeast form (4). Pathogenicity has been intimately associated with this process, since P. brasiliensis strains unable to differentiate into the yeast form are avirulent (5). Mammalian estrogens inhibit dimorphism, explaining the lower incidence of disease in females (6). The mycelium-to-yeast transition in P. brasiliensis is governed by the rise in temperature that occurs upon contact of mycelia or conidia with the human host. In vitro, it can be reversibly reproduced by shifting the growth temperature between 22 and 36 °C. Molecular events related to genes that control signal transduction, cell wall synthesis, and integrity are likely to be involved in this dimorphic transition.

The abbreviations used are: PCM, paracoccidioidomycosis; contig, group of overlapping clones; EST, expressed sequence tag; PbAEST, P. brasiliensis assembled EST sequence; MAPK, mitogen-activated protein kinase.

* This work was supported by MCT, CNPq, CAPES, FUB, UFG, and FUNDECT/MS.

The online version of this article (available at http://www.jbc.org) contains nine additional tables.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) CA580326-CA584263, CN238087-CN238093, and CN373644-CN373755.

Minimal information about cDNA microarray experiments was deposited in the MIAMExpress databank (EMBL) under the accession numbers E-MEXP-103 and A-MEXP-71. The sequences are also available at http://www.biomol.unb.br/Pb.

To whom correspondence should be addressed. Tel.: 55-307-2423; Fax: 55-61-3498411; E-mail: msueli@unb.br.

These authors contributed equally to this work.

**P. brasiliensis** Mycelium and Yeast Transcriptional Profiles

A study of *P. brasiliensis* gene density suggests that this fungus contains between 7,500 and 9,000 genes, which is in agreement with the estimated gene number for ascomycete fungi genomes.

Here are presented the results of an effort to achieve a comprehensive metabolic view of the *P. brasiliensis* dimorphic life cycle based on analysis of 6,022 groups generated from both mycelium and yeast phases. This view arises from both a general metabolism perspective and the identification of the precise metabolic points that distinguish both morphological phases. Overexpressed genes and those that are up- or downregulated in both stages were identified. Expression levels were assessed by cDNA microarrays and some were confirmed by Northern blot. Drug targets and genes related to virulence regulated in both stages were identified. Expression levels phases. Overexpressed genes and those that are up- or down-
cise metabolic points that distinguish both morphological
eral metabolism perspective and the identification of the pre-
cisely participating in cell differentiation and infection were an-
noted, and now we are able to describe the corresponding signaling systems in *P. brasiliensis*.

MATERIALS AND METHODS

**Fungus—** *P. brasiliensis* isolate Pbh1 (ATCC MYA-826) was grown at either 22 °C in the mycelium form (14 days) or 36 °C as yeast (7 days) in semisolid Fava Neto’s medium. Following incubation, cells were collected for immediate RNA extraction with Trizol reagent (Invitrogen).

**Construction of cDNA Libraries and Sequencing—** Poly(A)+ mRNAs was isolated from total mycelium and yeast RNA through oligo(dT) -cellulose columns (Stratagene). Unidirectional cDNA libraries were constructed in AZAPI following supplier’s instructions (Stratagene). Phagemids containing fungal cDNA were then mass-excised and replicated in XL-1 Blue MRF+ cells. In order to generate ESTs, single pass 5′-end sequencing of cDNAs was performed by standard fluorescence labeling dye terminator protocols with T7 flanking vector primer. Samples were loaded onto a MegaBACE 1000 DNA sequence (Amersham Biosciences) for automated sequence analysis.

**EST Processing Pipeline and Annotation—** PHRED quality assessment and computational analysis were carried out as previously described (8). EST assembly was performed using the software package CAP3 (9) plus a homemade scaffolding program. Sequences of at least 100 nucleotides, with PHRED > 20, were considered for clustering. A total of 20,271 ESTs were selected by those selection criteria. Containment and rRNA sequences were then removed to generate a set of 19,718 ESTs, which was submitted to CAP3 clustering, generating 2,655 contigs and leaving 3,367 ESTs as singlets. Contigs plus singlets comprise the base set of 6,022 *P. brasiliensis* assembled EST sequences (PhAESt) that underwent further analysis. Annotation was carried out using a system that essentially compared these assemblies with sequences available in public databases. The BLASTX program (10) was used for annotation along with GenBankTM nonredundant (nr), cluster of orthologous groups (COG), and gene ontology (GO) data bases. The GO data base was also used to assign EC numbers to clusters of orthologous groups (COG), and gene ontology (GO) data bases.

**RESULTS**

**Transcriptome Features—** In sequencing the *P. brasiliensis* transcriptome, EST data were generated from nonnormalized cDNA libraries of mycelium and yeast cells. The size range of the cDNA inserts ranged from 0.5 to 2.5 kb. Single pass 5′ sequencing was performed on 25,598 cDNA clones, randomly selected at CAPES/MEC - UFMT, UNIR, CEFET/BA on June 12, 2008

vector-PCR amplification with T3 forward and T7 reverse universal primers. Membranes were first hybridized against the T3-[α-32P]dCTP-labeled oligonucleotide. The amount of DNA deposited in each spot was estimated by the quantification of the obtained signals. B2Scan software was employed to identify hybridization spots. Membranes were used for hybridization against a [α-32P]-labeled cDNA complex probes. The latter were prepared by reverse transcription of 10 µg of filamentous or yeast *P. brasiliensis* total RNA using oligo(dT)12-18 primer. One hundred microliters of [α-32P]dCTP complex probe (30–50 million cpm) was hybridized against nylon microarrays. Imaging plates were scanned by a phosphor imager (Cyclone; Packard Instruments) to capture the hybridization signals. B2Scan software was employed to quantify the signals with background subtraction. Spots were matched with a template grid. The ratio between vector and cDNA complex probe hybridization values for each spot was used as the reference normalization value. Total intensity normalization using the median expression value was adopted as previously described (15). Gene expression data analyzed here were obtained from three independent determinations for each phase (filamentous or yeast). We used the significance analysis of microarrays method (16) to assess the significant variations in gene expression between both mycelium and yeast. Briefly, this method is based on t test statistics, specially modified to high throughput analysis. A global error chance, the false discovery rate, and a gene error chance (q value) are calculated by the software.

**Gene Ontology—** Total EST RNA (15 µg) was separated in a 1.5% denaturing formaldehyde agarose gel and transferred to a Hybond-N nylon membrane (GE Healthcare). Probes were radiolabeled with the random primers DNA labeling system (Invitrogen) using [α-32P]dATP. Membranes were incubated with the probes in hybridization buffer 50% formamide, 4× SSPE, 5× Denhardt’s solution, 0.1% SDS, 100 µg/ml herring sperm DNA at 42 °C overnight and then washed twice (2× SSC, 1% SDS) at 65 °C for 1 h. Signal bands were visualized using a Typhoon 9210 phosphor imager (GE Healthcare).

**URLs—** Details of the results and raw data are available for download from the World Wide Web: Phgenome project Web site (www.bi- omol.unib.hr/Pb); Gene Ontology Consortium (www.geneontology.org); Cluster of Orthologous Genes (www.ncbi.nlm.nih.gov/COG); INTERPROSCAN (www.ebi.ac.uk/interpro); National Center for Biotechnology Information (www.ncbi.nlm.nih.gov); KyriChrom (www.kyri.chrom.du/genes and Genomes (www.genome.ad.jp/kegg)); B2Scan Software (tagc.univ-mrs.fr); Audic and Claverie statistical test (telethon.bio.unipd.it/biocinfo/ IDEG6_form/); Significance Analysis of Microarrays method (www-stat.stanford.edu/~tibs/SAM/); Candia albicans data base (genolist.pasteur.fr/CandidaDB/); genomes from Aspergillus nidulans and Neurospora crassa (www.broad.mit.edu/annotation/fungi/aspergillus/).

**Highly and Differentially Expressed Genes—** The 27 highly expressed genes in *P. brasiliensis* are shown in Supplemental Table II. We had used MIPS functional categories to classify 2,931 PbAESTs into 12 major groups. *P. brasiliensis* showed a slightly higher percentage of PbAESts (4%) related to cellular communication and signal transduction (Fig. 1B) compared with *S. cerevisiae* functional categorization (3.4%).
P. brasilensis Mycelium and Yeast Transcriptional Profiles

**Fig. 1. P. brasilensis transcriptome characterization.** A, distribution of blast best hit among organisms. Each PbAEST was tested against the GenBank™ nr data base, and the best hit organism was computed. A PbAEST was considered as not assigned when the best hit exceed an E value of $10^{-10}$. B, functional categorization of the PbAESTs using MIPS classification. We included 2931 curator-reviewed annotations in this analysis.

In order to support the electronic subtraction data, cDNAs from each phase were used to probe cDNA microarrays membranes containing 1,152 clones, which were selected based on the following criteria: (i) ESTs exclusive for a particular morphotype; (ii) ESTs corresponding to genes more expressed in mycelium or yeast cells; and (iii) some ESTs equally expressed in both cell types. From the 1,152 clones, 328 genes were up-regulated during the dimorphic transition: 58 in mycelium or yeast cells; and (iii) some ESTs were selected based on the following criteria: (i) ESTs exclusive for a particular morphotype; (ii) ESTs corresponding to genes more expressed in mycelium or yeast cells; and (iii) some ESTs equally expressed in both cell types. From the 1,152 clones, 328 genes were up-regulated during the dimorphic transition: 58 in mycelium and 270 in yeast (data not shown).

The cDNA microarray experiment confirmed most of the electronic subtraction data and also points out to new differentially expressed genes. Among them, a subclass of about 40 up-regulated genes in mycelium and yeast are described in Table I, which includes M51, M32, hydrophobin 1/2, the highly expressed yeast PbY20 protein, and some other genes that have previously been described as differentially expressed in P. brasilensis by different approaches (17–20). Other key up-regulated genes related to the metabolism of P. brasilensis (Table I) are described and discussed elsewhere in this work. Interestingly, we have found a yeast phase preferentially expressed gene that possibly encodes a previously characterized P. brasilensis estradiol-binding protein (21), also described in C. albicans and in other fungi (22). It is speculated that the interaction of the 17-β-estriadiol hormone with a cytoplasmic protein inhibits the mycelium-to-yeast transition, explaining the lower incidence of PCM in females.

**Metabolic Overview—P. brasilensis seems to be capable of producing ATP from the classical pathways of glycolysis, alcohol fermentation, and oxidative phosphorylation, since alcohol dehydrogenase, cytochrome genes, ATP synthase subunits, and pyrophosphatase genes were annotated. All genes encoding glycolytic enzymes were identified in both mycelium and yeast. Genes corresponding to the citrate cycle enzymes and to the components of complexes I, II, III, and IV were found, reflecting the ability of the fungus to perform complete aerobic pyruvate degradation and oxidative phosphorylation. Its putative capacity to also grow in anaerobiosis was evidenced by the alternative conversion of pyruvate to ethanol. Last, it may be able to utilize two-carbon sources in the form of acetate and ethanol through the glyoxylate cycle and obtain sulfite and nitrite from the environment.**

In order to validate the carbon source utilization profile predicted by the transcriptome data, two P. brasilensis isolates (Pb01 and Pb18) were grown in McVeigh-Morton minimum medium supplemented with different carbon sources and growth patterns were qualitatively evaluated (Supplemental Table III). We observed that, in accordance to the transcriptome analysis prediction, several mono- and disaccharides, such as D-glucose, D-fructose, D-galactose, D-mannose, D-sorbitol, α-trehalose, maltose, and sucrose were indeed utilized. On the other hand, the predicted assimilation of D-inositol was not confirmed. Transcripts related to the consumption of L-sorbose and L-lactose were not detected; in fact, P. brasilensis was unable to grow in L-sorbose as the sole carbon source. We consider that the unpredicted fungal growth in L-lactose can be explained by the fact that the P. brasilensis cDNA libraries were not constructed under induction conditions. The observation that fructose, galactose, and glycerol were only utilized by Pb01 and not by Pb18 isolate may simply reflect strain biological variability as previously observed (7). A detailed description of P. brasilensis metabolism, including a list of PbAESTs, is shown in Supplemental Table IV.

**Differential Metabolism between Mycelium and Yeast—**The up-regulated genes encoding enzymes in mycelium and yeast cells listed in Table I are highlighted in Fig. 2. The differential expression pattern of these genes (with the exception of glucokinase from mycelium cells) was confirmed by Northern blot analysis (Fig. 3). In general, the gene overexpression pattern suggests that mycelium saprophytic cells possess an aerobic metabolism, in contrast with yeast cells. Actually, mycelium up-regulated genes correspond to the main regulatory points of the citrate cycle, such as the genes coding for isocitrate dehydrogenase and succinyl-CoA synthetase; this strongly suggests a metabolic shunt to oxidative phosphorylation. Also, glucokinase is induced, producing glucose 6-phosphate, which is possibly converted through the oxidative pentose phosphate pathway to ribose 5-phosphate, and then to salvage pathways of purine and pyrimidine biosynthesis. In fact, this correlates well with the overexpression of adenylate kinase and uridine kinase genes. The excess of ribose 5-phosphate is probably converted to fructose 6-phosphate and glyceraldehyde 3-phosphate by the nonoxidative pentose phosphate pathway catalyzed by the overexpressed transaldolase. Those sugars are converted to pyruvate and acetyl-CoA for the citrate cycle in aerobic conditions.

In contrast, P. brasilensis yeast cells overexpress the genes encoding alcohol dehydrogenase I and pyruvate dehydrogenase E1 subunit (Table I and Fig. 3); the latter can be detected in high levels in cultures of S. cerevisiae grown both anaerobically.
and aerobically in the presence of ethanol (23). The carbohydrate metabolism is probably shifted toward ethanol production, reflecting the anaerobic behavior of the yeast form as previously reported (24). Several pathways that provide substrates for the glyoxylate cycle are up-regulated in the yeast cells (Table I and Fig. 3). First, isocitrate lyaseredirects the metabolic flow using ethanol and acetate as two-carbon sources and generating oxaloacetate, which can be reconverted to glu-

<table>
<thead>
<tr>
<th>PhAESt</th>
<th>EC number</th>
<th>Annotated function</th>
<th>Number of reads</th>
<th>p value</th>
<th>-Fold change</th>
<th>Accession number/Best hit organism/ E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1092</td>
<td>4.2.1.22</td>
<td>Cystathionine ( \beta )-synthase( ^e )</td>
<td>4 0 0.030842</td>
<td>25.2</td>
<td>0.001602</td>
<td>NP_013458.1/S. cerevisiae-108</td>
</tr>
<tr>
<td>2356</td>
<td>2.2.1.2</td>
<td>Transaldolase( ^e )</td>
<td>4 0 0.030842</td>
<td>25.2</td>
<td>0.001602</td>
<td>NP_013458.1/S. cerevisiae-108</td>
</tr>
<tr>
<td>2476</td>
<td>3.1.2.22</td>
<td>Palmitoyl-protein thioesterase( ^e )</td>
<td>4 0 0.030842</td>
<td>25.2</td>
<td>0.001602</td>
<td>NP_013458.1/S. cerevisiae-108</td>
</tr>
<tr>
<td>4135</td>
<td>1.1.1.41</td>
<td>Isocitrate dehydrogenase( ^e )</td>
<td>1 0 2.4869000000000</td>
<td>2.7</td>
<td>T49777/N. crassa/1e-73</td>
<td>CAD21381.1/N. crassa/2e-46</td>
</tr>
<tr>
<td>5530</td>
<td>6.2.1.5</td>
<td>( \beta )-Succinyl CoA synthetase( ^e )</td>
<td>1 0 2.4869000000000</td>
<td>2.7</td>
<td>T49777/N. crassa/1e-73</td>
<td>CAD21381.1/N. crassa/2e-46</td>
</tr>
<tr>
<td>4749</td>
<td>2.7.1.2</td>
<td>Gluconokinase( ^e )</td>
<td>1 0 2.4869000000000</td>
<td>1.7</td>
<td>T47207/Aaspergillus niger/2e-50</td>
<td>CAD21381.1/N. crassa/2e-46</td>
</tr>
<tr>
<td>4246</td>
<td>2.7.1.48</td>
<td>Uridine kinase( ^e )</td>
<td>1 0 2.4869000000000</td>
<td>2.7</td>
<td>T47207/Aaspergillus niger/2e-50</td>
<td>CAD21381.1/N. crassa/2e-46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Yeast up-regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2536</td>
</tr>
<tr>
<td>2431</td>
</tr>
<tr>
<td>737</td>
</tr>
<tr>
<td>201</td>
</tr>
<tr>
<td>797</td>
</tr>
<tr>
<td>814</td>
</tr>
<tr>
<td>1704</td>
</tr>
<tr>
<td>1585</td>
</tr>
<tr>
<td>63</td>
</tr>
<tr>
<td>778</td>
</tr>
<tr>
<td>136</td>
</tr>
<tr>
<td>767</td>
</tr>
<tr>
<td>701</td>
</tr>
<tr>
<td>1724</td>
</tr>
<tr>
<td>171</td>
</tr>
<tr>
<td>1983</td>
</tr>
<tr>
<td>244</td>
</tr>
<tr>
<td>258</td>
</tr>
<tr>
<td>279</td>
</tr>
<tr>
<td>314</td>
</tr>
<tr>
<td>555</td>
</tr>
<tr>
<td>756</td>
</tr>
<tr>
<td>865</td>
</tr>
<tr>
<td>963</td>
</tr>
<tr>
<td>980</td>
</tr>
<tr>
<td>3073</td>
</tr>
</tbody>
</table>

\( ^a \) Number of mycelium (M)- and yeast (Y)-derived ESTs in the PhAESt.  
\( ^b \) p value for the Audic and Claverie test.  
\( ^c \) Fold change found for the microarray experiments.  
\( ^d \) Previously shown to be differential by Northern blot or proteome analysis.  
\( ^e \) Electronic subtraction and cDNA microarray analysis; differential pattern in both analyses.  
\( ^f \) Electronic subtraction differential pattern and not assayed in cDNA microarray analysis.  
\( ^g \) Singlets that are differential in cDNA microarray analysis.  

Differentially expressed genes in mycelium and yeast cells detected by electronic subtraction and cDNA microarray analysis

The PhAESts were analyzed as to their differential expression by two methods: a statistical analysis of the number of mycelium and yeast ESTs clustered in each PhAESt (14) and a cDNA microarray analysis of 1,152 PhAESts, chosen according to the electronic subtraction criteria. A differential pattern of genes encoding enzymes was used in the analysis of the differential metabolism.
cose. In addition, the branched-chain aminotransferase gene is also overexpressed (as are other aminotransferase genes, such as those of aspartate and histidinol-P) and converts valine, leucine, and isoleucine to acetyl-CoA, which is then fed to the cycle. The yeast differential acetamidase also contributes to this pathway by deriving acetate from acetamide. Furthermore, the up-regulated acyl-CoA synthetase generates acetyl-CoA in the first step of β-oxidation, which may also be taken up by the cycle. Finally, the generation of sulfite by phosphoadenylyl sulfate reductase provides acetate for the glyoxylate cycle as mentioned above. The overall analysis suggests that ATP production through alcohol fermentation and the respiratory chain occurs in a biased pattern, the former being preferential in the yeast form and the latter in mycelium.

Yeast cells are rich in chitin; the high expression of chitin deacetylase reveals its possible involvement in cell wall loosening, reorganization, and synthesis of newly components during cell growth and budding of yeast cells. This enzyme is not present in humans and thus represents a possible drug target. In mycelium, overexpression of cystathionine β-synthase and nitroalkane oxidase strongly suggests that mycelium cells take up sulfite and nitrogen, respectively, from the environment for metabolic processing. Finally, the probable role of the remaining overexpressed gene encoding palmitoylthioesterase remains unclear. In contrast, the enzymes isochorismatase and ubiquinone-reductase are greatly up-regulated in the yeast form, strongly suggesting a high production of ubiquinone by *P. brasiliensis*, which could be involved in cellular oxidative stress under anaerobic conditions. The high yeast expression of dihydropteroteinate synthase produces, as a consequence, high levels of tetrahydrofolate, which probably will increase the metabolic flow toward purine biosynthesis. The meaning of the high expression in yeast of choline sulfatase, gluconate dehydrogenase, glutamyl-tRNA amidotransferase, and phenylacetate hydroxylase also remains unclear.

**Cell Cycle and Genetic Information**—The main genes in-
volved in cell cycle and in the basic genetic information flow machinery (DNA replication, repair, recombination, transcription, RNA processing, translation, and post-translational modifications) are well conserved in comparison with their counterparts from *S. cerevisiae*. Also, sequences related to mitochondrial replication, budding, sporulation, and mating were also annotated (Supplemental Table V).

From the cell cycle-related orthologues identified in *P. brasiliensis*, those related to the structure and assembly of the cytoskeleton, chromatin structure, chromosome segregation, cyclins, and cell cycle control genes were highlighted. Genes related to the major DNA repair mechanisms found in yeast (mismatch, base excision, and recombination systems) were identified in *P. brasiliensis*, although not every component was represented, since cells were not subjected to DNA-damaging conditions. The RAD52 gene, which plays an essential role in *S. cerevisiae* recombination, is also present in the *P. brasiliensis* transcriptome.

Among the identified transcription factors, the orthologues for *MAT, MCM1*, and *NudD* are of relevance, since they are implicated in ascomycete sexual reproduction. These genes represent a strong evidence for mating in *P. brasiliensis*, so far not yet described, which is reinforced by the detection of six transcripts involved in meiotic recombination.

**Stress Responses**—Cell differentiation in *P. brasiliensis* requires a temperature shift, which might be associated with a stress response. We have found 48 sequences encoding molecular chaperones and their associated co-chaperones in *P. brasiliensis* transcriptome (Supplemental Table VI). These sequences were divided into nine groups: small chaperones (four genes), HSP40 (9), HSP60 (10), HSP70 (7), HSP90 (4), HSP100 (4), 14-3-3 (2), calnexin (1), and immunophilins (7). Eight of these are differentially expressed: calnexin, cct7 (cytoplasmic hsp60) and sba1 (HSP90/70 co-chaperone) for the mycelium form and cpr1 (HSP90/70 co-chaperone), hsp42, hsp60, ssc1 (HSP70), and hsp90 for the yeast form. From these, hsp60 and hsp70 had been previously characterized as differentially expressed in yeast (25, 26). cDNA microarray analysis confirmed the differential expression pattern of sba1. Furthermore, the number of chaperone and co-chaperone ESTs is 38% larger in the yeast cDNA library than in the mycelium library. These data represent an evidence of an altogether higher expression of HSPs in yeast cells, compatible with growth at 37 °C.

Oxidative agents may cause stress and damage to *P. brasiliensis* cells. They may originate from the activity of host macrophages or from intracellular oxidative species. *P. brasiliensis* contains several genes encoding enzymes with known or putative antioxidant properties, such as superoxide dismutases, catalases (two isoenzymes), peroxiredoxins, and a novel cytochrome c peroxidase (Supplemental Table VII). Homologues to genes encoding secondary antioxidant enzymes belonging to the glutathione S-transferase family were also found. Several transcription factors may be involved in the induction of antioxidant defenses in *P. brasiliensis*. Homologues to YAP1, HAP3, and SKN7 from *S. cerevisiae* (27) were discovered in the transcriptome, showing that the oxidative stress regulators from *P. brasiliensis* and baker’s yeast might be conserved.

**Signal Transduction Pathways**—Transcriptome analysis and reverse annotation revealed several putative components of the biosignaling pathways in *P. brasiliensis* (Supplemental Table VIII), such as (i) MAPK signaling for cell integrity, cell wall construction, pheromone/mating, and osmotic regulation; (ii) cAMP/protein kinase A, regulating fungal development and virulence, and (iii) calcium-calmodulin-calcinucin, controlling growth at high temperature. Furthermore, a ras homologue sequence was detected raising the possibility of cross-talk among the distinct signal transduction pathways (Fig. 4).

In budding yeast, the MAPK cascade responsible for cell integrity mediates cell cycle regulation and cell wall synthesis, responding to different signals including temperature, changes in external osmolarity, and mating pheromone. Components of this pathway identified in *P. brasiliensis* encompass the most classical steps, with the exception of a cell surface tyrosine kinase-like receptor that was not found in the transcriptome so far analyzed. Rho1p is a small GTP-binding protein of the *Rho* subfamily required for cell growth and coordinated regulation of cell wall construction (28) through the synthesis of β-1,3-glucan. It also activates Pkc1p, which in turn regulates the MAPK pathway.

Transcripts related to the pathway for activation by mating pheromone were identified in the *P. brasiliensis* transcriptome. The intermediary components appear to be constitutively expressed in both mycelium and yeast forms. Intriguingly, mating has not yet been described in *P. brasiliensis*. Conversely, the Hog1 MAPK cascade is activated when there is an increase in the environment osmolarity. One of its targets, Glo1p, which controls genes required for cell adaptation and survival upon osmotic stress in *S. cerevisiae* (29), was also detected in *P. brasiliensis*.

The cAMP/protein kinase A is a cascade known to regulate fungal differentiation and virulence. From the genes identified in *P. brasiliensis*, we highlight a homologue to several fungal adenylate cyclases; the low affinity cAMP phosphodiesterase, encoded by the gene Pde1; homologues to both the regulatory and the catalytic subunits of protein kinase A, which is involved in the regulation of the cell surface flocculin Flo11p/Muc1p (30). In *P. brasiliensis* exogenous cAMP is known to inhibit the process of filamentation (31). Both the catalytic (CnaA) and the Ca"2+"-binding regulatory B (CnaB) subunits of calcineurin were found in *P. brasiliensis*. In dimorphic fungi, cAMP- and calcineurin-dependent pathways seem to be involved in differentiation. As in the pathogenic fungus *Cryptococcus neoformans* (32), calcineurin might also play a role in mating of *P. brasiliensis*. In several pathogenic and nonpathogenic fungi, *RAS* is involved in filamentation, pseudohyphal/hyphal growth, and mating (33). A *RAS*-related transcript was identified in *P. brasiliensis*, but further studies are required to elucidate its function in mycelium-to-yeast transition and in the mechanism of pathogenicity.

**Virulence Genes, Drug Targets and Resistance**—In order to identify genes that could be related to *P. brasiliensis* virulence, its transcriptome has been searched for orthologues assigned as virulence factors in human pathogenic fungi, as defined by Falkow’s postulate (34). Table II lists 28 *P. brasiliensis* sequences, which were previously experimentally established as virulence or essential genes in *C. albicans*, *C. neoformans*, and *Aspergillus fumigatus*. They were subdivided into four classes: metabolism-, cell wall-, and signal transduction-related and others. Some of these genes has been considered for antifungal therapy and are also listed in Table III as potential drug targets.

MAPK-related sequences, whose orthologues in *C. albicans* were experimentally correlated to hyphal formation and virulence, were also detected. The extrapolation to the *P. brasiliensis* model is not direct, since yeast, not hyphae, is the pathogenic cell type, but several MAPK homologues are found in species exhibiting diverse morphology and infection habits (35). A *cavps34* orthologue, identified in *P. brasiliensis* transcriptome (ups34), is implicated in the protein/lipid transport from the Golgi apparatus/endosome to the vacuole and has been proved to be important to *C. albicans* virulence (36).

Noteworthy is the finding of glyoxylate cycle genes in
**P. brasiliensis**, since its activity has been reported as a fungal virulence requirement (37). The activity of the key enzymes malate synthase and isocitrate lyase was reported to be upregulated in *C. albicans* upon phagocytosis (38). Both enzymes were detected in the *P. brasiliensis* transcriptome, with isocitrate lyase being overexpressed in the yeast phase, as confirmed by Northern blot analysis (Fig. 3).

The cell wall, as the most obvious difference between human and fungal cells, represents a prime target for antifungals. Genes involved in its biogenesis and assembly can act as virulence factors and therefore are putative drug targets. We have identified orthologues to chitin synthase 3 (*chs3*), glucosamine-6-phosphate acetyltransferase (*gna1*), mannosyltransferase (*pmt1*), and 1,2-mannosyltransferase (*mnt1*) genes and glycosidases Phr1p and Phr2p. The expression of the two last genes in *C. albicans* is responsive to the product of *prr2* (39), a pH-related transcription factor also present in the *P. brasiliensis* transcriptome. The detection of chitin deacetylase, as an overexpressed yeast gene confirmed by cDNA microarray and Northern blot (data not shown), points out to a novel target for drug research in *P. brasiliensis*.

Microbe resistance to reactive oxygen and nitrogen intermediates plays an important role in virulence (40). We were able to identify sequences that are oxidative stress response orthologues, including an alternative oxidase (*aox1*), a copper/zinc superoxide dismutase (*sod1*), and two different catalase orthologues, one of them a peroxisomal *cat1*, as recently described (41).

The urate oxidase gene detected in the *P. brasiliensis* transcriptome, but not in *S. cerevisiae*, *C. albicans*, and *Homo sapiens* genomes, suggests that uric acid could be degraded to allantoin. In addition, the presence of a *C. neoformans* urease orthologue also probably reflects the degradation of urea to ammonia and carbamate. A role in virulence and sporulation has been assigned for both genes (42). The production of urea has been involved in an improved *in vitro* survival for those microorganisms exposed to an acidic environment. In this view, it could be related to the survival of the fungus in the host cells.

The development of new drugs is crucial, considering the problem of emerging drug resistance and toxicity (37). Novel drug targets have been found through the analysis of genome sequences. The genes listed in Table III have no homologues in the human genome and therefore could be considered for the development of new antifungal drugs. Most therapies designed to treat fungal infections target the ergosterol biosynthetic pathway (43). The orthologue of C-24 sterol methyltransferase (*ERG6*) is present in *P. brasiliensis*. In addition, modulation of sphingolipid metabolism exerts a deep impact on cell viability. The synthesis of inositol-phosphoryl-ceramide from phytoceramide catalyzed by the product of the *aur1* gene, present in *P. brasiliensis*, corresponds to the first specific step of this pathway (44). Translation elongation factors have also been pointed out as drug targets (37). In the *P. brasiliensis* transcriptome, we have found an elongation factor-3 sequence that is absent in human genome (45) and thus can be addressed for pharmaceutical purposes.

Twenty PbAESTs annotated as related to multiple drug resistance genes were identified (Supplemental Table IX). They include 12 *S. cerevisiae* orthologues, 10 of which are related to the ABC transporter and two to major facilitator superfamilies (46). One of them corresponds to Pfr1, a gene recently described in *P. brasiliensis* (47), and another is related to the CDR1 gene from *C. albicans*, which is up-regulated in the presence of human steroid hormones (48). It has been speculated that...
steroid hormones are involved in morphological changes as well as in pathogenicity in *P. brasiliensis* and also in drug resistance in *C. albicans*. Interestingly, the process of infection of *P. brasiliensis* is strongly biased toward males, albeit the role of steroid hormones in the expression of ABC transporters in this organism remains to be investigated.

**DISCUSSION**

The *P. brasiliensis* transcriptome described here is represented by 6,022 EST clusters that may cover about 80% of the fungal total genome, whose gene number has been estimated to be ~8,000 genes. This number greatly exceeds the previous EST studies in this fungus (8, 49). The analysis compares the two fungal cell types as well as their metabolic behavior. The results obtained probably reflect the adaptations associated with the mycelium (soil) and yeast (human host) environments. Most importantly, they provide new insights with respect to the translational machinery of both fungal cell types and also in drug resistance with the *C. albicans* orthologues, except *phr1* (marked with an asterisk).

### Table II

<table>
<thead>
<tr>
<th>Putative virulence or essential genes found in <em>P. brasiliensis</em> transcriptome related to the experimentally confirmed orthologues of <em>C. albicans</em>, <em>C. neoformans</em>, and/or <em>A. fumigatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PBAEST</strong></td>
</tr>
<tr>
<td>Metabolic genes</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Cell wall genes</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Signal transduction</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Other fungal virulence determinant genes</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

* All *P. brasiliensis* assembled ESTs are BBH with *C. albicans* orthologues, except *phr1* (marked with an asterisk).

### Table III

<table>
<thead>
<tr>
<th>Putative drug targets genes found in <em>P. brasiliensis</em> transcriptome with no homologues in the human genome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PBAEST</strong></td>
</tr>
<tr>
<td>Cell wall</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Glyoxylate cycle</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Other targets</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<sup>3</sup> G. San-Blas, personal communication.
signal transduction pathways, virulence genes, and drug targets for this pathogen.

The transcription profile of the mycelium infective phase suggests the shunting of pyruvate into aerobic metabolism, since the expression of the EST's encoding enzymes of the trichloroacetic acid cycle are up-regulated in this fungal phase. In contrast, the yeast transcription profile evidenced the deviation of pyruvate from the glycolytic pathway into anaerobic metabolism; this observation is consistent with a lower oxygen level in infected tissues. Its putative ability to produce ethanol suggests a potential anaerobic pathway for \textit{P. brasiliensis}.

Since \textit{P. brasiliensis} is a medical problem in Latin America, the prediction of new drug targets from sequence information is of great importance. Chitin deacetylase, which is absent in macrophages and human pulmonary epithelial cells infected by \textit{P. brasiliensis}, is a medical problem in Latin America, the prediction of new drug targets from sequence information is of great importance. Chitin deacetylase, which is absent in macrophages and human pulmonary epithelial cells infected by \textit{P. brasiliensis}, is likely to facilitate the development of new therapeutics to PCM and other medically relevant mycosis.

In conclusion, the knowledge of the transcribed sequences of \textit{P. brasiliensis} will most likely facilitate the development of new therapeutics to PCM and other medically relevant mycosis.

Acknowledgments—We are grateful to Hugo Costa Paes and Robert Miller for English text revision.
Kinases of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae*: An overview

Alexandre Melo Bailão, Juliana Alves Parente, Maristela Pereira and Célia Maria de Almeida Soares

Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Goiás, Brazil.

Abstract

*Mycoplasma synoviae* and *Mycoplasma hyopneumoniae* are wall-less eubacteria belonging to the class of Mollicutes. These prokaryotes have a reduced genome size and reduced biosynthetic machinery. They cause great losses in animal production. *M. synoviae* is responsible for an upper respiratory tract disease of chickens and turkeys. *M. hyopneumoniae* is the causative agent of enzootic pneumonia in pigs. The complete genomes of these organisms showed 17 ORFs encoding kinases in *M. synoviae* and 15 in each of the *M. hyopneumoniae* strain. Four kinase genes were restricted to the avian pathogen while three were specific to the pig pathogen when compared to each other. All deduced kinases found in the non pathogenic strain (J[ATCC25934]) were also found in the pathogenic *M. hyopneumoniae* strain. The enzymes were classified in nine families composing five fold groups.

Key words: Mycoplasma, kinases, genomes.

Received: April 12, 2006; Accepted: October 5, 2006.

Introduction

Edmond Nocard and Emile Roux successfully cultivated the agent of the contagious bovine pleuropneumonia, *Mycoplasma mycoides*, over a century ago (Nocard and Roux, 1898). Since that time, approximately 111 species of the genus *Mycoplasma* have been identified in animals. These and other 102 species comprise the class of Mollicutes (Minion et al., 2004). These prokaryotes are known as the smallest self replicating organisms (Glass et al., 2000; Westberg et al., 2004). Most members of this class are pathogenic and colonize a wide variety of hosts, such as animals, plants and insects. Mollicutes represent a group of Low-G+C-content eubacteria that are phylogenetically related to the *Clostridium-Streptococcus-Lactobacillus* branch of the phylum (Woese et al., 1980; Rogers et al., 1985; Maniloff, 1992). As a consequence of the reduced biosynthetic machinery, Mollicutes live in nature as obligate parasites and depend on the uptake of many essential molecules from their hosts (Papazisi et al., 2003). Thus, they have been considered model systems for defining the minimal set of genes required for a living cell (Morowitz, 1984). Although, Mollicutes have a simple genome, mycoplasma diseases are complex and relatively unknown (Minion et al., 2004). One hallmark of these diseases is the chronicity (Ross, 1992), but equally important is the ability to alter or circumvent the immune response and to potentiate diseases caused by other pathogens (Ciprian et al., 1988; Thacker et al., 1999; Muhlradt, 2002). A key factor in the ability of mycoplasmas to establish a chronic infection is their genome flexibility, which allows them to produce a highly variable mosaic of surface antigens (Citti and Rosengarten, 1997; Chambaud, et al., 1999; Shen et al., 2000 Assunção et al., 2005).

In the last years, the genomes of ten mycoplasma species have been completely sequenced (Himmelreich et al., 1996; Glass et al., 2000; Chamabaud et al., 2001; Sasaki et al., 2002; Berent and Messik, 2003; Papazisi et al., 2003; Westberg et al., 2004; Jaffe et al., 2004; Minion et al., 2004). Recently, the complete genomes of a pathogenic (7448) and nonpathogenic (J [ATCC 25934]) strains of *Mycoplasma hyopneumoniae*, as well as the complete genome of a strain (53) of *Mycoplasma synoviae* (Vasconcelos et al., 2005) were obtained. Both species cause great adverse impact on animal production. *M. hyopneumoniae* is the causative agent of porcine enzootic pneumonia, a mild, chronic pneumonia of swine, commonly complicated by opportunistic infections with other bacteria (Ross, 1992). Like most other members of the order *Mycoplasmatales*, *M. hyopneumoniae* is infective for a single species, but the mechanisms of host specificity are unknown. *M. synoviae* is the major poultry pathogen...
Kinases play indispensable roles in numerous cellular metabolic and signaling pathways, and they are among the best-studied enzymes at the structural, biochemical, and cellular levels. Despite the fact that all kinases use the same phosphate donor (in most cases, ATP) and catalyze apparently the same phosphoryl transfer reaction, they display remarkable diversity in their structural folds and substrate recognition mechanisms, probably due largely to the extraordinarily diverse nature of the structures and properties of their substrates (Cheek et al., 2005).

Complete genome sequencing identified 679, 681 and 694 Open Reading Frames (ORF) of *M. hyopneumoniae* strains J (Mhy-J), 7448 (Mhy-P) and *M. synoviae* strain 53 (Msy), respectively. Analysis of these mycoplasma genomes by bioinformatics tools identified 15 Mhy-J ORFs, 15 Mhy-P ORFs and 17 Msy ORFs, all of which encode kinases. Due to the biological importance of these enzymes we expect that their study will improve the comprehension of the reduced biosynthetic pathways in mollicutes.

**Methods**

By using previous results from the complete genomes of *M. synoviae* and *M. hyopneumoniae*, J and 7448 strains as input to BLAST search tools we obtained 17 ORFs encoding kinase homologues in *M. synoviae* and 15 in both strains of *M. hyopneumoniae*. Putative biological functions of the kinases were deduced by using Pfam interface and InterPro information. The classification of enzymes into fold groups and families was performed by following the described by Cheek et al. (2005). In brief, all kinase sequences from the NCBI non-redundant database were assigned to a set of 57 profiles describing catalytic kinase domains by using the hmmsearch program of the HMMR2 package (Eddy, 1998). Sequences from each Pfam/COG profile presenting significant PSI-BLAST (Altschul et al., 1997) hits to each other were clustered into the same family. Families in the same fold group share structurally similar nucleotide-binding domains that have the same architecture and topology (or are related by circular permutation) for at least the core of the domain. Multiple sequence alignments were generated using the ClustalX 1.81 software (Thompson et al., 1997). The amino acid sequence relationships were generated with the predicted protein sequences obtained from 47 kinase-encoding ORFs identified in the complete genome sequences of *M. synoviae* and *M. hyopneumoniae*. A phylogenetic tree was constructed by multiple sequence alignments (pairwise alignments) using the Clustal X 1.81 program (Thompson et al., 1997) and visualized by using the TreeView software. The tree was constructed by using the minimum evolution (neighbor-joining) method (Saitou and Nei, 1987).

**Results and Discussion**

**Mycoplasma kinases**

In this study we briefly review the kinase genes of *M. hyopneumoniae* and *M. synoviae*, and we describe a classification and metabolic comparative analysis of kinases of these organisms. In the genome sequences we identified a total of 47 kinase-encoding ORFs which are related to several different biosynthetic pathways, such as purine and pyrimidine metabolism, glycolysis, pyruvate metabolism, as well as cofactor metabolism and others (Table 1). The two *M. hyopneumoniae* strains have equal numbers of the same kinases-encoding ORFs. Three of these are absent in *M. synoviae* (glycerol kinase, glucokinase and 5-dehydro-2-deoxygluconokinase) which has an additional 17 ORFs that encode kinases. Four of them (three ORFs encoding deoxyguanosine kinase and one ORF encoding N-acetylmannosamine kinase) are exclusive to this species when compared to *M. hyopneumoniae* strains J and 7448 (Table 1). These differences between the two species could be related to specific nutritional requirements found by each pathogen in its respective host. All kinases found in the pathogenic strain

**Table 1 - Kinases identified in the *M. synoviae* and *M. hyopneumoniae* genomes.**

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Presence of ORFs encoding kinase in mycoplasmas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Msy ORF</td>
</tr>
<tr>
<td>Deoxyguanosine kinase</td>
<td>MS0380</td>
</tr>
<tr>
<td></td>
<td>MS0140</td>
</tr>
<tr>
<td></td>
<td>MS0141</td>
</tr>
<tr>
<td>N-acetylmannosamine kinase</td>
<td>MS0195</td>
</tr>
<tr>
<td>Serine/threonine-protein kinase</td>
<td>MS0121</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>MS0648</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>MS0580</td>
</tr>
<tr>
<td>Thymidine kinase</td>
<td>MS0521</td>
</tr>
<tr>
<td>Cytidylate kinase</td>
<td>MS0143</td>
</tr>
<tr>
<td>Guanylate kinase</td>
<td>MS0123</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>MS0114</td>
</tr>
<tr>
<td>Uridylate kinase smbA</td>
<td>MS0677</td>
</tr>
<tr>
<td>6-phosphofructokinase</td>
<td>MS0296</td>
</tr>
<tr>
<td>Acetate kinase</td>
<td>MS0652</td>
</tr>
<tr>
<td>Riboflavin kinase / FMN adenyllytransferase</td>
<td>MS0563</td>
</tr>
<tr>
<td>Thymidylate kinase</td>
<td>MS0052</td>
</tr>
<tr>
<td>Ribose-phosphate pyrophosphokinase</td>
<td>MS0130</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>-</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>-</td>
</tr>
<tr>
<td>5-dehydro-2-deoxygluconokinase</td>
<td>-</td>
</tr>
</tbody>
</table>
of *M. hyopneumoniae* (7448) were also identified in the nonpathogenic strain (J). This finding could be explained by the fact that such enzymatic activities may be essential to Mollicutes which have a reduced metabolism.

**Kinase classification**

The classification of kinases found in *M. hyopneumoniae* strains J and 7448, as well as in *M. synoviae* was performed according to the description of Cheek *et al.* (2005). Here, the definition of kinase was restricted to enzymes which catalyze the transfer of the terminal phosphate group from ATP to a substrate containing an alcohol, nitrogen, carboxyl or phosphate group as phosphoryl acceptor. The classification scheme lists a total of 25 kinase family homologues which are assembled into 12 groups based on the similarity of the structural fold. Within a fold group, the core of the nucleotide-binding domain of each family has the same architecture, and the topology of the protein core is either identical or related by circular permutation (Cheek *et al.*, 2005). In the two *M. hyopneumoniae* strains and in the *M. synoviae* strain the 47 identified ORFs code for 18 different kinases classified in nine families. These were grouped into five fold groups, as shown in Table 2. Fold Group 2 (Rossmann-like) contains 11 enzymes divided into five families, in which all the seven members of the P-loop kinase family are proteins involved in purine and pyrimidine metabolism. The remaining four members of this group are fall into four families which, together with four members of Group 4 and a member of Group 5 (TIM β/α barrel kinase) are involved in the carbohydrate metabolism. Group 1 (Protein S/T-Y kinase) and Group 8 (Riboflavin kinase) are each represented by one enzyme only, which participate in signaling cascades and riboflavin metabolism, respectively.

**Nucleotide metabolism and kinases**

Mollicutes are unable to synthesize purines and pyrimidines by *de novo* pathways, and guanine, guanosine, uracil, thymine, cytidine, adenine and adenosine may serve as precursors for nucleic acids, and nucleotide coenzymes in these organisms (Himmelreich *et al.*, 1996). They only synthesize ribonucleotides by the salvage pathway. In the complete genome of *M. hyopneumoniae* and *M. synoviae* we identified six kinases in the first one and seven kinases in the second one, all of which catalyze key steps in the nucleotide salvage pathway. Deoxyribonucleotides are produced from ribonucleotides by a ribonucleoside diphosphate reductase. Adenine, guanine and uracil can be metabolized to the corresponding nucleoside monophosphate by adenine phosphoribosyltransferase, hypoxanthine-guanine phosphoribosyltransferase and uracil phosphoribosyltransferase, respectively. ADP, GDP, UDP and CDP are generated by adenylate, guanylate, uridylylate and cytidylylate kinases. Only *M. synoviae* has three ORFs encoding deoxyguanosine kinase, which can convert deoxyguanosine to dGMP. However, a nucleotide diphosphate kinase (ndk), the main enzyme for the production of NTP from NDP, was not found in the *M. hyopneumoniae* and *M. synoviae* genomes. This finding is in agreement with data from other Mollicutes genome sequences. It was proposed that the absence of an ndk gene ortholog in Mollicutes could be compensated by 6-phos-

---

**Table 2 - Classification of *M. synoviae* and *M. hyopneumoniae* kinase activities by family and fold group**.

<table>
<thead>
<tr>
<th>Fold Group</th>
<th>Family</th>
<th>PFAM members</th>
<th>Kinase activity (EC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: protein S/T-Y kinase/ atypical protein kinase/ lipid kinase/ ATP-grasp</td>
<td>Protein S/T-Y kinase</td>
<td>PF00069</td>
<td>2.7.1.37 Serine/threonine protein kinase</td>
</tr>
<tr>
<td>Group 2: Rossmann-like</td>
<td>P-loop kinases:</td>
<td>PF00406</td>
<td>2.7.4.3 Adenylate kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF00265</td>
<td>2.7.1.21 Thymidine kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF01712</td>
<td>2.7.1.113 Deoxyguanosine kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF02224</td>
<td>2.7.1.41 Cytidylylate kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF00625</td>
<td>2.7.1.48 Guanylylate kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF00096</td>
<td>2.7.4.14 Uridylylate kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF02223</td>
<td>2.7.4.9 Thymidylylate kinase</td>
</tr>
<tr>
<td></td>
<td>Phosphoglycerate kinase:</td>
<td>PF00162</td>
<td>2.7.2.3 Phosphoglycerate kinase</td>
</tr>
<tr>
<td></td>
<td>Phosphofructokinase-like:</td>
<td>PF00365</td>
<td>2.7.1.11 6-phosphofructokinase</td>
</tr>
<tr>
<td></td>
<td>Ribokinase-like:</td>
<td>PF00294</td>
<td>2.7.1.92 5-dehydro-2-deoxygluconokinase</td>
</tr>
<tr>
<td></td>
<td>Thiamin pyrophosphokinase</td>
<td>PF00156</td>
<td>2.7.6.1 Ribose-phosphate pyrophosphokinase</td>
</tr>
<tr>
<td>Group 4: ribonuclease H-like</td>
<td>Ribonuclease H-like</td>
<td>PF00480</td>
<td>2.7.1.60 N-acetylmannosamine kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF00871</td>
<td>2.7.2.1 Acetate kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF00370</td>
<td>2.7.1.30 Glycerol kinase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF02685</td>
<td>2.7.1.2 Glucokinase</td>
</tr>
<tr>
<td>Group 5: TIM β/α? barrel kinase</td>
<td>TIM β/α? barrel kinase</td>
<td>PF00224</td>
<td>2.7.1.40 Pyruvate kinase</td>
</tr>
<tr>
<td>Group 8: riboflavin kinase</td>
<td>Riboflavin kinase</td>
<td>PF01687</td>
<td>2.7.1.26 Riboflavin kinase</td>
</tr>
</tbody>
</table>

*The classification was based on Cheek *et al.* (2005).*
phofructokinases, phosphoglycerate kinases, pyruvate kinases, and acetate kinases. In addition, besides reactant ADP/ATP, these organisms could use other ribo- and deoxyribo-purine and pyrimidine NDPs and NTPs (Pollack et al., 2002).

Like in *M. penetrans*, important enzymes such as uridine kinase and pyrimidine nucleoside phosphorylase, which convert cytosine to CMP, are also missing in the two species. The synthesis of CTP from UTP by CTP synthetase is possible only in two *M. hyopneumoniae* strains. The production of deoxynucleotide diphosphate from thymidine may be performed by thymidine and thymidylate kinases. A gene encoding ribose-phosphate pyrophosphokinase is present and this enzyme would produce 5-phosphoribosyl diphosphate, a crucial component in nucleotide biosynthesis. All kinases involved in the nucleotide salvage pathway fall into fold Group 2. Moreover, only ribose-phosphate pyrophosphokinase is not in the P-loop kinases family of this group.

Kinases involved in the metabolism of carbohydrates

Both *M. hyopneumoniae* and *M. synoviae* have the entire set of genes responsible for glycolysis. Like in *M. pulmonis* (Chambaud et al., 2001), *M. hyopneumoniae* strain 232 (Minion et al., 2004), and *M. mobile* (Jaffe et al., 2004), glycolysis in *M. hyopneumoniae* J and 7448 can begin by direct phosphorylation of glucose by glucokinase (Group 4; ribonuclease H-like family) activity. Alternatively, as described for other Mollicutes (Fraser et al., 1995; Himmelreich et al., 1996; Glass et al., 2000), *M. synoviae* produces glucose 6-phosphate only by the action of phosphoenolpyruvate-dependent sugar phosphotransferase system. The two species *M. hyopneumoniae* and *M. synoviae* have a 6-phosphofructokinase (Group 2; phosphofructokinase-like family), phosphoglycerate kinase (Group 2; phosphoglycerate kinase family) and pyruvate kinase (Group 5; TIM βατ? barrel kinase family). These three key enzymes also participate in the glycolysis pathway, like in other Mollicutes. In addition, they have an acetate kinase (Group 4; ribonuclease H-like family), an essential enzyme in the production of acetyl-CoA from acetate.

Even though, *M. synoviae* and *M. hyopneumoniae* strains have glycerol transporter-related proteins, only the second species presents a glycerol kinase (Group 4; ribonuclease H-like family) enzyme which could directly convert glycerol to glycerol 3-phosphate. This product is then converted into glyceraldehyde 3-phosphate.

In their amino sugar metabolism, mycoplasmas can produce fructose 6-phosphate (F6P) also from N-acetyl-D-glucosamine. In this pathway, *M. synoviae* N-acetylmannosamine kinase (Group 4; ribonuclease H-like family) catalyzes a key reaction in the production of F6P from N-acetyl-neuraminic. Even though both species lack the inositol metabolism pathway, only *M. hyopneumoniae* presents a 5-dehydro-2-deoxygluconokinase (Group 2; Thiamin pyrophosphokinase family), an enzyme which catalyzes a step in this pathway. The presence of specific kinases in the *M. synoviae* and *M. hyopneumoniae* (strain J and 7448) genomes shows the possibility for the use of different metabolic routes by each mycoplasma in response to the specific nutritional conditions found by each pathogen in its respective host environment.

Riboflavin metabolism and kinases

*M. hyopneumoniae* and *M. synoviae* lack enzymes that synthesize many coenzymes and cofactors. However, they produce Flavine Adenine Dinucleotide (FAD) from riboflavin. This process is performed in two steps where, in the first step, riboflavin kinase phosphorylates riboflavin to form flavin mononucleotide (FMN). Next, FMN is converted to flavin adenine dinucleotide (FAD) by a FMN adenylyltransferase (Karthikeyan, et al., 2003). FAD is an enzyme cofactor used in several metabolic pathways. In *M. synoviae* and *M. hyopneumoniae*, the two steps are performed by a single bifunctional enzyme riboflavin kinase/ FMN adenylyltransferase, as occurs also in bacteria (Mantstein et al., 1986; Mack et al., 1998). It is a unique enzyme and the only representative for fold Group 5.

Amino acid sequence relationships

In order to investigate the phylogenetic relationships of the kinase families of *M. synoviae* 53, *M. hyopneumoniae* J and *M. hyopneumoniae* 7448, the 47 deduced amino acid sequences of the ORFs encoding kinases were aligned using the ClustalX 1.81 program. Robustness of branches was estimated by using 100 bootstrap replicates.

Figure 1 shows the phylogenetic tree for kinases as calculated from the neighbour-joining method. The tree was rooted with Group 1 since it has only one representative. The kinase sequences were well resolved into clades. The P-loop kinase family of Group 2 (Rossmann-like) was clustered into four subclades (Figure 1, letters A, B, C and D). The subclades B and C comprise sequences from *M. synoviae*, *M. hyopneumoniae* J and *M. hyopneumoniae* 7448 implicated in phosphorylation of the monophosphate nucleotides. Thymidylate kinase and deoxiguanosine kinase convert TMP to TDP and deoxiguanosine to dGMP, respectively. Although these enzymes have different functions, they have structurally similar nucleotide-binding domains following the classification described by Cheek et al., (2005). The other members of the Rossmann-like Group, which are the phosphoglycerate kinase, ribokinase-like and thiamine pyrophosphokinase families, clustered in individual groups. The sequences from Group 4 formed four clades. Although belonging to the same fold group they are implicated in different metabolic pathways.

Concluding Remarks

In the complete genomes of *M. synoviae* strain 53, *M. hyopneumoniae* strains J and 7448 we identified kinases in-
involved in many essential metabolic pathways such as carbohydrates, purine, pyrimidine and cofactors metabolism. The presence of those enzymes evidenced the metabolic machinery utilized by these organisms which are considered minimalist models.

Acknowledgments
This work was performed within the Brazilian National Genome Program (Southern Network for Genome Analysis and Brazilian National Genome Project Consortium) with funding provided by MCT/CNPq and SCT/FAPERGS (RS).

References


Figure 1 - Phylogenetic tree obtained from kinase amino acid sequence relationships. The kinase fold groups and families are shown in brackets. The numbers on the branches are bootstrap values obtained with 100 replications. The kinase encoding ORFs are represented by MSkinase (M. synoviae), MHJKinase (M. hyopneumoniae J) and MHPKinase (M. hyopneumoniae 7448).


Internet Resources

M. synoviae complete genome database, http://www.brgene.lncc.br/finalMS/.


Database of protein families (Pfam), http://www.sanger.ac.uk/Software/Pfam/.

InterProScan software, http://www.ebi.ac.uk/InterProScan/.

Associate Editor: Arnaldo Zaha
Identification of the GTPase superfamily in *Mycoplasma synoviae* and *Mycoplasma hyopneumoniae*

Clayton Luiz Borges, Juliana Alves Parente, Maristela Pereira and Célia Maria de Almeida Soares

Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Goiás, Brazil.

**Abstract**

Mycoplasmas are the smallest known prokaryotes with self-replication ability. They are obligate parasites, taking up many molecules of their hosts and acting as pathogens in men, animals, birds and plants. *Mycoplasma hyopneumoniae* is the infective agent of swine mycoplasmosis and *Mycoplasma synoviae* is responsible for subclinical upper respiratory infections that may result in airsacculitis and synovitis in chickens and turkeys. These highly infectious organisms present a worldwide distribution and are responsible for major economic problems. Proteins of the GTPase superfamily occur in all domains of life, regulating functions such as protein synthesis, cell cycle and differentiation. Despite their functional diversity, all GTPases are believed to have evolved from a single common ancestor. In this work we have identified mycoplasma GTPases by searching the complete genome databases of *Mycoplasma synoviae* and *Mycoplasma hyopneumoniae*, J (non-pathogenic) and 7448 (pathogenic) strains. Fifteen ORFs encoding predicted GTPases were found in *M. synoviae* and in the two strains of *M. hyopneumoniae*. Searches for conserved G domains in GTPases were performed and the sequences were classified into families. The GTPase phylogenetic analysis showed that the subfamilies were well resolved into clades. The presence of GTPases in the three strains suggests the importance of GTPases in 'minimalist' genomes.

**Key words:** Mycoplasma, GTPase superfamily, genome.

Received: April 12, 2006; Accepted: October 10, 2006.

**Introduction**

Mycoplasmas are a genus of obligate parasites belonging to the Mollicutes class, the smallest known prokaryotes with self-replication ability (Razin *et al.*, 1998). They present a very small genome evolved to the minimalist status by losing non-essential genes, including those involved in cell wall synthesis, as well those related to catabolic and metabolic pathways (Himmelreich *et al.*, 1996). The two species, *Mycoplasma hyopneumoniae* and *Mycoplasma synoviae*, are responsible for significant economic impact on animal production. *M. hyopneumoniae* is the infective agent of swine mycoplasmosis (DeBey and Ross, 1994), which increases the susceptibility to secondary infections (Ciprian *et al.*, 1988). *M. synoviae* is responsible for subclinical upper respiratory infections, but may also result in airsaccultis and synovitis in chickens and turkeys (Kleven, 1997; Allen *et al.*, 2005).

Many crucial functions for life are provided by a single versatile mechanism that has evolved to fulfill many roles. A prime example is the GTPase superfamily of proteins that occurs in all domains of life, regulating functions such as protein synthesis, cell cycle and differentiation (Bourne *et al.*, 1990). Despite this extraordinary functional diversity, all GTPases are believed to have evolved from a single common ancestor, a fact which resulted in the conservation of their action mechanism, of the core structure and of sequence motifs (Bourne, 1995).

GTPases are often described as molecular switch proteins because of their particular mode of action. Each GTPase specifically binds and hydrolyzes GTP in a cyclic mechanism that activates and inactivates the GTPase protein (Bourne *et al.*, 1991). In this cycle, a GTPase passes through three conformational states. Initially, the GTPase is inactive and is not bound to any nucleotide. After binding GTP, the protein becomes active and changes its conformation, and as such its affinity to effector molecules or other enzymes. GTP is then hydrolyzed simultaneously, with an effect being generated in the GTPase target. Subsequently, GDP is released from the inactive GTPase, returning the protein to the empty state. This cycle allows the active GTPase to interact periodically with a target and, in this
way, to act as a timed switch in the cell (Bourne et al., 1990).

That cyclic reaction usually involves several other factors that either catalyze the hydrolysis step of the GTPase cycle or catalyze the release of bound GDP from the inactive state of the GTPase (Bourne, 1995). Each GTPase cycle appears to be unique. The rate of switch turnover is dependent on specific interaction factors, as well as on the intrinsic properties of each GTPase. Additionally, some GTPases interact with many different effectors and targets and, in that way, can coordinate cellular responses (Bourne et al., 1990; Bourne, 1995). A core domain that is able to bind either GDP or GTP confers the characteristic switch mechanism of GTPases. The folding of this domain is a defining feature of GTPases (Jurnak et al., 1990). In fact, X-ray crystallography of diverse GTPases shows that the folding of this G-domain is nearly invariant throughout the GTPase superfamily. GTPases can consist solely of the G-domain or may have additional domains on the amino- and carboxyl-terminal ends of the proteins (Sprang, 1997).

Due to the importance of the mycoplasmas, complete genome projects have been reported in the last years (Himmelreich et al., 1996; Hutchison et al. 1999; Glass et al., 2000; Chambaud et al., 2001; Papazisi et al., 2003; Sasaki et al., 2002; Jaffe et al., 2004; Minion et al., 2004; Westberg et al., 2004). Complete genomes of M. synoviae (strain 53), M. hyopneumoniae pathogenic strain (7448) and non-pathogenic strain (J [ATCC25934]) were recently described (Vasconcelos, et al., 2005) and the data are available in databases. The objective of this work is the identification and classification of the GTPase superfamily in the three complete genomes of M. synoviae strain 53 and M. hyopneumoniae (strains J and 7448).

Material and Methods

By using data from the complete genome of M. synoviae and M. hyopneumoniae, strains J and 7448 associated to BLAST search tools we have identified 15 ORFs encoding GTPase superfamily homologs in M. synoviae, as well as 15 ORFs in both strains of M. hyopneumoniae. Classification of the GTPase families and their putative function has been performed by using Pfam interface and InterPro homepage. Search for G-domains in mycoplasma GTPases was performed by alignment of described Escherichia coli GTPases sequences (Caldon et al., 2001) with those of M. synoviae and M. hyopneumoniae (strains J and 7448). Multiple sequence alignments were generated using the ClustalX 1.81 software (Thompson et al., 1997).

The phylogenetic relationships within the GTPase superfamily were inferred from all 33 sequences from M. synoviae strain 53 and M. hyopneumoniae strains J and 7448. A phylogenetic tree was constructed by multiple sequence alignments using the Clustal X program and visualized by using the Tree View software. Trees were constructed by using the neighbor-joining method (Saitou and Nei, 1987). Robustness of branches was estimated by using 100 bootstrap replicates.

Results and Discussion

Structural analysis of the GTPases superfamily

Searches for GTPases performed on M. synoviae and M. hyopneumoniae strains J and 7448 genome databases revealed the presence of 15 GTPase orthologs. These GTPases were classified into subfamilies, and the results are shown in Table 1. ORFs were classified as belonging to the Elongation factor, the Era, the FtsY/Ffh and the Obg/YchF subfamilies, or were annotated as unclassified proteins related to GTPases or GTP binding proteins.

Searches for the G-domain, described in all GTPase subfamilies, was performed by using the deduced protein sequences encoded by the identified ORFs presented in Table 1. Figure 1 presents the alignment of the G1-G4 motifs of the cited GTPases. The G-domain is divided into four G motifs: G1 (G/AXXXXGKT/S), G2 (not conserved), G3 (DXXG) and G4 (NKXD) sequence motifs, where X denotes any amino acid (Caldon, et al., 2001). The G1, G2 and G3 motifs were found in all mycoplasma GTPase subfamilies (Figure 1). The G4 motif was found in the EF-G, EF-Tu, IL-2, LepA, Era, EngA, ThdF/TmE, and OBG subfamilies. In the YchF, FtsY and Ffh subfamilies, the region of the G4 motif, although present, was not well conserved (Figure 1).

Functions ascribed to G-motifs include the mediation of interactions with the guanine nucleotides and effector proteins. It has been suggested that G1, G3 and G4 motifs could have evolved to bind and hydrolyze guanosine triphosphate and also for interacting with the cofactor Mg2+ (Bourne et al., 1991). The non conserved G2 motif is described as the effector domain that undergoes a conformational change necessary for GTPase function (Bourne et al., 1995, Sprang, 1997).

Elongation factor subfamily

The elongation factor subfamily (EF) is composed of the Elongation factor G (EF-G), Elongation factor-TU (EF-TU), Initiation factor-2 (IF-2) and GTP-binding protein LepA (LepA), (Caldon, et al. 2001). The EF family from bacteria is composed of multidomain GTPases with essential functions in the elongation and initiation phases of translation. EF-Tu catalyzes binding of aminoacyl-tRNA to the ribosomal A-site, while EF-G catalyses the translocation of peptidyl-tRNA from the A-site to the P-site (Rodnina et al., 2000; Nilsson and Nissen, 2005). The initiation factor-2 (IF-2) may be involved in introducing the initiator tRNA into the translation machinery and in performing the first step in the peptide chain elongation cycle (Kyprides and Woese, 1998). ORFs encoding all elongation factor members were present in M. synoviae and M. hyopneumoniae J and 7448 (Table 1). All G1-4 motifs were
Table 1 - ORFs encoding GTPases and GTP binding proteins from *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448, with putative functions.

<table>
<thead>
<tr>
<th>GTPase Family</th>
<th>ORF Product</th>
<th>EC /Cellular process involvement</th>
<th>ORFs encoding GTPases found in Mycoplasmas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Mycoplasma synoviae 53</em></td>
</tr>
<tr>
<td>Elongation factor subfamily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF-G</td>
<td>Elongation factor EF-G</td>
<td>3.6.1.48 / protein biosynthesis</td>
<td>MS0047</td>
</tr>
<tr>
<td>EF-TU</td>
<td>Elongation factor Tu</td>
<td>3.6.1.48 / protein biosynthesis</td>
<td>MS0067</td>
</tr>
<tr>
<td>IF-2</td>
<td>Translation initiation factor IF-2</td>
<td>/ / Binding / protein biosynthesis</td>
<td>MS0068</td>
</tr>
<tr>
<td>LepA</td>
<td>GTP-binding protein LepA</td>
<td>/ / Protein biosynthesis</td>
<td>MS0489</td>
</tr>
<tr>
<td>Era subfamily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Era</td>
<td>GTP-binding protein Era</td>
<td>/ / ATP Binding / nucleic acid binding</td>
<td>MS0387</td>
</tr>
<tr>
<td>EngA</td>
<td>GTP-binding protein EngA</td>
<td>/ / 70S ribosome stabilization</td>
<td>MS0142</td>
</tr>
<tr>
<td>ThdF/TrmE</td>
<td>Thiophene and furan oxidation protein ThdF</td>
<td>/ / rRNA processing / indirect Ribosome function</td>
<td>MS0362</td>
</tr>
<tr>
<td>FtsY/Ffh subfamily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FtsY</td>
<td>Cell division protein FtsY</td>
<td>/ / Cell division</td>
<td>MS0145</td>
</tr>
<tr>
<td>Ffh</td>
<td>Signal recognition particle, subunit FFH/SRP54</td>
<td>/ / Protein targeting to membrane</td>
<td>MS0201</td>
</tr>
<tr>
<td>OBG and YchF subfamily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OBG</td>
<td>GTP-binding protein Obg</td>
<td>/ / Ribosome maturation.</td>
<td>MS0168</td>
</tr>
<tr>
<td>YchF</td>
<td>GTP-binding protein YchF</td>
<td>/ / Putative ATP Binding</td>
<td>MS0663</td>
</tr>
<tr>
<td>Unclassified</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP-binding protein</td>
<td>/ / Cell division</td>
<td>MS0650 - YihA</td>
<td>MHJ0446 - YihA</td>
</tr>
<tr>
<td>Cell division protein</td>
<td>/ / Cell division</td>
<td>MS0340 - FtsZ</td>
<td>MHJ0406 - FtsZ</td>
</tr>
<tr>
<td>Probable GTPase</td>
<td>EC 3.6.1- / unknown</td>
<td>MS0120 - EngC</td>
<td>MHJ0148 - EngC</td>
</tr>
<tr>
<td>Putative GTP-binding</td>
<td>/ / ATP Binding</td>
<td>MS0664 - YlqF</td>
<td>MHJ0083 - YlqF</td>
</tr>
</tbody>
</table>

found in the ORFs encoding EF GTPases from both mycoplasma species (Figure 1), suggesting that the proteins can be functional in these organisms. Two truncated hypothetical EF-G proteins were also found in the *M. synoviae* genome. The ORFs present high homology to the 3' region of the complete EF-G ORF found in this organism, suggesting that they are not functional genes, in accordance with the ‘minimal genome’ characteristic of mycoplasmas.

**Era subfamily**

This family is comprised of the GTP binding protein ERA (ERA), the GTP binding protein EngA (EngA), as well as the Thiophene and furan oxidation protein (ThdF). Both *M. synoviae* and *M. hyopneumoniae* (J and 7448) present ORFs related to the Era subfamily. The Era member of the Era subfamily is an essential GTPase that probably regulates the cell cycle (Gollop and March, 1991; Britton et al., 1998) and is involved in regulating carbon (Lerner and Inouye, 1991) and nitrogen (Powell et al., 1995) metabolism. A second member of this group, EngA, has been suggested to be essential for growth in *Neisseria gonorrhoeae* (Mehrt al., 2000). ThdF may be involved in tRNA modification and in the direct or indirect regulation of ribosome function (Caldon, et al., 2001). The presence of all Era subfamily members (Table 1) with all G1-G4 motifs (Figure 1) in *M. synoviae* and *M. hyopneumoniae* (J and 7448) suggests that those ORF products are active and play biological functions in the analyzed organisms.

**FtsY/Ffh subfamily**

The FtsY/Ffh subfamily is represented by the cell division protein FtsY, termed FtsY, and by the signal recognition particle FFH/SRP54, termed Ffh. ORFs encoding for the two proteins of this subfamily have been reported in the *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448 (Table 1). The G1-G3 motifs were found in the deduced amino acid sequences for FtsY and Ffh of *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448, when compared with *E. coli* FtsY/Ffh sequences (Figure 1). The sequence corresponding to the G4 motif was found in the three analyzed mycoplasmas, even though this motif was not well conserved (NKXD). The amino acids K and D are present in mycoplasma FtsY and Ffh sequences in comparison to the *E. coli* ortholog predicted proteins. These proteins are described as essential to *E. coli* since Ffh/SRP mutants present a lethal phenotype and SRP subunit mutants present growth defects (Lu, et al., 2001).

**OBG and YchF subfamily**

The comparative analysis of *M. synoviae* strain 53, *M. hyopneumoniae* (strains J and 7448) showed the presence of the same ortholog ORFs encoding OBG and YchF proteins (Table 1). G1-G3 motifs were found in all ORF products. The G4 motif was found in the OBG member, but not in the YchF ORF product (Figure 1). Similarly, this motif was also not found well conserved in the *E. coli* YchF protein.
The function of the OBG subfamily remains elusive, although there is evidence for its involvement in the initiation of chromosome replication (Kok et al., 1994), in bacterial sporulation (Trach and Hoch, 1989; Vidwans et al., 1995), and in the activation of a transcription factor that controls the general stress response (Scott and Haldenwang, 1999). The YchF members of the OBG/YchF subfamily are also distributed in all domains of life (Mittenhuber, 2001), but the biological function of this protein has not been elucidated.

Unclassified GTPases

The GTPases found in the genomes of mycoplasmas which were not classified as belonging to one of the 11 universally conserved bacterial GTPases (Caldon, et al., 2001) were described here as unclassified. Four ORFs from M. synoviae strain 53 and M. hyopneumoniae strains J and 7448 were identified in this group: EngC, YlqF, FtsZ and YihA. The E. coli ortholog EngC is a GTPase with a predicted role as a regulator of translation (Daigle and Brown, 2004). The putative GTP binding protein YlqF is described as necessary for growth of Streptococcus pneumoniae and Staphylococcus aureus and may be involved in ribosomal assembly (Zalacain et al., 2003).

The cell division protein FtsZ was also found in M. synoviae strain 53 and M. hyopneumoniae strains J and 7448. This protein appears to act at the earliest step in cell septation and is required at the final steps of cytokinesis (Ma, et al., 1996; Jensen, et al., 2005). The GTPase YihA has been described as an essential gene of the bacterial “minimal genome”, even though it seems to be dispensable in some organisms, as described for Mycobacterium tuberculosis, Chlamydia trachomatis, Treponema pallidum, Borrelia burgdorferi and Synechocystis sp. (Dassain et al., 1999).

GTPase amino acid sequence relationships

To visualize the amino acid sequence relationship of Mycoplasma GTPase subfamilies, a phylogenetic tree was constructed by using the neighbour-joining method (Saitou and Nei, 1987). A total of 33 deduced amino acid sequences encoding GTPases from M. synoviae, M. hyopneumoniae J and M. hyopneumoniae 7448 were aligned using the CLUSTAL X program (Thompson et al., 1997). Robust-
ness of branches was estimated by using 100 bootstrap replicates. By using the Tree View software a deduced phylogeny was visualized and is shown in Figure 2. A close relationship among amino acid sequences of proteins which belong to the same subfamily can be observed in the three Mycoplasma species. GTPases that have similar functions were clustered into the same clade, suggesting a metabolic conservation in reactions involving GTPases. The bootstrap values reveal the high homology among the subfamilies of proteins of *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448. GTPases are classified into subfamilies based on the presence of different G-domains (G1, G2, G3 and G4). Since unclassified GTPases do not present conserved G-domains, and were not classified by Caldon et al. (2001), they were not included in our phylogenetic analysis.

**Concluding Remarks**

The GTPase superfamily, present in all domains of life, is related to many functions such as protein synthesis, cell cycle and differentiation. The presence of orthologs for all the subfamily members described in prokaryotes in the complete genome of *M. synoviae* and *M. hyopneumoniae* strains J and 7448, evidences the essential functions of GTPases in these ‘minimalist’ organisms.

**Acknowledgments**

This work was carried out in the context of the Brazilian National Genome Program (Southern Network for Genome Analysis and Brazilian National Genome Project Consortium) with funding provided by MCT/CNPq and SCT/FAPERGS (RS).

**Abbreviations**

EF-G (Elongation factor G).  
EF-TU (Elongation factor Tu).  
IF-2 (Translation initiation factor 2).  
MHJ (*Mycoplasma hyopneumoniae* strain J).  
MHP (*Mycoplasma hyopneumoniae* strain 7448).  
MS (*Mycoplasma synoviae* strain 53).  
ThdF (Thiophene and furan oxidation protein).

**References**


Internet Resources

M. synoviae complete genome database, http://www.brgene.lncc.br/finalMS/.


Database of protein families (Pfam), http://www.sanger.ac.uk/Software/Pfam/.

InterProScan software, http://www.ebi.ac.uk/InterProScan/.

Associate Editor: Darcy F. de Almeida
The transcriptional profile of Paracoccidioides brasiliensis yeast cells is influenced by human plasma

Alexandre Melo Bailão1, Augusto Shrank2, Clayton Luiz Borges1, Juliana Alves Parente1, Valéria Dutra2, Maria Sueli Soares Felipe4, Rogério Bento Fiúza1, Maristela Pereira1 & Célia Maria de Almeida Soares1

1Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Goiás, Brazil; 2Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil; and 3Laboratório de Biologia Molecular, Universidade de Brasília, Brazil

Correspondence: Almeida Soares, Célia Maria de Almeida Soares, Laboratório de Biologia Molecular, ICB II, Campus II-Universidade Federal de Goiás, 74001-970, Goiânia-Goiás, Brazil. Tel./fax: +55 62 3521 1110; e-mail: celia@icb.ufg.br

Received 24 January 2007; revised 17 April 2007; accepted 25 April 2007. First published online 30 June 2007.

DOI:10.1111/j.1574-695X.2007.00277.x

Editor: Alex van Belkum

Keywords
Paracoccidioides brasiliensis; transcription; human plasma.

Abstract
Paracoccidioides brasiliensis causes infection through host inhalation of airborne propagules of the mycelial phase of the fungus, which reach the lungs, and then disseminate to virtually all parts of the human body. Here we describe the identification of differentially expressed genes in P. brasiliensis yeast cells, by analyzing cDNA populations from the fungus treated with human plasma, mimicking superficial infection sites with inflammation. Our analysis identified transcripts that are differentially represented. The transcripts upregulated in yeast cells during incubation in human plasma were predominantly related to fatty acid degradation, protein synthesis, sensing of osmolarity changes, cell wall remodeling and cell defense. The expression pattern of genes was independently confirmed.

Introduction
Paracoccidioides brasiliensis is an important human pathogen causing paracoccidioidomycosis, a systemic mycosis with broad distribution in Latin America (Restrepo et al., 2001). Although the area of incidence ranges nonuniformly from Mexico to Argentina, the incidence of disease is higher in Brazil, Venezuela and Colombia (Blotta et al., 1999). The fungus is thermomorphic; that is, it grows as a yeast-like structure in the host tissue or when cultured at room temperature (18–23 °C). The infection is caused by inhalation of airborne propagules of the mycelial phase of the fungus, which reach the lungs and differentiate into the yeast parasitic phase (Lacaz, 1994).

During infection, P. brasiliensis can be exposed to human plasma. After host inhalation of mycelial propagules and fungal establishment in the lungs, it can be disseminated through the bloodstream. Additionally, the fungus can promote infection in superficial sites that contain plasma as a consequence of vascular leakage (Franco, 1987). We are just beginning to understand the fungal adaptations to the host during P. brasiliensis infection. We have previously identified a set of candidate genes that P. brasiliensis may express to adapt to the host conditions. We have demonstrated that P. brasiliensis switches gene expression in response to infection in mouse liver, resulting in the overexpression of transcripts coding mainly for genes involved in transport facilitation and cell defense. The yeast fungal cells adapt to the blood environment by overexpressing transcripts related to general metabolism, with emphasis on nitrogen metabolism, protein synthesis, and osmosensing (Bailão et al., 2006).

The present study examined the effects of human plasma on the P. brasiliensis transcriptional profile using cDNA representational difference analysis (cDNA-RDA), which is a powerful application of subtractive hybridization and is considered to reflect a large number of relevant gene transcripts (Hubank & Schatz, 1994). The results show a profound influence of plasma on P. brasiliensis gene expression, suggesting genes that could be essential for fungal adaptation to this host condition.

Materials and methods
Paracoccidioides brasiliensis growth conditions
Paracoccidioides brasiliensis isolate 01 (ATCC MYA-826) has been studied at our laboratory (Bailão et al., 2006; Barbosa et al., 2006). It was grown in the yeast phase at 36 °C, in...
Fava-Neto’s medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) protease peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 1% (w/v) agar; pH 7.2] for 7 days.

**Incubation of *P. brasiliensis* yeast cells in human plasma**

Human blood from 10 healthy donors was collected by venepuncture using heparinized syringes, and centrifuged at 1000 g. *Paracoccidioides brasiliensis* yeast cells were harvested from 7-day-old cultures, and washed twice with phosphate-buffered saline (PBS) (NaCl 137 mM, KCl 2.7 mM, NaH₂PO₄ 1.4 mM, Na₂HPO₄ 4.3 mM, pH 7.4). The fungal cells (5 × 10⁶ cells mL⁻¹) were inoculated into 7.5 mL of human plasma and incubated for several time intervals at 36 °C with shaking. The fungal cells were collected by centrifugation for 5 min at 1500 g, and washed five times with PBS. As controls, *P. brasiliensis* yeast cells from Fava-Neto’s cultures washed five times with PBS and 7.5 mL of the same plasma were taken to prepare control cDNA samples.

**RNA extractions, subtractive hybridization and generation of subtracted libraries**

Total RNA of the *P. brasiliensis* control yeast cells and of yeast cells incubated with human plasma for 10 and 60 min was extracted by the use of Trizol reagent (GIBCO, Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The quality of RNA was assessed by use of the A₂₆₀ nm/ A₂₈₀ nm ratio, and by visualization of rRNA on 1.2% agarose gel electrophoresis. The RNAs were used to construct double-stranded cDNAs. For subtractive hybridization, 1.0 μg of total RNAs was used to produce double-stranded cDNA using the SMART PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA). First-strand synthesis was performed with reverse transcriptase (RT Superscript II, Invitrogen, CA, USA), and the first strand was used as a template to synthesize the second strand of cDNA. The resulting cDNAs were digested with the restriction enzyme Sau3AI. Two subtracted cDNA libraries were made using driver cDNA from 7-day-old-cultures of yeast cells and tester cDNAs synthesized from RNAs extracted from *P. brasiliensis* obtained from yeast cells after incubation with human plasma for 10 and 60 min. The resulting products were purified using the GFX kit (GE Healthcare, Chalfont St Giles, UK). The cDNA representation analysis described by Hubank & Schatz (1994) was used, as modified by Dutra et al. (2004). The tester-digested cDNA was bound to adapters (a 24-mer annealed to a 12-mer). For generation of the differential products, ‘tester’ and ‘driver’ cDNAs were mixed, hybridized at 67 °C for 18 h, and amplified by PCR with the 24-mer oligonucleotide primer (Dutra et al., 2004; Bailão et al., 2006). Two successive rounds of subtraction and PCR amplification using hybridization tester/driver ratios of 1:10 and 1:100 were performed to generate second differential products. Adapters were changed between cross-hybridizations, and differential products were purified using the GFX kit. The adapters used for subtractive hybridizations were: NBam12, GATCTTCCTCTCGG; NBam24, AGGC AACGTGTCTTATCGAGGAGG; RBam12, GATCTTCGGTGGA; and RBam24, AGCACTCTCCGCGCTTTCTTCACCGAG.

After the second subtractive reaction, the final amplified cDNA pools were submitted to electrophoresis in 2.0% agarose gels, and the purified cDNAs were cloned directly into the pGEM-T Easy vector (Promega, Madison, USA). *Escherichia coli* XL1 Blue competent cells were transformed with the ligation products. Selected colonies were picked and grown in microliter plates. Plasmid DNA was prepared from clones using standard protocols. In order to generate the expressed sequence tags (ESTs), single-pass, 5’-end sequencing of cDNAs by standard fluorescence labeling dye-terminator protocols with T7 flanking vector primer was performed. Samples were loaded onto a MegaBACE 1000 DNA sequencer (GE Healthcare) for automated sequence analysis.

**Sequences, processing and EST database construction**

EST sequences were preprocessed using the phred (Ewing & Green, 1998) and crossmatch programs (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm). Only sequences with at least 100 nucleotides and phred quality ≥ 20 were selected. ESTs were screened for vector sequences against the UniVec data. The resulting sequences were then uploaded to a relational database (MySQL) on a Linux (Fedora Core 2) platform, and processed using a modified version of the PHOREST tool (Ahren et al., 2004). PHOREST is a web-based tool for comparative studies across multiple EST libraries/projects. It analyzes the sequences by running the BLAST (Altschul et al., 1990) program against a given database, and assembling the sequences using the CAP (Huang, 1992) program. PHOREST has been modified to store the BLAST results of many databases, to query translated frames against the InterPro database (Mulder et al., 2003), and to work with CAP3 (Huang & Madan, 1999) instead of CAP.

To assign functions, the valid ESTs and the assembled consensus sequences were locally compared against a non-redundant protein sequence database with entries from GO (http://www.geneontology.org), KEGG (http://www.genome.jp.kegg) and NCBI (http://www.ncbi.nlm.nih.gov), using the BLASTX algorithm with an e-value cut-off at 10⁻⁵. If the EST sequences did not match any database sequences, the Blastn algorithm was used (www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990).

Sequences were placed into three categories: (1) annotated, which corresponds to sequences showing significant
matches with protein sequences with an identified function in databanks; (2) hypothetical protein, which corresponds to sequences for which the e-value was >10\(^{-5}\), or for which no match was observed in databanks; or (3) conserved hypothetical protein, which corresponds to protein group sequences for which significant matches (e < 10\(^{-5}\)) and homology to a protein with no identified function was observed.

ESTs were grouped into 99 clusters, represented by 63 contigs and 36 singlets. With cap3 assembly information stored in the relational database, SQL queries were performed to determine transcripts unique to a certain EST library and/or present in two or more libraries. Sequences were grouped in functional categories according to the classification of the MIPS functional catalog (Munich Center for Protein Sequences; http://www.mips.gst.de/). The clusters were compared with P. brasiliensis ESTs upregulated during incubation of yeast cells with human blood (Bailão et al., 2006) (GenBank accession numbers EB085193–EB086102) and with the P. brasiliensis transcriptome database (https://dna.biomol.unibr.br/Pb/) using the BLAST program (Altschul et al., 1990). The nucleotide sequences reported here are available in the GenBank database under the accession numbers EH643296–EH643872.

**In silico determination of overexpressed genes in human plasma in comparison to human blood incubation of P. brasiliensis yeast cells by electronic Northern blotting**

To assign a differential expression character, the contigs formed with the human plasma and the human blood treatment ESTs were statistically evaluated using the method of Audic & Claverie (1997). Genes in the human plasma treatment that were more expressed as determined with a 95% confidence rate compared to human blood were considered overregulated. A website (http://igs-server.cnrs-mrs.fr) was used to compute the probability of differential regulation.

**Dot-blot analysis**

Plasmid DNAs of selected clones were obtained. Serial dilutions of DNAs were performed, and the material was applied, under vacuum, to Hybond-N+ nylon membranes (GE Healthcare). The DNAs were hybridized to cDNAs, which were obtained under specific conditions, labeled using the Random Prime labeling module (GE Healthcare). Detection was performed using the Gene Image CDP-Star detection module (GE Healthcare). The probes used were as follows: aromatic l-amino acid decarboxylase (ddc); translation elongation factor 1, gamma chain (eEF-1γ); serine proteinase (pr1H); glutamine synthetase (gln1); ferric reductase (fre2); transmembrane osmosensor (shol); acidic amino acid permease (dip5); and eukaryotic translation initiation factor 4A (eIF-4A).

**Semiquantitative reverse transcriptase (RT)-PCR analysis**

Semiquantitative RT-PCR experiments were also performed to confirm the RDA results and the reliability of our approaches. Yeast cells of P. brasiliensis treated with human plasma, as well as control yeast cells, were used to obtain total RNAs. These RNAs were obtained from experiments independent of those used in the cDNA subtraction. The single-stranded cDNAs were synthesized by reverse transcription towards total RNAs, using the Superscript II RNaseH reverse transcriptase, and PCR was performed using cDNA as the template in a 30-μL reaction mixture containing specific primers, sense and antisense, respectively, as follows: endoplasmic reticulum to Golgi transport vesicle protein (erv46), 5′-CTATTTTGTTGGTAGTTGTC-3′ and 5′-TTCTCTGGTGGTGGT-3′; pyridoxamine phosphate oxidase (ppol), 5′-CATCAGCAGACTGCCCTC-3′ and 5′-GGAGGTCCCTGTTGGTCT-3′; putative major facilitator protein (pmt1), 5′-CGATTCTCGGAA TTTGTCAC-3′ and 5′-GGTCGGCCCAATGAGTTC-3′; eukaryotic release factor 1 (epF-1), 5′-CAACGTTGACCT TGCATTGG-3′ and 5′-CCATGAGCTTGTATTGG-3′; eukaryotic translation initiation factor 4A (eIF-4A), 5′-GCTCTGGAGGGAGTGTC-3′ and 5′-CC TTTGTGGAGAGGAGTGTC-3′; and ribosomal L34 protein (l34), 5′-CAAGACTCAGCGCCCAAC-3′ and 5′-GCCGCCGCAATG ACTGAGC-3′. The reaction mixture was incubated initially at 95 °C for 1 min, and this was followed by 25–35 cycles of denaturation at 95 °C for 1 min, annealing at 55–65 °C for 1 min, and extension at 72 °C for 1 min. The annealing temperature and the number of PCR cycles were optimized in each case to ensure that the intensity of each product fell within the exponential phase of amplification. The DNA product was separated by electrophoresis in 1.5% agarose gel, stained, and photographed under UV light illumination. The analyses of relative differences were performed with the SCION IMAGE BETA 4.03 program (http://www.scioncorp.com).

**Protein extract preparation and Western blot analysis**

Protein extracts were obtained from P. brasiliensis yeast cells incubated with human plasma for 1 and 12 h. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% polyacrylamide gels. The protein extracts were electrophoresed and transferred to membranes. The membranes were incubated in 0.05%
Measurement of formamidase activity

Formamidase activity was measured by monitoring the appearance of ammonia, as previously described (Skouloubris et al., 1997; Borges et al., 2005). Briefly, samples of 50 μL (0.2 μg of total protein) were added to 200 μL of formamide substrate solution at a final concentration of 100 mM in 100 mM phosphate buffer (pH 7.4) and 10 mM EDTA. The reaction mixture was incubated at 37°C for 30 min; then, 400 μL of phenol-nitroprusside and 400 μL of alkaline hypochlorite (Sigma Aldrich, Co.) were added, and the samples were incubated for 6 min at 50°C. Absorbance was then read at 625 nm. The amount of ammonia released was determined from a standard curve. One unit (U) of formamidase activity was defined as the amount of enzyme required to hydrolyze 1 μmole of formamide (corresponding to the formation of 1 μmole of ammonia) per minute per milligram of total protein.

SDS sensitivity tests

For SDS sensitivity assays, yeast cells were incubated with human plasma for 1, 12 and 24 h. Cells were washed five times in 1 × PBS, and 10^2 cells were spotted in 5 μL onto Fava-Neto’s medium containing SDS at the indicated concentration. Plates were incubated at 36°C for 7 days. Controls were obtained using 10^2 cells of yeast forms grown for 7 days and subjected to the same washing conditions.

Results

Plasma incubation induces a specific transcriptional response in *P. brasiliensis* yeast cells

The RDA approach was performed between the yeast control fungal cells (driver) and the yeast cells treated with human plasma for 10 and 60 min (testers). Subtraction was performed by incubating the driver and the testers. Selection of the cDNAs was achieved by construction of subtracted libraries in pGEM-T Easy, as described earlier. Figure 1 shows the RDA products of the two conditions of subtraction. Different patterns of DNA amplification were observed after two cycles of RDA, as shown.

In total, 577 clones were successfully sequenced. Of these, 303 were obtained from incubation of fungus in human plasma for 10 min, and 274 were obtained from yeast cells after incubation in human plasma for 60 min. Using the BLASTX program, 2.25% of the ESTs would correspond to proteins of unknown function, with no matches in databases. In addition, 97.93% of the ESTs displayed significant similarity to genes in the *P. brasiliensis* database (https://dna.biomol.unb.br/Pb/), whereas 2.07% did not show similarity to known *P. brasiliensis* genes.

The nature of adaptations made by *P. brasiliensis* during treatment in human plasma can be inferred by classifying the ESTs into 11 groups of functionally related genes (Table 1). We analyzed the redundancy of the transcripts by determining the number of ESTs related to each transcript. The most redundant cDNAs appearing during human plasma treatment for 10 min were as follows: ddc (59 ESTs), eEF-1γ (38 ESTs), sho1 (18 ESTs), gln1 (18 ESTs), pr1H (13 ESTs), and Ap-1-like transcription factor (meab) (11 ESTs). After 60 min of incubation in human plasma, the most abundant transcripts were those encoding eIF-4A (35 ESTs), SHO1 (23 ESTs) eEF-1γ (19 ESTs), PR1H (14 ESTs), FRE2 (12 ESTs), and DIP5 (12 ESTs), as shown in Table 1.

In addition, a comparison was performed between upregulated transcripts appearing during human plasma incubation and those present during yeast cell incubation in human blood (Bailão et al., 2006). The same batch of blood was used to prepare human plasma and for the incubation of yeast cells in total blood. Table 1 gives the genes
<table>
<thead>
<tr>
<th>MIPS category</th>
<th>Gene product</th>
<th>Best hit/accession number</th>
<th>e-value</th>
<th>P10 (%)</th>
<th>P60 (%)</th>
<th>Redundancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td>2-Methylcitrate dehydratase (MCD)</td>
<td><em>Neurospora crassa</em> [C0]</td>
<td>1e−95</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-Aminolevulinic acid synthase*</td>
<td><em>Aspergillus oryzae</em> [C0]</td>
<td>6e−70</td>
<td>1</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetolactate synthase (ILV2)*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>3e−63</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adenine phosphoribosyltransferase*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>1e−60</td>
<td>−</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aldehyde dehydrogenase</td>
<td><em>Emericella nidulans</em> [C0]</td>
<td>4e−42</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anthranilate synthase component II*</td>
<td><em>Aspergillus fumigatus</em> [C0]</td>
<td>1e−58</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aromatic L-Amino-acid decarboxylase (DDC)*</td>
<td><em>Gibberella zeae</em> [C0]</td>
<td>5e−63</td>
<td>59</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Formamidase*</td>
<td><em>P. brasiliensis</em> [C0]</td>
<td>1e−82</td>
<td>−</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamine synthetase (GLN1)</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>1e−107</td>
<td>18</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inosine-5-monophosphate dehydrogenase*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>6e−71</td>
<td>1</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADPH-quinone reductase*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>2e−81</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oleate delta-12 desaturase*</td>
<td><em>Aspergillus fumigatus</em> [C0]</td>
<td>6e−85</td>
<td>3</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyridoxamine 5'-phosphate oxidase (PPO1)</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>3e−90</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sphingosine-1-phosphate lyase*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>2e−43</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thiamine-phosphate diphosphorylase*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>3e−33</td>
<td>4</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transglutaminase*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>3e−90</td>
<td>−</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetyl-CoA dehydrogenase (FADE1)</td>
<td><em>P. brasiliensis</em> [C0]</td>
<td>1e−100</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acyltransferase family protein (SMAI)*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>6e−27</td>
<td>1</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome c oxidase assembly protein (COX15)*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>1e−70</td>
<td>−</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome c oxidase subunit V*</td>
<td><em>Aspergillus niger</em> [C0]</td>
<td>2e−17</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P450 monoxygenase*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>1e−76</td>
<td>7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d-Lactate dehydrogenase*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>4e−76</td>
<td>1</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Long-chain fatty-acid CoA-ligase (FAA1)</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>1e−61</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Multifunctional β-oxidation protein (FOX2)</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>9e−83</td>
<td>−</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NADH-fumarate reductase (CFR)*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>2e−82</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetyl-CoA dehydrogenase (FADE1)</td>
<td><em>P. brasiliensis</em> [C0]</td>
<td>8e−88</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcription</td>
<td>Ap-1-like transcription factor (meab protein)</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>2e−35</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Cutinase-like transcription factor 1</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>2e−37</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Splicing factor U2 35-kDa subunit*</td>
<td><em>Magnaporthe griseae</em> [C0]</td>
<td>9e−64</td>
<td>1</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcription factor HACA</td>
<td><em>Aspergillus niger</em> [C0]</td>
<td>4e−59</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zinc finger (GATA type) family protein transcription factor</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>3e−28</td>
<td>−</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein synthesis</td>
<td>40S ribosomal protein S1B</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>2e−91</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Eukaryotic release factor 1 (eRF1)^</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>8e−99</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eukaryotic translation elongation factor 1 γ (eEF-1γ)^</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>4e−56</td>
<td>38</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eukaryotic translation initiation factor 4A(eEF-4A)^</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>1e−79</td>
<td>16</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eukaryotic translation initiation factor 4E(eEF-4E)^</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>1e−97</td>
<td>−</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Translation elongation factor 1 α chain</td>
<td><em>Ajiellomyces capsulata</em></td>
<td>5e−24</td>
<td>−</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Translation elongation factor 3</td>
<td><em>Ajiellomyces capsulata</em></td>
<td>1e−78</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Translation elongation factor Tu, mitochondrial</td>
<td><em>Aspergillus fumigatus</em> [C0]</td>
<td>1e−68</td>
<td>−</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protein sorting/modification</td>
<td>26S Proteasome non-ATPase regulatory subunit 9*</td>
<td><em>Kluveromyces lactis</em> [C0]</td>
<td>5e−12</td>
<td>−</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Golgi z-1,2-mannosyltransferase*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>1e−33</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitochondrial inner membrane protease, AAA family*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>2e−84</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probably protein involved in intramitochondrial protein sorting</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>2e−40</td>
<td>−</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellular transport/transport facilitation</td>
<td>Acidic amino acid permease (DIPS)</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>6e−73</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>ATP-binding cassette (ABC) transporter (MDR)</td>
<td><em>Venturia inaequalis</em> [C0]</td>
<td>5e−64</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABC multidrug transport protein</td>
<td><em>Gibberella zeae</em> [C0]</td>
<td>3e−43</td>
<td>−</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Coatomer protein*</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>1e−74</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endoplasmic reticulum calcium-transporting ATPase</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>6e−78</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endoplasmic reticulum–Golgi transport vesicle protein (ERV46)*</td>
<td><em>Gibberella zeae</em> [C0]</td>
<td>2e−69</td>
<td>1</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferric reductase (FRE2)^</td>
<td><em>Aspergillus nidulans</em> [C0]</td>
<td>8e−61</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GDP-mannose transporter</td>
<td><em>Cryptococcus neoformans</em> [C0]</td>
<td>1e−35</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
**Table 1.** Continued.

<table>
<thead>
<tr>
<th>MIPS category</th>
<th>Gene product</th>
<th>Best hit/accession number</th>
<th>e-value</th>
<th>P10</th>
<th>P60</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><em>H</em>/nucleoside cotransporter</em>*</td>
<td>Aspergillus nidulans/XP_409630.1</td>
<td>7e^-47</td>
<td>–</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>High-affinity zinc/ion permease (ZRT1)</strong></td>
<td>Candida albicans/EAK6396.1</td>
<td>6e^-57</td>
<td>3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Major facilitator family transporter</strong></td>
<td>Magnaporthe grisea/XP_365043.1</td>
<td>5e^-65</td>
<td>–</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Major facilitator superfamily protein</strong></td>
<td>Aspergillus nidulans/XP_410760.1</td>
<td>1e^-51</td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Mitochondrial carrier protein</strong></td>
<td>Neurospora crassa/XP_328128</td>
<td>3e^-76</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Potential low-affinity zinc/ion permease</strong></td>
<td>Aspergillus fumigatus/AAT11931</td>
<td>1e^-41</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Potential nonclassic secretion pathway protein</strong></td>
<td>Aspergillus nidulans/XP_411820.1</td>
<td>1e^-28</td>
<td>7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Putative major facilitator protein (PTM1)</strong></td>
<td>Neurospora crassa/EAA27169.1</td>
<td>3e^-33</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Putative transmembrane Ca^{2+} transporter protein</strong></td>
<td>Aspergillus nidulans/XP_407818.1</td>
<td>1e^-35</td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Signal transduction</strong></td>
<td>cAMP-dependent serine/threonine protein kinase</td>
<td>Aspergillus nidulans/AAK71879.1</td>
<td>1e^-86</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Leucine zipper-EF-hand-containing transmembrane protein</td>
<td>Aspergillus nidulans/XP_407076.1</td>
<td>1e^-76</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Protein with PYP-like sensor domain (PAS domain)</td>
<td>Neurospora crassa/EAA32992.1</td>
<td>4e^-45</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Putative cAMP-dependent protein kinase</td>
<td>Aspergillus nidulans/XP_412934.1</td>
<td>2e^-74</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ras small GTPase, Rab type</td>
<td>Aspergillus niger/CAC17832</td>
<td>7e^-80</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Transmembrane osmosensor (SHO1)</td>
<td>Aspergillus nidulans/XP_411835.1</td>
<td>1e^-38</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td><strong>Cell rescue and defense</strong></td>
<td>Catalase A</td>
<td>Ajellomyces capsulatus/AAF01462.1</td>
<td>2e^-74</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Chaperonin-containing T-complex</td>
<td>Aspergillus nidulans/XP_406286.1</td>
<td>3e^-74</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Heat shock protein 30 (HSP30)</td>
<td>Aspergillus oryzae/BAD02411</td>
<td>7e^-16</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Serine proteinase (PR1H)</td>
<td>P. brasiliensis/AAD37783</td>
<td>3e^-96</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td><strong>Cell wall biogenesis</strong></td>
<td>1,3-β-Glucan synthase</td>
<td>P. brasiliensis/AAD37783</td>
<td>3e^-96</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Putative glycosyl hydrolase family 76</td>
<td>Aspergillus nidulans/XP_408641.1</td>
<td>1e^-69</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Putative glycosyl transferase</td>
<td>Aspergillus nidulans/XP_409862.1</td>
<td>3e^-45</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td><strong>Unclassified</strong></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_411679.1</td>
<td>5e^-36</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_405564.1</td>
<td>5e^-53</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_412972.1</td>
<td>5e^-41</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_413281.1</td>
<td>7e^-54</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Neurospora crassa/XP_323499</td>
<td>3e^-25</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_405564.1</td>
<td>1e^-30</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_404965.1</td>
<td>3e^-43</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Magnaporthe grisea/XP_365936.1</td>
<td>2e^-41</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_407902.1</td>
<td>2e^-35</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_407958.1</td>
<td>1e^-10</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_410433.1</td>
<td>5e^-46</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Neurospora crassa/CAC28640.1</td>
<td>1e^-49</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_410463.1</td>
<td>5e^-34</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_407250.1</td>
<td>8e^-24</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_404476.1</td>
<td>1e^-22</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Conserved hypothetical protein</td>
<td>Aspergillus nidulans/XP_408657.1</td>
<td>6e^-27</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td><strong>Hypothetical protein</strong></td>
<td>No hits found</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Hypothetical protein</strong></td>
<td>Aspergillus nidulans/XP_410643.1</td>
<td>2e^-10</td>
<td>1</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td><strong>Hypothetical protein</strong></td>
<td>Aspergillus nidulans/XP_407811.1</td>
<td>1e^-10</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Hypothetical protein</strong></td>
<td>No hits found</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Hypothetical protein</strong></td>
<td>No hits found</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td><strong>Hypothetical protein</strong></td>
<td>No hits found</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Hypothetical protein</strong></td>
<td>Candida albicans/EAK91016</td>
<td>1e^-14</td>
<td>–</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Hypothetical protein</strong></td>
<td>No hits found</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td><strong>Hypothetical protein</strong></td>
<td>No hits found</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*Transcripts not detected during yeast cell incubation in human blood (Bailão et al., 2006).

Transcripts overexpressed in human plasma when compared to human blood treatment (see Bailão et al., 2006).

Novel genes detected in P. brasiliensis.
upregulated in plasma as compared to human blood. It is of special note that transcripts encoding several enzymes of metabolic pathways and other categories, such as transglutaminase (EC 2.3.2.13), NADPH-quinone reductase (EC 1.6.5.5), acetoacetate synthase (EC 2.2.1.6), D-lactate dehydrogenase (EC 1.1.2.4), acetyl-CoA synthetase (EC 6.2.1.1), NADH-fumarate reductase (EC 1.3.99.1), cytochrome P450 monoxygenase (EC 1.14.14.1), eukaryotic translation factor 4E, catalase A (EC 1.11.1.6), and formamidase (EC 3.5.1.49), are among the upregulated genes.

We also performed a global analysis of our unisequence set for homology against genes present in the *P. brasiliensis* transcriptome database at [https://dna.biomol.unb.br/Pb/](https://dna.biomol.unb.br/Pb/) and at the EST collections present in GenBank ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). The analysis of generated ESTs allowed for the identification of some new transcripts that have not been demonstrated previously for *P. brasiliensis*, as identified in Table 1.

**Analysis of the upregulated genes in* P. brasiliensis* yeast cells after human plasma treatment**

Figure 2 presents the classification of 99 clusters of *P. brasiliensis* ESTs according to the classification developed at MIPS. As observed, most of the ESTs generated in the human plasma treatment for 10 min corresponded to upregulated ESTs related to cell general metabolism (33.00% of the total ESTs), protein synthesis (18.81% of the total ESTs), and facilitation of transport (14.52% of the total ESTs). Also relevant is the abundance of transcripts related to signal transduction (7.59% of the total ESTs) and transcription (6.93% of the total ESTs), as shown in Fig. 2a. During the incubation of yeast cells in human plasma for 60 min, it was observed that most of the upregulated transcripts are related to protein synthesis (25.55% of the total ESTs) and cell metabolism (14.23% of the total ESTs), followed by the ESTs in the cellular transport (12.77% of the total) and energy production (13.14% of the total ESTs) categories (Fig. 2b).

The most redundant ESTs selected by RDA during human plasma treatment for 10 and 60 min are summarized in Table 2. The encoded products showed similarity to various proteins present in databases. The most upregulated transcripts in the host-like conditions studied encoded the following functional groups: eukaryotic translation factors, cell transporters, enzymes involved in cell metabolism, transcription regulators, factors involved in the response to stress, and osmosensors. This suggests that these are general phenomena associated with adaptation of the fungal cells to the host milieu.

Among the upregulated transcripts, some were previously shown to be also overexpressed during yeast cell treatment with human blood (Bailão et al., 2006). Among those transcripts were cDNAs encoding DIP5, DDC, translation factors, FRE2, SHO1, and PR1H, as shown in Table 2. It should be pointed out that among those transcripts, some showed higher redundancy in the human plasma treatment as compared to yeast cell incubation with human blood. This is particularly the case for the transcripts encoding DDC (EC 4.1.1.28), FRE2 (EC 1.16.1.7) and PR1H. Some abundant transcripts were not previously described as being upregulated during the incubation of yeast cells in human blood, e.g. acetyl-CoA synthase (EC 6.2.1.1) and cytochrome P450 monoxygenase (EC 1.14.14.1), as shown in Table 2. Some upregulated transcripts, such as those coding for eRF1, eEF1γ, GLN1, PR1H and SHO1, have been demonstrated previously to be overexpressed in yeast cells during infection in the blood of experimental mice (Bailão et al., 2006) (Table 2).

**Fig. 2.** Functional classification of *Paracoccidioides brasiliensis* cDNAs derived from RDA experiments using as testers the cDNAs obtained from RNA of *Paracoccidioides brasiliensis* yeast cells after incubation with human plasma for 10 min (a) or 60 min (b). The percentage of each functional category is shown (see Tables 1 and 2). The functional classification was based on BLASTX homology of each EST against the GenBank nonredundant database at a significant homology cut-off of \( \leq 10^{-6} \) and the MIPS functional annotation scheme. Each functional class is represented as a color-coded segment and expressed as a percentage of the total number of ESTs in each library.
Confirmation of the expression of selected genes of P. brasiliensis

To further define gene response patterns and corroborate the RDA findings, we initially performed dot-blot analysis of P. brasiliensis cDNA-RDA clones. Individual plasmid cDNA clones were blotted in serial dilutions and hybridized to labeled cDNAs obtained from the condition in which the transcript was indicated to be most upregulated. As shown in Fig. 3, the transcripts encoding DDC, eEF-1γ, PR1H and GLN1 were confirmed to be upregulated during human plasma incubation for 10 min (Fig. 3b). The transcripts encoding FRE2, SHO1, DIP5 and eIF-4A were upregulated during P. brasiliensis incubation in human plasma for 60 min (Fig. 3c).

Further confidence in our ability to infer relative expression-level data from EST redundancy analysis was provided by semiquantitative RT-PCR analysis on independently generated RNAs of yeast cells recovered after incubation with human plasma. The upregulation of seven genes was investigated. The transcripts encoding ERV46, PPO1 and PTM1 were upregulated during 10 min of incubation in human plasma (Fig. 4a). The transcript encoding eIF-1 was upregulated during 60 min of treatment of yeast cells with human plasma (Fig. 4b). On the other hand, transcripts encoding eEF-1γ, GLN1 and SHO1 were overexpressed in both conditions, after 10 and 60 min of incubation in human plasma (Fig. 4c). Figure 4 presents a representative profile of the RT-PCR experiments, confirming the upregulation of genes in the cited conditions, as demonstrated in the subtracted cDNA libraries.

Western blot analysis and an enzymatic activity assay were employed to further validate the RDA findings at the protein level. The formamidase protein was selected because it was overexpressed in yeast cells after 1 h of incubation in human plasma. As shown, formamidase can accumulate in yeast cells after 1 and 12 h of incubation in human plasma (Fig. 5a). The enzymatic activity of formamidase in yeast cell extracts is compatible with the accumulation of the protein detected in the Western blot assay, as demonstrated in Table 3.

An overview of the metabolic adaptations of P. brasiliensis upon incubation in human plasma

The most prominent adaptations undergone by P. brasiliensis during treatment with human plasma are summarized in Fig. 6. As observed, the degradation of fatty acids through β-oxidation, putatively generating acetyl-CoA and propionyl-CoA, could be inferred, as several enzymes are upregulated during the treatment. The flavoprotein dehydrogenase that introduces the double bond passes electrons directly to
O₂ during β-oxidation in peroxisomes, producing H₂O₂, a product that could be removed from peroxisomes by catalase A, which is overexpressed in the subtracted cDNA library. Additionally, the methylcitrate cycle could assimilate propionyl-CoA, generating pyruvate. Also, the synthesis of acetyl-CoA from pyruvate and acetate could be performed by the overexpressed enzyme acetyl-CoA synthase. Additionally, soluble fumarate reductase in the cytoplasm could catalyze the conversion of fumarate to succinate during the reoxidation of intracellular NADH, thus providing additional succinate.

**Sensitivity of yeast cells to SDS after incubation with human plasma**

We tested whether the incubation of yeast cells with human plasma could be reflected in the relative sensitivity of cells to SDS, an anionic detergent that destabilizes the cell wall at
very low concentrations. The yeast cells incubated with human plasma show greater sensitivity to this osmotic destabilizing agent when compared to the control cells (Fig. 7).

Discussion

Cellular organisms develop a myriad of strategies to maintain specific internal conditions when challenged by the host environment. The complexity of the *P. brasiliensis* system for detecting and responding to the host environment is only beginning to come to light. Survival and proliferation in the host are essential steps for *P. brasiliensis* to cause infection. *Paracoccidioides brasiliensis* alters the transcriptional profile in host-like conditions, as we have described previously (Bailão et al., 2006). To elucidate the influence of human plasma on transcript profiles, we attempted to isolate differentially regulated genes expressed in this condition. The fungus can be constantly exposed to human plasma during superficial infections, as a consequence of the local inflammatory response, although the effect of plasma on *P. brasiliensis* gene expression is not known.

Some metabolic enzymes were upregulated in the subtracted libraries. During plasma treatment of *P. brasiliensis*, the overexpression of transcripts encoding enzymes of β-oxidation was observed. All the enzymes related to the β-oxidation pathway are upregulated in the yeast cells of *P. brasiliensis* upon incubation with human plasma. It is of special note that a peroxisomal multifunctional enzyme is probably a 2-enoyl-CoA hydratase/3-hydroxyacyl-CoA 36 kDa

![Fig. 5. Validation of the RDA results by Western blot. Total cellular extracts were obtained from yeast cells incubated with human plasma for 1 and 12 h. The proteins (25 μg) were electrophoretically transferred to a nylon membrane and checked by Ponceau S to determine equal loading. The samples were reacted with: (a) a polyclonal antibody produced against the *Paracoccidioides brasiliensis* recombinant formamidase (dilution 1:1000); and (b) a polyclonal antibody raised to the recombinant GAPDH. After reaction with alkaline phosphatase-conjugated anti-mouse IgG (a) and alkaline phosphatase-conjugated anti-rabbit IgG (b), the reaction was developed with BCIP/NBT. The analyses of relative differences were performed with the SCION IMAGE BETA 4.03 program (http://www.scioncorp.com).](image)

**Table 3.** Formamidase activity of yeast cell protein extracts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.36 ± 0.0417</td>
</tr>
<tr>
<td>1 h of incubation in human plasma</td>
<td>2.09 ± 0.0707</td>
</tr>
<tr>
<td>12 h of incubation in human plasma</td>
<td>1.84 ± 0.0622</td>
</tr>
</tbody>
</table>

*One unit of FMD activity was defined as the amount of enzyme required to hydrolyze 1 μmole of formamide (corresponding to the formation of 1 μmole of ammonia) per minute per milligram of total protein.

![Fig. 6. Some metabolic pathways that are overexpressed during *Paracoccidioides brasiliensis* yeast cell incubation with human plasma. (A)Transcripts that are not overexpressed during *Paracoccidioides brasiliensis* treatment with human blood. (B)Transcripts present in database. FAA1, long-chain fatty acid-CoA ligase; FADE1, acyl-CoA dehydrogenase; FOX2, multifunctional β-oxidation protein; CATA, catalase A; SMA1, acyltransferase family protein; ACS, acetyl-CoA synthetase; CFR, NADH-fumarate reductase; MCS, methylcitrate synthase; MCD, methylcitrate dehydrogenase; CAN, aconitate; MCL, methylcitrate lyase; SDH, succinate dehydrogenase; FUM, fumarate reductase; MDH, malate dehydrogenase; ILV2, acetolactate synthase; Mcitrate, methylcitrate; Misocitrate, methylisocitrate.](image)
dehydrogenase, as described in *Saccharomyces cerevisiae*, *Candida tropicalis* and mammals (Moreno et al., 1985; Hiltunen et al., 1992; Breitling et al., 2001). β-Oxidation of even-chain-length fatty acids yields acetyl-CoA units exclusively, whereas β-oxidation of odd-chain-length fatty acids yields both acetyl-CoA and propionyl-CoA. In several bacteria and fungi, propionyl-CoA is assimilated via the methylcitrate cycle, which oxidizes propionyl-CoA to pyruvate (Brock et al., 2000). The growth of fungi on gluconeogenic compounds such as acetate or fatty acids positively regulates enzymes of the glyoxylate cycle, even in the presence of repressing carbon sources such as glucose (Cánovas & Andrianopoulos, 2006). Acetyl-CoA synthetases (EC 6.2.1.1) have been detected as isoforms in microorganisms such as the fungus *Phycomyces blakesleeanus*, in where they can use acetate and propionate as substrates (De Cima et al., 2005). Alternatively, conversion of pyruvate to acetyl-coenzyme A can be accomplished by the concerted action of the enzymes of the pyruvate dehydrogenase bypass: pyruvate decarboxylase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase (van den Berg et al., 2002). Consistently, the yeast cells of *P. brasiliensis* produce ATP preferentially through alcohol fermentation (Felipe et al., 2005). In this sense, aldehyde dehydrogenase (EC 1.2.1.3) can allow the conversion of ethanol into acetate via acetyldehyde, thus providing acetyl-CoA to the glyoxylate cycle. In *P. brasiliensis*, alcohol dehydrogenase is upregulated in the yeast cells, as previously demonstrated (Felipe et al., 2005).

Plasma significantly upregulated the expression of transcripts associated with protein biosynthesis. Among these are, for instance, eukaryotic translation factors. The enhanced expression of these factors suggests a general increase of protein synthesis in the plasma environment, as we had previously described for *P. brasiliensis* yeast cells treated with human blood (Bailão et al., 2006). This finding could reflect fungal passage to a nutrient-rich medium, as described for *C. albicans* (Fradin et al., 2003).

Plasma treatment also promotes upregulation of transcripts encoding facilitators of transport in *P. brasiliensis* yeast cells. The most upregulated transcripts encode for a
putative ferric reductase (FRE2) and for an acidic amino acid permease (DIP5) of \textit{P. brasiliensis}. During plasma treatment, the overexpression of the transcript encoding FRE2 could be related to the reduction of Fe(III), and the Fe(II) thus formed could be bound to a transporter permease, such as a zinc/iron permease (ZRT1), as suggested previously (Bailão et al., 2006). The high level of uptake of glutamate by DIP5 could result in chitin deposition, as will be discussed below.

Signal transduction pathways play crucial roles in cellular adaptation to environmental changes. The high-osmolarity glycerol (HOG) pathway in \textit{S. cerevisiae} and other fungi consists of two branches that seem to sense osmotic changes in different ways (Westfall et al., 2004). The SHO1 adapter protein role was characterized in \textit{C. albicans}, in which it is related to the fungal morphogenesis interconnecting two pathways involved in cell wall biogenesis and oxidative stress (Román et al., 2005). We have previously demonstrated the expression of the novel \textit{sho1} transcript homolog of \textit{P. brasiliensis} in yeast cells during human blood treatment, as well as in \textit{P. brasiliensis} yeast cells present in blood of infected mice, suggesting its involvement in the osmolarity sensing of \textit{P. brasiliensis} yeast cells during fungus dissemination through the blood. It is of special note that the transcript encoding this novel osmosensor of \textit{P. brasiliensis} (Bailão et al., 2006) is predominantly overexpressed in yeast cells during incubation with human plasma, vs. the incubation with human blood. In \textit{C. albicans}, the influence of blood cells in the transcriptional response has been described by Fradin et al. (2005).

Also, transcripts putatively related to cell defense are upregulated during human plasma treatment of \textit{P. brasiliensis} yeast cells. The gene encoding transglutaminase (TGAse) has been reported to insert an irreversible isopeptide bond within and or between proteins using specific glutamine residues on one protein and the primary amide group on the other molecule. The resultant molecules are resistant to proteinases and denaturants (Greenberg et al., 1991). In addition, a TGAse-like reaction has been associated with the attachment of Pir proteins to the β-1,3-glucan in \textit{S. cerevisiae} (Ecker et al., 2006). TGAse was found to be localized in the cell wall of fungi. In \textit{C. albicans}, TGAse was suggested to be important in the structural organization of the fungus by establishing crosslinks among structural proteins, and its inhibition resulted in increased sensitivity of protoplasts to osmotic shock (Ruiz-Herrera et al., 1995).

Glutamine synthetase is also upregulated in the human plasma incubation condition. We had hypothesized that the enzyme overexpression could be related to the chitin synthesis increase that could occur during osmotic stress (Bailão et al., 2006). In this way, chitin synthesis has been shown to be essential in the compensatory response to cell wall stress in fungi, preventing cell death (Popolo et al., 1997). The sugar donor for the synthesis of chitin is UDP-N-acetylglucosamine. The metabolic pathway leading to the formation of UDP-N-acetylglosamine from fructose 6-phosphate consists of five steps, of which the first is the formation of glucoseamine 6-phosphate from glucosate and fructose 6-phosphate, a rate-limiting step in the pathway. The cell wall stress response in \textit{Aspergillus niger} involves increased expression of the gene \textit{gfaA}, which encodes the glutamine: fructose-6-phosphate amidotransferase, and increased deposition of chitin in the cell wall (Ram et al., 2004). Similarly, we speculate that the increase in the glutamine synthetase transcript in \textit{P. brasiliensis} could be related to chitin deposition in response to the change in external osmolarity faced by the fungus in the superficial condition of infection as well as during the blood route of dissemination. The glutamine synthetase transcript was found to be expressed in \textit{P. brasiliensis} yeast cells infecting mice blood, reinforcing its role in fungal infection (Bailão et al., 2006). Corroborating our suggestion, fungal yeast cells were more sensitive to SDS upon incubation with human plasma, suggesting changes in the structural organization of the cell wall.

Also putatively related to the oxidative response stress, NADPH-quinone reductase (EC 1.6.5.5) catalyzes a two-electron transfer from NADPH to quinone, whose reduced status is undoubtedly important for managing oxidative stress. Oxidative stress resistance is one of the key properties that enable pathogenic microorganisms to survive the effects of the production of reactive oxygen by the host. In this sense, a homolog of the protein in \textit{Helicobacter pylori} is a potential antioxidant protein and is related to its ability to colonize mouse stomach (Wang & Maier, 2004). Catalase A is another transcript upregulated during yeast cell incubation with human plasma. Catalases are described as important factors conferring resistance to oxidative stress in fungi (Giles et al., 2006).

Several lines of evidence suggest that serine proteinases are required for the successful invasion of host cells by pathogens. An extracellular SH-dependent serine proteinase has been characterized from the yeast phase of \textit{P. brasiliensis}; it cleaves the main components of the basal membrane \textit{in vitro}, thus being potentially relevant to fungal dissemination (Puccia et al., 1999). Serine proteinases could have an important role in cleavage of host proteins, either during the invasion of a host cell or during dissemination through organs. It is of special note that a serine proteinase homolog of \textit{Bacillus subtilis} was able to facilitate siderophore-mediated iron uptake from transferrin via the proteolytic cleavage of the protein (Park et al., 2006). In addition, the incubation of \textit{A. fumigatus} in media containing human serum greatly stimulated proteinase secretion, and the serine proteinase catalytic class had the highest activity (Gifford et al., 2002). The serine proteinase transcript overexpressed during human plasma treatment of yeast cells was also
present during blood infection of mice by *P. brasiliensis*, as previously demonstrated (Bailão et al., 2006).

In fungi, several different types of melanin have been identified to date. The two most important types are DHN-melanin (named for one of the pathway intermediates, 1,8-dihydroxynaphthalene) and DOPA-melanin (named for one of the precursors, 1,3,4-dihydroxyphenylalanine). Both types of melanin have been implicated in pathogenesis (Hamilton & Gomez, 2002). With regard to *P. brasiliensis*, it has been demonstrated that growth of yeast cells in a defined medium with 1-DOPA resulted in melanization of the cells (Gomez et al., 2001). Furthermore, it has been reported that fungal melanin protects *P. brasiliensis* from phagocytosis and increases its resistance to antifungal drugs (Silva et al., 2006). Transcripts encoding DDC (EC 4.1.1.28) were predominantly upregulated in yeast cells upon incubation with human plasma. This finding could reflect the high levels of 1-DOPA in human plasma, as previously described (Machida et al., 2006), which can be converted to melanin by the yeast cells of *P. brasiliensis*.

We compared the profiles of upregulated genes during the present treatment (human plasma treatment of yeast cells) with those described during incubation with human blood, mimicking the effects of fungal dissemination through organs and tissues (Bailão et al., 2006). Blood contains different components, cellular and soluble, which have been demonstrated to affect *C. albicans* to different extents (Fradin et al., 2005). It has been demonstrated that neutrophils have the dominant influence on *C. albicans* gene expression in blood. Our comparative analysis demonstrated that 16.63% of the upregulated transcripts in human plasma were not present in human blood, suggesting the influence of blood cells in the transcriptional profile, as previously described (Bailão et al., 2006). In this sense, some genes are upregulated only during plasma treatment.

To our knowledge, this study is the first to use cDNA-RDA analysis to characterize changes in gene expression patterns during human plasma treatment of *P. brasiliensis*. The data that we have amassed are the first on the adaptation of *P. brasiliensis* to numerous stresses during human plasma treatment at the level of individual genes. The establishment of genetic tools for *P. brasiliensis*, such as DNA-mediated transformation and modulation of gene expression by gene knockout or RNA interference techniques, will be of great importance in establishing the roles of those genes that are highly expressed in response to host conditions.

**Acknowledgements**

This work at Universidade Federal de Goiás was supported by grants from CNPq (Conselho Nacional de Desenvolvi-

**References**


Occurrence of group A rotavirus mixed P genotypes infections in children living in Goiânia-Goiás, Brazil

E. R. L. Freitas · C. M. A. Soares · F. S. Fiaccadori · M. Souza · J. A. Parente · P. S. S. Costa · D. D. P. Cardoso

Received: 21 December 2007 / Accepted: 28 April 2008 © Springer-Verlag 2008

Abstract Group A rotaviruses (RVA) are the main causing agents of acute gastroenteritis worldwide, having a great impact on childhood mortality in developing countries. The objective of this study was to identify RVA-positive fecal samples with mixed P genotypes by hemi-nested reverse transcriptase-polymerase chain reaction (RT-PCR), followed by sequencing confirmation. Our results showed that, from the 81 RVA-positive samples, 25 were positive for more than one P genotype by hemi-nested RT-PCR. Of these 25 samples, 12 (48%) had their mixed P genotypes confirmed by sequencing and, from these, 10 were identified as P[6]P[8], one as P[4]P[6], and one as P[4]P[6]P[8]. Our results confirm the occurrence of RVA mixed infections among children in Brazil and reinforce the importance of the constant monitoring of RVA circulating strains for the efficacy of control/prevention against these agents.

Introduction

Gastroenteritis is an important cause of morbidity and childhood mortality, especially in developing countries, where it is estimated that 1.5 billion cases occur per year in children less than five years of age, with about three million deaths [1]. Group A rotaviruses (RVA) belong to the Reoviridae family, genus Rotavirus, and are the main etiological agents for acute viral gastroenteritis in children [2]. They are responsible, annually, for approximately 111 million episodes of gastroenteritis, 2 million hospitalizations, and 440 thousand deaths of children up to five years of age [1]. The RVA capsid is formed by three concentrical protein layers that surround the viral genome, composed by 11 segments of double-stranded RNA (dsRNA). The external capsid layer is formed by proteins VP7 and VP4, which are both immunogenic and define the G and P genotypes, respectively. Currently, there are at least 15 G and 27 P genotypes described for RVA [2–8]. The VP4 and VP7 genes segregate independently, resulting in several G and P combinations [9, 10], with P[8]G1, P[4]G2, P[8]G3, P[8]G4, and P[8]G9 being the most commonly found worldwide [11, 12].

The segmented nature of the rotavirus genome allows for genomic reassortment, which may result in mixed infec-
tions by uncommon G and P combinations, such as P[4]P
[6], G2G8 and P[4]P[6], G2G9. Natural genomic reassort-
ment usually occurs after the same cell is co-infected by
samples of common occurrence, such as P[6]G8 with P[4]
prevention measures against RVA, vaccination has the most
potential to succeed, and because RVA immunity seems to
be type-specific [14], knowledge about the circulating G
and P genotypes/serotypes before, during, and after the
vaccination period is highly important. Mixed infections
can also have an impact on vaccination effectiveness. This
study presents novel information about the occurrence of
RVA mixed P genotypes infections in children living in the
city of Goiânia in the state of Goiás, Brazil.

Materials and methods

Fecal samples

A total of 81 RVA-positive fecal samples from children, 49
from males and 32 from females, were evaluated. These
samples were collected from children up to 5 years of age
with acute gastroenteritis and who lived in the city of
Goiânia. The samples were collected from April 1998 to
August 2003, after written authorization by the parents or
legal guardians was obtained. The study was approved by
the Ethics in Research Committee of the Federal University
of Goiás (no. 004/2000).

RVA detection

The fecal samples were first screened for RVA by
immunoenzymatic assay combined for rotavirus and ade-
novirus (EIARA) [15] and polyacrylamide gel electropho-
resis (PAGE) [16] in a previous study conducted in our
laboratory.

Viral dsRNA extraction

The viral dsRNA was extracted from 20% fecal suspensions
using silica and guanidinium isothiocyanate, as described
by Boom et al. [17], following modifications by Cardoso
et al. [18].

Table 1 Hemi-nested reverse transcriptase-polymerase chain reaction (RT-PCR) primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5′-3′)</th>
<th>Positions (nt)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con2</td>
<td>ATT TCG GAC CAT TTA TAA CC</td>
<td>868–887</td>
<td>–</td>
</tr>
<tr>
<td>Con3</td>
<td>TGG CTT CGC CAT TTT ATA GAC A</td>
<td>11–32</td>
<td>876</td>
</tr>
<tr>
<td>1T-1 P[8]</td>
<td>TCT ACT TGG ATA ACG TGC</td>
<td>339–356</td>
<td>345</td>
</tr>
</tbody>
</table>

P genotyping

The samples were submitted to hemi-nested reverse
transcriptase-polymerase chain reaction (RT-PCR) P geno-
typing using a pair of consensus primers (Con2 and Con3)
that correspond to conserved nucleotide sequences of VP4.
The resulting ampiclons of 876 bp were then used as a
template in a second PCR, with a mixture of genotype-specific
primers (2T-1 P[4], 3T-1 P[6], 1T-1 P[8]) complementary to
variable regions of the VP4 genes. The hemi-nested RT-PCR
P genotyping was performed according to Gentsch et al. [19].
The primers’ designation, sequence, position, and product
length are shown in Table 1.

The reverse transcription and the amplification reaction
were performed in one stage. The viral dsRNA was
combined with dimethyl sulfoxide and incubated at 97°C
for 5 min, followed by the addition of the reaction mixture,
in a final volume of 100 μl: 1× PCR buffer (20 mM Tris-HCl
(pH8.0) and 50 mM KCl; Invitrogen™, Life Technologies,
Carlsbad, CA), 2 mM MgCl₂, dNTPs mix (dATP 0.8 mM,
dCTP 0.8 mM, dTTP 0.8 mM, dGTP 0.8 mM), 2.5 U Taq-
DNA polymerase (Invitrogen™), 200 U Reverse Transcrip-
tase SuperScript II (Invitrogen™, Life Technologies), and
the consensual primers (0.2 μM each). The cycling
parameters used were: 42°C for 60 min, 99°C for 5 min,
followed by 30 cycles at 94°C for 1 min, 50°C for 2 min,
and 72°C for 1 min, and a final 7-min extension cycle at
72°C.

For the hemi-nested PCR, 1 μL of the product of the first
amplification was added to the same reaction mixture,
minus the reverse transcriptase, described above using the
Con3 as consensual primer, together with the specific
primers. The cycling parameters used were: 15 cycles at
94°C for 1 min, 42°C for 2 min, 72°C for 1 min, and a final
7-min extension cycle at 72°C. In all of the reactions, the
Wa (human P[8] genotype) prototype sample was used as
the positive control and sterile Milli-Q water was used as
the negative control. All of the samples were re-tested,
under the same conditions, using the specific primers
separately.

The amplified product was visualized by gel electropho-
resis using 1.5% agarose gel containing ethidium bromide
(1 μg/mL). The 123 pb DNA ladder (Invitrogen™, Life
Technologies) was used as a molecular weight standard.
DNA sequencing and phylogenetic analysis

The purification of the hemi-nested RT-PCR products was performed using the QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA), following the manufacturer’s instructions. Sequencing of the purified PCR products was performed using the primer Con3 (VP4 gene) and the DYEEnamic™ ET Dye Terminator Kit (Amersham Biosciences, Piscataway, NJ), by automatic sequencing using the MegaBACE 1000 DNA Sequencer (Amersham Biosciences).

The nucleotide sequences obtained were analyzed and compared with sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and aligned using the Clustal X program [20]. The phylogenetic tree was constructed by the neighbor joining method using the TreeView program.

Results

Genotyping by hemi-nested RT-PCR

After hemi-nested RT-PCR reactions using a pool of the specific primers were performed, each sample’s result was confirmed in a second hemi-nested RT-PCR reaction using each of the specific primers separately (Fig. 1). From the 81 confirmed in a second hemi-nested RT-PCR reaction using than one P genotype, 21 with enough DNA concentration were sequenced. When analyzed by the BLAST program and compared with the sequences deposited in GenBank, 12 samples showed a mixed pattern of P genotypes distributed as follows: ten were P[6]P[8], one P[4]P[6], and one P[4]P[6] P[8] (Table 2). The identity values ranged from 91–100%. Two P[6]P[8] samples (19,316 and 19,608) showed homology to both genotypes, but they were not considered for further analysis because of their sequence size. From all of the samples submitted to sequencing, nine were characterized only as P[8]. Phylogenetic tree construction was performed as follows: a sequence of 143 nucleotides (194–336 nt) for P[4], 153 (111–263 nt) for P[6], and 143 (194–263 nt) for P[8], based on the sequence of the prototype samples RV-5, 1076, and Wa, respectively (Fig. 2).

Sequencing of RVA-positive samples

Of the 25 RVA samples that were identified as having more than one P genotype, 22 reacted with more than one P genotype, 22 as having a mixed P profile by hemi-nested RT-PCR, 12 (48%) were confirmed by genome sequencing and nine (36%) were considered to be positive only for P[8]. One of the reasons why some of the samples did not have their mixed profile confirmed could be the low DNA concentration of the samples after amplification of the P[6] and P[4] fragments, resulting in sub-optimal conditions for sequencing.

Of the 25 RVA-positive samples that were considered as having a mixed P profile by hemi-nested RT-PCR, 12 (48%) were confirmed by genome sequencing and nine (36%) were considered to be positive only for P[8]. One of the reasons why some of the samples did not have their mixed profile confirmed could be the low DNA concentration of the samples after amplification of the P[6] and P[4] fragments, resulting in sub-optimal conditions for sequencing.

In this study, the predominant combinations of P genotypes found were P[6]P[8], followed by P[4]P[6] and P[4]P[6]P[8]. Our results are similar to those of a study conducted in Guinea Bissau, where 38% of the fecal samples collected from children with acute gastroenteritis had mixed P genotypes, with P[4]P[6] being the most predominant [22]. Another study from Denmark showed that 21% of all samples analyzed had mixed P genotypes, with P[4]P[8], followed by P[4]P[6] and P[6]P[8] being the most common [23]. Similar results were also reported in Belém, Brazil, where Mascarenhas et al. [24] detected 23% of mixed P genotypes infections. Another study conducted in Rio de Janeiro, Brazil, revealed that only 16% of the

![Fig. 1](image-url)
samples analyzed had mixed P genotypes, with the predominance of P[4]P[8], followed by P[6]P[8] [21]. The occurrence of mixed infections by RVA in a population may be very important when considering the potential for genetic reassortment among distinct samples, which may result in the emergence of unusual G and P genotype combinations, leading to increased genetic diversity of these agents and, in this way, it can have an impact on the vaccination effectiveness [11, 21, 25].

The G genotyping results of the 12 mixed samples used in this study were published in a previous study conducted in our laboratory [26]. In the present study, from ten of those samples that had been previously genotyped as G1, one was characterized as P[4]P[6]P[8] and nine as P[6]P[8], whereas the one sample with the G2 genotype was identified as P[4]P[6] and the G3 as P[6]P[8].

This is the first study to describe the occurrence of mixed RVA infections in the Central-West region of Brazil. Our data provide important information on the identity of the RVA circulating strains in the region, which will be useful for a better understanding of the impact of RVA mixed infections in childhood gastroenteritis. The findings can also

<table>
<thead>
<tr>
<th>Hemi-nested RT-PCR</th>
<th>Sequencing genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>P[4]P[8]</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>

**Fig. 2** Phylogenetic analysis of VP4 nucleotide sequences of genotype P mixed samples. The phylogenetic tree was constructed by the neighbor joining method using the Clustal X and TreeView programs. GenBank access numbers: P[8] [OP601 (AJ302153), F45 (U30716), BrH8 (U41006), L8 (AF061358), Wa (L34161), Br1054 (U41004)]; P[6] [ST3 (L33895), M37 (L20877), 1076 (M88480), Se585 (AJ311737)]; P[4] [RV5 (U59103), L26 (M58292), and I200–1997 (DQ172840)]

**Table 2** Comparison between the hemi-nested RT-PCR and sequencing results of mixed group A rotaviruses (RVA) samples

<table>
<thead>
<tr>
<th>Hemi-nested RT-PCR</th>
<th>Sequencing genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>P[4]P[8]</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>
be used for the evaluation of previous vaccines’ efficacy and for the development of future control/prevention strategies.

Acknowledgment The authors thank the National Counsel of Technological and Scientific Development (CNPq) of Brazil for providing financial support.

References

Molecular characterization of the NSP4 gene of human group A rotavirus samples from the West Central region of Brazil

Talissa de Moraes Tavares, Wilia Marta Elsner Diederichsen de Brito, Fabiola Souza Fiaccadori, Erika Regina Leal de Freitas, Juliana Alves Parente, Paulo Sérgio Sucasas da Costa, Loreny Gimenes Giugliano, Márcia Sueli Assis Andreais, Célia Maria Almeida Soares, Divina das Dôres de Paula Cardoso

Laboratório de Virologia, Instituto de Patologia Tropical e Saúde Pública 1Laboratório de Biologia Molecular, Instituto de Ciências Biológicas 2Faculdade de Medicina, Universidade Federal de Goiás, Rua 235 s/n, Setor Universitário, 74605-050 Goiânia, GO, Brasil 3Departamento de Biologia Celular, Universidade de Brasília, Brasília, DF, Brasil 4Departamento de Patologia, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brasil

Nonstructural protein 4 (NSP4), encoded by group A rotavirus genome segment 10, is a multifunctional protein and the first recognized virus-encoded enterotoxin. The NSP4 gene has been sequenced, and five distinct genetic groups have been described: genotypes A-E. NSP4 genotypes A, B, and C have been detected in humans. In this study, the NSP4-encoding gene of human rotavirus strains of different G and P genotypes collected from children between 1987 and 2003 in three cities of West Central region of Brazil was characterized. NSP4 gene of 153 rotavirus-positive fecal samples was amplified by reverse transcriptase-polymerase chain reaction and then sequenced. For phylogenetic analysis, NSP4 nucleotide sequences of these samples were compared to nucleotide sequences of reference strains available in GenBank. Two distinct NSP4 genotypes could be identified: 141 (92.2%) sequences clustered with NSP4 genotype B, and 12 sequences (7.8%) clustered with NSP4 genotype A. These results reinforce that further investigations are needed to assess the validity of NSP4 as a suitable target for epidemiologic surveillance of rotavirus infections and vaccine development.

Key words: group A rotavirus - NSP4 gene - genotypes - West Central region - Brazil

Group A rotaviruses are a major cause of gastro-enteritis in infants and young children throughout the world. Each year, these viruses cause approximately 111 million episodes of severe diarrhea, which results in 611,000 deaths (Parashar et al. 2006). In Brazil, the frequency of group A rotavirus infection among young children was found to be between 12 and 42% (Linhares 2000, Cardoso et al. 2003, Costa et al. 2004, Cauãs et al. 2006, Munford et al. 2007), and about 80,000 children are hospitalized for the infection yearly (Linhares 2000). Viral particles consist of a non-enveloped, triple-layer protein capsid structure that surrounds a genome composed of 11 segments of double-stranded RNA. The genome encodes six structural proteins (VP1-VP4, VP6 and VP7) and six nonstructural proteins (NSP1-NSP6) (Estes & Kapikian 2007).

Variability in the genes encoding VP7 and VP4 proteins forms the basis of the current strain typing of group A rotaviruses into G and P genotypes, respectively. Studies of rotavirus infections in humans have identified distinct G and P genotypes circulating simultaneously in different parts of the world (Santos & Hoshino 2005, Estes & Kapikian 2007, Gulati et al. 2007, Martella et al. 2007, Munford et al. 2007, Matthijnssens et al. 2008). There is currently only limited information available on the detection or genetic variability of the gene that encodes nonstructural protein 4 (NSP4) (Carlet et al. 2000, Mori et al. 2002, Iturriza-Gómez et al. 2003, Araújo et al. 2007, Mascarenhas et al. 2007).

NSP4, encoded by segment 10, is a transmembrane glycoprotein of 175 amino acids (aa) (Estes & Kapikian 2007). NSP4 serves as an intracellular receptor for the budding of subviral double-layered particles into the endoplasmic reticulum, a step that is critical for the acquisition of a transient viral membrane and viral particle maturation (Taylor & Bellamy 2003). In addition, NSP4 has been found to have an enterotoxin-like activity that was originally mapped between aa 114 and 135. Modifications in the toxigenic activity and virulence of rotavirus have been associated with aa changes in this region (Ball et al. 1996, Zhang et al. 1998). Finally, it has been proposed that antibodies against NSP4 might reduce both the frequency and severity of diarrhea in mice. Together with studies in human infants, these data suggest that the immune response to NSP4 could modulate rotavirus-induced diarrhea in human disease (Ball et al. 1996, Yuan et al. 2004, Vizzi et al. 2005).

The NSP4 genes of animal and human rotavirus have been sequenced and compared. Sequence analyses have revealed the existence of five distinct NSP4 genotypes: A (KUN), B (Wa), C (AU-I), D (EW) and E (avian-like).
Genotypes A, B, C and D have been determined from mammalian rotavirus strains, while genotype E has been identified from avian rotavirus strains. Genotypes A, B and C have been detected in humans (Carlet et al. 2000, Mori et al. 2002, Lin & Tian 2003).

In Brazil, there are a few molecular studies of the rotavirus NSP4 gene from strains of diverse origin and various G and P genotypes (Cunliffe et al. 1997, Mascara-renhas et al. 2006, 2007, Araújo et al. 2007). As yet, however, there has been no investigation into the detection rate and the genetic diversity of NSP4 genes in the West Central region of Brazil. This study presents novel epidemiological data regarding the circulation of NSP4 genotypes of rotavirus strains recovered from children in three cities of the West Central region of Brazil.

PIATES, MATERIALS AND METHODS

Samples - The NSP4 gene was investigated in 330 rotavirus A-positive fecal samples that were previously obtained from infected children during surveillance studies performed at the Laboratory of Virology of Universidade Federal de Goiás (UFG) in the city of Goiânia, Brazil. These samples were collected from children up to five years of age with (n = 325) or without (n = 5) acute gastroenteritis between August 1987 and September 2003 in three cities of West Central region, Brazil: Goiânia (GO), Campo Grande (CG) and Brasília (BRA).

A total of 202 group A rotavirus samples were identified in GO from 1987 to 2001, 81 samples in CG between 2000 and 2003 and 47 samples in BRA in 2001 and 2002. All samples from GO and CG were collected “in nature”, whereas, of the samples collected in BRA, only eight were collected “in nature” and 39 were collected by rectal swab. All of the collected samples had been previously identified as group A rotavirus (Cardoso et al. 2003, Souza et al. 2003, Costa et al. 2004, Andreasi et al. 2007) with a combined enzyme immunoassay for rotavirus and adenovirus (Pereira et al. 1985) and/or by polyacrylamide gel electrophoresis (Pereira et al. 1983).

Specimens were collected from children after signed written consent was provided by their parents or other legal guardians. This study was approved by the Ethics Committee of the Research of UFG (Protocol nº.004/2000).

RNA extraction - The viral dsRNA was extracted from 20% fecal suspension by the glass powder method, using guanidine isothiocyanate buffer and silica as described by Boom et al. (1990) with modifications (Cardoso et al. 2002).

Reverse transcription-polymerase chain reaction (RT-PCR) amplification - The RT-PCR followed the protocol described by Lee et al. (2000). The purified viral double-stranded RNA (dsRNA) was denatured at 97°C for 10 min and then used as template for the RT-PCR. The RT of dsRNA was carried out with SuperScript™ (Invitrogen Carlsbad, CA, USA), and PCR amplification was performed with Taq DNA polymerase (Invitrogen Carlsbad, CA, USA). Fragments of the NSP4 gene of 725 bp were amplified using forward (10BEG16) and reverse (10END722) primers as described by Lee et al. (2000).

Sequencing reaction - The PCR products were purified using the QIAquick® PCR purification kit (Qiagen, São Paulo, Brazil). The PCR-purified products were sequenced by a MegaBACE 1000 automatic sequencer (GE Healthcare, Sunnyvale, USA), using a DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Buckinghamshire, United Kingdom). The primers used were the same as for PCR amplification. The products were further purified by ethanol precipitation and resuspended in formamide.

Sequence analysis - The sequences obtained were analyzed with PHRED/PHRAP/CONSED (http://www.phrap.org) and pre-processed using the Phred (Ewing & Green 1998) and Crossmatch (http://wwwgenome.washington.edu/UGWC/analysisistools/Swat.cfm) programs. Only sequences with at least 100 nucleotides and Phred quality greater than or equal to 20 were considered for further analysis with the Blastn program (Altschul et al. 1990) in the National Center for Biotechnology Information Database (http://www.ncbi.nlm.nih.gov). NSP4 sequences were aligned and compared to NSP4 sequences of standard reference strains [AU-1 (D89873), AU32 (D88830), Ch-1 (AB065287), EW (U96335), KUN (D88829), RV5 (U59103), OSU (D88831), Wa (AF093199) and Brazilian strains [j15348 (D498179) and r7363 (D498192)] available in GenBank using Clustal X software (Thompson et al. 1997). NSP4 genotypes were determined by phylogenetic analysis using the neighbor-joining algorithm method (Saitou & Nei 1987).

Nucleotide sequence accession numbers - The NSP4 nucleotide sequence data determined in this study were deposited into the GenBank sequence database with accession numbers: EU620072-EU620111.

Statistical analysis - The statistical analysis was performed with the Epi Info version 6 program, using the chi-squared (χ²) test for proportion comparison. Statistical significance was assessed at a p value of < 0.05.

RESULTS

The NSP4 gene was detected in 259 (78.5%) of 330 rotavirus A-positive fecal samples. Of them, rates of 82.7% (167/202), 85.2% (69/81) and 48.9% (23/47) were observed in fecal specimens from children in GO, CG and BRA, respectively (χ² = 28.55, p = 0.000).

A total of 231 samples that had an amplified NSP4 gene were submitted to sequencing. NSP4 nucleotide sequences of 153 (66.2%) samples could be analyzed and were compared to nucleotide sequences of prototype strains. Of those, 141 sequences (92.2%) clustered with NSP4 genotype B with degrees of identity ranging from 89.0% to 99.0%; and the remaining 12 sequences (7.8%) clustered with NSP4 genotype A (93.0% - 95.0% of identity). Genotype A samples were identified only in children from GO, while genotype B samples were identified in samples from all three cities (Table I). In GO, the only sample collected in 1987 was genotype B; in the 1990s, however, both genotypes A and B were detected in the 45 samples analyzed, with genotype B (73.3%) predominating. From 2000 to 2003, only genotype B samples were found to be circulating in the three cities analyzed (Table II).
Rotavirus samples P[8]G1, P[6]G1, P[8]G2, P[8]G3, P[8]G4 and P[8]G9 were more closely related to NSP4 genotype B, whereas P[6]G9 rotavirus samples were closely related to NSP4 genotype A. All of these samples that clustered into genotype A or B had human origin (Fig. 1, Table III).

NSP4 genotypes A and B were identified in children with or without acute gastroenteritis. Of the 153 samples, 151 were from children with diarrhea, and two were from children without diarrhea. No significant differences were found in the nucleotide sequences of the NSP4 genes from symptomatic and asymptomatic samples.

The deduced aa sequences of the NSP4 genes of 15 human group A rotavirus samples were aligned with aa sequences of reference prototype strains (Fig. 2). Variations between sequences of genotypes A and B were found in the H3 cytoplasmic domain (aa 63-80), amphipathic alpha-helix domain (aa 93-133), VP4 binding site (aa 112-146), interspecies variable domain (aa 131-141) and VP6 binding site (aa 156-175). Changes were also identified in aa 89, 148, 153 and 154.

Several significant aa differences were observed between distinct NSP4 genotypes, mainly in the interspecies variable domain (Fig. 2, marked in gray box) and in the VP6 binding site (Fig. 2, indicated by diamonds).

In the region proposed to be the enterotoxigenic domain (aa 114-135), the following changes were observed: (Y-H) and (K-N) at aa 131 and 133, respectively (Fig. 2, indicated by asterisks). No differences in the aa sequences of NSP4 were observed between samples from children with or without diarrhea (Fig. 2).

<table>
<thead>
<tr>
<th>Cities-States</th>
<th>Samples</th>
<th>Genotype A</th>
<th>Genotype B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goiânia-GO</td>
<td>91</td>
<td>12 13.2</td>
<td>79 86.8</td>
</tr>
<tr>
<td>Campo Grande-MS</td>
<td>51</td>
<td>- -</td>
<td>51 100.0</td>
</tr>
<tr>
<td>Brasília-DF</td>
<td>11</td>
<td>- -</td>
<td>11 100.0</td>
</tr>
<tr>
<td>Total</td>
<td>153</td>
<td>12 7.8</td>
<td>141 92.2</td>
</tr>
</tbody>
</table>

Note: a: children with (n = 151) and without (n = 2) diarrhea.

Although five rotavirus NSP4 genotypes have been identified to date (Ciarel et al. 2000, Mori et al. 2002, Lin & Tian 2003), most of the diversity in the NSP4-encoding gene among human rotaviruses lies in genotypes A and B (Iturriza-Gómez et al. 2003). Other studies, however, have detected unusual strains. Cho et al. (2006) observed that two human rotavirus samples in Seoul had a low degree of homology with the currently described NSP4 genotypes, suggesting a possible new NSP4 genotype.

In this study, NSP4 genotypes A and B could be recognized in human group A rotavirus-positive fecal samples. These results are similar to those described by other authors in Brazil (Mascarenhas et al. 2006, 2007, Araújo et al. 2007) and in other countries (Kirkwood et al. 1999, Lee et al. 2000, Iturriza-Gómez et al., Cho et al. 2006). None of the samples analyzed in our study were closely related to the AU-1 prototype strain, which was isolated from humans and described as NSP4 genotype C (Iturriza-Gómez et al. 2003).

Our data show that genotype B was the most frequently detected (92.2%) genotype in the West Central region. Similar data were also observed in the Southeast region of Brazil (Araújo et al. 2007) as well as in other countries (Kirkwood et al. 1999, Lee et al. 2000, Iturriza-Gómez et al. 2003, Cho et al. 2006); however, different results were observed in the Northern region of Brazil, where genotype A was predominant (Mascarenhas et al. 2006, 2007). Genotype A samples were identified only in children from GO, while genotype B samples were identified in all three cities. In GO, genotype A was found only in the 1990s; however, after 2000, it seemed to be replaced by genotype B. Furthermore, the only sample identified in 1987 characterized as NSP4 genotype B was not included.

DISCUSSION

In this study, a detection rate of 78.5% was observed for the NSP4-encoding gene from rotavirus-positive fecal samples collected in three cities located in the West Central region of Brazil. We speculate that the NSP4 gene could not be detected in all samples due to degradation of the RNA probably as a result of: i) low number of particles present in fecal specimens from rectal swabs; ii) RNA degradation by RNAses; iii) repeated freezing and unfreezing of these samples; iv) preservation of fecal specimens at -20°C and not at -70°C; v) defective particle; presence and/or vi) eventual inhibitor persistence of the RT-PCR.

Although five rotavirus NSP4 genotypes have been identified to date (Ciarel et al. 2000, Mori et al. 2002, Lin & Tian 2003), most of the diversity in the NSP4-encoding gene among human rotaviruses lies in genotypes A and B (Iturriza-Gómez et al. 2003). Other studies, however, have detected unusual strains. Cho et al. (2006) observed that two human rotavirus samples in Seoul had a low degree of homology with the currently described NSP4 genotypes, suggesting a possible new NSP4 genotype.

In this study, NSP4 genotypes A and B could be recognized in human group A rotavirus-positive fecal samples. These results are similar to those described by other authors in Brazil (Mascarenhas et al. 2006, 2007, Araújo et al. 2007) and in other countries (Kirkwood et al. 1999, Lee et al. 2000, Iturriza-Gómez et al., Cho et al. 2006). None of the samples analyzed in our study were closely related to the AU-1 prototype strain, which was isolated from humans and described as NSP4 genotype C (Iturriza-Gómez et al. 2003).

Our data show that genotype B was the most frequently detected (92.2%) genotype in the West Central region. Similar data were also observed in the Southeast region of Brazil (Araújo et al. 2007) as well as in other countries (Kirkwood et al. 1999, Lee et al. 2000, Iturriza-Gómez et al. 2003, Cho et al. 2006); however, different results were observed in the Northern region of Brazil, where genotype A was predominant (Mascarenhas et al. 2006, 2007). Genotype A samples were identified only in children from GO, while genotype B samples were identified in all three cities. In GO, genotype A was found only in the 1990s; however, after 2000, it seemed to be replaced by genotype B. Furthermore, the only sample identified in 1987 characterized as NSP4 genotype B was not included.

In this study, a detection rate of 78.5% was observed for the NSP4-encoding gene from rotavirus-positive fecal samples collected in three cities located in the West Central region of Brazil. We speculate that the NSP4 gene could not be detected in all samples due to degradation of the RNA probably as a result of: i) low number of particles present in fecal specimens from rectal swabs; ii) RNA degradation by RNAses; iii) repeated freezing and unfreezing of these samples; iv) preservation of fecal specimens at -20°C and not at -70°C; v) defective particle; presence and/or vi) eventual inhibitor persistence of the RT-PCR.

Although five rotavirus NSP4 genotypes have been identified to date (Ciarel et al. 2000, Mori et al. 2002, Lin & Tian 2003), most of the diversity in the NSP4-encoding gene among human rotaviruses lies in genotypes A and B (Iturriza-Gómez et al. 2003). Other studies, however, have detected unusual strains. Cho et al. (2006) observed that two human rotavirus samples in Seoul had a low degree of homology with the currently described NSP4 genotypes, suggesting a possible new NSP4 genotype.

In this study, NSP4 genotypes A and B could be recognized in human group A rotavirus-positive fecal samples. These results are similar to those described by other authors in Brazil (Mascarenhas et al. 2006, 2007, Araújo et al. 2007) and in other countries (Kirkwood et al. 1999, Lee et al. 2000, Iturriza-Gómez et al. 2003, Cho et al. 2006). None of the samples analyzed in our study were closely related to the AU-1 prototype strain, which was isolated from humans and described as NSP4 genotype C (Iturriza-Gómez et al. 2003).

Our data show that genotype B was the most frequently detected (92.2%) genotype in the West Central region. Similar data were also observed in the Southeast region of Brazil (Araújo et al. 2007) as well as in other countries (Kirkwood et al. 1999, Lee et al. 2000, Iturriza-Gómez et al. 2003, Cho et al. 2006); however, different results were observed in the Northern region of Brazil, where genotype A was predominant (Mascarenhas et al. 2006, 2007). Genotype A samples were identified only in children from GO, while genotype B samples were identified in all three cities. In GO, genotype A was found only in the 1990s; however, after 2000, it seemed to be replaced by genotype B. Furthermore, the only sample identified in 1987 characterized as NSP4 genotype B was not included.
sequences of symptomatic and asymptomatic samples. These results are consistent with other investigations (Horie et al. 1997, Lee et al. 2000, Mascarenhas et al. 2007); however, since only two samples from children without diarrhea were analyzed, the role of NSP4 as a possible pathogenic determinant of rotavirus could not be assessed in this work.


### TABLE III

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>NSP4 genotypes</th>
<th>1990-1999</th>
<th>2000-2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>P[8]G1</td>
<td>B</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>P[6]G1</td>
<td>B</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>P[8]G2</td>
<td>B</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>P[8]G3</td>
<td>B</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>P[8]G4</td>
<td>B</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>P[8]G9</td>
<td>B</td>
<td>-</td>
<td>4</td>
</tr>
</tbody>
</table>

*a: see Cardoso et al. (2003), Souza et al. (2003), Costa et al. (2004), Andreassi et al. (2007).*

Amino acid variations between genotypes A and B were concentrated mainly in the interspecies variable domain (aa 131-141) and in the VP6 binding region (aa 156-175) (Estes & Kapikian 2007). Our data on the region of aa 131-141 are similar to a study from Brazil (Araújo et al. 2007), considering the occurrence of extensive variation in this region, as well as to a study performed in the UK (Iturriza-Gómarra et al. 2003). In addition, in the present study, it was observed that aa 131 was identified as tyrosine (genotype A) or histidine (genotype B), similar to the results of Mascarenhas et al. (2007), but contrary to the common postulate that diarrheal samples have a tyrosine at this position (Ball et al. 1996). Our results are also in agreement with other investigations (Cunlife et al. 1997, Iturriza-Gómarra et al. 2003, Araújo et al. 2007), and they suggest that at these NSP4 protein regions are distinct between genotypes.

The immune response to A and B genotypes of the NSP4 gene has not yet been defined, and it is still unknown whether the inclusion of this gene in rotavirus vaccination strategies is important (Araújo et al. 2007). In this context, the possible selection of NSP4 as a target for vaccine development requires further investigation (Lee et al. 2000, Araújo et al. 2007). In this study, the nucleotide sequence of the NSP4 gene was determined for the first time in 153 human rotavirus strains belonging to genotypes of different G and P combinations recovered from children with or without diarrhea in West Central region of Brazil. The NSP4 gene

---

**Fig. 2:** multiple alignment of the partial deduced amino acid sequence of the NSP4 protein of 15 human rotaviruses samples with human prototype strains grouped in genotypes A, B and C. Dots indicate identity to the KUN/Hu strain. Gray box indicates interspecies variable domain. Diamonds indicate VP6 binding site. Asterisks indicate enterotoxigenic domain, asymptomatic child.
analysis performed in our study provides insight into the genetic relationships between different rotavirus samples circulating in a particular region of Brazil.

REFERENCES


Souza MBLD, Rácz ML, Leite JGP, Soares CMA, Martins RMB, Munford V, Cardoso DDP 2003. Molecular and serological characterization of group A rotavirus isolates obtained from hospital-


Capítulo VI

Perspectivas
VI – PERSPECTIVAS

1. Análise da expressão gênica da serino protease em condições de limitação de nitrogênio e durante a infecção em diferentes sítios de infecção em camundongos através da técnica de PCR em tempo real;

2. Análise de atividade proteolítica da serino protease em gel de atividade contendo gelatina;

3. Análises proteômicas de sobrenadante de cultura de *P. brasiliensis* em condições de limitação de nitrogênio;

4. Ampliar estudos de interações intermoleculares de serino protease de *P. brasiliensis* através da técnica de duplo-híbrido em sistema *S. cerevisiae*;

5. Desenvolvimento de ferramentas genéticas para análise do papel das proteases identificadas diferencialmente expressas durante a transição dimórfica de *P. brasiliensis*.
Referências Bibliográficas
VII – REFERÊNCIAS BIBLIOGRÁFICAS


BAGAGLI E, BOSCO SM, THEODORO RC, FRANCO M. Phylogenetic and evolutionary aspects of Paracoccidioides brasiliensis reveal a long coexistence with animal hosts that explain several biological features of the pathogen. Infect Genet Evol. 2006. 6: 344-51.


DAVE JA, GEY VAN PITTIUS NC, BEYERS AD, EHLERS MR, BROWN GD. Mycosin-1, a subtilisin-like serine protease of *Mycobacterium tuberculosis*, is cell wall-


MARSH JA, KALTON HM, GABER RF. Cns1 is an essential protein associated with the hsp90 chaperone complex in *Saccharomyces cerevisiae* that can restore cyclophilin 40-dependent functions in cpr7Delta cells. *Mol Cell Biol.* 1998. 18: 7353–9.


NICHOLSON DW, ALI A, THORNBERRY NA, VAILLANCOURT JP, DING CK, GALLANT M, GAREAU Y, GRIFFIN PR, LABELLE M, LAZEBNIK YA, MUNDAY NA, RAJU SM, SMULSON ME, YU TY, MILLER DK. Identification


PARENTE JA, BORGES CL, BAILÃO AM, FELIPE MSS, PEREIRA M, SOARES CMA. Comparison of transcription of multiple genes during mycelia transition to yeast


SILVA MB, MARQUES AF, NOSANCHUK JD, CASADEVALL A, TRAVASSOS LR, TABORDA CP. Melanin in the dimorphic fungal pathogen Paracoccidioides brasiliensis: effects on phagocytosis, intracellular resistance and drug susceptibility. Microbes Infect. 2006. 8: 197-205.


TERÇARIOLI GR, BAGAGLI E, REIS GM, THEODORO RC, BOSCO SDE M, MACORIS SA, RICHIINI-PEREIRA VB. Ecological study of Paracoccidioides...


VIII. Anexos

Produção bibliográfica durante o doutorado

Artigos completos publicados em periódicos


6. FREITAS ER, SOARES CMA, FIACCADORI FS, SOUZA M, PARENTE JA, COSTA PS, CARDOSO DD. Occurrence of group A rotavirus mixed P genotypes


**Manuscritos in press ou em revisão**

1. TACCO BACA, PARENTE JA, BARBOSA MS, BÁO SN, GÓES TS, PEREIRA M, SOARES CMA. Characterization of a secreted aspartyl protease of the fungal pathogen *Paracoccidioides brasiliensis*. Medical Mycology. *In press*.

Manuscritos

1. **PARENTE JA**, SALEM-IZZAC SM, SANTANA JM, BAILÃO AM, SOARES CMA. Characterization of a secreted serine protease from *Paracoccidioides brasiliensis*.

2. BORGES CL, **PARENTE JA**, BARBOSA MS, SANTANA JM, BAO SN, SOUSA MV, SOARES CMA. Characterization of the formamidase of *Paracoccidioides brasiliensis*: protein analysis, localization and intermolecular interactions.
VIII. Anexos

Produção bibliográfica durante o doutorado

Artigos completos publicados em periódicos


6. FREITAS ER, SOARES CMA, FIACCADORI FS, SOUZA M, PARENTE JA, COSTA PS, CARDOSO DD. Occurrence of group A rotavirus mixed P genotypes


**Manuscritos in press ou em revisão**

1. TACCO BACA, PARENTE JA, BARBOSA MS, BÁO SN, GÓES TS, PEREIRA M, SOARES CMA. Characterization of a secreted aspartyl protease of the fungal pathogen *Paracoccidioides brasiliensis*. Medical Mycology. *In press.*

Manuscritos

1. **PARENTE JA**, SALEM-IZZAC SM, SANTANA JM, BAILÃO AM, SOARES CMA. Characterization of a secreted serine protease from *Paracoccidioides brasiliensis*.

2. BORGES CL, **PARENTE JA**, BARBOSA MS, SANTANA JM, BAO SN, SOUSA MV, SOARES CMA. Characterization of the formamidase of *Paracoccidioides brasiliensis*: protein analysis, localization and intermolecular interactions.