Genomic DNA microarray comparison of gene expression patterns in *Paracoccidioides brasiliensis* mycelia and yeasts *in vitro*

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Paracoccidioides brasiliensis is a thermally dimorphic fungus, and causes the most prevalent systemic mycosis in Latin America. Infection is initiated by inhalation of conidia or mycelial fragments by the host, followed by further differentiation into the yeast form. Information regarding gene expression by either form has rarely been addressed with respect to multiple time points of growth in culture. Here, we report on the construction of a genomic DNA microarray, covering approximately 25 % of the genome of the organism, and its utilization in identifying genes and gene expression patterns during growth *in vitro*. Cloned, amplified inserts from randomly sheared genomic DNA (gDNA) and known control genes were printed onto glass slides to generate a microarray of over 12 000 elements. To examine gene expression, mRNA was extracted and amplified from mycelial or yeast cultures grown in semi-defined medium for 5, 8 and 14 days. Principal components analysis and hierarchical clustering indicated that yeast gene expression profiles differed greatly from those of mycelia, especially at earlier time points, and that mycelial gene expression changed less than gene expression in yeasts over time. Genes upregulated in yeasts were found to encode proteins shown to be involved in methionine/cysteine metabolism, respiratory and metabolic processes (of sugars, amino acids, proteins and lipids), transporters (small peptides, sugars, ions and toxins), regulatory proteins and transcription factors. Mycelial genes involved in processes such as cell division, protein catabolism, nucleotide biosynthesis and toxin and sugar transport showed differential expression. Sequenced clones were compared with *Histoplasma capsulatum* and *Coccidioides posadasii* genome sequences to assess potentially common pathways across species, such as sulfur and lipid metabolism, amino acid transporters, transcription factors and genes possibly related to virulence. We also analysed gene expression with time in culture and found that while transposable elements and components of respiratory pathways tended to increase in expression with time, genes encoding ribosomal structural proteins and protein catabolism tended to sharply decrease in expression over time, particularly in yeast. These findings expand our knowledge of the different morphological forms of *P. brasiliensis* during growth in culture.

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Abbreviations: gDNA, genomic DNA; PCA, principal components analysis.

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The microarray data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO series accession number GSE15511 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15511>).

Supplementary methods, two supplementary figures and a supplementary table are available with the online version of this paper.

INTRODUCTION

Paracoccidioidomycosis is the most prevalent systemic mycosis in Latin America and is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis* (Mackinnon, 1970; Wanke & Londero, 1994). The infection originates with the inhalation of airborne mycelial particles or conidia. The infecting propagules subsequently transform into multiply budding yeasts in tissues. Conversion of the mycelial form (or conidia) to yeast is necessary for establishing infection (Borges-Walmsley *et al.*, 2002; Brummer *et al.*, 1993; Rooney & Klein, 2002). In addition to differences in morphology, yeast and mycelia also differ in nutritional requirements (Arraes *et al.*, 2005; Paris *et al.*, 1985), cell wall composition (more β -glucan in mycelia versus more α -glucan in yeasts, as well as higher amounts of chitin in yeasts) (Kanetsuna *et al.*, 1969; San-Blas & Vernet, 1977; San Blas & San Blas, 1977), respiratory activity (Felipe *et al.*, 2005; Kanetsuna & Carbonell, 1966) and membrane lipid composition (Leverly *et al.*, 1996, 1998, 2000; Manocha, 1980; Toledo *et al.*, 1999).

Isolation and characterization of genes is a relatively recent trend in *P. brasiliensis* research. The first gene to be cloned and characterized was *gp43*, which encodes the primary diagnostic antigen of *P. brasiliensis*, a 43 kDa glycoprotein (Cisalpino *et al.*, 1996). In addition, expression levels of *gp43* vary among isolates, independently of virulence and morphology (Mattar-Filho *et al.*, 1997). A number of genes, such as *hsp70*, *hsp60*, *lon*, mannosyltransferase, *clpB*, *l35* and hydrophobin, have been identified and characterized through conventional methods, facilitating identification of potential virulence factors and morphology-associated differentially expressed genes (Albuquerque *et al.*, 2004; Barros & Puccia, 2001; Costa *et al.*, 2002; da Silva *et al.*, 1999; Izacc *et al.*, 2001; Jesuino *et al.*, 2002, 2004).

Recently, two cDNA sequencing projects for *P. brasiliensis* were completed by the Brazilian scientific community, and the genomes of three isolates (Pb18, Pb01 and Pb03) are currently being sequenced by the Broad Institute (Cambridge, MA, USA). These cDNA projects have greatly increased the number of gene sequences in the public databases and, through genomic methodologies, have identified genes potentially implicated in the dimorphic switch (e.g. MEKK, MAPK, and Ras-like proteins), respiratory processes (i.e. aerobic in mycelia and anaerobic in yeast), heat-shock responses, drug resistance (e.g. ABC-type transporters), cell wall biosynthesis (e.g. α -glucan synthase), sulfur metabolism, virulence, pathogenicity (e.g. phospholipase, cell wall biosynthesis and adhesion) and as potential drug targets (e.g. chitin deacetylase and 4-hydroxyphenyl pyruvate dehydrogenase) (Felipe *et al.*, 2003, 2005; Ferreira *et al.*, 2006; Goldman *et al.*, 2003; Marques *et al.*, 2004; Nunes *et al.*, 2005). Although these studies have added knowledge about the biology of *P. brasiliensis*, other approaches can also prove useful to shed light on the genetic composition of this organism. We describe here the construction of a random-shear genomic

DNA microarray, and its application in the characterization of gene expression of the morphological forms of *P. brasiliensis* during growth in semi-synthetic culture media, and in the subsequent identification of differentially expressed genes. These data complement published studies and extend our understanding of the biology of mycelia and yeast forms of *P. brasiliensis*.

METHODS

Organisms and maintenance. *P. brasiliensis* isolate Pb01 (ATCC MYA-826) was selected for study because it has been used in a number of molecular biological studies (da Silva *et al.*, 1999; Felipe *et al.*, 2003). Yeast cultures were maintained on modified McVeigh/Morton (MVM) agar slants (Restrepo & Jimenez, 1980) at 36 °C and were transferred every 10–15 days. Mycelial cultures were maintained on MVM slants at ambient temperature and were transferred monthly.

***P. brasiliensis* growth and nucleic acid extraction.** Spent culture filtrate was used as a growth supplement (2:1 ratio of fresh liquid MVM to spent culture filtrate), as previously described (Clemons *et al.*, 1989; Stover *et al.*, 1986) for the growth of mycelial and yeast liquid cultures prior to DNA and RNA extractions.

Genomic DNA (gDNA) was extracted from yeasts collected from 4-day-old liquid cultures (36 °C, shaking at 150 r.p.m.) using previously described methodology (McCullough *et al.*, 2000) (see Supplementary Methods for detailed descriptions). The quantity and quality of the extracted DNA were assessed using a spectrophotometric absorbance ratio ($A_{260}:A_{280}$) and by electrophoresis through a 0.8% agarose gel stained with ethidium bromide and visualized by UV transillumination.

For total RNA extractions, one yeast or two mycelial 5-day-old slants were used to inoculate 150 ml supplemented liquid MVM medium, which was incubated on a gyratory shaker at 150 r.p.m. at 36 °C for yeast, or at ambient temperature for mycelia, for 5, 8 or 14 days. Liquid cultures were evaluated for contaminating organisms by microscopy and by plating samples on 5% sheep blood agar followed by incubation for 2–3 days at 36 °C. Total RNA was extracted using TRIzol reagent as per the manufacturer's instructions (see Supplementary Methods for details). The quantity and quality of the extracted RNA were assessed by $A_{260}:A_{280}$ ratio and electrophoresis through a 1.5% agarose gel stained with ethidium bromide (examining the presence of intact 18S and 28S rRNA bands) and visualized by UV transillumination at 302 nm. Contaminating gDNA was removed using RNase-free DNase (RQ1 RNase-free DNase, Promega) as per the manufacturer's instructions.

Library construction. Genomic DNA was sheared hydrodynamically into ~1.5 kb fragments using a HydroShear apparatus (Genomic Solutions) as described elsewhere (Oefner *et al.*, 1996; Thorstenson *et al.*, 1998). Adequacy of DNA shearing was assessed by 1.5% agarose gel electrophoresis and visualized by UV after ethidium bromide staining. Sheared DNA was used as inserts for the genomic library.

The genomic library was assembled using the λ ZAP II phage as vector (λ ZAP II Predigested *Eco*RI/CIP-treated Vector kit, Stratagene) and a modification of the linker-adaptor strategy described by Thorstenson *et al.* (1998) (also see: <http://www-sequence.stanford.edu/protocols/library.html>). A diagram of the library construction is presented in Supplementary Fig. S1.

Phage insert and control gene amplifications. Individual DNA inserts in the phage were amplified using PCR as follows: phage

capsids were denatured at 70 °C for 5 min to release viral DNA. DNA inserts were amplified using Platinum PCR Supermix 96 (Invitrogen Life Technologies) with primers complementary to the vector (primer SK, 5'-cgctctagaactagtgatc-3', and primer KS, 5'-tcgaggtcgacggatc-3') following the manufacturer's instructions (see Supplementary Methods for details).

A total of 34 genes from *P. brasiliensis* were selected for inclusion as known controls on the array. Genes were chosen based on known or expected expression patterns in mycelia or yeast or during form transition from mycelia to yeast. Template sequences were obtained from GenBank and primer pairs were designed for each gene using Primer 3 software, considering an amplicon size of ~1 kb (Rozen & Skaletsky, 2000). Nine primer pairs were designed from regulatory gene sequences provided by Dr Adrian R. Walmsley (University of Durham, UK) (see Supplementary Table S1 for genes and primers). The microarray contained a total of 40 control features based on the above-mentioned genes.

All amplified fragments were electrophoresed through 1.0 % agarose gels for 12 min using an E-Gel 96 High-Throughput Agarose Electrophoresis System (Invitrogen) and photographed. Amplicon lengths were estimated using a 1 kb Plus DNA Ladder (Invitrogen) (Supplementary Fig. S1). Amplicons were purified using a Nucleofast 96 PCR Clean-up kit (BD Biosciences).

Microarray construction. Purified PCR products from the cloned fragments and controls (12 243 inserts from the library plus 40 features representing 34 known genes from *P. brasiliensis* for a total of 12 283 elements) were spotted onto aminosilane-coated glass slides (UltraGAPS Slides, Corning) using custom-built robots at the Stanford Microarray Core Facility (<http://www.microarray.org>).

Nucleic acid labelling. Cyanine 5 (Cy5)-labelled cDNA was synthesized from mycelial or yeast total RNA as per a protocol provided by the Stanford Microarray Core Facility, with modifications (see Supplementary Methods for full details).

Cyanine 3 (Cy3)- or Cy5-labelled *P. brasiliensis* gDNA was synthesized from 250 ng sheared gDNA using the BioPrime Array CGH Genomic Labelling System (Invitrogen) following the manufacturer's instructions. Cy3-labelled gDNA was used as a control to facilitate comparisons between slides and data integration in future experiments (Gadgil *et al.*, 2005; Talaat *et al.*, 2002; Williams *et al.*, 2006). Cy5-labelled gDNA was used in self-to-self hybridizations along with Cy3-labelled gDNA during microarray validation procedures.

Hybridization procedure and image acquisition. Samples of labelled nucleic acids for hybridization were concentrated using a Microcon YM-30 Centrifugal Filter Device (Millipore) and suspended to 40 µl with 3 × saline-sodium citrate buffer (Sigma-Aldrich) containing 0.1 % SDS (Sigma-Aldrich). These samples were incubated at 100 °C for 2 min, 37 °C for 15–25 min, transferred onto a microarray slide and covered with a coverslip and placed in a hybridization chamber (see Supplementary Methods for details). Hybridization was carried out at 50 °C for 14–18 h followed by washing and scanning within 24 h (GenePix 4000B Microarray Scanner, Axon Instruments). All hybridization experiments were done in duplicate from each of two independent cultures.

Microarray data analysis. Cy3-labelled gDNA was used as the control for comparisons, as it provides a stable and practical reference to hybridize against labelled cDNA in competitive hybridizations (Gadgil *et al.*, 2005; Talaat *et al.*, 2002) and enables determination of an absolute value of gene expression. Furthermore, the use of a more stable reference, such as gDNA, as opposed to experiment-specific cDNA pools, provides a useful link to compare arrays across different

experiments, adding to the usefulness of any given array dataset (Williams *et al.*, 2006). Cy5-labelled cDNA was synthesized from mycelial or yeast total RNA obtained after days 5, 8 and 14 of culture.

Spots with more than 50 % of their pixel values below the background median plus one standard deviation on the gDNA channel (i.e. Cy3), that were shown to contain more than one insert by agarose gel electrophoresis of amplified clones, or whose insert PCR failed, were eliminated from the analysis. There were 1846 spots with these criteria. Overall, this microarray contained 10 437 elements, which we estimate covers approximately 25 % of the *P. brasiliensis* genome considering an average insert size of about 1 kb and a genome size for *P. brasiliensis* of 30 Mb (Almeida *et al.*, 2007; Cano *et al.*, 1998; Montoya *et al.*, 1999).

Gene expression data underwent global normalization using the R statistical package to perform quantile normalization of the global reference channel by computing the centroid of all global reference signatures, and performing a LOESS fit of each individual global reference signature to the centroid. The LOESS curve for a given sample was then used to scale both channels for each gene in that sample. Data were further normalized using GeneSpring GX 7.3.1 (Agilent Technologies) as follows: per spot to the gDNA channel, and per gene to the respective median of each gene (Talaat *et al.*, 2002). For mycelial versus yeast gene expression, per-slide normalization consisted of selecting a list of spots where gDNA intensities showed a linear relationship with cDNA intensities in *M* versus *A* plots [$M = \log_2(\text{Cy5}/\text{Cy3})$ versus $A = \log_2(\text{Cy5} \times \text{Cy3})^{1/2}$] across all time points in both mycelia and yeast, as well as being below a twofold change in gene expression values. A total of 102 spots satisfied these criteria and were used as positive controls for the per-slide normalization process.

Principal components analysis (PCA) using the conditions of time and morphology was used to find primary tendencies in the dataset (Alter *et al.*, 2000). A twofold cutoff for mycelial versus yeast differences was applied to all time points to focus on broader changes in expression levels. Array features were identified as representing differentially expressed genes after parametric two-way ANOVA analysis on log-transformed values ($P < 0.05$), considering time and morphology as the parameters analysed and using Benjamini and Hochberg's method for multiple-test control of false discovery rate (Manly *et al.*, 2004). Clones of interest were sequenced and analysed as described in Supplementary Methods. Sequenced genes were further analysed by hierarchical clustering using Pearson's correlation as the similarity measure and the unweighted pair group method with arithmetic mean (UPGMA) as the linkage method (Eisen *et al.*, 1998).

For interspecies microarray data comparison, protein sequences were obtained from GenBank corresponding to the accession numbers listed by Hwang *et al.* (2003) and Johannesson *et al.* (2006). Each protein sequence was compared against our sequenced microarray probes using BLASTX. The top-scoring BLASTX hit was considered a match if its E-value was less than $1e-5$.

Gene expression data validation. Microarray results were verified using real-time RT-PCR (Brilliant QRT-PCR Core Reagent kit and MX3000P QPCR System, Stratagene). Eight genes of known expression pattern were examined (see Supplementary Methods for details) and results were extracted using MX3000P v1.20 software (Stratagene). These results were analysed by two-way ANOVA with Bonferroni correction (GraphPad Prism v3.0, GraphPad Software), as well as for Spearman and Pearson's correlation coefficients in GeneSpring GX 7.3.1 (Agilent). GeneSpring data transformations included normalizing each gene to its corresponding median across all samples to centre the data around zero and used *I35* (for comparisons with microarray data) or α -tubulin (for comparisons between *I35*- and α -tubulin-normalized real-time RT-PCR datasets)

as reference genes. No differences were observed using either gene as reference. All real-time RT-PCRs were done in triplicate and used the same RNA samples that were used in microarray hybridizations.

RESULTS

Random shear genomic library

During the course of this study a total of 19 800 clones of sheared gDNA were isolated and stored. The insert sizes ranged from 500 to 3000 bp (Supplementary Fig. S1b). A total of 481 clones were selected for sequencing and Fig. 1 presents an overview of the gene classifications after sequence analysis. The predicted functions contained a variety of categories, such as transposable elements, transporters, respiratory processes, metabolism (protein, amino acid, sugar and lipid), cell division, morphogenesis, RNA processing and transcription. DNA sequences with a match were generally closely related to genes of fungal origin, and most matched with *P. brasiliensis* or *Ajellomyces capsulatus* expressed sequence tags (ESTs). These results demonstrated that the library construction methodology worked as expected.

Microarray validation

A gDNA microarray was assembled containing 12 243 inserts from the library plus 40 features representing known genes from *P. brasiliensis*. Supplementary Fig. S2 presents a scatter plot of log₂-transformed intensities of hybridized Cy3-labelled gDNA versus Cy5-labelled gDNA. Test results routinely showed ~99% of spots with fluorescence levels above the background and similar fluorescence intensities for both channels in each spot, validating that the microarray was functioning. Single-probe hybridization results showed that when a single cloned insert was labelled and hybridized to the array, the

highest fluorescence reading was found at its corresponding spot, indicating that the microarray elements were correctly spotted at their assigned positions (results not shown). Quality control filters described above (see Methods, Microarray data analysis) were used to eliminate 1846 spots from the analysis process.

Gene expression validation

The expression of eight genes was validated using real-time RT-PCR. These were hydrophobin, *y20*, mannosyltransferase, *hsp70*, α -tubulin, *l35*, *pfr1* and a toxin efflux pump [major facilitator superfamily (MFS) pump] gene. Fig. 2 presents the estimated quantities of mRNA transcripts for each of these genes as determined by real-time RT-PCR. All selected genes showed significant differences in expression between mycelia and yeast. *l35*, *pfr1*, α -tubulin and hydrophobin had significant differences over time and all, except the MFS pump, showed significant changes with time and morphological form. Statistical analyses showed that all, except the MFS pump, had similar expression levels in both forms for at least one time point. *l35* and α -tubulin, genes previously used as controls in *P. brasiliensis* gene expression studies (Goldman *et al.*, 2003; Jesuino *et al.*, 2002, 2004), were shown to have similar expression levels only on day 5. Correlation coefficients of real-time RT-PCR and microarray data using *l35* as the normalizer were all positive and in general above 0.7 (Table 1), indicating that the gDNA microarray platform is useful for gene expression experiments.

Differential gene expression

PCA of expression using log-transformed data was performed to determine general tendencies present in our dataset. The first two principal components captured 81.75% of the variance present in the data. The PCA plot shown in Fig. 3 indicates that mycelial gene expression was

Fig. 1. Pie chart representing 481 sequenced clones showing the general distribution of categories after sequence analysis. Y, yeast, M, mycelia.

Fig. 2. Real-time RT-PCR results for eight specific genes obtained from the same RNA samples used in the microarray hybridizations; each group of bars represents days 5, 8 and 14 from left to right. *mannosyl transf*, mannosyltransferase gene.

generally different from that in yeast, and that mycelial expression was generally unchanged over the selected time points. In contrast, the day 8 and day 14 time points of gene expression by yeast were different from the day 5 time point (Fig. 3).

A more detailed view of general gene expression is presented in Fig. 4, where hierarchical clustering of all genes present on the array showed that yeast had more upregulated genes than mycelia (cluster 1). Clusters 2, 3 and 4 illustrate that yeast tends to vary expression more with time than do mycelia. Upregulated genes in mycelia tended to concentrate in clusters 3 and 4. The control genes of known expression present in these clusters were used to determine their general nature. Thus, clusters 1 and 2 contained controls known to have higher expression in yeast (*hsp104/clpa*, mannosyltransferase, *kex2* and *lon1*), while clusters 3 and 4 contained known mycelial controls

(*m51*, hydrophobin, 40S ribosomal protein and *gp43*). Clones were chosen for sequencing based on ANOVA analysis for significance, using time and morphology as parameters and their presence in each of these clusters. We focused on genes that were overexpressed at all time points in either mycelia or yeast (morphology related genes) and genes that showed variations with time (metabolism- and stationary-phase-related genes).

Interspecies comparison, overview of some genes overexpressed in mycelia and yeast, and related biological processes

Fig. 5 shows the clustering results of 521 sequenced features present in the array and illustrates the gene expression patterns that were investigated in this study, while a more detailed description of some differentially expressed genes is given in Table 2. Mycelia and yeast are known to differ in

Table 1. Correlation coefficients of log-transformed *I*35-normalized relative intensities between microarray and real-time RT-PCR datasets

Gene*	log ₁₀ values from array/log ₁₀ values from real-time RT-PCR at:			Correlation coefficient	
	Day 5	Day 8	Day 14	Pearson	Spearman
<i>hsp70</i> (M)	−0.2/−0.69	−0.202/−0.545	−0.033/−0.213	0.689	0.543
<i>hsp70</i> (Y)	−0.296/0.127	0.096/0.537	0.21/0.491		
α-Tubulin (M)	−0.096/−0.154	0.105/0.136	−0.056/−0.097	0.549	0.314
α-Tubulin (Y)	−0.196/−0.001	−0.072/−0.085	−0.078/−0.156		
Hydrophobin (M)	1.034/0.892	0.896/0.983	0.297/0.229	0.887	0.771
Hydrophobin (Y)	−0.592/−0.358	−0.119/−0.738	0.072/−0.455		
Mannosyltransferase (M)	−0.345/−0.548	−0.289/−0.065	−0.087/−0.102	0.948	0.943
Mannosyltransferase (Y)	0.222/0.335	0.412/0.576	0.304/0.442		
MFS pump (M)	0.047/0.451	0.199/0.566	0.052/0.233	0.709	0.943
MFS pump (Y)	−0.502/−0.752	−0.025/−0.652	0.024/−0.502		
Pfr1 (M)	−0.158/−0.413	−0.087/−0.102	−0.088/−0.078	0.37	0.543
Pfr1 (Y)	−0.288/0.437	0.070/0.541	0.087/0.491		
Y20 (M)	−0.088/−0.504	−0.161/−0.219	−0.061/−0.037	0.827	0.6
Y20 (Y)	−0.181/0.075	0.258/0.712	0.167/0.434		

*M, mycelium; Y, yeast.

cell wall structure and composition (Kanetsuna *et al.*, 1969; San-Blas & Vernet, 1977; San Blas & San Blas, 1977), nutritional requirements (yeast requires sulfur-containing amino acids) (Arraes *et al.*, 2005; Paris *et al.*, 1985), membrane lipid composition (Leverly *et al.*, 1996, 1998,

2000; Manocha, 1980; Toledo *et al.*, 1999) and respiratory activity (Felipe *et al.*, 2005; Kanetsuna & Carbonell, 1966).

Analysing the yeast cluster in light of these functional categories we found a cysteine desulfurase, an aminopeptidase (homocysteine catabolism) and two genes (*sah1* and a SAM-dependent methyltransferase) involved in metabolism of sulfur-containing amino acids. Cysteine desulfurase, aminopeptidase and *sah1* were upregulated in yeast only on day 5, whereas SAM-dependent methyltransferase was upregulated in yeast at all time points. Interestingly we also found an N-terminal peptidyl-methionine acetyltransferase (*nat2*), required for survival under heat stress in *Saccharomyces cerevisiae*, as well as two clones containing the dipeptide transporter *ptr2*. We also found that a homologue of *met17*, which catalyses the addition of inorganic sulfur into *O*-acetyl-L-homoserine generating homocysteine, was upregulated in mycelia only on day 5, with yeast catching up at later time points. The expression of inorganic sulfur assimilation genes in yeast has been previously reported, but it is still unknown whether the pathway is functional (Ferreira *et al.*, 2006). Similarly, overexpression of sulfur metabolism genes and amino acid transporters has been detected in the pathogenic form of *Histoplasma capsulatum* (Hwang *et al.*, 2003).

Expression changes in genes related to respiratory processes, such as NAD-dependent isocitrate dehydrogenase, which has been reported to be more highly expressed in yeast (Kanetsuna & Carbonell, 1966), were also detected. Our results are in agreement, as this gene showed constant overexpression in yeast in comparison with mycelia. Contrasting data have shown this gene to be upregulated in mycelia (Felipe *et al.*, 2005), perhaps due to differences in experimental design, as reported elsewhere (Marques

Fig. 3. Correlation plot of mycelial and yeast datasets with eigenarrays as determined by PCA. Correlations with eigenarray 1 (*x* axis), which captured 60.84% of the variance present in the data, show opposing behaviours in mycelia and yeast, which could be related to morphology and/or temperature. Correlations with eigenarray 2 (*y* axis), which captured 20.91% of the variance present in the data, show that mycelial datasets are more closely related to each other than to yeast datasets, suggesting more changes in yeast over the sample times examined.

Fig. 4. Hierarchical clustering of all genes present in the microarray using Pearson correlation as the similarity measure. Relative gene expression is centred around the median expression value per gene, where bright red is twofold expression or greater with respect to the median, black is expression equal to the median value and bright green is twofold expression or less with respect to the median. M, mycelia; Y, yeast; Time, days of growth in culture. Cluster 1, genes overexpressed in yeast at all time points; cluster 2, genes overexpressed in yeast with increased levels over time; cluster 3, genes overexpressed in mycelia with decreased levels over time; cluster 4, genes that show stable expression in mycelia and increased expression in yeast over time.

et al., 2004). Alternatively, the different results may be attributable to variations between fungal isolates, as observed in *Coccidioides posadasii* (Johannesson *et al.*, 2006). In addition, we found that a cytochrome c_1 haem protein precursor, which is related to aerobic respiratory processes, was constantly overexpressed in yeast. Our data also indicated the overexpression of two subunits of NADH:ubiquinone oxidoreductase, involved in electron transport for oxidative phosphorylation by yeast. Although components of complexes I and III were found to be upregulated in yeast, complex IV components (cytochrome c oxidase subunits 1, 2 and 5a) were upregulated in mycelia at earlier time points or had equivalent levels. Malate synthase and malate dehydrogenase homologues belonging to the glyoxylate cycle were also detected. The first was constantly upregulated in yeast and the latter upregulated in yeast on day 5 only, which is in agreement with previously published data. These findings further support the importance to *P. brasiliensis* of the glyoxylate cycle, which allows the utilization of two-carbon compounds for the production of glucose through gluconeogenesis during systemic infection and within phagocytic cells, thus improving pathogen survival chances against host defences in the nutrient-poor environment of the phagolysosome (Derengowski *et al.*, 2008; Felipe *et al.*, 2005; Ferreira *et al.*, 2006; Nunes *et al.*, 2005; Tavares *et al.*, 2007). The importance of the glyoxylate cycle has been established in plant and animal pathogens such as *Candida albicans*, *Penicillium marneffeii*, *Trichophyton rubrum* and *Magnaporthe grisea*. For fungi such as *Candida albicans*, the glyoxylate cycle is thought to be most important during the early stages of infection after internalization by macrophages. However, studies with *Aspergillus fumigatus* have indicated that the glyoxylate cycle is not necessary for virulence (Ebel *et al.*, 2006; Schobel *et al.*, 2007). As the glyoxylate cycle is not present in humans it is considered an attractive target for drug development (Barelle *et al.*, 2006; Fradin *et al.*, 2003; Lorenz & Fink, 2001, 2002; Lorenz *et al.*, 2004; Thirach *et al.*, 2008; Wang *et al.*, 2003; Zaugg *et al.*, 2009).

Among the genes involved in lipid metabolism we identified a transcription factor homologue *hac1*, upregulated at all times in yeast. In *S. cerevisiae* this gene is involved in the unfolded protein response in the endoplasmic reticulum (ER), as well as in phospholipid biosynthesis. The unfolded protein response can be caused by stresses such as lipid deprivation, heat, drug treatment, and mutations in or overexpression of wild-type secretory proteins, and involves increased production of proteins that increase lipid biosynthesis or restore homeostasis in the ER, as needed (Kaufman, 1999). The unfolded protein response has also been associated with virulence and susceptibility to antifungals in *A. fumigatus* (Richie *et al.*, 2009). Upregulation of the gene in yeast could be a response to growth at higher temperature or could be related to the distinct lipid composition of yeast cells (Manocha, 1980). We also found a homologue of *A.*

Fig. 5. Clustering of 521 known genes (on the left) present in the microarray using Pearson correlation as the similarity measure. Relative gene expression is centred around the median expression value per gene, where bright red is twofold expression or greater with respect to the median, black is expression equal to the median value and bright green is twofold expression or less with respect to the median. All genes have more than 50 % of pixels in each spot above the Cy5 background by one standard deviation in at least one time point. M, mycelia; Y, yeast; Time, days of growth in culture. The Y Genes centre panel is a close-up view of the corresponding cluster on the tree to the left. The M Genes panel on the right is a close-up view of the corresponding cluster on the tree to the left.

Table 2. DNA sequence analysis of some differentially expressed genes

The E-value column is the result of comparison of *P. brasiliensis* sequences against the listed orthologues using TBLASTX.

Gene product (name)	Accession number of orthologue	E-value	Y : M ratio		
			Day 5	Day 8	Day 14
RNA-binding protein (<i>rrp4</i>)	154278203	8e-81	5.5594	3.7742	0.6789
Calcium-binding protein 1 (<i>cbp1</i>)	37287441	4e-18	3.8482	3.5586	0.9247
S-Adenosylmethionine (SAM)-dependent methyltransferases	154272540	1e-49	10.0487	3.5275	0.3510
cAMP-dependent protein kinase regulatory subunit PKAR	119187826	2e-21	4.4675	4.8792	1.0922
Malate dehydrogenase	47119067	1e-127	2.4800	0.4437	0.1789
Dipeptide/tripeptide permease (<i>ptr2</i>)	154276227	1e-137	10.1514	15.2549	1.5027
met17	154277107	1e-59	0.4580	0.8945	1.9530
Malate dehydrogenase	154276901	2e-49	0.9264	0.5109	0.5515
Proteasome component (<i>pre1</i>)	154279089	3e-88	1.4101	0.8867	0.6288
Phosphate permease	154270785	4e-112	0.2756	0.5916	2.1465
Global nitrogen regulator (<i>gat1</i>)	154277445	1e-115	5.8787	5.1120	0.8696
N-terminal peptidyl-methionine acetyltransferase (<i>nat2</i>)	169774028	9e-26	3.5137	3.9654	1.1286
Cytidylyltransferase family	154273632	8e-24	7.7649	5.2304	0.6736
3-Isopropylmalate dehydrogenase A	154282586	4e-38	0.6888	1.6274	2.3628
Ergosterol biosynthesis-related protein (<i>erg28</i>)	119178773	6e-46	2.2496	1.3669	0.6076
Signal transduction protein (<i>syg1</i>)	154287187	3e-40	2.7428	2.4294	0.8857
bZIP transcription factor HaCa (<i>hac1</i>)	121711847	1e-14	5.2024	1.5962	0.3068
Serine/threonine protein kinase	154281766	6e-58	6.4503	4.9595	0.7689
Phosphatidylinositol-4-phosphate 5-kinase (<i>fab1</i>)	119178397	3e-122	3.9312	2.6804	0.6818
Nitrogen assimilation transcription factor (<i>nirA</i>)	119174461	1e-151	5.4128	6.9235	1.2791
Multidrug efflux system protein (MFS pump)	115433858	2e-123	0.4003	0.3632	0.9074
Proteasome component (<i>pup1</i>)	154281652	9e-97	1.3060	0.7628	0.5841
Acetyl-CoA synthetase 2	154287527	2e-110	1.0267	2.9621	2.8852
Phosphatidylethanolamine methyltransferase (<i>cho2</i>)	121716690	2e-87	4.3969	2.3518	0.5349
Acetyl-CoA synthetase	154271371	4e-19	0.7472	1.1888	1.5911
Heat-shock protein CLPA	29570851	4e-170	1.6413	2.2682	1.3820
Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	119194576	2e-45	10.4890	5.6809	0.5416
Bifunctional pyrimidine biosynthesis protein	154271443	0.0	2.9832	1.4934	0.5006
mak10	119182512	2e-46	5.2276	4.4244	0.8464
Transposase	119192871	5e-5	2.4936	1.4600	0.5855
HSP 70	154285929	4e-97	0.9884	1.3417	1.3575
Aminopeptidase Y (<i>ape3</i>)	154279319	8e-110	0.1816	0.1012	0.5570
YL1 nuclear protein	134110309	4e-8	3.4008	8.1215	2.3881
srb5	154278540	1e-63	3.6601	4.0000	1.0929
tfc3	154276065	9e-47	4.5067	4.6859	1.0398
Cysteine protease (OTU family)	154275449	2e-72	7.4170	3.7188	0.5014
bZIP transcription factor	154271591	3e-86	8.4127	6.7116	0.7978
RNA polymerase III (<i>rpc4</i>)	119175302	1e-43	4.2021	7.8540	1.8691
NAD-dependent isocitrate dehydrogenase	119495396	3e-68	7.1235	4.6404	0.6514
Heat-shock protein CLPA	29570851	0.0	2.5000	3.5207	1.4083
Dipeptide/tripeptide permease (<i>ptr2</i>)	154276227	2e-5	5.8696	3.3763	0.5752
Cysteine desulfurase	154286611	4e-34	2.0398	0.5638	0.2764
6-Phosphofructo-2-kinase	154287085	1e-12	0.2520	0.7974	3.1638
S-Adenosyl-L-homocysteine hydrolase (<i>sah1</i>)	119173081	1e-18	1.7895	0.4398	0.2457
C4-dicarboxylate transporter/malic acid transport protein	121709273	2e-9	8.4042	4.6131	0.5489
U1 snRNP-associated protein Usp105 (<i>prp39</i>)	121707302	1e-142	7.2775	4.7781	0.6566
Succinyl-CoA : 3-ketoacid-coenzyme A transferase 1	119194818	1e-76	12.4411	3.5945	0.2889
Ubiquitin C-terminal hydrolase (<i>yuh1</i>)	19113320	5e-79	2.5323	2.9002	1.1453
Serine/threonine-protein kinase (<i>nrc-2</i>)	154286021	9e-43	9.8590	7.6698	0.7779
Aminopeptidase (bleomycin resistance)	154270890	1e-163	1.8652	0.8604	0.4613
Phosphoribosylformylglycinamide synthase (<i>ade6</i>)	154285197	5e-53	0.2440	0.1417	0.5807
Malate synthase A	154277551	2e-90	4.5375	3.0169	0.6649

fumigatus fab1 that encodes 1-phosphatidylinositol-3-phosphate 5-kinase, responsible for the generation of phosphatidylinositol 3,5-bisphosphate. In *S. cerevisiae*, *fab1* is involved in vacuolar sorting and homeostasis (Cooke *et al.*, 1998). Also of interest is the requirement for *fab1* for growth at elevated temperatures in *S. cerevisiae* (Yamamoto *et al.*, 1995), which may explain its constitutively high expression in the yeast form. We also found a homologue to *S. cerevisiae erg28*, whose protein product is reported to be the anchor to the ER of the *erg25*, *erg26* and *erg27* protein products and is also involved in interaction with the protein of *erg6*; all are involved in ergosterol biosynthesis (Mo *et al.*, 2004). These findings, along with the previously reported upregulation of *erg25* in the yeast form of *P. brasiliensis*, further support the hypothesis that membrane lipid reorganization in yeast is a response to a higher-temperature environment or oxidative stress (Marques *et al.*, 2004). Interestingly, we found a glycerol-3-phosphate dehydrogenase. This gene is over-expressed in *H. capsulatum* yeasts (Table 3), with an important role in glycerol metabolism during growth on

non-fermentable carbon sources and in nutrient-poor environments, as may occur during infection of the mammalian host (Hwang *et al.*, 2003; Sprague & Cronan, 1977).

Other genes differentially regulated in yeast included those involved in mRNA transcription, processing and degradation, as our results indicated that mRNA populations are very different between mycelia and yeast. The isolation of differentially regulated transcription factors from yeast (YL1, serine/threonine protein kinases and cAMP-dependent protein kinase, among others) provides a number of candidates for future experiments on genes encoding proteins that regulate morphogenesis of the pathogenic form of this fungus. Serine/threonine kinases, for example, have been found to be upregulated in the pathogenic form of *Coccidioides posadasii* (Johannesson *et al.*, 2006). We also found genes directly associated with transcription from RNA polymerase II and III promoters (*rpc4*, *srb5* and *tfc3*), mRNA export from the nucleus (*sac3*), splicing (*prp39*) and degradation (*rrp4*), which may have roles in

Table 3. Results of a comparison of phase-specific genes from *P. brasiliensis*, *H. capsulatum* (Hwang *et al.*, 2003) and *Coccidioides posadasii* (Johannesson *et al.*, 2006)

S, saprobic form (mycelial); P, pathogenic form (yeast for *P. brasiliensis* and *H. capsulatum*, spherule for *Coccidioides posadasii*). The E-value column shows the results of the comparison of *P. brasiliensis* sequences with the listed orthologues using BLASTX.

Gene product (name)	Accession number of orthologue	E-value	Differential expression		
			<i>P. brasiliensis</i>	<i>H. capsulatum</i>	<i>C. posadasii</i>
Phosphate permease	66844646	1e-64	S		P
Adhesin/tyrosinase	24371014	1e-08	S	S	
Multidrug efflux system protein (MFS pump)	14389419	1e-34	S	S	P
Hypothetical protein	67538290	9e-59	S		P
Proteasome component (<i>ecm29</i>)	66848179	1e-76	S		P
Aminopeptidase Y (<i>ape3</i>)	45758834	2e-05	S		S
Trichothecene C-15 hydroxylase	21429419	8e-15	S	P	
Similar to clock-controlled gene 9 protein (<i>ccg-9</i>)	66852048	7e-44	None		P
Copper amine oxidase 1	66846018	2e-47	None		S
Translation initiation factor eIF-2 gamma subunit	603617	5e-42	None	P	
ABC transporter 1	20530737	1e-09	None	P	
Bicyclomycin/multidrug efflux system	28881194	5e-15	P		P
Nitrate reductase, putative	66851237	3e-20	P		P
Transposable element (retrotransposon; Ylt1)	50554773	3e-28	P		P
Phosphatidylinositol kinase (<i>tel1</i>)	66851023	2e-33	P		P
Hypothetical protein [exocyst complex component (<i>sec3</i>)]	129557017	3e-08	P		P
Heat-shock protein CLPA (heat-shock protein 78)	28629052	2e-91	P		P
Intermembrane space AAA protease (IAP-1)	66847034	4e-38	P		P
Ca ²⁺ /H ⁺ antiporter (ChaA)	66844276	5e-10	P		P
High-affinity nickel transporter	66852855	5e-82	P		P
Pirin-like protein (iron-binding nuclear protein)	66844884	3e-95	P		P
YL1 nuclear protein	50555151	2e-09	P		P
Nitrogen assimilation transcription factor	67515633	1e-110	P		S
Succinyl-CoA:3-ketoacid-coenzyme A transferase 1	37992813	2e-05	P		S
Glycerol-3-phosphate dehydrogenase (<i>glpA</i>)	16416069	9e-99	P	P	
Copper resistance-associated P-type ATPase	6164949	2e-28	P	P	P

yeast having a larger number of genes differentially expressed than did mycelia.

Putative virulence factors, such as *cbp1*, were overexpressed in yeast at all time points. In *H. capsulatum*, this gene encodes a calcium-binding protein identified as overexpressed in yeasts and considered a virulence factor. It would be interesting to study whether *P. brasiliensis cbp1* aids survival within macrophages in the same way as the equivalent protein in *H. capsulatum* (Hwang *et al.*, 2003; Patel *et al.*, 1998; Sebgathi *et al.*, 2000). Other interesting virulence candidates were the *nir* gene, encoding a nitrate reductase also found in the parasitic form of *Coccidioides posadasii*, and urease (a nickel-dependent enzyme) (Table 3) (Johannesson *et al.*, 2006; Rappleye & Goldman, 2006). Urease activity has been detected before in *P. brasiliensis*, giving further support to the suggestion that such a gene is functional and important in this species (Bagagli *et al.*, 1998).

Comparisons with related fungal species also pointed towards common expression by pathogenic forms of a number of transporters, such as drug-resistance proteins and copper, nickel and calcium transporters, all of which may have essential roles in pathogen survival during infection (Table 3).

The mycelial form of *P. brasiliensis* had few differentially overexpressed genes. The mycelial genes included trichothecene C-15 hydroxylase and a putative gene encoding a trichothecene efflux pump; the MFS pump was also shown to be upregulated in mycelia by real-time RT-PCR. Whether *P. brasiliensis* produces trichothecene, a mycotoxin, remains to be determined. However, these genes have been identified in the ongoing *P. brasiliensis* genomic sequencing project of the Broad Institute (entries PADG_03582.1, PABG_01811.1, PAAG_01242.1 and PABG_05447.1). We also detected a hypothetical protein containing RNA recognition motifs and a putative splicing factor, which may influence the overall mycelial gene expression program. Other genes expressed by mycelia included a sugar transporter, phosphoribosylformylglycinamide synthase (*ade6*), involved in *de novo* purine nucleotide biosynthesis, and a pseudouridine synthase. Comparisons with related fungal pathogens showed that the MFS pump and an adhesin/tyrosinase were also overexpressed in the saprobic phase of *H. capsulatum*, while *ape3* (involved in protein catabolic processes) was detected in the mycelial phase of *Coccidioides posadasii* (Table 3) (Hwang *et al.*, 2003; Johannesson *et al.*, 2006).

Interspecies comparison also yielded genes that differed in their expression patterns across species, including nitrogen assimilation transcription factor, succinyl-CoA:3-ketoacid-coenzyme A transferase 1 and *cgg9*, among others. These differences may reflect changes caused by experimental conditions or reflect specific metabolic processes of each fungal species (Table 3).

Genes that changed expression with time were also selected for analysis. The first group comprised genes that were

upregulated with time in both forms and were also more highly expressed in yeast (i.e. the transposon cluster). These were the two above-mentioned subunits of NADH:ubiquinone oxidoreductase, 3-isopropyl malate dehydrogenase A (leucine biosynthesis), glutamate dehydrogenase (amino acid transport and metabolism) and *rxt2*. The most notable feature of this cluster was the presence of the majority of clones (29 out of 35) that contain transposable element sequences. Similarly, transposable elements have been detected as overexpressed in *H. capsulatum* yeasts (Hwang *et al.*, 2003), although their role in morphogenesis and growth in culture remains unclear. Recently, it has been established that *S. cerevisiae* Ty long terminal repeat (LTR) retrotransposon transcription is activated by adenine starvation, among other stresses, and in some cases can also affect transcription of neighbouring genes (Servant *et al.*, 2008).

The second cluster with time-related variations was the ribosomal structure cluster, containing most of the ribosomal proteins detected in this study (seven genes plus one control). This cluster showed a marked decrease in gene expression with time, especially in yeast. Components of the proteasome, *pre1* and *pup1*, and a ubiquitin ligase, *ubr1*, behaved in the same manner, which could be a reaction to medium depletion or reaching stationary phase, especially in the yeast form.

Finally, among the 481 sequenced clones, the presence of phase-specific hypothetical and novel genes with no matches in analysed databases was assessed, and the results are shown in Fig. 1. Phase-specific hypothetical genes represent 13% of the identified genes (11% in yeast and 2% in mycelia), and may have roles in morphogenesis that are also important in other dimorphic pathogenic fungi, such as *H. capsulatum* and *Coccidioides* spp. Genes with no match in analysed databases may represent species-specific biochemical processes, with 6% present in yeast and 1% present in mycelia. These sets of genes are possible candidates for further studies on *P. brasiliensis* morphogenesis.

DISCUSSION

The primary goal of these studies was that of building a randomly sheared genomic DNA library for use in a microarray-based approach to assess gene expression patterns in the pathogenic fungus *P. brasiliensis*. We examined gene expression of both mycelia and yeast during growth in semi-synthetic media over a period of 14 days to identify differentially expressed genes. Our results demonstrate that the methodology used for library and microarray construction is useful and applicable to studies of *P. brasiliensis*. Furthermore, we found previously undescribed genes that were differentially expressed by the two forms and gained insights regarding the behaviour of this fungus during growth in culture. These results are similar to those for other genomic shotgun approaches for

microarray construction, which have been used successfully in studies involving pathogens such as *Plasmodium falciparum* and *H. capsulatum* (Hayward *et al.*, 2000; Hwang *et al.*, 2003).

The utilization of gDNA for library construction reduces the cloning bias seen in cDNA libraries. The adaptor–linker strategy minimizes the generation of chimeric inserts and is useful for the cloning of blunt-end DNA fragments. However, such gDNA libraries, and the microarrays assembled from them, also contain non-coding sequences. These may be uninformative in gene expression studies, but may nevertheless be useful in studies such as comparative genomic hybridization. Another drawback is that two genes may be present in a single insert if they are positioned closely in the genome (~6.4% of sequenced clones in our study contained two genes), which makes it difficult to distinguish expression of the adjacent genes.

Global gene expression and gene-specific analysis showed that mycelia and yeast forms had distinct behaviours during growth in culture. Yeast showed a larger number of upregulated genes than mycelia, and also showed more rapid and distinct changes in gene expression with time. Although not previously reported for *P. brasiliensis*, these differences might be predictable, since mycelia and yeast exist in thermally distinct environments and differ in cell wall structure and composition, nutritional requirements, membrane lipid composition and respiratory activity. Form-specific genes related to these differences, as well as genes related to growth in culture, were detected, and require further experimentation for full characterization. For the most part, our results correlate with previous genome-wide studies in *P. brasiliensis* and add further knowledge about the biology of this fungus. The decreased number of differentially expressed genes by yeast on day 14 is likely associated with the cultures attaining stationary phase due to exhaustion of essential nutrients (Arraes *et al.*, 2005; Ferreira *et al.*, 2006; Paris *et al.*, 1985), and indicate that differentially expressed genes are more readily observed at earlier phases of growth.

In conclusion, we assembled a random-shear shotgun gDNA microarray that is useful for studies of gene expression in *P. brasiliensis*. Our validation of the methods used, and our findings of differentially expressed genes involved in various functional categories, demonstrate the utility of the array. We found that yeast has a larger number of upregulated genes than do mycelia, particularly early in growth at day 5. Evidence presented here suggests that the thermal dimorphism of *P. brasiliensis* and growth in culture, in the mycelial or yeast form, are complex phenomena that involve a variety of biological processes. The identified genes include potential cell surface proteins, genes belonging to pathways that do not occur in humans, and transcription regulators that may be used as targets for the development of future therapies. Comparisons among different fungal species demonstrated a number of common metabolic processes across species in the

morphological form found in tissues, as well as in the saprobic mycelial form, contributing to the growing set of general characteristics of these closely related dimorphic fungi. Further experimentation and the application of gene disruption tools are essential to assess the exact functions and behaviours of the reported genes and how they relate to dimorphism.

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REFERENCES

- Albuquerque, P., Kyaw, C. M., Saldanha, R. R., Brigido, M. M., Felipe, M. S. & Silva-Pereira, I. (2004). Pbhyd1 and Pbhyd2: two mycelium-specific hydrophobin genes from the dimorphic fungus *Paracoccidioides brasiliensis*. *Fungal Genet Biol* **41**, 510–520.
- Almeida, A. J., Matute, D. R., Carmona, J. A., Martins, M., Torres, I., McEwen, J. G., Restrepo, A., Leão, C., Ludovico, P. & Rodrigues, F. (2007). Genome size and ploidy of *Paracoccidioides brasiliensis* reveals a haploid DNA content: flow cytometry and GP43 sequence analysis. *Fungal Genet Biol* **44**, 25–31.
- Alter, O., Brown, P. O. & Botstein, D. (2000). Singular value decomposition for genome-wide expression data processing and modeling. *Proc Natl Acad Sci U S A* **97**, 10101–10106.
- Arraes, F. B., Benoliel, B., Burtet, R. T., Costa, P. L., Galdino, A. S., Lima, L. H., Marinho-Silva, C., Oliveira-Pereira, L., Pfrimer, P. & other authors (2005). General metabolism of the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis*. *Genet Mol Res* **4**, 290–308.
- Bagagli, E., Sano, A., Coelho, K. I., Alquati, S., Miyaji, M., de Camargo, Z. P., Gomes, G. M., Franco, M. & Montenegro, M. R. (1998). Isolation of *Paracoccidioides brasiliensis* from armadillos (*Dasypus novemcinctus*) captured in an endemic area of paracoccidioidomycosis. *Am J Trop Med Hyg* **58**, 505–512.
- Barelle, C. J., Priest, C. L., Maccallum, D. M., Gow, N. A., Odds, F. C. & Brown, A. J. (2006). Niche-specific regulation of central metabolic pathways in a fungal pathogen. *Cell Microbiol* **8**, 961–971.
- Barros, T. F. & Puccia, R. (2001). Cloning and characterization of a LON gene homologue from the human pathogen *Paracoccidioides brasiliensis*. *Yeast* **18**, 981–988.
- Borges-Walmsley, M. I., Chen, D., Shu, X. & Walmsley, A. R. (2002). The pathobiology of *Paracoccidioides brasiliensis*. *Trends Microbiol* **10**, 80–87.
- Brummer, E., Castaneda, E. & Restrepo, A. (1993). Paracoccidioidomycosis: an update. *Clin Microbiol Rev* **6**, 89–117.
- Cano, M. I., Cisalpino, P. S., Galindo, I., Ramirez, J. L., Mortara, R. A. & da Silveira, J. F. (1998). Electrophoretic karyotypes and genome sizing of the pathogenic fungus *Paracoccidioides brasiliensis*. *J Clin Microbiol* **36**, 742–747.
- Cisalpino, P. S., Puccia, R., Yamauchi, L. M., Cano, M. I., da Silveira, J. F. & Travassos, L. R. (1996). Cloning, characterization, and epitope expression of the major diagnostic antigen of *Paracoccidioides brasiliensis*. *J Biol Chem* **271**, 4553–4560.
- Clemons, K. V., Feldman, D. & Stevens, D. A. (1989). Influence of oestradiol on protein expression and methionine utilization during

- morphogenesis of *Paracoccidioides brasiliensis*. *J Gen Microbiol* 135, 1607–1617.
- Cooke, F. T., Dove, S. K., McEwen, R. K., Painter, G., Holmes, A. B., Hall, M. N., Michell, R. H. & Parker, P. J. (1998). The stress-activated phosphatidylinositol 3-phosphate 5-kinase Fab1p is essential for vacuole function in *S. cerevisiae*. *Curr Biol* 8, 1219–1222.
- Costa, A. A., Gomez, F. J., Pereira, M., Felipe, M. S., Jesuino, R. S., Deepe, G. S., Jr & de Almeida Soares, C. M. (2002). Characterization of a gene which encodes a mannosyltransferase homolog of *Paracoccidioides brasiliensis*. *Microbes Infect* 4, 1027–1034.
- da Silva, S. P., Borges-Walmsley, M. I., Pereira, I. S., Soares, C. M., Walmsley, A. R. & Felipe, M. S. (1999). Differential expression of an *hsp70* gene during transition from the mycelial to the infective yeast form of the human pathogenic fungus *Paracoccidioides brasiliensis*. *Mol Microbiol* 31, 1039–1050.
- Derengowski, L. S., Tavares, A. H., Silva, S., Procopio, L. S., Felipe, M. S. & Silva-Pereira, I. (2008). Upregulation of glyoxylate cycle genes upon *Paracoccidioides brasiliensis* internalization by murine macrophages and *in vitro* nutritional stress condition. *Med Mycol* 46, 125–134.
- Ebel, F., Schwienbacher, M., Beyer, J., Heesemann, J., Brakhage, A. A. & Brock, M. (2006). Analysis of the regulation, expression, and localisation of the isocitrate lyase from *Aspergillus fumigatus*, a potential target for antifungal drug development. *Fungal Genet Biol* 43, 476–489.
- Edgar, R., Domrachev, M. & Lash, A. E. (2002). Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 30, 207–210.
- Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 95, 14863–14868.
- Felipe, M. S., Andrade, R. V., Petrofeza, S. S., Maranhão, A. Q., Torres, F. A., Albuquerque, P., Arraes, F. B., Arruda, M., Azevedo, M. O. & other authors (2003). Transcriptome characterization of the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis* by EST analysis. *Yeast* 20, 263–271.
- Felipe, M. S., Andrade, R. V., Arraes, F. B., Nicola, A. M., Maranhão, A. Q., Torres, F. A., Silva-Pereira, I., Poças-Fonseca, M. J., Campos, E. G. & other authors (2005). Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. *J Biol Chem* 280, 24706–24714.
- Ferreira, M. E., Marques Edos, R., Malavazi, I., Torres, I., Restrepo, A., Nunes, L. R., de Oliveira, R. C., Goldman, M. H. & Goldman, G. H. (2006). Transcriptome analysis and molecular studies on sulfur metabolism in the human pathogenic fungus *Paracoccidioides brasiliensis*. *Mol Genet Genomics* 276, 450–463.
- Fradin, C., Kretschmar, M., Nichterlein, T., Gaillardin, C., d'Enfert, C. & Hube, B. (2003). Stage-specific gene expression of *Candida albicans* in human blood. *Mol Microbiol* 47, 1523–1543.
- Gadgil, M., Lian, W., Gadgil, C., Kapur, V. & Hu, W. S. (2005). An analysis of the use of genomic DNA as a universal reference in two channel DNA microarrays. *BMC Genomics* 6, 66.
- Goldman, G. H., dos Reis Marques, E., Duarte Ribeiro, D. C., de Souza Bernardes, L. A., Quiapin, A. C., Vitorelli, P. M., Savoldi, M., Semighini, C. P., de Oliveira, R. C. & other authors (2003). Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. *Eukaryot Cell* 2, 34–48.
- Hayward, R. E., Derisi, J. L., Alfaradhi, S., Kaslow, D. C., Brown, P. O. & Rathod, P. K. (2000). Shotgun DNA microarrays and stage-specific gene expression in *Plasmodium falciparum* malaria. *Mol Microbiol* 35, 6–14.
- Hwang, L., Hocking-Murray, D., Bahrami, A. K., Andersson, M., Rine, J. & Sil, A. (2003). Identifying phase-specific genes in the fungal pathogen *Histoplasma capsulatum* using a genomic shotgun microarray. *Mol Biol Cell* 14, 2314–2326.
- Izacc, S. M., Gomez, F. J., Jesuino, R. S., Fonseca, C. A., Felipe, M. S., Deepe, G. S. & Soares, C. M. (2001). Molecular cloning, characterization and expression of the heat shock protein 60 gene from the human pathogenic fungus *Paracoccidioides brasiliensis*. *Med Mycol* 39, 445–455.
- Jesuino, R. S., Azevedo, M. O., Felipe, M. S., Pereira, M. & De Almeida Soares, C. M. (2002). Characterization of a chaperone ClpB homologue of *Paracoccidioides brasiliensis*. *Yeast* 19, 963–972.
- Jesuino, R. S., Pereira, M., Felipe, M. S., Azevedo, M. O. & Soares, C. M. (2004). Molecular cloning and characterization of a cDNA encoding the *Paracoccidioides brasiliensis* L35 ribosomal protein. *Med Mycol* 42, 217–221.
- Johannesson, H., Kasuga, T., Schaller, R. A., Good, B., Gardner, M. J., Townsend, J. P., Cole, G. T. & Taylor, J. W. (2006). Phase-specific gene expression underlying morphological adaptations of the dimorphic human pathogenic fungus, *Coccidioides posadasii*. *Fungal Genet Biol* 43, 545–559.
- Kanetsuna, F. & Carbonell, L. M. (1966). Enzymes in glycolysis and the citric acid cycle in the yeast and mycelial forms of *Paracoccidioides brasiliensis*. *J Bacteriol* 92, 1315–1320.
- Kanetsuna, F., Carbonell, L. M., Moreno, R. E. & Rodriguez, J. (1969). Cell wall composition of the yeast and mycelial forms of *Paracoccidioides brasiliensis*. *J Bacteriol* 97, 1036–1041.
- Kaufman, R. J. (1999). Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 13, 1211–1233.
- Leverly, S. B., Toledo, M. S., Suzuki, E., Salyan, M. E., Hakomori, S., Straus, A. H. & Takahashi, H. K. (1996). Structural characterization of a new galactofuranose-containing glycolipid antigen of *Paracoccidioides brasiliensis*. *Biochem Biophys Res Commun* 222, 639–645.
- Leverly, S. B., Toledo, M. S., Straus, A. H. & Takahashi, H. K. (1998). Structure elucidation of sphingolipids from the mycopathogen *Paracoccidioides brasiliensis*: an immunodominant β -galactofuranose residue is carried by a novel glycosylinositol phosphorylceramide antigen. *Biochemistry* 37, 8764–8775.
- Leverly, S. B., Toledo, M. S., Doong, R. L., Straus, A. H. & Takahashi, H. K. (2000). Comparative analysis of ceramide structural modification found in fungal cerebrosides by electrospray tandem mass spectrometry with low energy collision-induced dissociation of Li^+ adduct ions. *Rapid Commun Mass Spectrom* 14, 551–563.
- Lorenz, M. C. & Fink, G. R. (2001). The glyoxylate cycle is required for fungal virulence. *Nature* 412, 83–86.
- Lorenz, M. C. & Fink, G. R. (2002). Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryot Cell* 1, 657–662.
- Lorenz, M. C., Bender, J. A. & Fink, G. R. (2004). Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* 3, 1076–1087.
- Mackinnon, J. E. (1970). On the importance of South American blastomycosis. *Mycopathol Mycol Appl* 41, 187–193.
- Manly, K. F., Nettleton, D. & Hwang, J. T. (2004). Genomics, prior probability, and statistical tests of multiple hypotheses. *Genome Res* 14, 997–1001.
- Manocha, M. S. (1980). Lipid composition of *Paracoccidioides brasiliensis*: comparison between the yeast and mycelial forms. *Sabouraudia* 18, 281–286.
- Marques, E. R., Ferreira, M. E., Drummond, R. D., Felix, J. M., Menossi, M., Savoldi, M., Travassos, L. R., Puccia, R., Batista, W. L. &

- other authors (2004). Identification of genes preferentially expressed in the pathogenic yeast phase of *Paracoccidioides brasiliensis*, using suppression subtraction hybridization and differential macroarray analysis. *Mol Genet Genomics* **271**, 667–677.
- Mattar-Filho, R., Azevedo, M. O., Pereira, M., Jesuino, R. S., Salem-Izacc, S. M., Brito, W. A., Gesztes, J. L., Soares, R. B., Felipe, M. S. & Soares, C. M. (1997). Expression of glycoprotein gp43 in stage-specific forms and during dimorphic differentiation of *Paracoccidioides brasiliensis*. *J Med Vet Mycol* **35**, 341–345.
- McCullough, M. J., DiSalvo, A. F., Clemons, K. V., Park, P. & Stevens, D. A. (2000). Molecular epidemiology of *Blastomyces dermatitidis*. *Clin Infect Dis* **30**, 328–335.
- Mo, C., Valachovic, M. & Bard, M. (2004). The *ERG28*-encoded protein, Erg28p, interacts with both the sterol C-4 demethylation enzyme complex as well as the late biosynthetic protein, the C-24 sterol methyltransferase (Erg6p). *Biochim Biophys Acta* **1686**, 30–36.
- Montoya, A. E., Alvarez, A. L., Moreno, M. N., Restrepo, A. & McEwen, J. G. (1999). Electrophoretic karyotype of environmental isolates of *Paracoccidioides brasiliensis*. *Med Mycol* **37**, 219–222.
- Nunes, L. R., Costa de Oliveira, R., Leite, D. B., da Silva, V. S., dos Reis Marques, E., da Silva Ferreira, M. E., Ribeiro, D. C., de Souza Bernardes, L. A., Goldman, M. H. & other authors (2005). Transcriptome analysis of *Paracoccidioides brasiliensis* cells undergoing mycelium-to-yeast transition. *Eukaryot Cell* **4**, 2115–2128.
- Oefner, P. J., Hunicke-Smith, S. P., Chiang, L., Dietrich, F., Mulligan, J. & Davis, R. W. (1996). Efficient random subcloning of DNA sheared in a recirculating point-sink flow system. *Nucleic Acids Res* **24**, 3879–3886.
- Paris, S., Duran-Gonzalez, S. & Mariat, F. (1985). Nutritional studies on *Paracoccidioides brasiliensis*: the role of organic sulfur in dimorphism. *Sabouraudia* **23**, 85–92.
- Patel, J. B., Batanghari, J. W. & Goldman, W. E. (1998). Probing the yeast phase-specific expression of the *CBP1* gene in *Histoplasma capsulatum*. *J Bacteriol* **180**, 1786–1792.
- Rappleye, C. A. & Goldman, W. E. (2006). Defining virulence genes in the dimorphic fungi. *Annu Rev Microbiol* **60**, 281–303.
- Restrepo, A. & Jimenez, B. E. (1980). Growth of *Paracoccidioides brasiliensis* yeast phase in a chemically defined culture medium. *J Clin Microbiol* **12**, 279–281.
- Richie, D. L., Hartl, L., Aimaniananda, V., Winters, M. S., Fuller, K. K., Miley, M. D., White, S., McCarthy, J. W., Latgé, J. P. & other authors (2009). A role for the unfolded protein response (UPR) in virulence and antifungal susceptibility in *Aspergillus fumigatus*. *PLoS Pathog* **5**, e1000258.
- Rooney, P. J. & Klein, B. S. (2002). Linking fungal morphogenesis with virulence. *Cell Microbiol* **4**, 127–137.
- Rozen, S. & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**, 365–386.
- San Blas, G. & San Blas, F. (1977). *Paracoccidioides brasiliensis*: cell wall structure and virulence. *Mycopathologia* **62**, 77–86.
- San-Blas, G. & Vernet, D. (1977). Induction of the synthesis of cell wall α -1,3-glucan in the yeastlike form of *Paracoccidioides brasiliensis* strain IVIC Pb9 by fetal calf serum. *Infect Immun* **15**, 897–902.
- Schobel, F., Ibrahim-Granet, O., Avé, P., Latgé, J. P., Brakhage, A. A. & Brock, M. (2007). *Aspergillus fumigatus* does not require fatty acid metabolism via isocitrate lyase for development of invasive aspergillosis. *Infect Immun* **75**, 1237–1244.
- Sebghati, T. S., Engle, J. T. & Goldman, W. E. (2000). Intracellular parasitism by *Histoplasma capsulatum*: fungal virulence and calcium dependence. *Science* **290**, 1368–1372.
- Servant, G., Penner, C. & Lesage, P. (2008). Remodeling yeast gene transcription by activating the Ty1 long terminal repeat retrotransposon under severe adenine deficiency. *Mol Cell Biol* **28**, 5543–5554.
- Sprague, G. F. & Cronan, J. E. (1977). Isolation and characterization of *Saccharomyces cerevisiae* mutants defective in glycerol catabolism. *J Bacteriol* **129**, 1335–1342.
- Stover, E. P., Schar, G., Clemons, K. V., Stevens, D. A. & Feldman, D. (1986). Estradiol-binding proteins from mycelial and yeast-form cultures of *Paracoccidioides brasiliensis*. *Infect Immun* **51**, 199–203.
- Talaat, A. M., Howard, S. T., Hale, W., 4th, Lyons, R., Garner, H. & Johnston, S. A. (2002). Genomic DNA standards for gene expression profiling in *Mycobacterium tuberculosis*. *Nucleic Acids Res* **30**, e104.
- Tavares, A. H., Silva, S. S., Dantas, A., Campos, E. G., Andrade, R. V., Maranhão, A. Q., Brígido, M. M., Passos-Silva, D. G., Fachin, A. L. & other authors (2007). Early transcriptional response of *Paracoccidioides brasiliensis* upon internalization by murine macrophages. *Microbes Infect* **9**, 583–590.
- Thirach, S., Cooper, C. R., Jr & Vanittanakom, N. (2008). Molecular analysis of the *Penicillium marneffe* glyceraldehyde-3-phosphate dehydrogenase-encoding gene (*gpdA*) and differential expression of *gpdA* and the isocitrate lyase-encoding gene (*acuD*) upon internalization by murine macrophages. *J Med Microbiol* **57**, 1322–1328.
- Thorstenon, Y. R., Hunicke-Smith, S. P., Oefner, P. J. & Davis, R. W. (1998). An automated hydrodynamic process for controlled, unbiased DNA shearing. *Genome Res* **8**, 848–855.
- Toledo, M. S., Levery, S. B., Straus, A. H., Suzuki, E., Momany, M., Glushka, J., Moulton, J. M. & Takahashi, H. K. (1999). Characterization of sphingolipids from mycopathogens: factors correlating with expression of 2-hydroxy fatty acyl (*E*)- Δ^3 -unsaturation in cerebroside of *Paracoccidioides brasiliensis* and *Aspergillus fumigatus*. *Biochemistry* **38**, 7294–7306.
- Wang, Z. Y., Thornton, C. R., Kershaw, M. J., Debaio, L. & Talbot, N. J. (2003). The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*. *Mol Microbiol* **47**, 1601–1612.
- Wanke, B. & Londero, A. T. (1994). Epidemiology and paracoccidioidomycosis infection. In *Paracoccidioidomycosis*, pp. 109–120. Edited by M. Franco, C. da Silva Lacaz, A. Restrepo-Moreno & G. Del Negro. Boca Raton, FL: CRC Press.
- Williams, B. A., Gwartz, R. M. & Wold, B. J. (2006). Genomic DNA as a general cohybridization standard for ratiometric microarrays. *Methods Enzymol* **410**, 237–279.
- Yamamoto, A., DeWald, D. B., Boronenkov, I. V., Anderson, R. A., Emr, S. D. & Koshland, D. (1995). Novel PI(4)P 5-kinase homologue, Fab1p, essential for normal vacuole function and morphology in yeast. *Mol Biol Cell* **6**, 525–539.
- Zaugg, C., Monod, M., Weber, J., Harshman, K., Pradervand, S., Thomas, J., Bueno, M., Giddey, K. & Staib, P. (2009). Gene expression profiling in the human pathogenic dermatophyte *Trichophyton rubrum* during growth on proteins. *Eukaryot Cell* **8**, 241–250.

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