Capítulo V

Artigos em colaboração

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Transcriptional Profiles of the Human Pathogenic Fungus *Paracoccidioides brasiliensis* in Mycelium and Yeast Cells*[S]

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***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 overexpressed transcripts, stage-specific genes, and also those that are up- or down-regulated as assessed by *in silico* electronic 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] (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.

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[S] The on-line 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 GenBank™/EBI Data Bank with accession number(s) CA580326-CA584263, CN238087-CN253933, 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 https://www.biomol.unb.br/Pb.

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[1] 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.

P. brasiliensis genome size was estimated to be ~30 Mb (7). A study of *P. brasiliensis* gene density suggests that this fungus contains between 7,500 and 9,000 genes,[2] 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 down-regulated 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 were also detected in several metabolic pathways. Finally, the majority of genes involved in signal transduction pathways (cAMP/protein kinase A, Ca^{2+}/calmodulin, and MAPKs) possibly participating in cell differentiation and infection were annotated, and now we are able to describe the corresponding signaling systems in *P. brasiliensis*.

MATERIALS AND METHODS

Fungus—*P. brasiliensis* isolate Pb01 (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)$^+$ mRNA was isolated from total mycelium and yeast RNA through oligo(dT)-cellulose columns (Stratagene). Unidirectional cDNA libraries were constructed in λZAPII 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 sequencer (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 these exclusion criteria. Contaminant 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 (PbAESTs) 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 GenBank™ 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 assemblies. Additionally, we used the FASTA program (11) to compare assemblies with *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* predicted polypeptides. The INTERPROSCAN program (12) was used to obtain domain and family classification of the assemblies. Metabolic pathways were analyzed using maps obtained in the KEGG Web site (13) with annotated EC numbers, and this information was used to help in assigning function to PbAESTs.

Differential Expression Analysis in Silico by Electronic Subtraction—To assign a differential expression character, the contigs formed with mycelium and yeast ESTs were statistically evaluated using a test previously described (14) with a confidence of 95%.

cDNA Microarrays and Data Analysis—A set of two microarrays containing a total of 1,152 clones in the form of PCR products was spotted in duplicate on 2.5 × 7.5-cm Hybond N$^+$ nylon membranes (Amersham Biosciences). Arrays were prepared using a Generation III Array Spotter (Amersham Biosciences). Complementary DNA inserts of both *P. brasiliensis* libraries were amplified in 96-well plates using vector-PCR amplification with T3 forward and T7 reverse universal primers. Membranes were first hybridized against the T3 [α-^{33}P]dCTP-labeled oligonucleotide. The amount of DNA deposited in each spot was estimated by the quantification of the obtained signals. After stripping, membranes were used for hybridization against α-^{33}P-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-^{33}P]cDNA 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. BZScan 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.

Northern Blot Analysis—Total 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-^{32}P]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: Pbgenome project Web site (www.biomol.unb.br/Pb); Gene Ontology Consortium (www.geneontology.org); Cluster of Ortologous Genes (www.ncbi.nlm.nih.gov/COG); INTERPROSCAN (www.ebi.ac.uk/interpro/); National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/); Kyoto Encyclopedia of Genes and Genomes (www.genome.ad.jp/kegg); BZScan Software (tagc.univ-mrs.fr); Audic and Claverie statistical test (telethon.bio.unipd.it/bioinfo/IDEG6_form/); Significance Analysis of Microarrays method (www-stat.stanford.edu/~tibs/SAM/); *Candida albicans* data base (genolist.pasteur.fr/CandidaDB/); genomes from *Aspergillus nidulans* and *Neurospora crassa* (www.broad.mit.edu/annotation/fungi/aspergillus/).

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 from both libraries. Upon removal of bacterial and rRNA contaminant sequences, a total of 19,718 high quality ESTs underwent CAP3 assembly, yielding 2,655 contigs and 3,367 singlets, which constitute the so-called 6,022 *P. brasiliensis* Assembled EST (PbAEST) data base. Contigs presented an average size of 901 bp, and the number of ESTs assembled into contigs varied from 2 to 657 in the largest one (PbAEST 1068), which corresponds to M51, a previously reported *P. brasiliensis* mycelium-specific transcript (17). Of the 6,022 PbAESTs, 4,198 (69.4%) showed a probable homologue in GenBank™, and 4,130 (68.3%) showed a fungus homologue (Fig. 1*A* and Supplemental Table I). 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. 1*B*) compared with *S. cerevisiae* functional categorization (3.4%).

Highly and Differentially Expressed Genes—The 27 highly transcribed genes found in the *P. brasiliensis* transcriptome, using a cut-off of 50 reads, are shown in Supplemental Table II. Some of them were previously reported (8). Also, up- and down-regulated genes in mycelium and yeast cells were detected by statistical comparison of the number of sequences in corre-

[2] C. Reinoso, G. Niño-Vega, G. San-Blas, and A. Dominguez (2003) IV Congreso Virtual de Micologia, personal communication.

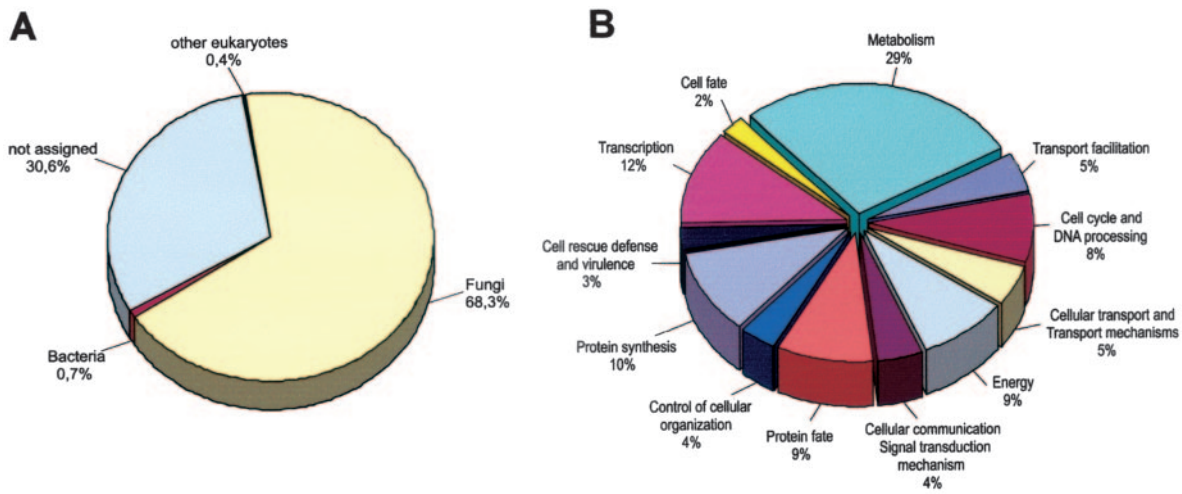

FIG. 1. ***P. brasiliensis* transcriptome characterization.** *A*, distribution of blast best hit among organisms. Each PbAEST was tested against the GenBank™ nr data base, and the best hit organism was computed. A PbAEST was considered as not assigned when the best hit exceed an *E* value of 10^{-10}.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

sponding PbAESTs (Table I). 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 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. brasiliensis* by different approaches (17–20). Other key up-regulated genes related to the metabolism of *P. brasiliensis* (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. brasiliensis* estradiol-binding protein (21), also described in *C. albicans* and in other fungi (22). It is speculated that the interaction of the 17-β-estradiol hormone with a cytoplasmic protein inhibits the mycelium-to-yeast transition, explaining the lower incidence of PCM in females.

Metabolic Overview—P. brasiliensis 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. brasiliensis* 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. brasiliensis* was unable to grown 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. brasiliensis* 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. brasiliensis* 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. brasiliensis* 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

TABLE I

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

The PbAESTs were analyzed as to their differential expression by two methods: a statistical analysis of the number of mycelium and yeast ESTs clustered in each PbAEST (14) and a cDNA microarray analysis of 1,152 PbAESTs, chosen according to the electronic subtraction criteria. A differential pattern of genes encoding enzymes was used in the analysis of the differential metabolism.

PbAEST	EC number	Annotated function	Number of reads[a]		*p* value[b]	-Fold change[c]	Accession number/Best hit organism/ *E* value
			M	Y			
Mycelium up-regulated genes							
1068		M51[d,e]	653	4	0.000000	41666.0	BE758605/*P. brasiliensis* /0.0
2274	4.4.1.5	Lactoylglutathione lyase[e]	75	0	0.000000	7.0	NP_105614.1/*Mesorhizobium loti*/ 1e-11
2521		Hydrophobin 1[d,f]	56	0	0.000000		AAM88289.1/*P. brasiliensis*/2e-51
1789		HSP90 co-chaperone[f]	19	10	0.018169		CAD21185.1/*N. crassa*/4e-48
2509	1.15.1.1	Copper-zinc superoxide dismutase[f]	14	5	0.010801		Q9Y8D9/*A. fumigatus*/1e-68
2458		Unknown[f]	13	6	0.025336		
2478		Hydrophobin 2[d,f]	9	0	0.000951		AAR11449.1/*P. brasiliensis*/2e-70
1287	1.13.11.32	2-nitropropane dioxygenase[f]	8	1	0.008606		CAB91335.2/*N. crassa*/e-133
1318		Amino acid permease[e]	8	0	0.001907	50.4	CAD21063.1/*N. crassa*/0.0
1470		Unknown[e]	8	2	0.021572	20.1	
2269	2.7.4.3	Adenylate kinase[f]	5	1	0.046263		NP_011097.1/*S. cerevisiae*/1e-42
2364		Unknown[e]	5	1	0.046263	3.6	
379		Unknown[e]	5	1	0.046263	4.9	
1092	4.2.1.22	Cystathionine β-synthase[f]	4	0	0.030842		AAL09565.1/*Pichia pastoris*/4e-96
2356	2.2.1.2	Transaldolase[f]	4	0	0.030842		NP_013458.1/*S. cerevisiae*/e-108
2476	3.1.2.22	Palmitoyl-protein thioesterase[f]	4	0	0.030842		I58097/*H. sapiens*/8e-42
4135	1.1.1.41	Isocitrate dehydrogenase[g]	1	0	0.248690	3.1	O13302/*Acetobacter capsulatum*/6e-31
5530	6.2.1.5	β-Succinyl CoA synthetase[g]	1	0	0.248690	2.7	T49777/*N. crassa*/9e-73
4749	2.7.1.2	Glucokinase[g]	1	0	0.248690	1.7	Q92407/*Aspergillus niger*/2e-50
4246	2.7.1.48	Uridine-kinase[g]	1	0	0.248690	2.7	T41020/*S. pombe*/3e-28
Yeast up-regulated genes							
2536		Y20 protein[e,d]	27	88	0.000000	8.7	AAL50803.1/*P. brasiliensis*/e-106
2431	1.1.1.1	Alcohol dehydrogenase I[f]	2	45	0.000000		P41747/*Aspergillus flavus*/e-129
737	3.5.1.41	Xylanase/chitin deacetylase[e]	8	33	0.000023	2.8	NP_223015.1/*Helicobacter pylori*/ e-113
201		Putative membrane protein Nce2[e]	0	27	0.000000	25.2	NP_015475.1/*S. cerevisiae*/5e-08
797	3.1.6.6	Choline sulfatase[e]	3	15	0.001602	4.8	NP_248721.1/*P. aeruginosa*/e-104
814		Glyoxylate pathway regulator[e]	0	15	0.000016	17.7	NP_009936.1/*S. cerevisiae*/4e-37
1704		60S ribosomal protein L19[f]	0	14	0.000032		NP_596715.1/*S. pombe*/6e-49
1585	1.8.4.8	PAPS reductase[e]	1	12	0.000815	5.1	AAG24520.1/*Penicillium chrysogenum*/e-121
63		Putative methyltransferase[e]	3	11	0.011314	2.5	CAD21381.1/*N. crassa*/2e-46
778		Putative estradiol-binding protein[e]	3	11	0.011314	29.5	NP_012049.1/*S. cerevisiae*/1e-31
136		Unknown[d,f]	4	10	0.030950	3.9	
767		Unknown[e]	3	10	0.017732	2.2	
701	1.2.4.1	Pyruvate dehydrogenase[f]	1	9	0.004973		Q10489/ *S. pombe*/1e-72
1724		Putative sterol transporter[e]	0	6	0.007915	29.3	NP_013748.1/*S. cerevisiae*/4e-12
171	2.6.1.42	Branched-chain aminotransferase[f]	0	5	0.015790		NP_012078.1/*S. cerevisiae*/7e-87
1983	1.6.5.3	NADH dehydrogenase (ubiquinone reductase)[f]	0	4	0.031496		S47150/*N. crassa*/1e-19
244	1.1.1.69	Gluconate dehydrogenase[f]	0	4	0.031496		NP_471610.1/*Listeria innocua*/ 1e-09
258	3.3.2.1	Isochorismatase[f]	0	4	0.031496		NP_436193.1/*Sinorhizobium meliloti*/1e-20
279	2.5.1.15	Dihydropteroate synthase[f]	0	4	0.031496		T49535/*N. crassa*/1e-38
314	2.6.1.1	Aspartate aminotransferase[f]	0	4	0.031496		NP_509047.1/*Caenorhabditis elegans*/4e-96
555	6.2.1.3	Acyl-CoA synthetase[f]	0	4	0.031496		NP_275799.1/*Methanothermobacter thermautotrophicus*/9e-89
756	6.3.5.7	Glutamyl-tRNA amidotransferase[f]	0	4	0.031496		Q33446/*A. nidulans*/1e-15
865	4.1.3.1	Isocitrate lyase[f]	0	4	0.031496		AAK72548.2/*Coccidioides immitis*/e-119
963	2.6.1.9	Histidinol-phosphate aminotransferase[f]	0	4	0.031496		P36605/*S. pombe*/4e-87
980	3.5.1.4	Acetamidase[f]	0	4	0.031496		AAK31195.1/*Aspergillus terreus*/2e-09
3073	1.14.13.3	Phenylacetate hydroxylase[g]	0	1	0.249998	2.3	AAF21760.1/*P. chrysogenum*/2e-48

[a] Number of mycelium (M)- and yeast (Y)-derived ESTs in the PbAEST.
[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.

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 lyase redirects the metabolic flow using ethanol and acetate as two-carbon sources and generating oxaloacetate, which can be reconverted to glu-

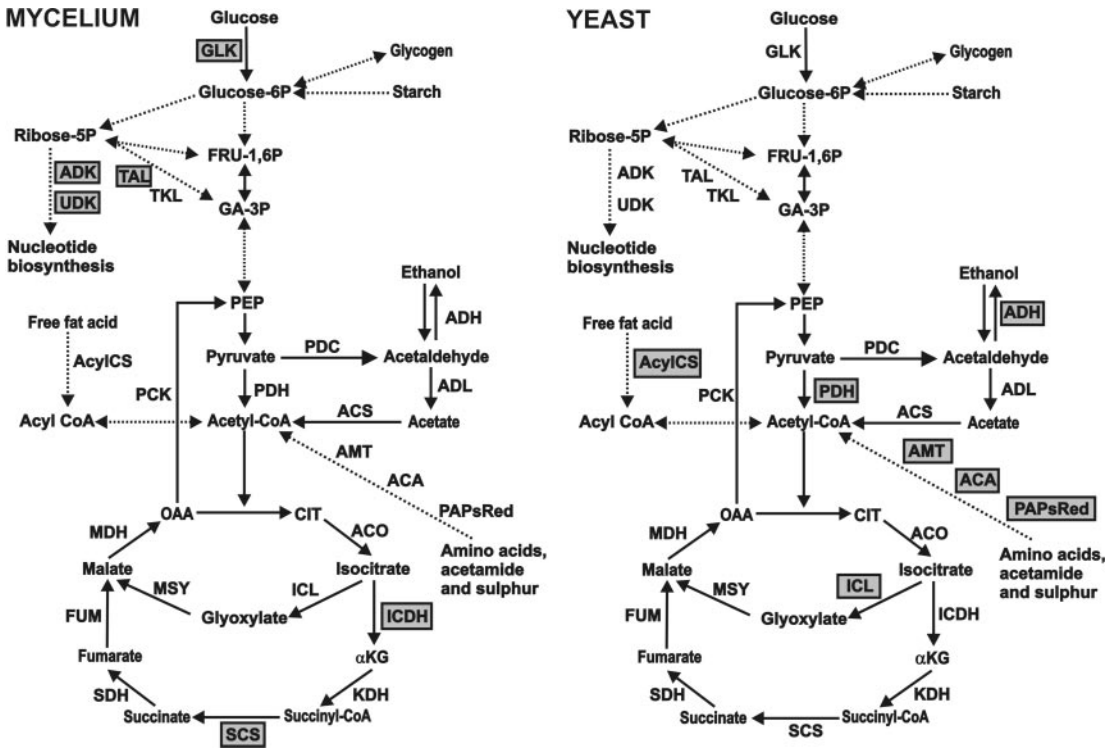

FIG. 2. **Comparison of the expression pattern of genes encoding for enzymes in mycelium-to-yeast cell differentiation of *P. brasiliensis*.** For the detailed metabolic comparison between mycelium and yeast metabolism, see Supplemental Table IV, since we have presented in this figure only the central pathways for carbohydrate metabolism and citrate cycle. Genes that are overexpressed are *boxed*, either in mycelium or yeast cells, according to the criteria described in Table I.

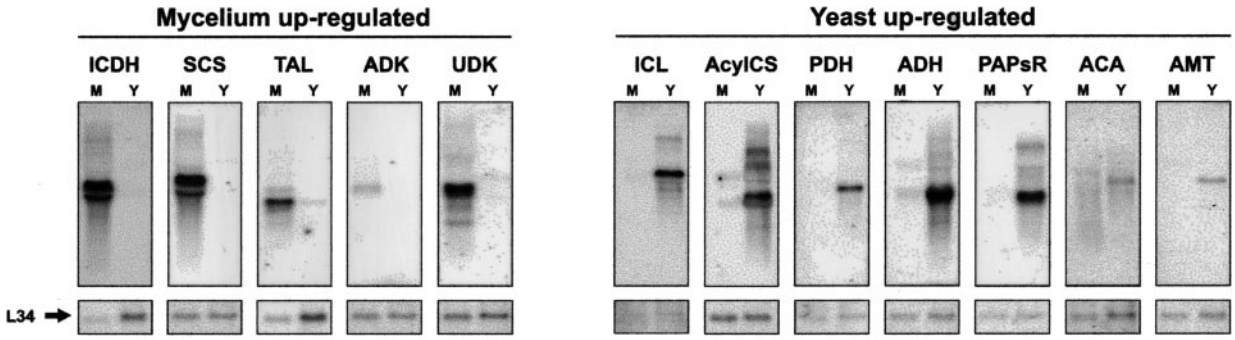

FIG. 3. **Northern blot analysis of mycelium and yeast up-regulated genes of *P. brasiliensis*.** Total RNA samples from both mycelium (*M*) and yeast (*Y*) were blotted onto nylon membranes and hybridized against gene-specific radiolabeled probes. *ICDH*, isocitrate dehydrogenase; *SCS*, β-succinyl-CoA-synthetase, *TAL*, transaldolase; *ADK*, adenylate kinase; *UDK*, uridylate kinase; *ICL*, isocitrate lyase; *acyl-CS*, acyl-CoA synthetase; *PDH*, pyruvate dehydrogenase; *ADH*, alcohol dehydrogenase; *PAPsR*, phosphoadenylyl sulfate reductase; *ACA*, acetamidase; *AMT*, aminotransferase. The constitutive 60 S ribosomal protein L34 was used as a loading control.

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 dihydropteroate 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 *NsdD* 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-calcineurin, 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 (*vps34*), 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

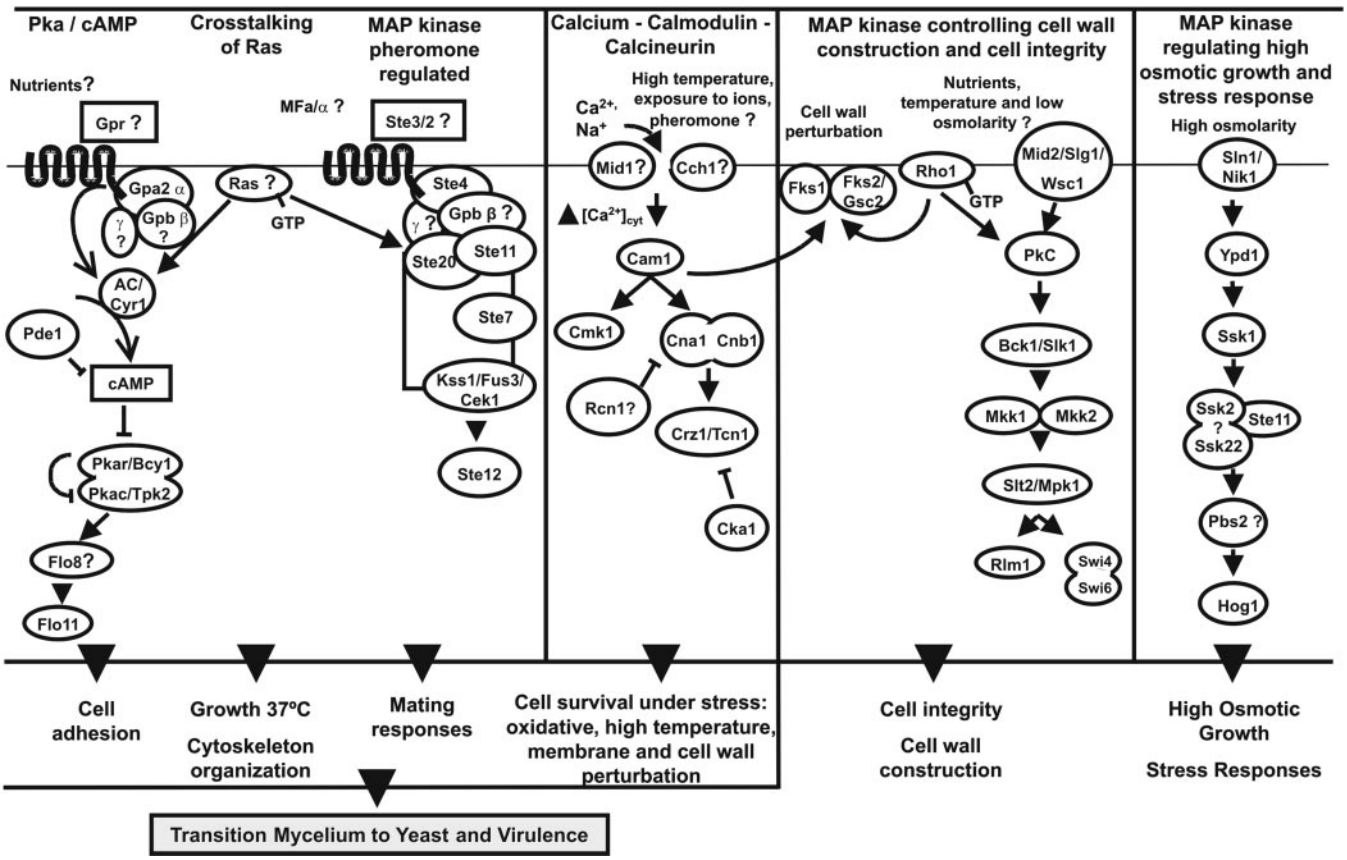

FIG. 4. **Signaling pathways in *P. brasiliensis*.** Shown are cAMP/protein kinase A regulating fungal development and virulence; MAPK signaling for cell integrity, cell wall construction, pheromone/mating, and osmoregulation; calcium-calmodulin-calcineurin controlling cell survival under stress conditions; and Ras allowing cross-talk of extracellular signals. For abbreviations of gene names see Supplemental Table VIII.

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 up-regulated 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 α1,2-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

TABLE II

Putative virulence or essential genes found in P. brasiliensis transcriptome related to the experimentally confirmed orthologues of C. albicans, C. neoformans, and/or A. fumigatus

PBAEST	Orthologue name	AC number/Organism	*E* value[a]	Remarks
Metabolic genes				
2403	*ura3*[b]	DCCKA (*C. albicans*)	3e-41	
		O13410 (*A. fumigatus*)	2e-83	
0670	*nmt*[b]	AAA34351 (*C. albicans*)	8e-60	Lipid synthesis
		AAA17547 (*C. neoformans*)	1e-60	
3750	*fas2*[b]	JC4086 (*C. albicans*)	7e-33	
1224	*hem3*	094048 (*C. albicans*)	1e-58	Hemosynthesis
3819	*tps1*[b]	CAA69223 (*C. albicans*)	1e-36	Glucose metabolism
1693	*icl1*	AAF34690 (*C. albicans*)	1e-112	Glyoxylate cycle
0831	*mls1*	AAF34695 (*C. albicans*)	1e-122	Glyoxylate cycle
1735	*pabaA*[b]	AAD31929 (*A. fumigatus*)	1e-12	Purine synthesis
Cell wall genes				
4346	*chs3*	P30573 (*C. albicans*)	7e-22	Potential drug targets
4968	*gna1*[b]	BAA36496 (*C. albicans*)	4e-16	
1067	*mnt1*	CAA67930 (*C. albicans*)	9e-49	
2980	*pmt1*	AAC31119 (*C. albicans*)	4e-46	
2382	*phr1*	AAF73430 (*C. albicans*)	2e-40*	
1375	*phr2*	AAB80716 (*C. albicans*)	1e-114	
Signal transduction				
4452	*cek1*	A47211 (*C. albicans*)	3e-30	Hyphal formation
1110	*cpp1*	P43078 (*C. albicans*)	6e-16	
267	*cst20*	AAB38875 (*C. albicans*)	6e-48	
358	*hog1*[b]	Q92207 (*C. albicans*)	2e-59	Osmoregulation
988	*nik1*[b]	AAC72284 (*C. albicans*)	7e-37	Hyphal development
Other fungal virulence determinant genes				
623	*cat1*[b]	CAA07164 (*C. albicans*)	1e-172	Peroxisomal catalase
3553	*mdr1*[b]	CAA76194 (*C. albicans*)	2e-27	
3306	*plb1*[b]	AAF08980 (*C. albicans*)	2e-38	Important in host cell penetration
4267	*top1*[b]	Q00313 (*C. albicans*)	4e-56	
5012	*vps34*[b]	CAA70254 (*C. albicans*)	2e-29	Vesicle trafficking
2516	*sod1*[b]	AAK01665 (*C. neoformans*)	4e-51	Nitric oxide detoxification
2463	*ure1*[b]	AAC62257 (*C. neoformans*)	6e-76	
1102	*aox1*[b]	AAM22475 (*C. neoformans*)	2e-48	Resistance to oxidative stress

[a] All *P. brasiliensis* assembled ESTs are BBH with *C. albicans* ortologues, except *phr1* (marked with an asterisk).
[b] Putatively novel *P. brasiliensis* virulence genes.

TABLE III

Potential drug targets genes found in P. brasiliensis transcriptome with no homologues in the human genome

PbAEST	Annotated function	Orthologue accession numbers	*E*-value	Remarks
Cell wall				
5198	β-1,3-glucan synthase	AAD37783	2e-108	Preferentially expressed in mycelium
4988	α-1,3-glucan synthase	AAL18964	2e-70	Preferentially expressed in yeast
0265	Rho	AAK08118	2e-92	Signal transduction
1147	Chitin synthase I	AAF82801	2e-81	
1927	Chitin synthase II	Q92444	3e-66	
4346	Chitin synthase IV	AF107624	2e-65	
3958	Chitin synthase asmA	JC5546	1e-64	
0737	Xylanase/Chitin deacetylase	ZP_00126582	1e-12	Up-regulated in *P. brasiliensis* yeast cells
5473	Bud neck involved	NP_014166	1e-12	Required to link CHS3p and CHS4p to the septins
1063	α-1,2-Mannosyltransferase	NP_009764	1e-20	Involved in protein glycosylation
Glyoxylate cycle				
2402	Malate synthase	P28344	1e-37	
1688	Isocitrate lyase	AAK72548.2	1e-144	Up-regulated in *P.brasiliensis* yeast cells
Other targets				
1959	Δ(24)-Sterol C-methyltransferase	T50969	4e-44	Ergosterol biosynthesis
0200	Aureobasidin resistance protein	AAD22750	1e-43	Sphingolipid synthesis
0845	Elongation factor 3	BAA33893	1e-142	Unique and essentially required for fungal translational machinery
4129	Urate oxidase	P33282	6e-77	Sporulation and pathogenesis
2456	Urease	AAC49868	3e-94	Sporulation and pathogenesis

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.[3] 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

[3] 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 ESTs 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 *P. brasiliensis*, which is dependent on the metabolic state of the cell. It seems that the main regulatory effector on the shunting of the end product of glycolysis into aerobic or anaerobic metabolism is temperature; therefore, it can be hypothesized that this physical factor is the central trigger of all of these molecular events, since it was the only parameter changed in the *in vitro* cultivation of yeast and mycelium of *P. brasiliensis*. Experiments are currently being carried out in order to confirm the *in vivo* expression profile of the differentially expressed genes in macrophages and human pulmonary epithelial cells infected by *P. brasiliensis*.

Since *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 humans and highly expressed in the parasitic yeast, could be a specific drug target for PCM therapy if it is shown to play a key role in the fungal metabolism during human infection. Functional analysis of the *P. brasiliensis* genes described in this work will lead to important information on cellular differentiation, pathogenicity, and/or virulence. These issues can only be addressed when molecular tools are developed for this organism. In conclusion, the knowledge of the transcribed sequences of *P. brasiliensis* will most likely facilitate the development of new therapeutics to PCM and other medically relevant mycosis.

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Research Article

Kinases of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae*: An overview

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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.

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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

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throughout the world, causing chronic respiratory disease and arthritis in infected chickens and turkeys (Allen *et al*., 2005).

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 scheme 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). Robustness of branches was estimated using 100 bootstrap replicates.

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.

Gene product	Presence of ORFs encoding kinase in mycoplasmas		
	Msy ORF	Mhy-J ORF	Mhy-P ORF
Deoxyguanosine kinase	MS0380 MS0140 MS0141	-	-
N-acetylmannosamine kinase	MS0195	-	-
Serine/threonine-protein kinase	MS0121	-	-
Pyruvate kinase	MS0648	MHJ0122	MHP0126
Adenylate kinase	MS0580	MHJ0170	MHP0174
Thymidine kinase	MS0521	MHJ0610	MHP0608
Cytidylate kinase	MS0143	MHJ0065	MHP0069
Guanylate kinase	MS0123	MHJ0149	MHP0153
Phosphoglycerate kinase	MS0114	MHJ0487	MHP0490
Uridylate kinase smbA	MS0677	MHJ0536	MHP0535
6-phosphofructokinase	MS0296	MHJ0107	MHP0111
Acetate kinase	MS0652	MHJ0505	MHP0508
Riboflavin kinase / FMN adenylyltransferase	MS0563	MHJ0270	MHP0278
Thymidylate kinase	MS0052	MHJ0251	MHP0259
Ribose-phosphate pyrophosphokinase	MS0150	MHJ0654	MHP0654
Glycerol kinase	-	MHJ0355	MHP0359
Glucokinase	-	MHJ0515	MHP0517
5-dehydro-2-deoxyglucono kinase	-	MHJ0220	MHP0226

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 b /a 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, thymidine, 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, uridylate and cytidylate 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*.

Fold Group	Family	PFAM members+	Kinase activity (EC)
Group 1: protein S/T-Y kinase/ atypical protein kinase/ lipid kinase/ ATP-grasp	Protein S/T-Y kinase	PF00069	2.7.1.37 Serine/threonine protein kinase
Group 2: Rossmann-like	P-loop kinases:	PF00406	2.7.4.3 Adenylate kinase
		PF00265	2.7.1.21 Thymidine kinase
		PF01712	2.7.1.113 Deoxyguanosine kinase
		PF02224	2.7.4.14 Cytidylate kinase
		PF00625	2.7.4.8 Guanylate kinase
		PF00696	2.7.4.- Uridylate kinase
		PF02223	2.7.4.9 Thymidylate kinase
	Phosphoglycerate kinase:	PF00162	2.7.2.3 Phosphoglycerate kinase
	Phosphofructokinase-like:	PF00365	2.7.1.11 6-phosphofructokinase
	Ribokinase-like:	PF00294	2.7.1.92 5-dehydro-2-deoxygluconokinase
	Thiamin pyrophosphokinase	PF00156	2.7.6.1 Ribose-phosphate pyrophosphokinase
Group 4: ribonuclease H-like	Ribonuclease H-like	PF00480	2.7.1.60 N-acetylmannosamine kinase
		PF00871	2.7.2.1 Acetate kinase
		PF00370	2.7.1.30 Glycerol kinase
		PF02685	2.7.1.2 Glucokinase
Group 5: TIM β/α? barrel kinase	TIM β/α? barrel kinase	PF00224	2.7.1.40 Pyruvate kinase
Group 8: riboflavin kinase	Riboflavin kinase	PF01687	2.7.1.26 Riboflavin kinase

*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 in 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 deoxythymidine 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 are 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-posphate 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 β/α? 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-neuraminate. 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 (Manstein *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-

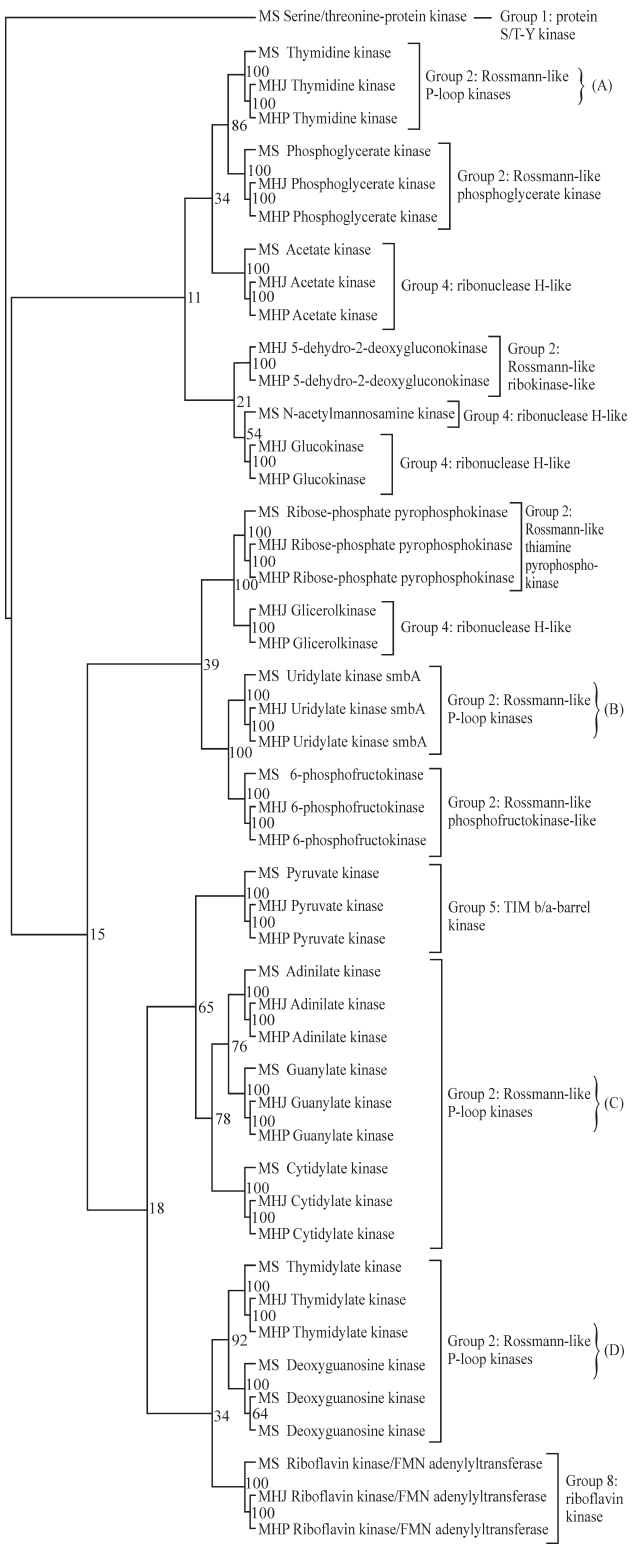

Figure 1 - Phylogenetic tree obtained from kinase amino acid sequence relationships. The kinase fold groups and families are shown in brackets on the right side. The Group 2: Rossmann-like P-loop kinases were clustered into four sub-groups (A, B, C and D). The numbers on the branches are bootstrap values obtained with 100 replications. The kinase encoding ORFs are represented by MSkinase (*M. synoviae*), MHJkinase (*M. hyopneumoniae* J) and MHPkinase (*M. hyopneumoniae* 7448).

volved 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.

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Internet Resources

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

M. hyopneumoniae strain J and *M. hyopneumoniae* strains 7448 complete genomes databases, http://www.genesul.lncc.br.

BLAST tools, http://www.ncbi.nlm.nih.gov/blast.

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

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

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Research Article

Identification of the GTPase superfamily in *Mycoplasma synoviae* and *Mycoplasma hyopneumoniae*

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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.

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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 airsacculitis 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

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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 GTP or GDP 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. hyopneomoniae* 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 mg^{2+} (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 (Kyrpides 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.

GTPase Family	ORF Product	EC /Cellular process involvement	ORFs encoding GTPases found in Mycoplasmas		
			Mycoplasma synoviae 53	*Mycoplasma hyopneumoniae -J*	*Mycoplasma hyopneumoniae -7448*
Elongation factor subfamily					
EF-G	Elongation factor EF-G	3.6.1.48 / protein biosynthesis	MS0047	MHJ0071	MHP0075
EF-TU	Elongation factor Tu	3.6.1.48 / protein biosynthesis	MS0667	MHJ0524	MHP0523
IF-2	Translation initiation factor IF-2	- / Binding / protein biosynthesis	MS0686	MHJ0585	MHP0584
LepA	GTP-binding protein LepA	- / Protein biosynthesis	MS0489	MHJ0069	MHP0073
Era subfamily					
Era	GTP-binding protein Era	- / ATP Binding / nucleic acid binding	MS0387	MHJ0152	MHP0156
EngA	GTP-binding protein EngA	- / 70s ribosome stabilization	MS0142	MHJ0066	MHP0070
ThdF/TrmE	Thiophene and furan oxidation protein ThdF	- / tRNA processing - indirect Ribosome function	MS0362	MHJ0205	MHP0209
FtsY/Ffh subfamily					
FtsY	Cell division protein FtsY	- / Cell division	MS0145	MHJ0008	MHP0008
Ffh	Signal recognition particle, subunit FFH/SRP54	- / Protein targeting to membrane	MS0021	MHJ0053	MHP0057
Obg and YchF					
OBG	GTP-binding protein Obg	- / Ribosome maturation.	MS0168	MHJ0037	MHP0041
YchF	GTP-binding protein YchF	- / Putative ATP Binding	MS0663	MHJ0284	MHP0293
Unclassified	GTP-binding protein	- / Cell division	MS0650 - YihA	MHJ0446 - YihA	MHP0449 - YihA
	Cell division protein FtsZ	- / Cell division	MS0340 - FtsZ	MHJ0406 - FtsZ	MHP0393 - FtsZ
	Probable GTPase EngC	EC 3.6.1.- / unknown	MS0120 - EngC	MHJ0148 - EngC	MHP0152 - EngC
	Putative GTP-binding protein	- / ATP Binding	MS0664 - YlqF	MHJ0083 - YlqF	MHP0087 - YlqF

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 furam oxidation protein (ThdF). Both *M. synoviae* and *M. hypneumoniae* (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* (Mehr *et 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.

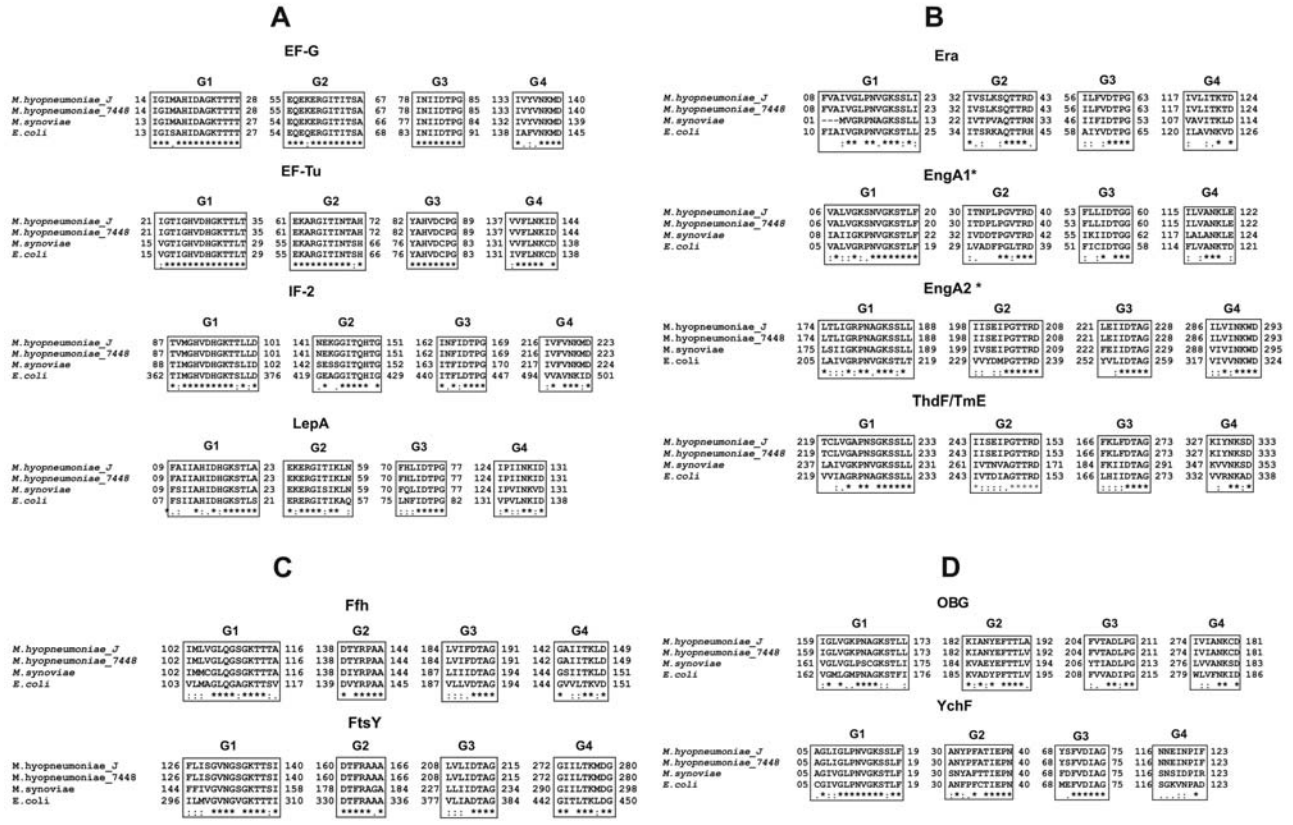

Figure 1 - Alignment of G1, G2, G3 and G4 motifs of the GTPase subfamilies. Panel A: Elongation factor subfamily. Panel B: Era subfamily. Panel C: FtsY/Ffh subfamily. Panel D: OBG YchF subfamily. The sequences used in the alignment are listed in Table 1 and were obtained from: *M. hyopneumoniae J* (*Mycoplasma hyopneumoniae J* GenBank accession number NC-007295), *M. hyopneumoniae 7448* (*Mycoplasma hyopneumoniae 7448*, GenBank accession number NC-007332), *M. synoviae* (*Mycoplasma synoviae* GenBank accession number NC-007294) and *E. coli* (*Escherichia coli*, GenBank accession number NC-000913). The positions of the G1-G4 motifs were obtained by comparison with the most highly conserved regions of *E. coli* orthologs.
*EngA1 and EngA2 refer to the two different G-domains found in all EngA orthologues.

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-

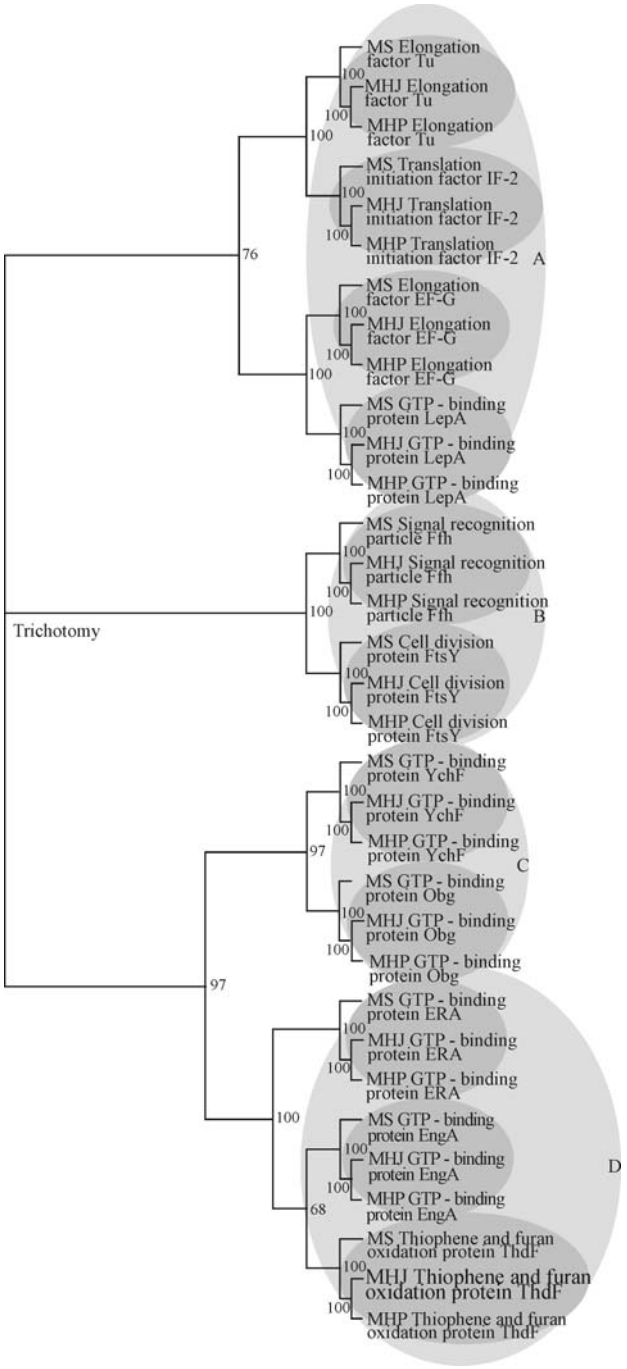

Figure 2 - Amino acid sequence relationship of the GTPase superfamily. (A) Elongation factor subfamily. (B) FtsY/Ffh subfamily. (C) OBG/YchF subfamily. (D) Era subfamily. The numbers on the branches are bootstrap values obtained with 100 replications. Members of each family are described as MS for *M. synoviae* strain 53, MHJ for *M. hyopneumoniae* strain J and MHP for *M. hyopneumoniae* strain 7448.

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.

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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).

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Internet Resources

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

M. hyopneumoniae strain J and *M. hyopneumoniae* strains 7448 complete genome databases, http://www.genesul.lncc.br.

BLAST tools, http://www.ncbi.nlm.nih.gov/blast.

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

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

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RESEARCH ARTICLE

The transcriptional profile of *Paracoccidioides brasiliensis* yeast cells is influenced by human plasma

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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 thermodimorphic; that is, it grows as a yeast-like structure in the host tissue or when cultured at 35–36 °C, and as mycelium in the saprobic condition 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) proteose 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 venepunctures 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, NaH_2PO_4 1.4 mM, Na_2HPO_4 4.3 mM, pH 7.4). The fungal cells (5×10^6 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, Carlsbard, CA) according to the manufacturer's instructions. The quality of RNA was assessed by use of the $A_{260\,nm}/A_{280\,nm}$ 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 (Clonetech 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 representational 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, GATCCTCCCTCG; NBam24, AGGC AACTGTGCTATCCGAGGGAG; RBam12, GATCCTCGGT GA; and RBam24, AGCACTCTCCAGCCTCTCTCACCGAG.

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.unb.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-1*γ); serine proteinase (*pr1H*); glutamine synthetase (*gln1*); ferric reductase (*fre2*); transmembrane osmosensor (*sho1*); 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′-CCTTATATGGGGTGAGTGGT-3′ and 5′-CCTCTCGTTCGCACTGCTC-3′; pyridoxamine phosphate oxidase (*ppo1*), 5′-CATCGACGACTGCCTCC TC-3′ and 5′-GGACGGCTTCTGGGTGCT-3′; putative major facilitator protein (*ptm1*), 5′-CGATTCCTCGCAA TTGGTCA-3′ and 5′-CGTTGCGCCCAATGAGTTC-3′; eukaryotic release factor 1 (*eRF-1*), 5′-CAACGTTGACTT TGTCATTGG-3′ and 5′-CCATGGACTTGTCATATACTG-3′; *eEF-1*γ, 5′-GGCTTGGAGAGGGAGTCG-3′ and 5′-CC CTTGTTGGACGAGACCC-3′; *gln1*, 5′-CGTTACCCTCA CCGTAGAC-3′ and 5′-CATACGGCTGGCCCAAGG-3′; *sho1*, 5′-CCACCACCGGCCACTGAC-3′ and 5′-CCCGAAA CAACTGTCTCCG-3′; and ribosomal L34 protein (*l34*), 5′-CAAGACTCCAGGCGGCAAC-3′ and 5′-GCACCGCCATG ACTGACG-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%

(v/v) Tween-20 plus Tris-buffered saline containing 1% (w/v) dry fat milk, and were then incubated with a polyclonal antibody raised to the recombinant formamidase of *P. brasiliensis* (Borges *et al.*, 2005). The secondary antibody was alkaline phosphatase-conjugated anti-(mouse IgG). Control reactions were performed with a primary antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *P. brasiliensis* (Barbosa *et al.*, 2006). The secondary antibody was alkaline phosphatase-conjugated anti-(rabbit IgG) (diluted 1 : 3000). Reactions were developed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT).

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

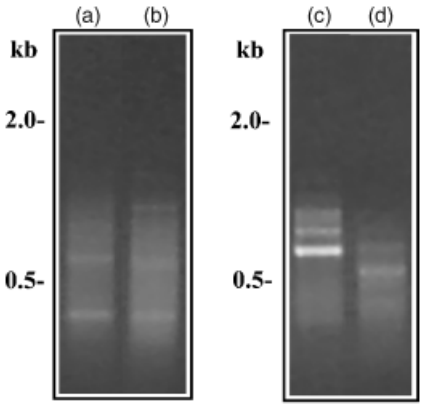

Fig. 1. Agarose gel electrophoresis of subtracted differential cDNA pools derived from *Paracoccidioides brasiliensis* yeast cells incubated with human plasma. Products of the first and second rounds of subtraction performed using as testers the cDNA obtained from RNAs of yeast cells incubated with human plasma for 10 min (lanes a and c, respectively) or for 60 min (lanes b and d, respectively). The numbers on the left side are molecular size markers.

Table 1. Annotated ESTs with high abundance in yeast cells during incubation in human plasma vs. control yeast cells

MIPS category	Gene product	Best hit/accession number	e-value	Redundancy P10	Redundancy P60
Metabolism	2-Methylcitrate dehydratase (MCD)	*Neurospora crassa*/EAA36584.1	$1e^{-95}$	2	3
	5-Aminolevulinic acid synthase*	*Aspergillus oryzae*/AAD38391	$6e^{-70}$	1	–
	Acetolactate synthase (ILV2)*	*Aspergillus nidulans*/XP_409093.1	$3e^{-63}$	3	1
	Adenine phosphoribosyltransferase*	*Aspergillus nidulans*/XP_413220.1	$1e^{-60}$	–	2
	Aldehyde dehydrogenase	*Emericella nidulans*/AAK18073	$4e^{-42}$	–	1
	Anthranilate synthase component II*	*Aspergillus fumigatus*/CAF32024	$1e^{-58}$	–	1
	Aromatic L-Amino-acid decarboxylase (DDC)†	*Gibberella zeae*/XP_385471.1	$5e^{-63}$	59	16
	Formamidase*	*P. brasiliensis*/AAT11170.1	$1e^{-82}$	–	3
	Glutamine synthetase (GLN1)	*Aspergillus nidulans*/XP_408296.1	$1e^{-107}$	18	9
	Inosine-5-monophosphate dehydrogenase*	*Gibberella zeae*/XP_381037.1	$1e^{-54}$	1	–
	NADPH-quinone reductase*	*Aspergillus nidulans*/XP_411331.1	$6e^{-71}$	1	–
	Oleate delta-12 desaturase*	*Aspergillus fumigatus*/CAE47978	$2e^{-81}$	–	1
	Pyridoxamine 5′-phosphate oxidase (PPO1)	*Aspergillus nidulans*/XP406447.1	$6e^{-85}$	3	–
	Sphingosine-1-phosphate lyase*	*Aspergillus nidulans*/XP406126.1	$3e^{-90}$	5	1
	Thiamine-phosphate diphosphorylase*	*Aspergillus nidulans*/XP_408015.1	$2e^{-43}$	3	1
	Transglutaminase*	*Aspergillus nidulans*/XP_405385.1	$3e^{-33}$	4	–
Energy	Acetyl-CoA synthetase (ACS)*	*Aspergillus nidulans*/EAA62719	$3e^{-90}$	–	9
	Acyl-CoA dehydrogenase (FADE1)	*P. brasiliensis*/AAQ04622	$1e^{-100}$	1	4
	Acyltransferase family protein (SMA1)*	*Aspergillus nidulans*/XP_412367.1	$6e^{-27}$	1	–
	Cytochrome c oxidase assembly protein (COX15)*	*Aspergillus nidulans*/XP406052.1	$1e^{-70}$	–	3
	Cytochrome c oxidase subunit V*	*Aspergillus niger*/CAA10609	$2e^{-17}$	1	2
	Cytochrome P450 monooxygenase*	*Aspergillus nidulans*/XP412215.1	$1e^{-74}$	7	4
	D-Lactate dehydrogenase*	*Aspergillus nidulans*/XP413203.1	$4e^{-76}$	1	–
	Long-chain fatty-acid CoA-ligase (FAA1)	*Aspergillus nidulans*/XP410151.1	$1e^{-61}$	1	4
	Multifunctional β-oxidation protein (FOX2)	*Aspergillus nidulans*/XP411248.1	$9e^{-83}$	–	2
	NADH-fumarate reductase (CFR)*	*Aspergillus nidulans*/XP405680.1	$2e^{-82}$	4	8
Cell cycle	Septin-1	*Coccidioides immitis*/AAK14772.1	$8e^{-88}$	1	1
Transcription	Ap-1-like transcription factor (meab protein)	*Aspergillus nidulans*/XP_411679.1	$2e^{-35}$	11	4
	Cutinase-like transcription factor 1	*Aspergillus nidulans*/XP_405562.1	$2e^{-37}$	3	2
	Splicing factor U2 35-kDa subunit*	*Magnaporthe grisea*/XP_365103.1	$9e^{-64}$	1	–
	Transcription factor HACA	*Aspergillus niger*/AAQ73495	$4e^{-59}$	6	3
	Zinc finger (GATA type) family protein transcription factor	*Aspergillus nidulans*/XP407289.1	$3e^{-29}$	–	3
Protein synthesis	40S ribosomal protein S1B	*Aspergillus nidulans*/XP_413007.1	$2e^{-91}$	1	3
	Eukaryotic release factor 1 (eRF1)†	*Aspergillus nidulans*/EAA60141	$8e^{-99}$	2	5
	Eukaryotic translation elongation factor 1 γ (eEF-1γ)†	*Aspergillus nidulans*/XP_410700.1	$4e^{-56}$	38	19
	Eukaryotic translation initiation factor 4A(eEIF-4A)	*Aspergillus nidulans*/XP_407069.1	$1e^{-79}$	16	35
	Eukaryotic translation initiation factor 4E (eEIF-4E)*	*Aspergillus nidulans*/XP_407548.1	$1e^{-97}$	–	3
	Translation elongation factor 1 α chain	*Ajellomyces capsulata*/AAB17119	$5e^{-24}$	–	2
	Translation elongation factor 3	*Ajellomyces capsulatus*/AAC13304	$1e^{-78}$	–	1
	Translation elongation factor Tu, mitochondrial	*Aspergillus fumigatus*/CAD27297	$1e^{-68}$	–	2
Protein sorting/modification	26S Proteasome non-ATPase regulatory subunit 9*	*Kluyveromyces lactis*/CAH00789.1	$5e^{-12}$	–	1
	Golgi α-1,2-mannosyltransferase*	*Aspergillus nidulans*/XP_410994.1	$1e^{-33}$	–	1
	Mitochondrial inner membrane protease, AAA family*	*Aspergillus nidulans*/XP_409725.1	$2e^{-84}$	–	1
	Probable protein involved in intramitochondrial protein sorting	*Aspergillus nidulans*/XP_408432.1	$2e^{-40}$	–	2
Cellular transport/transport facilitation	Acidic amino acid permease (DIP5)	*Aspergillus nidulans*/XP_410255.1	$6e^{-73}$	6	12
	ATP-binding cassete (ABC) transporter (MDR)	*Venturia inaequalis*/AAL57243	$5e^{-64}$	–	1
	ABC multidrug transport protein	*Gibberella zeae*/XP_382962.1	$3e^{-43}$	–	2
	Coatomer protein*	*Aspergillus nidulans*/XP_405059.1	$1e^{-74}$	1	–
	Endoplasmic reticulum calcium-transporting ATPase	*Aspergillus nidulans*/XP_409880.1	$6e^{-78}$	5	1
	Endoplasmic reticulum–Golgi transport vesicle protein (ERV46)*	*Gibberella zeae*/XP_380545.1	$2e^{-69}$	1	–
	Ferric reductase (FRE2)†	*Aspergillus nidulans*/XP_409043.1	$8e^{-61}$	10	12
	GDP-mannose transporter	*Cryptococcus neoformans*/AAW44189	$1e^{-35}$	2	1

Table 1. Continued.

MIPS category	Gene product	Best hit/accession number	e-value	Redundancy P10	Redundancy P60
	H*/nucleoside cotransporter	*Aspergillus nidulans*/XP_409630.1	$7e^{-47}$	–	1
	High-affinity zinc/iron permease (ZRT1)	*Candida albicans*/EAK96396.1	$6e^{-57}$	3	–
	Major facilitator family transporter	*Magnaporthe grisea*/XP_369043.1	$5e^{-65}$	–	1
	Major facilitator superfamily protein*,‡	*Aspergillus nidulans*/XP_410760.1	$1e^{-51}$	2	–
	Mitochondrial carrier protein	*Neurospora crassa*/XP_328128	$3e^{-76}$	4	1
	Potential low-affinity zinc/iron permease*	*Aspergillus fumigatus*/AAT11931	$1e^{-41}$	2	1
	Potential nonclassic secretion pathway protein*	*Aspergillus nidulans*/XP_411820.1	$1e^{-28}$	7	–
	Putative major facilitator protein (PTM1)	*Neurospora crassa*/EAA27169.1	$3e^{-33}$	1	–
	Putative transmembrane Ca^{2+} transporter protein CCC1	*Aspergillus nidulans*/XP_407818.1	$1e^{-35}$	–	2
Signal transduction	cAMP-dependent serine/threonine protein kinase SCH9	*Aspergillus nidulans*/AAK71879.1	$1e^{-86}$	–	1
	Leucine zipper-EF-hand-containing transmembrane protein 1*,‡	*Aspergillus nidulans*/XP_407076.1	$1e^{-76}$	–	1
	Protein with PYP-like sensor domain (PAS domain)	*Neurospora crassa*/EAA32992.1	$4e^{-45}$	–	2
	Putative cAMP-dependent protein kinase	*Aspergillus nidulans*/XP_412934.1	$2e^{-74}$	3	1
	Ras small GTPase, Rab type	*Aspergillus niger*/CAC17832	$7e^{-80}$	2	–
	Transmembrane osmosensor (SHO1)†	*Aspergillus nidulans*/XP_411835.1	$1e^{-38}$	18	23
Cell rescue and defense	Catalase A*	*Ajellomyces capsulatus*/AAF01462.1	$2e^{-74}$	2	–
	Chaperonin-containing T-complex*	*Aspergillus nidulans*/XP_406286.1	$3e^{-74}$	2	1
	Heat shock protein 30 (HSP30)	*Aspergillus oryzae*/BAD02411	$7e^{-16}$	–	1
	Serine proteinase (PR1H)†	*P. brasiliensis*/AAP83193	$6e^{-95}$	13	14
Cell wall biogenesis	1,3-β-Glucan synthase*	*P. brasiliensis*/AAD37783	$3e^{-96}$	–	1
	Putative glycosyl hydrolase family 76*,‡	*Aspergillus nidulans*/XP_408641.1	$1e^{-69}$	–	1
	Putative glycosyl transferase*	*Aspergillus nidulans*/XP_409862.1	$3e^{-45}$	–	1
Unclassified	Conserved hypothetical protein	*Aspergillus nidulans*/XP_411679.1	$5e^{-36}$	1	1
	Conserved hypothetical protein	*Aspergillus nidulans*/XP_405564.1	$5e^{-53}$	1	–
	Conserved hypothetical protein*	*Aspergillus nidulans*/XP_412972.1	$5e^{-41}$	1	1
	Conserved hypothetical protein	*Aspergillus nidulans*/XP_413281.1	$7e^{-54}$	4	3
	Conserved hypothetical protein	*Neurospora crassa*/XP_323499	$3e^{-25}$	1	1
	Conserved hypothetical protein*	*Aspergillus nidulans*/XP_405564.1	$1e^{-30}$	–	2
	Conserved hypothetical protein*	*Aspergillus nidulans*/XP_404965.1	$3e^{-43}$	4	–
	Conserved hypothetical protein*	*Magnaporthe grisea*/XP_365936.1	$2e^{-41}$	1	–
	Conserved hypothetical protein*	*Aspergillus nidulans*/XP_407902.1	$2e^{-35}$	–	5
	Conserved hypothetical protein*	*Aspergillus nidulans*/XP_407958.1	$1e^{-10}$	–	1
	Conserved hypothetical protein*	*Aspergillus nidulans*/XP_410433.1	$5e^{-46}$	1	–
	Conserved hypothetical protein*	*Neurospora crassa*/CAC28640.1	$1e^{-49}$	–	1
	Conserved hypothetical protein*	*Aspergillus nidulans*/XP_410463.1	$5e^{-34}$	1	–
	Conserved hypothetical protein*	*Aspergillus nidulans*/XP_407250.1	$8e^{-24}$	–	2
	Conserved hypothetical protein‡	*Aspergillus nidulans*/XP_404476.1	$1e^{-22}$	–	2
	Conserved hypothetical protein‡	*Aspergillus nidulans*/XP_408657.1	$6e^{-27}$	–	2
	Hypothetical protein	No hits found	–	1	1
	Hypothetical protein	*Aspergillus nidulans*/XP_410643.1	$2e^{-10}$	1	–
	Hypothetical protein	*Aspergillus nidulans*/XP_407811.1	$1e^{-10}$	1	1
	Hypothetical protein	No hits found	–	2	2
	Hypothetical protein*	No hits found	–	1	–
	Hypothetical protein	No hits found	–	–	1
	Hypothetical protein*	*Candida albicans*/EAK91016	$1e^{-14}$	–	1
	Hypothetical protein	No hits found	–	1	–
	Hypothetical protein‡	No hits found	–	2	2

*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), acetolactate 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 monooxygenase (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/ and at the EST collections present in GenBank (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 monooxygenase (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).

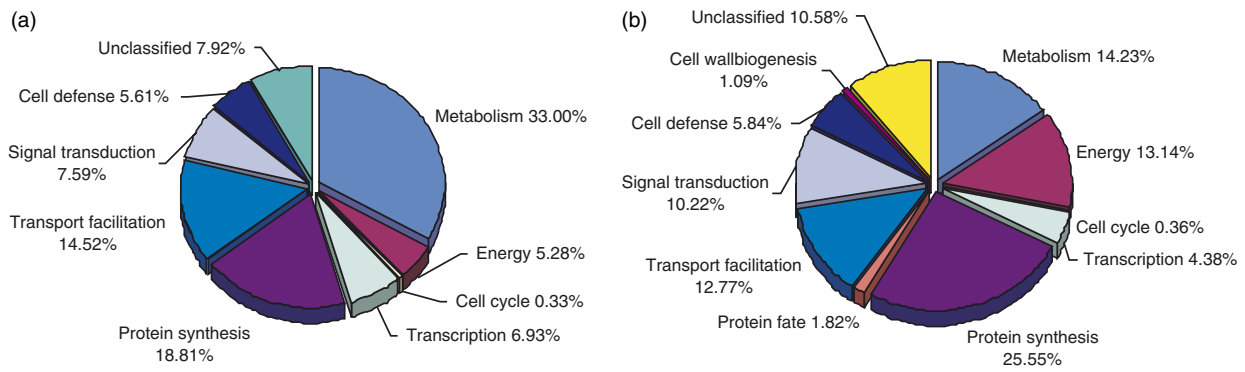

Fig. 2. Functional classification of *Paracoccidioides brasiliensis* cDNAs derived from RDA experiments using as testers the cDNAs obtained from RNA of *Paracoccidioides brasiliensis* yeast cells after incubation with human plasma for 10 min (a) or 60 min (b). The percentage of each functional category 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 $\leq 1e^{-05}$ 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.

Table 2. The most abundant cDNAs expressed during yeast cell incubation in human plasma

			Redundancy	
			Incubation in human plasma	
Gene product	Organism	e-value	10 min	60 min
Acetyl-CoA synthetase*	*Aspergillus nidulans*	$3e^{-90}$	–	9
Acidic amino acid permease†	*Aspergillus nidulans*	$6e^{-73}$	6	12
Ap-1-like transcription factor (meab protein)	*Aspergillus nidulans*	$2e^{-35}$	11	4
Aromatic-L-amino-acid decarboxylase†,‡	*Gibberella zeae*	$5e^{-63}$	59	16
Cytochrome P450 monooxygenase*	*Aspergillus nidulans*	$1e^{-74}$	7	4
Endoplasmic reticulum calcium-transporting ATPase	*Aspergillus nidulans*	$6e^{-78}$	5	1
Eukaryotic release factor 1‡,§	*Aspergillus nidulans*	$8e^{-99}$	2	5
Eukaryotic translation elongation factor 1 gamma chain†,‡,§,¶	*Aspergillus nidulans*	$4e^{-56}$	38	19
Eukaryotic translation initiation factor 4A‡	*Aspergillus nidulans*	$1e^{-79}$	16	35
Ferric reductase†,‡	*Aspergillus nidulans*	$8e^{-61}$	10	12
Fumarate reductase (NADH)*	*Magnaporthe grisea*	$2e^{-82}$	4	8
Glutamine synthetase†,§,¶	*Aspergillus nidulans*	$1e^{-107}$	18	9
Potential nonclassic secretion pathway protein*	*Aspergillus nidulans*	$1e^{-28}$	7	–
Serine protease†,‡,§	*P. brasiliensis*	$6e^{-95}$	13	14
Sphingosine-1-phosphate lyase*	*Aspergillus nidulans*	$3e^{-90}$	5	1
Transcription factor HACA	*Aspergillus niger*	$4e^{-59}$	6	3
Transmembrane osmosensor†,‡,§,¶	*Aspergillus nidulans*	$1e^{-38}$	18	23

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

†Transcripts validated by dot blot.

‡Transcripts more abundant in yeast cells during incubation in human plasma than during incubation in human blood (Bailão *et al.*, 2006).

§Transcripts detected in blood of infected mice, as previously demonstrated (Bailão *et al.*, 2006).

¶Transcripts validated by semiquantitative RT-PCR.

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 eRF-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

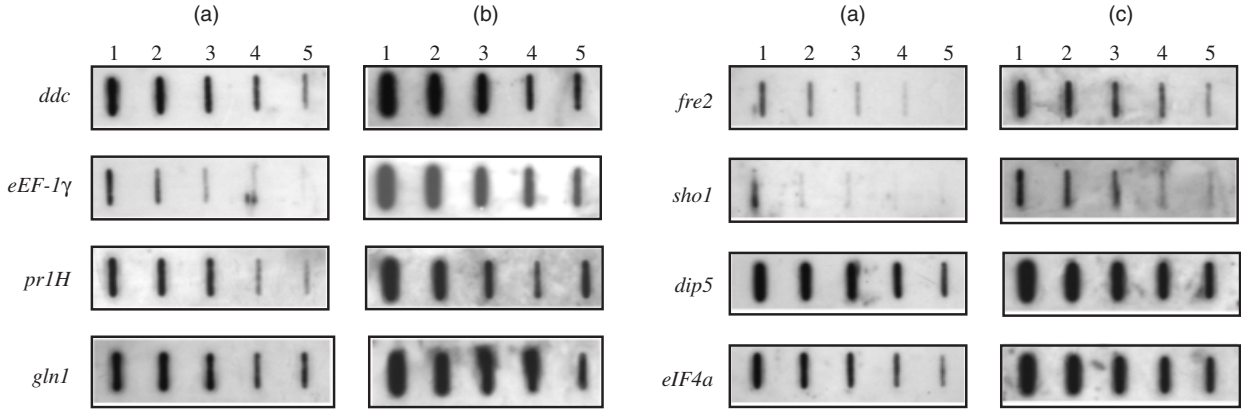

Fig. 3. Dot-blot analysis of *Paracoccidioides brasiliensis* cDNA-RDA clones. DNAs of individual clones were prepared and blotted in several dilutions (1–5). Individual clones were blotted and hybridized to the labeled cDNAs obtained from the control yeast cells (a), and labeled cDNAs obtained from *Paracoccidioides brasiliensis* after 10 min (b) or 60 min (c) of treatment with human plasma. The clones were: aromatic L-amino acid decarboxylase (*ddc*); eukaryotic elongation factor 1, gamma chain (*eEF1-γ*); serine protease (*pr1H*); glutamine synthetase (*gln1*); ferric reductase (*fre2*); transmembrane osmosensor (*sho1*); acidic amino acid permease (*dip5*); and eukaryotic initiation factor 4a (*eIF-4a*).

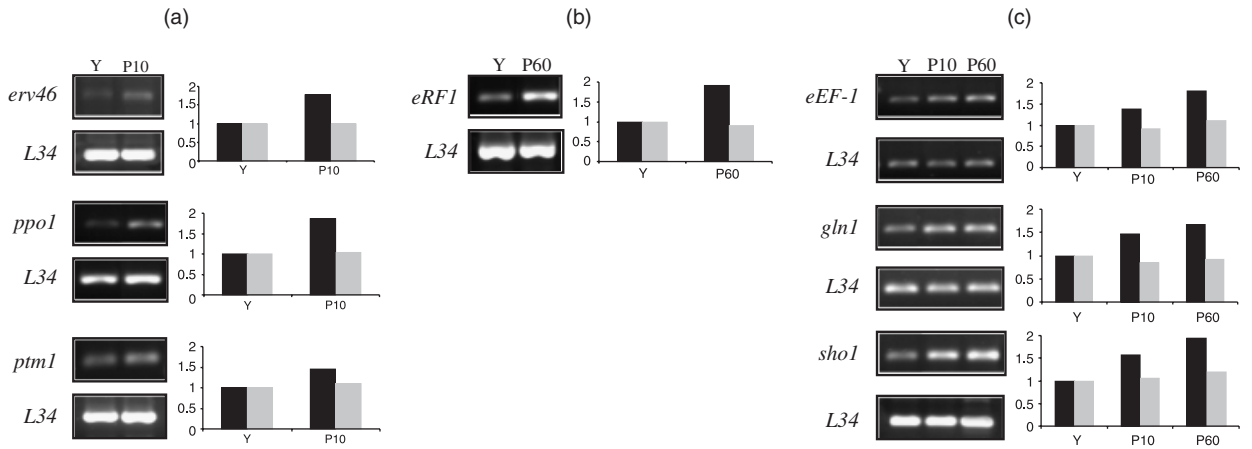

Fig. 4. Validation of RDA results by semiquantitative RT-PCR of RNAs obtained from yeast cells during incubation with human plasma. Semiquantitative RT-PCR analysis was carried out with specific primers, as described. Numbers associated with the bars indicate fold differences relative to the data for the reference *in vitro* cultured yeast cells, which were established by densitometry analysis. Using varied cycle numbers, the exponential phase of each primer was determined and used to allow semiquantitative analysis of the respective reactions. The same amounts of cDNAs were used for all PCR reactions. The RNAs used for RT-PCR were obtained from an independent sample of control yeast cells, and from an independent sample of the yeast cell incubation with human plasma, from those samples used for the RDA experiments. Clone names are given on the left side of the figure. The sizes of the amplified DNA fragments are as follows: *erv46*, 519 bp; *ppo1*, 394 bp; *ptm1*, 166 bp; *eRF1*, 392 bp; *eEF-1γ*, 438 bp; *gln1*, 494 bp; *sho1*, 386 bp. The RNA samples were obtained from: control yeast cells (Y); yeast cells treated with human plasma for 10 min (P10) and 60 min (P60). (a) Transcripts overexpressed during human plasma incubation for 10 min. (b) Transcripts overexpressed during human plasma incubation for 60 min. (c) Transcripts overexpressed in both conditions.

O_2 during β-oxidation in peroxisomes, producing H_2O_2, 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

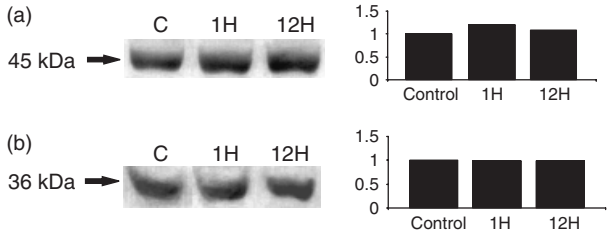

Fig. 5. Validation of the RDA results by Western blot. Total cellular extracts were obtained from yeast cells incubated with human plasma for 1 and 12 h. The proteins (25 μg) were electrophoretically transferred to a nylon membrane and checked by Ponceau S to determine equal loading. The samples were 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).

Table 3. Formamidase activity of yeast cell protein extracts

Treatment	Specific activity*
Control	1.36 ± 0.0417
1 h of incubation in human plasma	2.09 ± 0.0707
12 h of incubation in human plasma	1.84 ± 0.0622

*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.

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

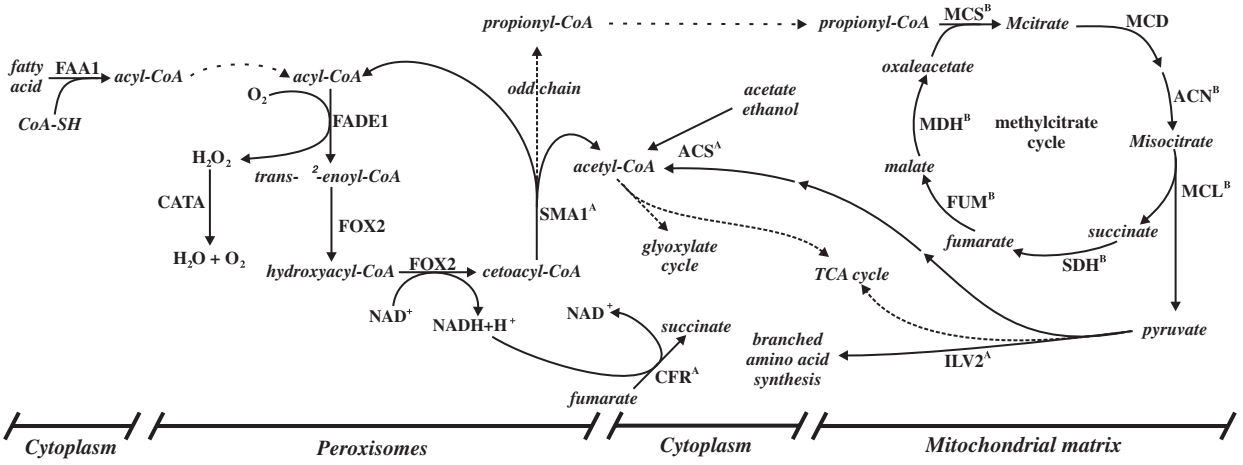

Fig. 6. Some metabolic pathways that are overexpressed during *Paracoccidioides brasiliensis* yeast cell incubation with human plasma. [(A)]Transcripts that are not overexpressed during *Paracoccidioides brasiliensis* treatment with human blood. [(B)]Transcripts present in database. FAA1, long-chain 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 dehydroghenase; CAN, aconitase; MCL, methylcitrate lyase; SDH, succinate dehydrogenase; FUM, fumarate reductase; MDH, malate dehydrogenase; ILV2, acetolactate synthase; Mcitrate, methylcitrate; Misocitrate, methylisocitrate.

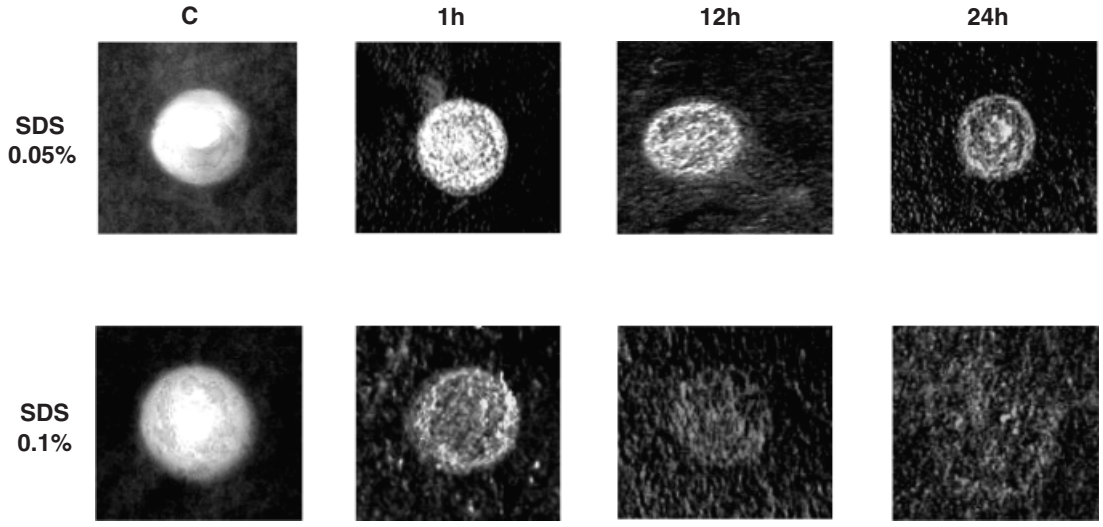

Fig. 7. Phenotypic analysis of *Paracoccidioides brasiliensis* yeast cells after incubation in human plasma for different time periods. Approximately 10^2 cells were spotted onto Fava-Neto's medium plates containing the indicated concentrations of SDS. Plates were incubated at 36 °C for 7 days. Experiments were performed in triplicate.

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*., 1996).

Mycobacterium tuberculosis genes involved in fatty acid metabolism are upregulated during infection of macrophages and mice, and the methylcitrate cycle is also required for growth of *M. tuberculosis* in murine bone marrow-derived macrophages (Muñoz-Elias *et al*., 2006). It is of special note that the methylcitrate dehydratase transcript is upregulated during *P. brasiliensis* yeast cell treatment with human plasma, and could provide pyruvate for the biosynthetic processes through the methylcitrate cycle.

Acetolactate synthase (EC 2.2.1.6) catalyzes the first common step in the biosynthesis of the branched amino acids isoleucine, valine and leucine, starting from pyruvate. Mutants for the homologous gene in *Cryptococcus neoformans* are avirulent and unable to survive in mice (Kingsbury *et al*., 2004). Also, fumarate reductase (EC 1.3.1.6) is upregulated during human plasma incubation of yeast cells of *P. brasiliensis*. In *S. cerevisiae*, two fumarate reductase isoenzymes are required for the reoxidation of intracellular NADH under anaerobic conditions (Enomoto *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 those 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 *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 *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 *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 *sho1* transcript homolog of *P. brasiliensis* in yeast cells during human blood treatment, as well as in *P. brasiliensis* yeast cells present in blood of infected mice, suggesting its involvement in the osmolarity sensing of *P. brasiliensis* yeast cells during fungus dissemination through the blood. It is of special note that the transcript encoding this novel osmosensor of *P. brasiliensis* (Bailão *et al.*, 2006) is predominantly overexpressed in yeast cells during incubation with human plasma, vs. the incubation with human blood. In *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 *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 *S. cerevisiae* (Ecker *et al.*, 2006). TGAse was found to be localized in the cell wall of fungi. In *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*-acetylglucosamine from fructose 6-phosphate consists of five steps, of which the first is the formation of glucosamine 6-phosphate from glutamine and fructose 6-phosphate, a rate-limiting step in the pathway. The cell wall stress response in *Aspergillus niger* involves increased expression of the gene *gfa*A, which encodes the glutamine: fructose-6-phoshate 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 *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 *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 *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 *P. brasiliensis*; it cleaves the main components of the basal membrane *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 *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 *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, L-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 L-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 L-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 of the roles of those genes that are highly expressed in response to host conditions.

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ARTICLE

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

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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.

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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 reassortment usually occurs after the same cell is co-infected by samples of common occurrence, such as P[6]G8 with P[4]G2 and P[6]G9 with P[4]G2 [13]. From all of the control/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 adenovirus (EIARA) [15] and polyacrylamide gel electrophoresis (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].

P genotyping

The samples were submitted to hemi-nested reverse transcriptase-polymerase chain reaction (RT-PCR) P genotyping using a pair of consensus primers (Con2 and Con3) that correspond to conserved nucleotide sequences of VP4. The resulting amplicons 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 $MgCl_2$, 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 Transcriptase 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 electrophoresis 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.

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

Primers	Sequence (5'-3')	Positions (nt)	Product length (bp)
Con2	ATT TCG GAC CAT TTA TAA CC	868–887	–
Con3	TGG CTT CGC CAT TTT ATA GAC A	11–32	876
2T-1 P[4]	CTA TTG TTA GAG GTT AGA GTC	474–494	483
3T-1 P[6]	TGT TGA TTA GTT GGA TTC AA	259–278	267
1T-1 P[8]	TCT ACT TGG ATA ACG TGC	339–356	345

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 DYEnamic® ET Dye Terminator Kit (Amersham Bioscences, 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 samples, 25 reacted with more than one P genotype, 22 were identified as P[6]P[8], one as P[4]P[8], one P[4]P[6], and one was reactive for P[4]P[6]P[8] (data not shown).

Sequencing of RVA-positive samples

Of the 25 RVA samples that were identified as having more 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).

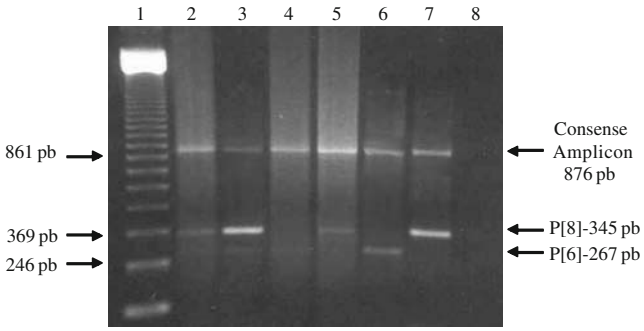

Fig. 1 1.5% agarose gel electrophoresis of hemi-nested reverse transcriptase-polymerase chain reaction (RT-PCR) using a pool of the specific primers and specific primers separately (1T-1 P[8]e, 3T-1 P[6]). Lane 1: 123 bp molecular weight ladder. Lanes 2 and 3: samples 19,318 and 17,158, respectively, positive for both P[6] (267 pb) and P[8] (345 pb) using primers specific for P[6] and P[8] genotypes in the same reaction. Lanes 4 and 5: sample 17,158, positive for P[6] and P[8], respectively. Lanes 6 and 7: sample 19,318, positive for both P[6] (lane 6) and P[8] (lane 7), using primers specific for P[6] and P[8] genotypes separately. Lane 8: negative control

Discussion

The continued monitoring of RVA genotypes in Brazil has allowed the identification of four epidemiological characteristics of these agents: (i) the great diversity of G and P genotypes circulating simultaneously in a determined place and time period; (ii) the emergence of unusual G and/or P genotypes; (iii) the detection of unusual G+P combinations; (iv) an elevated occurrence of rotavirus G and/or P mixed infections. It is now accepted that the combination of one or more of these characteristics may influence vaccination effectiveness [21]. In this context, the main objective of our study was to evaluate the occurrence of RVA mixed infections in children living in the city of Goiânia in the state of Goiás, Brazil, by characterizing the mixed P genotypes of these samples.

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

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

Hemi-nested RT-PCR	Sequencing genome				
	P[6]P[8]	P[4]P[6]	P[4]P[6]P[8]	P[8]	Total
P[6]P[8]	10	–	–	8	18
P[4]P[8]	–	–	–	1	1
P[4]P[6]	–	1	–	–	1
P[4]P[6]P[8]	–	-	1	–	1
Total	10	1	1	9	21

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

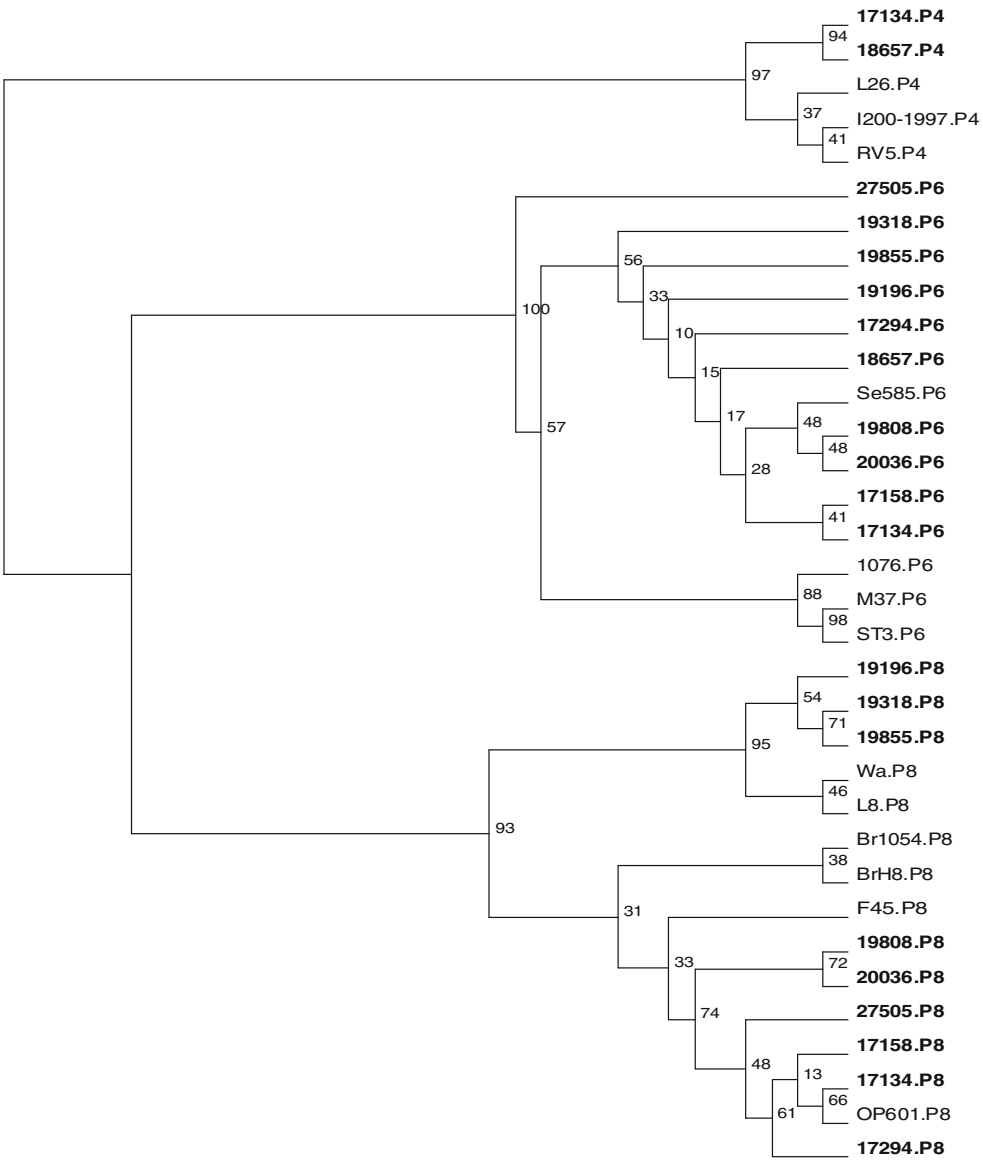

Fig. 2 Phylogenetic analysis of VP4 nucleotide sequences of genotype P mixed samples. The phylogenetic tree was constructed by the neighbor joining method using the Clustal X and TreeView programs. GenBank access numbers: P[8] [OP601 (AJ302153), F45 (U30716), BrH8 (U41006), L8 (AF061358), Wa (L34161),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

be used for the evaluation of previous vaccines' efficacy and for the development of future control/prevention strategies.

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

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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 gastroenteritis 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, Cauás 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) (Ciarlet et al. 2000, Mori et al. 2002, Iturriza-Gómara 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-1), D (EW) and E (avian-like).

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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 (Ciarlet 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, Masca-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 rotaviruses samples recovered from children in three cities of the West Central region of Brazil.

PATIENTS, 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 97ºC 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://www.genome.washington.edu/UWGC/analysistools/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 [rj5348 (DQ498179) and rj7363 (DQ498192)] 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 (χ^2) 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 ($\chi^2 = 28.55$, $p = 0.000$).

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).

TABLE I

Distribution of nonstructural protein (NSP4) genotypes of group A rotaviruses samples from infected children[a] from three cities of West Central region of Brazil

	Samples	Genotype A		Genotype B	
Cities-States	n	n	%	n	%
Goiânia-GO	91	12	13.2	79	86.8
Campo Grande-MS	51	-	-	51	100.0
Brasília-DF	11	-	-	11	100.0
Total	153	12	7.8	141	92.2

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

TABLE II

Distribution of NSP4 genotypes of group A rotaviruses samples from infected children[a] from West Central region of Brazil considering the year of collection

	Samples	Genotype A		Genotype B	
Year of collection	n	n	%	n	%
1990-1999	45	12	26.7	33	73.3
2000-2003	107	-	-	107	100.0
Total	152[b]	12	7.9	140[b]	92.1

a: children with (n = 150) and without (n = 2) diarrhea; *b*: the only sample identified in 1987 characterized as NSP4 genotype B was not included.

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).

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 (Ciarlet 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-Gómara 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-Gómara 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-Gómara 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-Gómara 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 1980s was genotype B, suggesting that the circulation of NSP4 genotypes changes over time. After 2000, genotype B was also found in CG and BRA. In Rio de Janeiro (RJ), it was shown that genotype B circulated during the years 1986-1988, 1990 and 2001-2004, while genotype A circulated only in 2002 (Araújo et al. 2007). Both genotypes A and B were identified during the 1990s and in 2000 from children in Belém (Mascarenhas et al. 2007).

Worldwide, several studies have compared the NSP4 genes of different rotavirus strains isolated from diarrheic and non-diarrheic children (Cunliffe et al. 1997, Lee et al. 2000, Mascarenhas et al. 2007). In this study, NSP4 genes were identified in children with or without diarrhea, but no differences were observed in the nucleotide

Fig. 1: phylogenetic analysis of nucleotide sequences of group A rotavirus nonstructural protein (NSP4) gene. Nucleotide sequences of prototype strains of NSP4 genotypes A-E obtained from GenBank are represented in bold. Nucleotide sequences of OSU/Po prototype, rj5348/Hu and rj7363/Hu strains obtained from GenBank were also included to better understand the phylogenetic relationships among strains. For each sample, the designation in relation to geographical location (BRA: Brasília; CG: Campo Grande; GO: Goiânia), sample number, year of collection, and G and P genotypes are shown. Group samples in which NSP4 nucleotide sequences showed 100% identity were designated as haplotypes (HP) (I-VII). HP.I: is represented by BRA266/01/P[8]G1, BRA270/01/P[8]G1, GO27440/01/P[8]G1, GO27783/01/P[8]G1 and GO27821/01/P[8]G1 samples; HP.II: is represented by BRA275/01/P[8]G1 and GO27892/01/P[8]G9 samples; HP.III: is represented by CG1676/01/P[8]G4 and CG2670/01/P[8]G4 samples; HP.IV: is represented by CG423535/03/P[8]G1, CG513102/03/P[8]G1, CG520839/03/P[8]G1, CG795087/03/P[8]G1, CG816414/03/P[8]G1, CG817198/03/P[8]G1 and CG827082/03/P[8]G1 samples; HP.V: is represented by CG518035/03/P[8]G1 and CG842284/03/P[8]G1 samples; HP.VI: is represented by CG785797/03/P[8G1], CG789219/03/P[8]G1, CG794537/03/P[8]G1 and CG837155/03/P[8]G1 samples; HP.VII: is represented by CG819946/03/P[8]G3, CG826796/03/P[8]G9 and CG857722/03/P[8]G1 samples.

TABLE III

Combinations of NSP4 and G and P genotypes of group A rotaviruses samples obtained from children at West Central region of Brazil considering the years of sample collection

Genotypes[a] G and P	NSP4 genotypes	1990-1999	2000-2003
		n	n
P[6]G9	A	2	-
P[8]G1	B	13	35
P[6]G1	B	1	1
P[8]G2	B	1	-
P[8]G3	B	-	1
P[8]G4	B	-	3
P[8]G9	B	-	4

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

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.

Surveys around the world indicate that P[8]G1, P[4]G2, P[8]G3 and P[8]G4 are the most common G and P genotypes combinations isolated in children with diarrhea caused by group A rotavirus. More recent studies have shown the emergence of P[8]G9 and P[6]G9 genotypes in cases of severe diarrhea in children (Santos & Hoshino 2005, Estes & Kapikian 2007, Munford et al. 2007, Matthijnssens et al. 2008). In this study, rotavirus samples representing the P[8]G1, P[6]G1, P[8]G2, P[8]

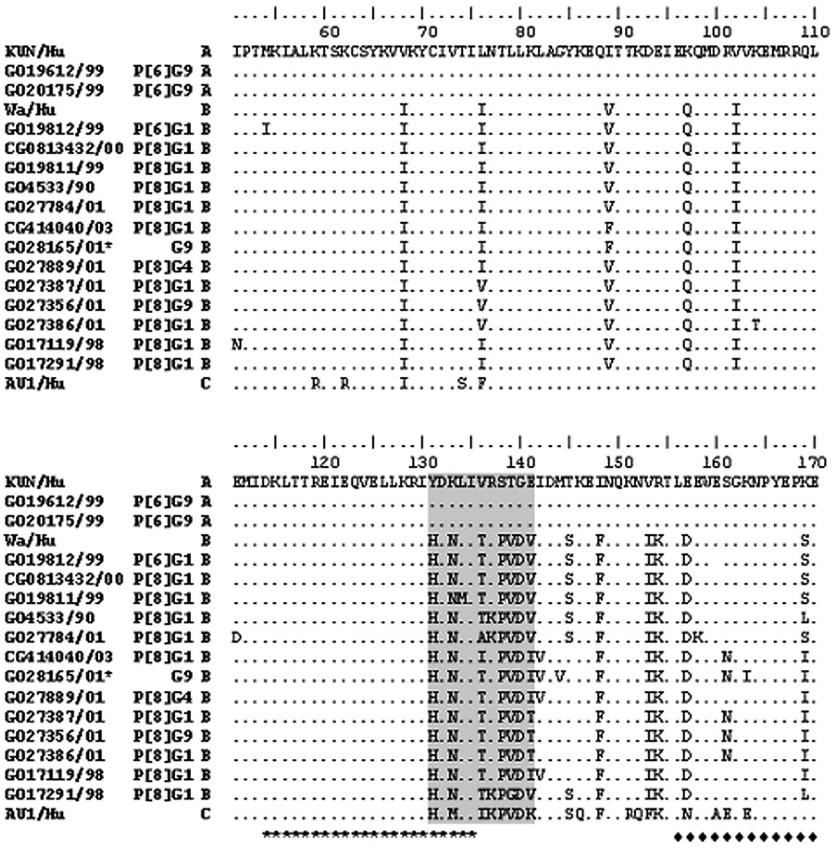

Fig. 2: multiple alignment of the partial deduced amino acid sequence of the NSP4 protein of 15 human rotaviruses samples with human prototype strains grouped in genotypes A, B and C. Dots indicate identity to the KUN/Hu strain. Gray box indicates interspecies variable domain. Diamonds indicate VP6 binding site. Asterisks indicate enterotoxigenic domain, assymptomatic child.

G3, P[8]G4 and P[8]G9 genotypes were more closely related to NSP4 genotype B comprising human reference strains Wa and AU32 (bootstrap value of 100%), whereas P[6]G9 rotavirus samples were closely related to NSP4 genotype A, comprising human reference strains RV5 and KUN (bootstrap value of 100%). In RJ, it was observed that P[8]G1, P[8]G5 and P[8]G9 rotavirus strains from children were also more closely related to NSP4 genotype B, while P[4]G2 strains were associated with genotype A (Araújo et al. 2007). Mascarenhas et al. (2007) observed that, among children in nurseries in Belém, P[6]G9 samples were also associated with genotype A, while P[6]G4 strains were associated with genotype B. In addition, P[6]G2 rotavirus strains clustered with genotype A in previous investigations (Mascarenhas et al. 2006). Interestingly, some P[4]G2 and P[6] G9 rotavirus strains characterized as NSP4 genotype A, and P[8]G1, P[6]G1, P[8]G3, P[8]G4 and P[8]G9 strains characterized as genotype B recovered from children in studies from Brazil, including the present study, were also identified from patients in the United States (Kirkwood et al. 1999), Taiwan (Lee et al. 2000), the United Kingdom (UK) and India (Iturriza-Gómara et al. 2003).

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-Gómara 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 diarrheic 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-Gómara 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

analysis performed in our study provides insight into the genetic relationships between different rotaviruses samples circulating in a particular region of Brazil.

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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*.

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VIII. Anexos

Produção bibliográfica durante o doutorado

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Anexos

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Produção bibliográfica durante o doutorado

Artigos completos publicados em periódicos

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8. **PARENTE JA**, BORGES CL, BAILÃO AM, FELIPE MSS, PEREIRA M, SOARES CMA. Comparison of transcription of multiple genes during mycelia transition to yeast cells of *Paracoccidioides brasiliensis* reveals insights to fungal differentiation and pathogenesis. *Mycopathologia.* 2008. 165: 259-73.

9. TAVARES T M, DE BRITO WM, FIACCADORI FS, **PARENTE JA**, DA COSTA PS, GIUGLIANO LG, ANDREASI MS, SOARES CMA, CARDOSO DD. Molecular characterization of VP6-encoding gene of group A human rotavirus samples from central west region of Brazil. *J Med Virol.* 2008. 80: 2034-9.

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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.*

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