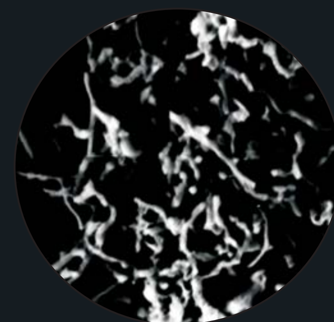
UNIVERSIDADE DE BRASÍLIA
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO
EM PATOLOGIA MOLECULAR

Genes diferencialmente expressos em *Paracoccidioides brasiliensis*

TESE DE DOUTORADO



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PÓS–GRADUAÇÃO EM PATOLGIA MOLECULAR

Genes diferencialmente expressos em *Paracoccidioides brasiliensis*

Candidato: Clayton Luiz Borges

Orientadora: Prof[a]. Dr[a]. Célia Maria de Almeida Soares

Tese apresentada ao Programa de Pós-Graduação em Patologia Molecular como requisito para obtenção do grau de Doutor em Patologia Molecular

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FACULDADE DE MEDICINA
PÓS–GRADUAÇÃO EM PATOLGIA MOLECULAR

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"Superação é poder fazer acontecer com as ferramentas que temos em mãos,

Superação é trabalhar da melhor forma possível independentemente do que pensem ou falem.

Superação é irmos além do que os outros acham que somos capazes!!!"

(Autor desconhecido)

"Vencer a si próprio é a maior de todas as vitórias"

Platão

Para ser feliz basta ter amigos,

família e orgulho do que se faz...

...sou feliz!

Claytim

Amar é sentir na felicidade do outro a própria felicidade.

Gottfried Wilhelm von Leibnitz

Dedico este trabalho à minha família:

Ao meu pai, minha referência de honestidade, amor e dedicação; e que mesmo ausente sempre esteve conosco;

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ABREVIATURAS E SIGLAS

4-HPPD - 4-hidroxil-fenil-piruvato dioxigenase

ACN - acetonitrila

AD - domínio de ativação da transcrição

Aox1 - oxidase alternativa

APS1 - 5'adenilil sulfato quinase

Atg4 - cisteína protease

ATP - adenosina trifosfato

BCIP - 5-bromo-4-chloro-3-indolil fosfato

BSA - soro albumina bovina

CTAB - brometo hexadecil trimetil amônio catiônico

BD - domínio de ligação ao DNA

cAMP - adenosina monofosfato cíclico

Cap20 - fator de virulência cap20

cDNA - DNA complementar

CEK1 - proteína quinase ativada por mitose

ClpA - chaperona ClpA

Cne1 - calnexina

CPP1 - fosfatase relacionada com formação de hifa

CST20 - quinase relacionada com formação de hifa

CTAB - brometo hexadecil trimetil amônio catiônico

Ctf1b - fator de transcrição C6 1-b

CTS - quitinase

DDC - descarboxilase de aminoácidos aromáticos

DDR48 - proteína relacionada ao estresse Ddr48

Dfg5 - glicosil hidrolase

DIP5 - permease de aminoácido ácido

DNA - ácido desoxirribonucléico

DTT - di-tiotreitol

EBP – proteína que se liga ao estradiol

EDTA - ácido etileno-diamino-tetra acético

Eef1a - fator de elongação da tradução, subunidade 1-alfa

ERG 6 - esterol c-metil-transferase

ERG 9 - esqualeno sintetase

ERG25 - C-4-esterol metil oxidase

EST – etiqueta de seqüência expressa

FKBP- peptidil prolil isomerase

Fmd - formamidase

GAPDH - gliceraldeído 3-fosfato desidrogenase

GEL - glucanosil transferases

GP - glicoproteína

GLK - glucoquinase

GLNA - glutamina sintase

GPI - glicosil-fosfatidil inositol

GST - glutationa S-transferase

HSP - proteína de choque térmico

IgG - imunoglobulina G

IPTG - isopropil-β-D-tiogalactopiranosídeo

ITS - seqüência espaçadora interna – “internally transcribed sequence”

Kb - Kilobases

kDa - KiloDalton

L-DOPA - L - Di-hidroxi fenil alanina

LEU2 - isopropilmalato desidrogenase

LYS21 - homocitrato sintase

MAPK - proteína quinase ativada por mitose

MEC - matriz extracelular

MUP - permease de alta afinidade para metionina

NADH - nicotinamida adenina dinucleotídeo reduzido

NBT - nitro blue tatrazólio

NP40 - nonidete P-40

Nsdd - GATA fator de transcrição *N*sdd

NTBC - 2-(2-nitro-4-trifluorometilbenzoil)-ciclohexano-1,3-diona

OLE1 - delta-9-desaturase

Oxr1 - proteína de resistência à oxidação

PAGE - eletroforese em gel de poliacrilamida

pb/bp - pares de bases

Pb – Paracoccidioides brasiliensis

Pb Y20 – proteína homóloga à flavodoxina

Pb M46 – homóloga à enolase

PBS - solução de tampão fosfato

PCM - paracoccidioidomicose

PCR - reação em cadeia da polimerase

PFGE - gel em eletroforese de pulso alternado

pH - potencial hidrogeniônico

pI - ponto isoelétrico

PKC - proteína quinase C

PMA1 - ATPase H+ de membrana plasmática

Rad6 - enzima E2 conjugada à ubiquitina

RAP-PCR - PCR de oligonucletídeo-ligado arbitrário

RDA - análise de diferença representacional

rDNA - DNA ribossomal

RNA - ácido ribonucleico

RNAm - RNA mensageiro

Rps27 - proteína ribossomal S27

Rps4 - 40S ribosomal protein S4

rRNA - RNA ribossomal

RT-PCR – PCR acoplado a transcrição reversa

SDS - dodecil sulfato de sódio

Sed1p - proteína de parede celular

SH - hibridização subtrativa

TPI - triose - fosfato isomerase

Ubq10 - poli-ubiquitina

UDP - uridina difosfato

URE - urease

RESUMO

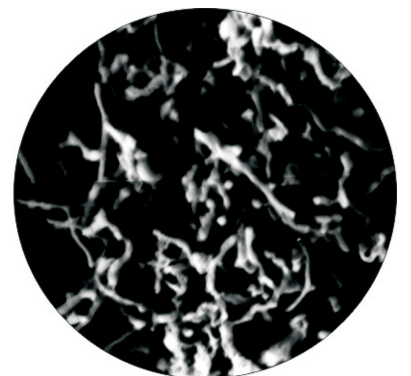
Paracoccidioides brasiliensis é um fungo termodimórfico causador da paracoccidioidomicose, uma doença endêmica na America Latina. A transição da forma miceliana (22°C) para forma levedura (36 °C) é induzida pela mudança da temperatura do ambiente para aquela no hospedeiro mamífero. Genes que são diferencialmente expressos nas fases de *P. brasiliensis* podem ser relevantes para o dimorfismo, e para o estabelecimento da infecção. No presente trabalho descrevemos a análise de genes diferencialmente expressos de *P. brasiliensis*. O gene da formamidase foi descrito como altamente expresso na fase miceliana de *P. brasiliensis*, isolado *Pb*01. Formamida aminohidrolase (formamidase, EC 3.5.1.49) catalisa a hidrólise específica da formamida para produção de amônia e formato. Nós identificamos a formamidase de *P. brasiliensis*, a qual reage com anticorpos presentes em soro de pacientes infectados com *P. brasiliensis*. O cDNA do gene foi clonado e a proteína heteróloga foi produzida e purificada. Adicionalmente, a proteína recombinante purificada foi reconhecida por soro de pacientes diagnosticados com paracoccidioidomicose e não reagiu com soro de indivíduos saudáveis. A proteína recombinante de 45-kDa foi cataliticamente ativa e sua atividade foi detectada em extratos protéicos das fases miceliana e leveduriforme de *P. brasiliensis*. A formamidase recombinante foi utilizada na produção de anticorpo policlonal em camundongos, que mostrou uma alta especificidade em ensaios de *Western blot*. A purificação da proteína nativa foi realizada em dois passos de cromatografia. As frações com atividade de formamidase foram selecionadas e analisadas por SDS-PAGE, o qual revelou uma proteína com massa molecular de 180-kDa. A proteína nativa purificada foi submetida à espectrometria de massas e foi identificada como formamidase. Adicionalmente, ensaios de SDS-PAGE com extratos protéicos desnaturados por calor revelaram uma proteína com massa molecular de 45-kDa. Estes resultados sugerem que a formamidase de *P. brasiliensis* é uma proteína tetramérica. A localização celular da proteína nativa em células fúngicas leveduriformes foi realizada através de microscopia confocal e de transmissão. A formamidase de *P. brasiliensis* foi encontrada no citoplasma e parede celular. Com o objetivo de elucidar o papel desta molécula, o cDNA codificante para formamidase foi utilizado para rastrear uma biblioteca construída com cDNAs de leveduras de *P. brasiliensis* utilizando o sistema de duplo-híbridos em *Saccharomyces cerevisiae*. Proteínas relacionadas com enovelamento protéico, processamento e destinação foram encontradas, as quais podem estar relacionadas com a localização da formamidase na parede celular, bem como com seu envolvimento no metabolismo de nitrogênio de *P. brasiliensis*. Um modelo das interações da formamidase foi construído. Nós avaliamos genes possivelmente envolvidos no estabelecimento da fase leveduriforme de *P. brasiliensis*. Mudanças na expressão gênica na fase levedura foram analisadas por meio da comparação do isolado *Pb*01 com um isolado que mantém a forma miceliana a 37 °C, utilizando a estratégia metodológica subtrativa cDNA-RDA. Em esforço para ajudar a identificação dos produtos gênicos associados com a fase leveduriforme parasita, perfis de cDNAs foram gerados de células leveduras do isolado *Pb*01 e do isolado *Pb*4940, o último cresce *in vitro* como micélio na temperatura do hospedeiro. O isolado *Pb*01 induziu a expressão de uma variedade de transcritos codificantes para proteínas relacionadas com resposta ao estresse, composição da parede/membrana celular, quando comparado com o isolado *Pb*4940, provavelmente refletindo o estabelecimento e manutenção da fase leveduriforme parasitária de *P. brasiliensis.*

ABSTRACT

Paracoccidioides brasiliensis is a thermally dimorphic fungus causing paracoccidioidomycosis, an endemic disease widespread in Latin America. The dimorphic transition from the mycelia (22 ºC) to the yeast (36 ºC) form is induced by a shift from the environmental temperature to that of the mammalian host. Genes that are differentially expressed in phases of *P. brasiliensis* can be relevant to the dimorphism, and to the establishment of infection. Here we describe the analysis of differentially expressed genes of *P. brasiliensis*. The formamidase gene of *P. brasiliensis* was described as highly expressed in mycelia of *P. brasiliensis,* isolate *Pb*01. Formamide aminohydrolase (formamidase, EC 3.5.1.49) catalyzes the specific hydrolysis of formamide to produce ammonia and formate. We identified the formamidase of *P. brasiliensis*, which reacts with antibodies present in sera of *P. brasiliensis* infected patients. The cDNA of the gene was cloned and the heterologous protein was produced and purified. Also, the purified recombinant protein was recognized by sera of patients with proven paracoccidioidomycosis and not by sera of healthy individuals. The recombinant 45-kDa protein was shown to be catalytically active and formamidase activity was also detected in *P. brasiliensis* yeast and mycelium protein extracts. The recombinant formamidase was used to produce polyclonal antibody in mice, which showed high specificity in western blot assay. We also performed purification of the native protein in two steps of chromatography. Fractions with formamidase activity were selected and analysed by SDS-PAGE, which revealed a protein with molecular mass of 180-kDa. The purified native protein was submitted to mass spectrometry and was identified as formamidase. Additionally, SDS-PAGE assay with heat-denatured protein extracts revealed a protein with molecular mass of 45-kDa. Those results suggest that *P. brasiliensis* formamidase is a tetrameric protein. The cellular localization of the native protein in fungal yeast cells was performed by confocal and transmission electron microscopy. The *P. brasiliensis* formamidase was found in cytoplasm and cell wall. In order to elucidate the role of this molecule, the cDNA encoding formamidase was used to screen a library constructed with *P. brasiliensis* yeast cells cDNAs using a *Saccharomyces cerevisiae* two-hybrid system. Proteins related with protein folding, processing and destination were found, which can be related to the cell wall localization of formamidase, as well as with the protein involvement in nitrogen metabolism of *P. brasiliensis.* A model for formamidase interactions is provided. We evaluated genes putatively involved in the establishment of yeast phase of *P. brasiliensis*. Changes in gene expression in the yeast phase were analyzed by comparison of the isolate *Pb*01 to a non-differentiating mycelia-like isolate using subtractive the cDNA-RDA methodological strategy. In an effort to help identify gene products associated with the yeast parasitic phase, cDNAs profiles were generated from yeast cells of isolate *Pb*01 and from isolate *Pb*4940, the last growing as mycelia at the host temperature. The isolate *Pb*01 induced the expression of a variety of transcripts encoding some proteins related to stress response, cell wall/membrane composition, compared to isolate *Pb*4940, probably reflecting the establishment/maintenance of the *P. brasiliensis* yeast parasitic phase.

Capítulo I

Introdução



I. Introdução

I.1. Aspectos Gerais

Paracoccidioides brasiliensis é um fungo termodimórfico, agente etiológico da Paracoccidioidomicose (PCM), doença endêmica na América Latina (Restrepo & Tobón, 2005). O Brasil é responsável por 80% dos casos descritos na literatura, seguido por Colômbia e Venezuela (Coutinho *et al.*, 2002). No Brasil, os estados das regiões Sul, Sudeste e Centro-Oeste são os locais onde a doença tem mais casos relatados (Paniago *et al.*, 2003).

O fungo cresce como levedura, a 36 ºC no hospedeiro humano ou quando cultivado *in vitro* e como micélio, forma infectiva e saprobiótica, no ambiente em temperaturas inferiores a 28 ºC (Bagagli *et al.*, 2006). As leveduras de *P. brasiliensis* são caracterizadas por apresentarem múltiplos brotamentos formados por evaginações da célula-mãe, revelando uma célula central circundada por várias células periféricas, microscopicamente similar à roda de leme de navio, o que caracteriza a presença de *P. brasiliensis* em materiais biológicos. A forma miceliana pode ser identificada por filamentos septados com conídeos terminais ou intercalares (Queiroz-Telles, 1994; Restrepo-Moreno, 2003).

I.2. Paracoccidioidomicose

A PCM é uma micose humana sistêmica granulomatosa. *P. brasiliensis* infecta hospedeiros humanos usualmente pela inalação de propágulos do micélio, como artroconídeos (San Blas *et al*., 2002). A patogênese da paracoccidioidomicose ainda não está bem definida, embora, em animais experimentais infectados por via inalatória com conídeos, ocorra diferenciação destes para forma leveduriforme. Observa-se também invasão tecidual e disseminação pela via hematogênica ou linfática para órgãos e tecidos extrapulmonares como fígado, baço e sistema nervoso central (San-Blas, 1993; Camargo & Franco, 2000; Valera *et al.*, 2008).

É possível que a inalação de propágulos de *P. brasiliensis* resulte na formação de um foco inflamatório nos pulmões, que geralmente regride com a imunidade do hospedeiro, constituindo a forma subclínica ou assintomática, conhecida como PCM infecção. A PCM pode ocorrer na forma juvenil, representando de 3 a 5% dos relatos descritos da doença, de desenvolvimento rápido e com o pior dos prognósticos, sendo a maioria de indivíduos constituída por crianças ou adultos jovens. Já a forma crônica ou adulta da doença acomete geralmente homens adultos, com idade entre 30 e 60 anos, progride vagarosamente, havendo comprometimento pulmonar evidente (Brummer *et al.*, 1993). A evolução da patologia e a

manifestação das formas clínicas dependem de fatores imunológicos do hospedeiro (Franco, 1987) e dos diferentes níveis de virulência de isolados do fungo (San-Blas & Nino-Vega, 2001).

Estima-se que 10 milhões de indivíduos no mundo estejam infectados e que 2 % das pessoas acometidas possam desenvolver a doença (McEwen *et al.* 1995). No Brasil, em áreas classificadas como altamente endêmicas, presume-se que a taxa de incidência anual da doença seja de 1 a 3 casos por 100.000 habitantes (Restrepo-Moreno & Greer 1983, Londero & Ramos 1990). Entre os anos de 1980 a 1995, a PCM foi descrita como a oitava causa de mortalidade por doença predominantemente crônica ou repetitiva, entre as infecciosas e parasitárias, e com a mais elevada taxa de mortalidade entre as micoses sistêmicas, apresentando uma taxa de mortalidade média anual de 1,45/milhão de habitantes, sendo que o estado de São Paulo apresentou maior freqüência (29,86 %), seguido por Paraná (14,52 %), Minas Gerais (12,51 %), Rio Grande do Sul (8,80 %) e Rio de Janeiro (7,92 %) (Coutinho *et al.* 2002). A incidência da doença até a puberdade é a mesma em ambos os sexos, porém na fase adulta, mais de 80% dos pacientes são do sexo masculino na faixa etária entre 30 e 60 anos (Martinez, 1997). A maior prevalência da doença em indivíduos do sexo masculino pode ser justificada pela inibição da transição de micélio para levedura pelo hormônio feminino 17-β-estradiol presente em mulheres em idade reprodutiva (Restrepo *et al.*, 1984; Sano *et al.*, 1999), ou pelo maior contato de indivíduos do sexo masculino com as fontes de infecção (Marques *et al.*, 1983).

Casos de PCM foram diagnosticados fora de áreas endêmicas como Europa, Estados Unidos da América e Ásia (Joseph *et al.*, 1966; Chikamori *et al.*, 1984; Ajello & Polonelli, 1985). Estes relatos possibilitaram a determinação de um período assintomático médio de 15,3 anos após o contato do paciente com a área endêmica até a manifestação da doença, o que dificulta a determinação do real período em que ocorreu a infecção (Brummer *et al.*, 1993).

I.3. Classificação Taxonômica

A primeira descrição da PCM foi realizada em São Paulo, em 1908, por Adolpho Lutz, com relatos das características do agente etiológico, previamente denominado de *Zymonema brasiliensis* por Splendore em 1912. Almeida (1930), após comparações com *Coccidioides immitis*, caracterizou o fungo como do gênero *Paracoccidioides* e da espécie *brasiliensis*. A classificação de *P. brasiliensis* como pertencente ao Reino: Fungi; Divisão: Eumycota; Sub-divisão: Deuteromycotina; Classe: Hyphomycetes; Ordem: Moniliales; Família: Moniliaceae; Gênero: *Paracoccidioides*; Espécie: *brasiliensis*, foi considerada devido ao não conhecimento de sua fase sexuada (Lacaz *et al.,* 1991). A caracterização do fungo como pertencendo ao filo

Ascomycota, mesmo não se conhecendo a fase teleomórfica, foi realizada por Leclerc *et al.* (1994) através de comparação entre seqüências de DNA da subunidade ribossomal 28S de dermatófitos e fungos dimórficos. Guého *et al.* (1997), realizaram posteriormente comparações entre seqüências parciais de rDNA de fungos dimórficos, classificando o fungo *P. brasiliensis*, juntamente com os gêneros *Histoplasma*, *Emmonsia* e *Blastomyces*, como pertencentes à família Onygenaceae. Outras análises filogenéticas realizadas com seqüências da subunidade ribossomal menor de fungos corroboraram a inserção de *P. brasiliensis* na ordem Onygenales (Bialek *et al.,* 2000).

Atualmente, *P. brasiliensis* é classificado no reino Fungi, filo Ascomycota, subdivisão Euascomycotina, classe Plectomyceto, subclasse Euascomycetidae, ordem Onygenales, família Onygenaceae, subfamília Onygenaceae Anamórficos, gênero Paracoccidioides, espécie *Paracoccidioides brasiliensis* (San-Blas *et al*., 2002, Bagagli *et al*., 2006). Matute *et al*., (2006, 2006a) descreveram a existência de três diferentes espécies filogenéticas de *P. brasiliensis*: S1 (espécie 1), PS2 (espécie filogenética 2) e PS3 (espécie filogenética 3). A espécie filogenética PS3 está geograficamente restrita à Colômbia, enquanto S1 está distribuída no Brasil, Argentina, Paraguai, Peru e Venezuela. Alguns isolados da espécie filogenética PS2 foram encontrados na Venezuela e no Brasil, nos Estados de São Paulo e Minas Gerais (Matute *et al*., 2006). Isolados classificados nas três espécies foram capazes de induzir a doença em hospedeiros humanos e animais; no entanto PS2 apresentou menor virulência (Matute *et al*., 2006a). A fim de analisar as relações filogenéticas entre os isolados de *P. brasiliensis*, foi realizada a comparação de seqüências de regiões codantes, não codantes e ITS (seqüência espaçadora interna - "internally transcribed sequence") de 7 novos isolados e 14 isolados já estudados de *P. brasiliensis* oriundos do Brazil, Colômbia e Venezuela. Vinte isolados se agruparam nos três grupos filogenéticos descritos anteriormente (S1, PS2 e PS3), com exceção do isolado *Pb*01 (objeto de nossos estudos). Este isolado claramente separa-se de todos os outros, apresentando uma alta variabilidade genética quando comparado com os outros isolados de *P. brasiliensis*, podendo constituir uma nova espécie no gênero Paracoccidioides (Carrero *et al*., 2008; Theodoro *et al*., 2008).

A análise da transmissão de genes, como o que codifica para a glicoproteína gp43 (Travassos *et al*., 1995; Cisalpino *et al*., 1996), uma candidata a vacina (Buissa-Filho *et al*., 2008; Travassos *et al*., 2008) e genes relacionados a fatores de virulência, foi realizada em isolados de *P. brasiliensis*, revelando que o gene que codifica para a glicoproteína gp43 está sobre seleção positiva dentre os isolados e apresentou pequena variação entre as seqüências codificantes, o que reforça a hipótese de utilização de marcadores moleculares para a

identificação de isolados de *P. brasiliensis* ou de grupos filogenéticos identificados no fungo. Além disso, esses estudos auxiliam na compreensão da patogênese e dos processos adaptativos dos grupos filogenéticos de *P. brasiliensis* (Matute *et al.,* 2008; Puccia *et al*., 2008).

I.4. Biologia de *P. brasiliensis*

O fungo *P. brasiliensis* é encontrado em locais com características ambientais comuns denominados de reservárias, os quais apresentam peculiaridades microambientas, tais como condições do solo e clima (Restrepo-Moreno, 1994). O nicho ecológico de *P. brasiliensis* não está bem estabelecido em virtude de seu raro e não reprodutível isolamento do ambiente, o prolongado período de latência no hospedeiro humano, bem como indefinição do hospedeiro intermediário do fungo (Bagagli *et al*., 1998; Bagagli *et al*., 2003). Contudo, foi identificado o crescimento de *P. brasiliensis* em meio de cultura com amostras de solo de regiões endêmicas (Terçarioli, *et al*., 2007), em habitats exógenos ao homem (Borelli, 1972) como fezes de pingüim e de uma amostra comercial de ração canina (Gezuele, 1989; Ferreira *et al.*, 1990), solo, água e plantas (Restrepo *et al*., 2001). O fungo foi isolado de tatus das espécies *Dasypus novemcinctus, Dasypus septemcinctus*, *Cabassous centralis* e de outros animais na natureza, mostrando que a infecção em animais silvestres é comum em áreas endêmicas (Bagagli *et al.* 2003, Corredor *et al.* 2005), assim como em animais domésticos, a qual deve se tratar de infecção acidental e não natural do fungo (Conti-Diaz, 2007). Através da técnica de PCR, utilizando-se amostras de tecidos de animais silvestres mortos acidentalmente, *P. brasiliensis* foi identificado em porco-da-índia (*Cavia aperea*), porco espinho (*Sphigurrus spinosus*), guaxinim (*Procyon cancrivorus*) e furão (*Gallictis vittata*), retratando um novo perfil de prevalência do fungo em animais silvestres (Richini-Pereira *et al*., 2008).

A organização genômica de *P. brasiliensis* era desconhecida pelo fato de não se conhecer a fase telemórfica do fungo. Estudos utilizando-se a técnica de gel em eletroforese de pulso alternado (PFGE), identificaram entre 4 ou 5 cromossomos com 2-10 Mb tanto de isolados do meio ambiente, quanto de isolados clínicos. *P. brasiliensis* apresentaria um genoma entre 23-31 Mb (Montoya *et al*., 1997, Feitosa *et al*., 2003). Estudos realizados utilizando-se citometria de fluxo em isolados de *P. brasiliensis* demonstraram que leveduras uninucleadas apresentam um genoma variando de 26,3 a 35,5 Mb. Quando foram considerados os conídios, nenhuma diferença significativa com a forma de levedura foi identificada (30,2 a 30,9 Mb) (Almeida *et al*., 2007). O genoma estrutural de três isolados de *P. brasiliensis* (*Pb*01, *Pb*03 e *Pb*18) foi realizado, confirmando a presença de 5 cromossomos em cada isolado. O genoma do isolado *Pb*01 apresentou 32,94 Mb, com um total de 9.132 genes. Essas informações auxiliarão

na compreensão das diferenças existentes entre os isolados, na caracterização de genes e suas regiões promotoras, e no desenvolvimento de novas ferramentas biomoleculares e genéticas importantes para a elucidação da biologia de *P. brasiliensis.* (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html).

I.5. Dimorfismo em *P. brasiliensis*

P. brasiliensis é fungo termodimórfico, pois tem como característica a alternância entre duas formas, micélio no ambiente e levedura quando submetido a ambientes hostis como o do hospedeiro humano (Kurokawa *et al.*, 1998; San-Blas *et al.*, 2002). A transição dimórfica é considerada parte importante na fase inicial da interação de fungos com o hospedeiro, sendo considerada etapa essencial para o estabelecimento de infecções (San-Blas *et al.*, 2002; Nemecek *et al.*, 2006).

A temperatura é um dos estímulos ambientais mais notórios para o dimorfismo de *P. brasiliensis*, o qual cresce como micélio em temperaturas inferiores a 28 °C e como levedura a 36 °C (Bagagli *et al.*, 2006). Além da temperatura, fatores nutricionais podem interferir no processo dimórfico de *P. brasiliensis*. O crescimento a 25 °C em meio de cultura quimicamente definido adicionado de soro fetal de bezerro, permitiu preservar a expressão fenotípica de *P. brasiliensis* na forma de levedura (Villar *et al.*, 1988).

Outro fator relacionado ao dimorfismo de *P. brasiliensis* é a presença do hormônio feminino 17-β-estradiol, o qual inibe a transição de micélio para levedura *in vitro* (Restrepo *et al.*, 1984) e *in vivo* (Sano *et al.*, 1999), sendo esse fato relacionado como possível fator de proteção contra a infecção em mulheres. Uma proteína de ligação ao estradiol (EBP – Estradiol binding protein), cujo transcrito foi identificado como preferencialmente expresso no transcritoma de *P. brasiliensis* na fase leveduriforme (Felipe *et al.*, 2005), foi anteriormente descrita como possuidora de sítio de ligação ao 17-β-estradiol (Loose *et al.*, 1983; Clemons *et al.*, 1989). Acredita-se que a interação do hormônio 17-β-estradiol com a EBP citoplasmática iniba a transição de micélio para levedura, explicando a diferença na incidência da PCM em mulheres. Aristizabal *et al.*, (2002) observaram, *in vivo*, a participação do hormônio feminino na resistência de fêmeas de rato ao desenvolvimento inicial da PCM.

Durante a transição de micélio para levedura, conversão morfogenética de *P. brasiliensis*, ocorrem mudanças na composição da parede celular. Análises citoquímicas e estruturais da parede celular do fungo em suas duas formas foram realizados confirmando a presença de polímeros de α-1,3-glicana em leveduras, prioritariamente, e β-1,3-glicana em micélio (Paris *et al.* 1986). Durante a transição de micélio para levedura ocorre a mudança do

polímero de β-1,3-glicana para α-1,3-glicana (Kanetsuna *et al.* 1969). Como provável meio de defesa, fagócitos humanos produzem β-1,3-glicanase capaz de digerir somente β-1,3-glicana. Contudo, durante a diferenciação de micélio para levedura logo no início da infecção, há maior teor α-1,3-glicana, a qual deve proteger o fungo contra a ação das enzimas β-glicanases fagocitárias permitindo a instalação da forma patogênica do fungo. Quitina é encontrada em ambas as formas do fungo apresentando um maior teor em levedura quando comparado a micélio (San-Blas *et al.* 1987, Kurokawa *et al.* 1998).

O gene codificante para a Dfg5, uma proteína de parede celular membro da família das glicosil hidrolases foi caracterizado. Ensaios de RT-PCR identificaram o transcrito como mais expresso na fase leveduriforme de *P. brasiliensis*. Ensaios de interação com proteínas de matriz extracelular (MEC) mostraram que a Dfg5 de *P. brasiliensis* interage com fibronectina, laminina e colágeno tipo I e IV. A interação de Dfg5 com proteínas da matriz extracelular deve estar relacionada com o provável papel da proteína na interação de *P. brasiliensis* com o hospedeiro (Castro *et al*., 2008). Castro e colaboradores (2005) identificaram transcritos que codificam para proteínas potencialmente ancoradas na membrana/parede celular via âncora GPI (glicosil-fosfatidil inositol). Os transcritos que codificam para as enzimas glicanosil-transferases (Gel1, Gel2 e Gel3) foram identificados no transcritoma de *P. brasiliensis*. A Gel3 está relacionada à morfogênese da parede celular na forma miceliana (Castro *et al*., 2008, *in press*).

Estudos de genes/proteínas, estágio específicos ou com expressão diferencial durante a transição dimórfica do fungo foram realizados por diferentes grupos de pesquisa. Alterações na expressão de proteínas em *P. brasiliensis* foram detectadas nos estágios iniciais do processo de diferenciação celular no isolado *Pb*01 (Silva *et al.* 1994). Utilizando técnicas de proteômica (eletroforese bidimensional), observou-se variação nos padrões de síntese protéica em diferentes isolados na fase miceliana e um perfil protéico mais similar de isolados na fase leveduriforme, mostrando que leveduras de diferentes isolados do fungo parecem apresentar um perfil protéico mais similar do que o perfil protéico presente em isolados da forma miceliana (Salem-Izacc *et al.*, 1997).

Cunha *et al.* (1999), detectaram, por meio de seqüenciamento aminoterminal, a proteína *Pb*Y20 similar à flavodoxinas, exclusiva da fase de levedura. O gene que codifica para a *Pb*Y20 foi caracterizado e apresenta baixos níveis de transcrito na fase miceliana do fungo, sendo induzido durante a diferenciação de micélio para levedura. A proteína foi detectada imunologicamente em extrato protéico total obtido de células em fase leveduriforme (Daher *et al*., 2005).

O cDNA e o gene que codificam para a enzima formamidase de *P. brasiliensis* foi caracterizado. A Formamidase de *P. brasiliensis* é preferencialmente expressa na fase miceliana do fungo (Felipe *et al*., 2003; Borges *et al*., 2005). A proteína recombinante foi produzida, apresentando atividade enzimática de formamidase. A proteína recombinante mostrou-se reativa com soros de pacientes com paracoccidioidomicose. A formamidase de *P. brasiliensis* deve ser importante para o metabolismo de nitrogênio e possivelmente deve estar relacionada com estratégias de sobrevivência e interação do fungo com o hospedeiro (Borges *et al*., 2005, 2008-presente trabalho).

Antígenos de *P. brasiliensis* foram identificados por meio de imunobloting utilizando-se soros de pacientes com PCM. As proteínas identificadas foram submetidas à seqüenciamento parcial de aminoácidos. Análises em banco de dados revelaram homologia com os antígenos catalase, malato desidrogenase, frutose-1-6-bifosfato aldolase (FBA), gliceraldeído-3-fosfato desidrogenase (GAPHD) e triose fosfato isomerase (TPI), preferencialmente expressos na fase leveduriforme de *P. brasiliensis* (Fonseca *et al*., 2001). Os genes codificantes dos antígenos foram caracterizados.

O cDNA codificante para a catalase P (peroxissomal) foi caracterizado e análises por *Northern blot* e *western blot* revelaram que o RNAm e o produto protéico apresentaram menor expressão na fase miceliana quando comparada à fase leveduriforme de *P. brasiliensis* (Moreira *et al*., 2004). Dois cDNAs codificantes da FBA foram caracterizados e apresentaram diferenças significativas, o que sugere a ocorrência de duplicação gênica em gene ancestral em *P. brasiliensis* (Carneiro *et al*., 2005). O cDNA e o gene que codifica para a enzima da via glicolítica GAPDH foram caracterizados. A análise da expressão do gene e da proteína foi avaliada revelando que, em *P. brasiliensis*, há um aumento da expressão de GAPDH durante a diferenciação de micélio para levedura bem como no processo reverso (Barbosa *et al*., 2004). A proteína recombinante foi produzida em modelo heterólogo e o anticorpo policlonal contra GAPDH foi produzido. Por meio de imunocitoquímica a proteína GAPDH foi localizada tanto no citoplasma quanto na parede celular de leveduras de *P. brasiliensis*. A proteína GAPDH se liga à componentes matriz extracelular, o que sugere seu papel como uma adesina de *P. brasiliensis*. A proteína recombinante e o anticorpo anti-GAPDH inibem a adesão e internalização de à pneumócitos cultivados *in vitro*, o que reforça o papel da GAPDH como molécula de interação *P. brasiliensis*-hospedeiro (Barbosa *et al*., 2006). O gene e o cDNA codificantes para TPI de *P. brasiliensis* foram caracterizados. A proteína, bem como o anticorpo policlonal produzido contra a proteína recombinante inibem a interação de *P. brasiliensis* com células epiteliais (Pereira *et al*., 2004, 2007).

Proteínas de choque térmico provavelmente relacionadas com a sobrevivência do fungo no ambiente do hospedeiro foram descritas, tais como HSP70 (Silva *et al.*, 1999), relacionada com a termotolerância de *P. brasiliensis*, porém, aparentemente sem correlação com virulência (Theodoro *et al.,* 2008a). O cDNA codificante para a proteína de choque térmico HSP90 foi seqüenciado. Análises por *northern blot* revelaram que a HSP90 é mais expressa na fase leveduriforme e durante a transição dimórfica de micélio para levedura. A HSP90 deve estar relacionada com a transição dimórfica e com o estresse oxidativo de *P. brasiliensis* (Nicola *et al*., 2008). Outras proteínas de estresse como HSP60 (Salem-Izacc *et al.*, 2001), ClpB (Jesuino *et al.*, 2002) foram descritas, as quais apresentam baixos níveis de expressão na forma miceliana, quando comparado com a forma leveduriforme de *P. brasiliensis*. A expressão diferencial de tais moléculas pode estar relacionada com estratégias de sobrevivência, adaptação e diferenciação de *P. brasiliensis* no ambiente do hospedeiro. A proteína de choque térmico HSP60, um antígeno de *P. brasiliensis* (Cunha *et al*., 2002) apresenta efeito imunoprotetor em infecção experimental com dose letal de *P. brasiliensis* em camundongos (Soares *et al*., 2008).

Chagas e colaboradores (2008) analisaram três catalases (*Pb*CatA, *Pb*CatP e *Pb*CatC), diferencialmente expressas nas fases de *P. brasiliensis*. Chagas e colaboradores (2008) identificaram atividades diferenciais em micélio e levedura das catalases A, P e C de *P. brasiliensis*. A espécie A predomina na fase miceliana e está associada ao estresse oxidativo endógeno. A espécie P predomina na fase leveduriforme de *P. brasiliensis* e está preferencialmente associada ao estresse oxidativo exógeno (Chagas *et al*., 2008).

I.6 Projetos Transcritomas de *P. brasiliensis*

Pesquisadores da região Centro-Oeste do Brasil realizaram o Projeto Genoma Funcional e Diferencial de *P. brasiliensis*, o qual resultou no seqüenciamento de 6.022 genes expressos nas fases miceliana e leveduriforme do isolado *Pb*01, possibilitando a detecção de genes diferencialmente expressos (Felipe *et al.*, 2003; 2005). A diferenciação celular em *P. brasiliensis* requer mudança na temperatura, o que pode ser associado com a resposta ao estresse. Dessa forma, foram identificados 48 transcritos codificando chaperonas ou proteínas envolvidas no processo de estresse, sendo oito desses transcritos diferencialmente expressos. A análise do transcritoma também revelou alguns prováveis componentes das vias de sinalização e seqüências gênicas consideradas como potenciais alvos para drogas antifúngicas em *P. brasiliensis*, não possuindo nenhum homólogo no genoma humano, como quitina deacetilase, isocitrato liase e α-1,3-glicana sintase, todos preferencialmente expressas na fase

leveduriforme. A fase miceliana, infectiva, utiliza o piruvato no metabolismo aeróbio, uma vez que as enzimas relacionadas ao ciclo do ácido cítrico estão induzidas nesta fase. Já a fase leveduriforme, utiliza preferencialmente o piruvato para o metabolismo anaeróbio, evidenciando possivelmente a condição de baixo teor de oxigênio nos tecidos do hospedeiro.

Pesquisadores do Estado de São Paulo realizaram o transcritoma do isolado *Pb*18, sendo identificados 4.692 genes (Goldman *et al.* 2003). Foram identificados potenciais fatores de virulência em *P. brasiliensis*, homólogos à *Candida albicans*. Genes da via de transdução de sinal foram implicados na transição dimórfica. A identificação de genes de *P. brasiliensis,* homólogos aos envolvidos em vias de transdução de sinal em *C. albicans,* controlados por MAPK e cAMP, sugerem que possam estar também atuando em *P. brasiliensis*. Uma quinase (CST20) homóloga à MEKK quinase e uma tirosina-fosfatase (CPP1) relacionadas com formação de hifa em *C. albicans*; e uma proteína quinase ativada durante a mitose (CEK1) homóloga a MAPK foram identificadas, o que sugere o papel dessas moléculas na mudança morfológica de *P. brasiliensis*.

Marques *et al.* (2004), identificaram, por meio de análises de ESTs, genes preferencialmente expressos na fase leveduriforme de *P. brasiliensis* (isolado *Pb*18). Dentre os genes identificados como diferencialmente expressos estão α-1,3-glicana sintase, envolvida no metabolismo de parede celular. A mudança morfológica de *P. brasiliensis* é acompanhada pela mudança na composição de monômeros de glicana da parede celular que passam de β-1,3-glicana para α-1,3-glicana, assim que o fungo adota a forma de levedura (San-Blas & Nino-Vega, 2001). Outro gene preferencialmente expresso na fase leveduriforme de *P. brasiliensis* foi identificado como homólogo a uma C-4 esterol metil oxidase (ERG25), a qual realiza o primeiro passo enzimático da síntese de ergosterol de fungos. O aumento na expressão de ERG25 deve estar relacionado com a maior utilização de ergosterol na membrana celular, onde mudanças são fatores importantes na transição morfológica a 36 °C (Goldman *et al*., 2003). Também foram identificados como induzidos na fase leveduriforme, genes envolvidos na assimilação de aminoácidos que contêm enxofre, tais como metionina permease (Marques *et al*., 2004), o qual deve estar relacionado à manutenção no morfotipo de levedura, como descrito para *Histoplasma capsulatum*. Durante a transição de micélio para levedura em *H. capsulatum*, grupos sulfidrílicos, principalmente na forma de cisteína parecem ser determinantes para o processo (Maresca & Kobayashi, 2000). Marques *et al*., (2004) confirmam, em seus experimentos, a auxotrofia do isolado *Pb*18 para o enxofre orgânico, sugerindo que o mesmo seja um importante estímulo para manter a fase leveduriforme patogênica de *P. brasiliensis*, isolado *Pb*18. O metabolismo de enxofre parece ser importante

para a transição dimórfica do isolado *Pb*01, visto que transcritos relacionados ao metabolismo e transporte de enxofre foram identificados. O transcrito que codifica para transportador de ferro/enxofre (*isc*) foi identificado como induzido em micélio. Transcritos que codificam para enzimas relacionadas à via *de novo* de biossíntese de cisteína tais como ATP sulfurilase, APS quinase e PAPS redutase, induzidas na fase leveduriforme e possivelmente relacionadas à transição dimórfica em *P. brasiliensis* (Andrade *et al.,* 2006).

A avaliação da expressão de genes de *P. brasiliensis*, isolado *Pb*18, durante a transição de micélio para levedura foi realizada por meio de microarranjos (Nunes *et al.* 2005) de DNA. Vários genes diferencialmente expressos durante a transição morfológica, como aqueles que codificam para enzimas envolvidas no metabolismo de aminoácidos, síntese de proteínas, proteínas de transdução de sinal, enzimas do metabolismo da parede celular, proteínas de resposta ao estresse oxidativo, controle do crescimento e desenvolvimento do fungo foram identificadas. Durante a transição da fase miceliana para a leveduriforme verificou-se alta expressão do gene que codifica para a 4-hidroxil-fenil-piruvato dioxigenase (4-HPPD), proteína envolvida no catabolismo de aminoácidos. Este gene pode ser inibido pela adição de NTBC [2-(2-nitro-4-trifluorometilbenzoil)-ciclohexano-1,3-diona], provocando bloqueio do crescimento e da diferenciação *in vitro* do fungo para a fase leveduriforme, o que indica papel da 4-HPPD na transição de micélio para levedura.

A análise do perfil transcricional de *P. brasiliensis* durante a diferenciação morfológica de micélio para levedura foi realizada por Bastos *et al.* (2007), em nosso laboratório. Durante os estágios iniciais (22 horas) do processo de diferenciação, genes relacionados com a síntese e remodelamento de membrana e parede celulares são induzidos, bem como transcritos que codificam para enzimas relacionadas com a síntese e degradação de carboidratos de membrana e para transportadores de precursores para a síntese desses carboidratos de membrana. Genes codificantes de enzimas relacionadas com glicosilação de proteínas, síntese e correto enovelamento de proteínas (Parente *et al*., 2008) e síntese de lipídeos são também induzidos na diferenciação de micélio para levedura. Os dados sugerem ser o remodelamento da membrana e parede celulares os eventos mais evidentes no início da transição dimórfica, uma vez que 34 genes relacionados à síntese/remodelamento da parede celular/membrana foram induzidos nesta condição (Bastos *et al*., 2007).

Outros genes relacionados à enzimas da síntese de componentes da parede celular, como fosfoglicomutase, UDP-glucose pirofosforilase e alfa-1,3 glicana sintase, foram induzidos, assim como um novo transcrito que codifica para alfa-glicosidase, possivelmente relacionado ao processamento de beta-1,6 glicana. A quitina é o maior componente da parede

celular. Transcritos que codificam para quitinase 1 (CTS1) e 3 (CTS3) possivelmente relacionados ao remodelamento da parede celular na transição dimórfica, foram induzidos nos estágios iniciais da diferenciação de micélio para levedura, bem como um transcrito que codifica para amino ácido permease ácida, relacionada à captação de glutamato, precursor para a síntese de quitina (Bastos *et al.*, 2007).

Genes relacionados à proteínas/enzimas da síntese de lipídeos de membrana são induzidos durante a transição dimórfica (Bastos *et al*., 2007). Genes envolvidos na via de assimilação do enxofre como a sulfito redutase mostraram-se super expressos durante a transição. Foram identificados genes codificantes de enzimas que participam do ciclo do glioxalato, como a isocitrato liase, malato desidrogenase, citrato sintase e aconitase, sugerindo um ativo ciclo do glioxalato durante a transição dimórfica. Proteínas relacionadas com vias de transdução de sinal tais como MAPK e histidina quinase foram também identificadas, sugerindo que a transição morfológica em *P. brasiliensis* é mediada por vias de transdução de sinais que controlam a adaptação ao ambiente para a sobrevivência do fungo no hospedeiro.

I.7. Genes diferencialmente expressos no processo infeccioso

A expressão de genes durante o processo infeccioso por *P. brasiliensis* tem sido investigada. Um projeto transcritoma de células leveduriformes de *P. brasiliensis* recuperadas de fígado de camundongos infectados foi realizado em nosso laboratório (Costa *et al*., 2007). Foram seqüenciadas 4.932 ESTs, as quais foram agrupadas num total de 1041 contigs e 561 singletons, sendo do total, 35,47% relacionadas a novos genes e 23,75% pertencentes a genes induzidos durante o processo infeccioso em fígado de animais experimentais. Neste trabalho, Costa *et al.* (2007), identificaram genes super expressos codificantes de enzimas de várias vias metabólicas, como fermentação alcoólica, biossíntese de aminoácidos, metabolismo de nitrogênio e biossíntese de lipídeos e esteróis, importantes na biossíntese/remodelamento da membrana, que devem ser relevantes para o processo infeccioso.

Foram identificados vários genes codificantes de enzimas do metabolismo de carboidratos, como aquele que codifica para álcool desidrogenase, a qual deve ser responsável por um comportamento anaeróbio de *P. brasiliensis* no tecido hepático (Costa *et al*., 2007). A enzima acilfosfatase, com papel ainda não estabelecido completamente, a qual é importante na regulação da via glicolítica e aumenta a taxa de fermentação em *Saccharomyces cerevisiae* (Raugei *et al*., 1996), encontra-se altamente expressa durante o processo infeccioso. A indução da expressão de genes codificantes das enzimas glucoquinase (GLK), altamente induzida na presença de etanol (Herrero *et al*. 1999), glicogênio fosforilase I, requerida para mobilização de

glicogênio e fosfoglicerato mutase (GPM1P), sugerindo que o metabolismo de carboidratos é bastante ativo durante o processo infeccioso no fígado (Costa *et al*., 2007). O fungo *P. brasiliensis*, no ambiente hepático, deve exacerbar o metabolismo de etanol dado o ambiente anaeróbio e rico em carboidratos, como descrito para *C. albicans* em infecção experimental em rim de camundongo (Barelle *et al*., 2006).

Assim como para o metabolismo de carboidratos, a expressão coordenada de genes codificantes para enzimas da biossíntese de lipídeos foi evidente durante o processo infeccioso em fígado de animais (Costa *et al*., 2007). A indução de genes relacionados à biossíntese de ácidos graxos sugere um ambiente rico em carboidratos e energia, condição supostamente presente no tecido hepático. A enzima málica é regularória no metabolismo de lipídeos e foi induzida. Transcritos codificantes da enzima anidrase carbônica, bem como aqueles que codificam para enzimas relacionadas à biossíntese de ergosterol (ERG6, ERG9) e síntese/remodelamento de membrana (OLE1) devem estar relacionadas às prováveis modificações de membrana do fungo no processo infeccioso. Esses dados sugerem que a biossíntese de lipídeos deve ocorrer em *P. brasiliensis* nas condições de infecção experimental de fígado, dado o alto suprimento de carboidratos presente neste nicho de infecção (Costa *et al*., 2007).

O ciclo do glioxalato é induzido em *P. brasiliensis* na condição experimental de infecção em fígado de camundongo. O ciclo do glioxalato é requerido para o crescimento de microrganismos em fontes de três carbonos, como etanol ou acetato. A enzima isocitrato liase, induzida em *P. brasiliensis* recuperado de fígado de camundongo infectado, é regulatória do ciclo do glioxalato, sugerindo regulação positiva dessa via metabólica. Os produtos de genes associados ao ciclo do glioxalato como o que codifica para hidroximetil glutaril-CoA liase, que fornece acetil CoA derivada de β-hidroxi-β-metilglutaril-CoA, um intermediário na biossíntese de esteróis, reforçam a relevância do ciclo do glioxalato no processo infeccioso por *P. brasiliensis*.

Transcritos codificantes para glutamina sintase (GLNA), urease (URE), homocitrato sintase (LYS21), isopropilmalato desidrogenase (LEU2), cistationa B-sintase, assim como uma permease de alta afinidade para metionina (MUP1) são induzidos em células leveduriformes recuperadas de fígado de camundongos infectados. Costa *et al*., (2007) descreveram 14 genes novos/induzidos relacionados à biossíntese/assimilação de aminoácidos, sugerindo que este aspecto do metabolismo deva ser importante para a sobrevivência do fungo no ambiente hepático. Transcritos relacionados à patogênese de fungos também foram identificados, tais como aqueles relacionados com a síntese de melanina, de urease que alcaliniza os tecidos,

favorecendo a disseminação da infecção por *Coccidioides posadassi* (Mirbod-Donovan *et al.*, 2006) e uma proteína de resistência à oxidação (Oxr1) a qual protegeria o fungo contra peróxido de hidrogênio tecidual (Elliott & Volkert, 2004).

O perfil de transcritos de *P. brasiliensis* internalizados em macrófagos murinos foi descrito (Tavares *et al.*, 2007). Genes relacionados à biossíntese de aminoácidos, como metionina, e o gene codificante da proteína de choque térmico HSP60 foram induzidos nessa condição, sugerindo serem os processos de síntese de alguns aminoácidos e de resposta ao estresse, relevantes durante a fagocitose por macrófagos (Tavares *et al.*, 2007).

I.8. RDA (Análise de Diferença Representacional) no estudo de genes diferencialmente expressos

Técnicas subtrativas têm sido utilizadas na identificação de genes diferencialmente expressos em condições experimentais (Diatchenko *et al.*, 1996). Essas técnicas permitem a identificação de alterações no padrão de expressão gênica por meio de enriquecimento seletivo de genes (Pastorian *et al.*, 2000). Dentre as técnicas mais utilizados podemos citar a Análise de Diferença Representacional (RDA) (Hubank & Schatz, 1994), a reação de PCR que utiliza pequenos oligonucleotídeos que se ligam arbitrariamente ao RNAm para a transcrição reversa (RAP-PCR) (Mathieu-Daude *et al.,* 1999), e a hibridização subtrativa (SH) (Diatchenko *et al.*, 1996). Tais técnicas visam à identificação de seqüências de ácidos nucléicos presentes em uma condição e ausentes ou menos abundantes em outra e apresentam a vantagem de identificar genes novos/induzidos sem a necessidade de um grande número de reações de seqüenciamento de DNA.

A análise de genes diferencialmente expressos através da técnica de RDA foi originalmente utilizada na identificação de diferenças entre populações de DNA (Lisitsyn, 1995) e foi posteriormente adaptada para a identificação de genes diferencialmente expressos em amostras de RNAm (Hubank & Schatz, 1994). A técnica é baseada em etapas de hibridização subtrativa seguida de reações de PCR, nas quais os produtos diferenciais são amplificados (Pastorian *et al.*, 2000). A Figura 1 ilustra as etapas experimentais da metodologia de RDA.

Condições que mimetizam nichos de parasitas no hospedeiro têm sido utilizadas nestas análises subtrativas por meio de RDA.

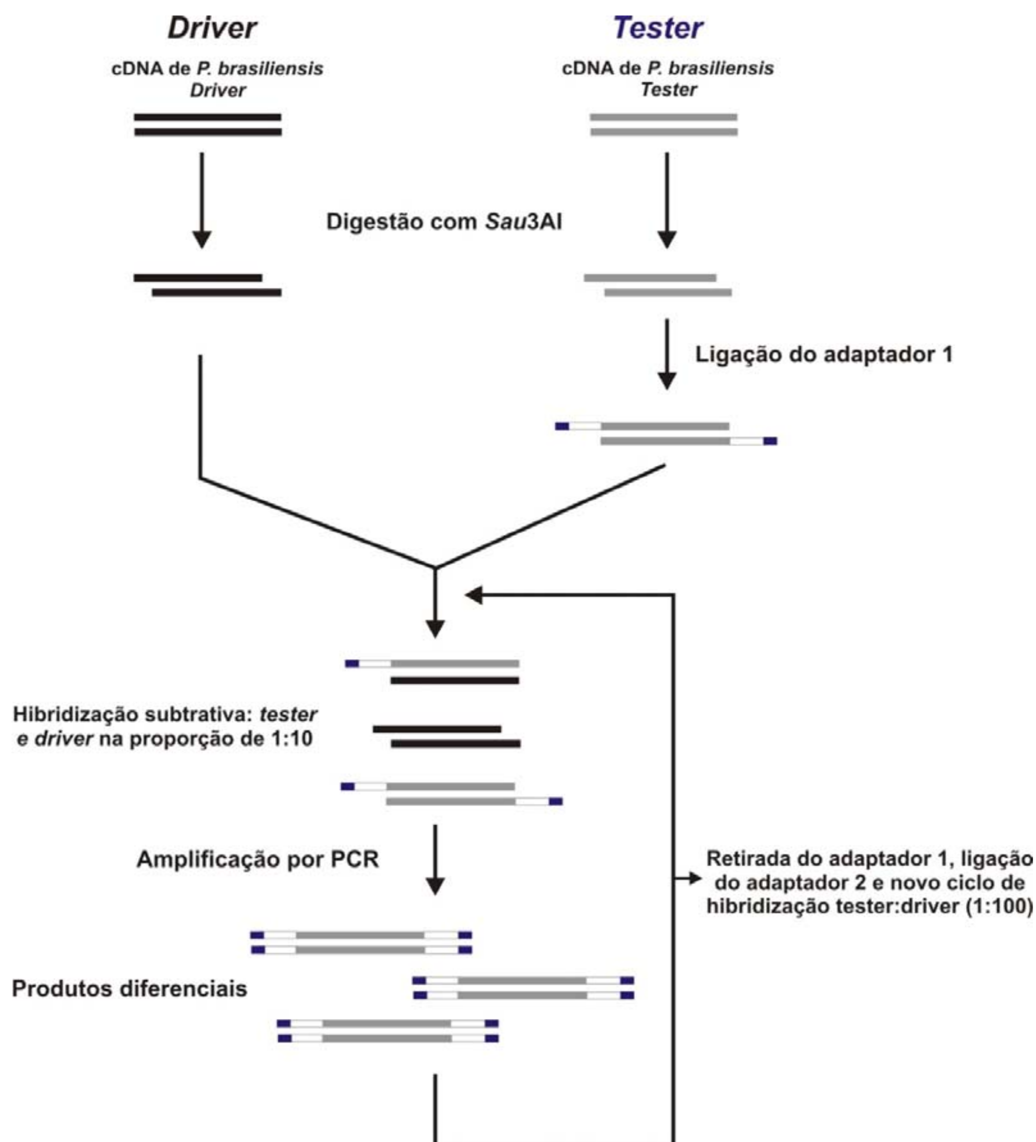


Figura 1 – Esquema representativo das etapas da metodologia do RDA. RNA total de células de *P. brasiliensis driver* e *tester* foram utilizados para a síntese de cDNA. Os cDNAs foram digeridos com a enzima de restrição *Sau*3AI. O primeiro par de adaptadores foi ligado aos cDNAs digeridos, na amostra *Tester*. As populações de cDNA foram misturadas para a realização de cada um dos dois ciclos de hibridização subtrativa com taxas de cDNAs tester: driver de 1:10 e 1:100, respectivamente.

Dutra *et al.* (2004) identificaram, por meio da técnica de cDNA-RDA, 34 genes de *Metarhizium anisopliae* super expressos quando este organismo foi cultivado com carapaças de carrapato, o hospedeiro natural do fungo. O transcrito que codifica para a subtilisina protease, envolvida na degradação de proteínas da cutícula, na penetração do fungo e na obtenção de nutrientes. O transcrito que codifica para a GAPDH foi identificado. A GAPDH foi descrita como adesina em outros organismos e pode desempenhar o mesmo papel em *M. anisopliae*. Transcritos que codificam para proteínas antioxidantes como catalases e HSP70 foram identificadas e devem estar envolvidas na patogênese de *M. anisopliae* (Dutra *et al.*, 2004).

Bailão *et al.* (2006, 2007), em nosso laboratório, utilizaram a técnica de cDNA-RDA para identificar genes de *P. brasiliensis* induzidos durante o processo infeccioso, em condições que mimetizam a via hematogênica de disseminação fúngica e sítios de infecção com inflamação. A captação de micronutrientes, resposta ao estresse térmico, a degradação de lipídeos, através da β-oxidação são presumivelmente induzidos. Todos os transcritos codificantes de enzimas da β-oxidação foram induzidos em condições que mimetizam a via hematogênica de disseminação do fungo no hospedeiro e sítios de infecção com inflamação, sugerindo que *P. brasiliensis* nesta condição, desvie o seu metabolismo para a degradação de lipídeos (Bailão *et al*., 2006, 2007). Em bactérias e fungos, propionil-CoA é assimilado através do ciclo do metilcitrato, onde o propionil-CoA é oxidado a piruvato (Brock *et al*., 2000). Os transcritos que codificam para a enzima 2-metil-citrato desidratase, que converte metil-citrato em metil-isocitrato, foram induzidos nos tratamentos com sangue e plasma, corroborando a relevância do metabolismo de lipídeos nesses possíveis nichos do hospedeiro (Bailão *et al.* 2006; 2007).

Genes relacionados com a captação de ferro, importantes para a sobrevivência de patógenos nos tecidos do hospedeiro onde a quantidade de ferro deve ser limitante, foram induzidos durante a infecção em fígado de camundongos, como demonstrado por meio de análises dos experimentos de RDA (Bailão *et al*., 2006). A aquisição de ferro tem sido descrita como fator de virulência em patógenos (Ratledge & Dover 2000). Transcritos codificantes para transportadores de ferro de alta e baixa afinidade, para membros da família das cobre oxidases foram encontrados com alta redundância em ensaios de cDNA-RDA de células recuperadas de fígado de camundongo infectado (Bailao *et al*., 2006). Transportador de ferro de alta afinidade é requerido para virulência de *C. albicans* em infecção de camundongos (Ramanan & Wang 2000). Os dados indicam a relevância da captação do micronutriente durante o processo infeccioso (Bailão *et al*., 2006).

A síntese de melanina está implicada na patogênese de fungos (Hamilton & Gomez 2002, Taborda *et al*., 2008). O crescimento de *P. brasiliensis* com L-DOPA (precursor da melanina) resulta na melanização das células fúngicas (Gomez *et al*., 2001), bem como a melanina protege *P. brasiliensis* de fagocitose (Silva *et al*., 2006). Os transcritos que codificam para L-amino ácido decarboxilase aromática, para tirosinase e para policetídeo sintase foram induzidos em células leveduriformes de *P. brasiliensis* recuperadas de fígado de camundongos e demonstram a relevância da síntese de melanina no processo (Bailão *et al*., 2006).

I.9. Formamidase: gene diferencialmente expresso em *P. brasiliensis*

A enzima formamidase, formamida amidohidrolase (EC 3.5.1.49), catalisa a hidrólise de formamida, produzindo formato e amônia (Hynes, 1975; Fraser *et al.*, 2001). A hidrólise de amidas, através da atividade de amidases é importante para microrganismos na obtenção de nitrogênio do solo, como já caracterizado em *Aspergillus nidulans* (Hynes 1975) e é descrita em organismos que apresentam, pelo menos, uma etapa do seu ciclo de vida no ambiente (Bury-Moné *et al.*, 2003).

A atividade de amidases também é importante para a defesa de patógenos. Um dos produtos finais da ação da formamidase, a amônia, tem papel importante na patogênese de *Helicobacter pylori,* auxiliando na destruição tecidual e em sua resistência ao pH ácido estomacal (Bury-Moné *et al.*, 2004). O outro produto, o formato, pode ser oxidado em dióxido de carbono, processo que utiliza o NAD^+ produzindo NADH, o qual pode ser utilizado em etapas metabólicas e na cadeia respiratória.

Formamidases são descritas como enzimas da família de amidases/nitrilases como enzimas homodiméricas, homotriméricas e homotetraméricas (Wyborn *et al*., 1996), bem como apresentando dois tetrâmeros ligados (O'Hara *et al.*1994). A cristalografia da formamidase de *H. pylori* revelou estrutura homo-hexamérica (Hung *et al.,* 2007).

O transcrito codificante da enzima formamidase de *P. brasiliensis* é altamente expresso na fase miceliana do fungo (Felipe, *et al.*, 2003; Borges *et al*., 2005). A proteína isolada por meio de focalização isoelétrica líquida é reativa com anticorpos presentes em soros de pacientes com PCM, sugerindo um papel potencial da proteína na interação do fungo com o hospedeiro. A proteína purificada foi digerida por endoproteinase Lys-C, obtendo-se quatro fragmentos peptídicos internos, os quais foram seqüenciados através do método de degradação de Edman e caracterizados. O cDNA codificante da formamidase de *P. brasiliensis* foi clonado, caracterizado e a expressão heteróloga da proteína foi obtida em modelo bacteriano. A proteína heteróloga purificada mostrou-se reativa com anticorpos presentes em soros de pacientes e apresentou atividade enzimática de formamidase. A atividade de formamidase também foi demonstrada em extrato protéico total de células leveduriformes e micelianas de *P. brasiliensis* (Borges, *et al.*, 2005).

O papel desempenhado pela formamidase de *P. brasiliensis* ainda não é elucidado. O sistema de duplo híbrido em levedura (*S. cerevisiae*) é uma metodologia que foi desenvolvida para identificar genes que codificam proteínas (presas) que interagem com uma proteína alvo (isca) *in vivo* (Fields e Song, 1989; Chien *et al.*, 1991; Fields e Sternglanz, 1994). A base para o sistema está na estrutura de um fator de transcrição que tem dois domínios fisicamente

separados: o domínio de ligação ao DNA (BD) e o domínio de ativação da transcrição (AD). Os genes codificantes para estes domínios estão presentes em plasmídeos diferentes onde são clonados genes de uma biblioteca de cDNA (presa) e o gene para o qual se deseja rastrear a interação (isca). Caso as proteínas façam interação *in vivo* o fator de transcrição atua sobre o promotor que se situa no início de um gene repórter, o qual determina o crescimento das leveduras em meio seletivo, onde crescem apenas as leveduras que apresentam proteínas que interagem. A Figura 2 ilustra o princípio da estratégia que caracteriza a metodologia de duplo híbrido. A identificação de proteínas que tenham interação *in vivo* com a formamidase no sistema de *S. cerevisiae* poderá auxiliar na compreensão do papel da proteína na biologia de *P. brasiliensis*.

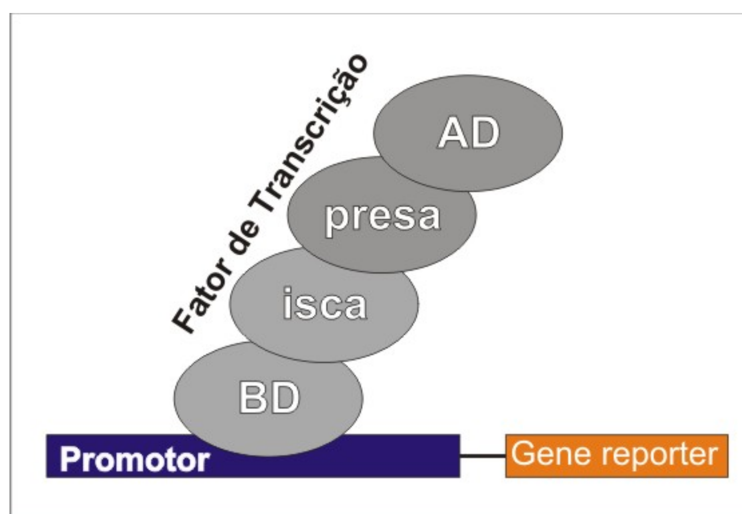
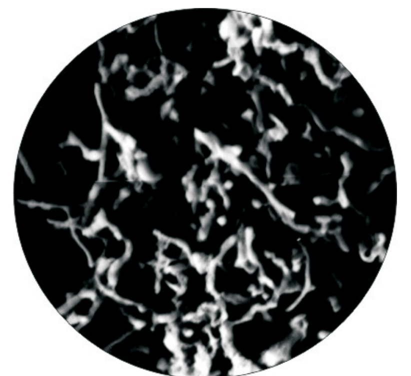


Figura 2 – Esquema da técnica do duplo híbrido em leveduras. BD – Domínio de ligação à região promotora do DNA, o qual é fusionado à formamidase (isca). AD – Domínio de ativação da transcrição, o qual é fusionado às proteínas (presas) de *P. brasiliensis*. A interação de proteínas fusionadas aos domínios (BD e AD) compõe o fator de transcrição que ativa a expressão do gene repórter permitindo o crescimento das leveduras em meio seletivo.

Neste trabalho, o sistema de duplo híbrido em levedura foi utilizado para identificar proteínas que interagem com a formamidase de *P. brasiliensis*. Proteínas relacionadas com enovelamento, processamento e destinação de proteínas foram encontradas, as quais podem estar relacionadas com o correto enovelamento, com a localização da formamidase na parede celular, bem como com seu envolvimento no metabolismo de nitrogênio de *P. brasiliensis*. Um modelo das interações da formamidase com proteínas de *P. brasiliensis* foi construído.

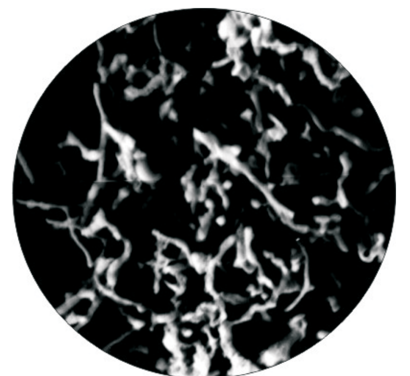
Justificativa



II. Justificativas

A capacidade de *P. brasiliensis* de provocar doença com grande variedade de manifestações clínicas depende da complexidade de interações entre *P. brasiliensis* e o hospedeiro humano. O laboratório de Biologia Molecular do Instituto de Ciências Biológicas da Universidade Federal de Goiás está inserido em um programa que visa à identificação e caracterização de moléculas potencialmente associadas à transição dimórfica e à interação do fungo *P. brasiliensis* com o hospedeiro humano. Neste contexto, moléculas diferencialmente expressas nas fases miceliana e leveduriforme, moléculas reconhecidas por anticorpos presentes em soro de pacientes com paracoccidioidomicose, assim como moléculas diferencialmente expressas em modelos experimentais de infecção são objeto de estudo, destacando-se como estratégias interessantes para o entendimento da biologia do fungo *P. brasiliensis*.

Objetivos



III. Objetivo Geral

Caracterizar genes e proteínas diferencialmente expressas pelo fungo patogênico humano *P. brasiliensis*.

III.1. Objetivos Específicos

1. **Caracterização da formamidase de *P. brasiliensis*:**
   Analise proteômica, localização celular e interações intermoleculares.

   - Estratégias:

   ✔ Produção da proteína recombinante em sistema heterólogo;

   ✔ Produção de anticorpo policlonal em camundongo;

   ✔ Purificação da proteína nativa e identificação dos peptídeos obtidos por digestão tríptica utilizando-se espectrometria de massas;

   ✔ Citolocalização da proteína nativa por microscopia eletrônica de transmissão e microscopia confocal.

   ✔ Construção de biblioteca de duplo híbrido em *S. cerevisiae*;

   ✔ Seqüenciamento dos produtos de PCR obtidos nas interações identificadas pela técnica de duplo híbrido;

   ✔ Confirmação das interações protéicas por meio de co-imunoprecipitação;

2. Avaliação de transcritos potencialmente relevantes para o estabelecimento/manutenção da fase leveduriforme.
   Análise de ESTs diferencialmente expressas em isolados de *P. brasiliensis* com e sem capacidade de termo-dimorfismo *in vitro.*

   - Estratégias:

   ✔ Microscopia eletrônica de varredura dos isolados;

   ✔ Construção de bibliotecas subtrativas de cDNA, por meio de RDA, e seqüenciamento de DNA;

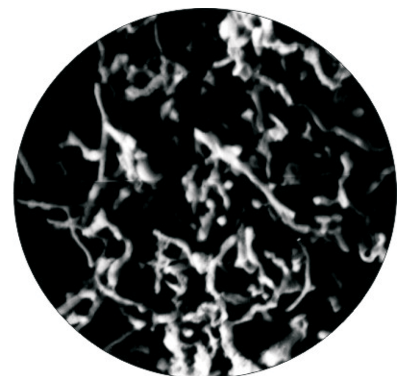
   ✔ Análise de seqüências e geração de ESTs;

   ✔ Análises comparativas utilizando-se ferramentas de bioinformática;

   ✔ Confirmação dos resultados obtidos por meio de *northern-blot* reverso e RT-PCR.

Capítulo II

Formamidase



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Original article

The antigenic and catalytically active formamidase of *Paracoccidioides brasiliensis*: protein characterization, cDNA and gene cloning, heterologous expression and functional analysis of the recombinant protein

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Abstract

Paracoccidioides brasiliensis is a well-characterized pathogen of humans. To identify proteins involved in the fungus–host interaction, *P. brasiliensis* yeast proteins were separated by liquid isoelectric focusing, and fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. Immunoreactive bands were detected with pooled sera of patients with *P. brasiliensis* infection. A protein species with a molecular mass of 45 kDa was subsequently purified to homogeneity by preparative gel electrophoresis. The amino acid sequence of four endoproteinase Lys-C-digested peptides indicated that the protein was a formamidase (FMD) (E.C. 3.5.1.49) of *P. brasiliensis*. The complete cDNA and a genomic clone (*Pbfmd*) encoding the isolated FMD were isolated. An open reading frame predicted a 415-amino acid protein. The sequence contained each of the peptide sequences obtained from amino acid sequencing. The *Pbfmd* gene contained five exons interrupted by four introns. Northern and Southern blot analysis suggested that there is one copy of the gene in *P. brasiliensis* and that it is preferentially expressed in mycelium. The complete coding cDNA was expressed in *Escherichia coli* to produce a recombinant fusion protein with glutathione S-transferase (GST). The purified recombinant protein was recognized by sera of patients with proven paracoccidioidomycosis and not by sera of healthy individuals. The recombinant 45-kDa protein was shown to be catalytically active; FMD activity was detected in *P. brasiliensis* yeast and mycelium.
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Keywords: Paracoccidioides brasiliensis; Serum reactive protein, formamidase; Immunological reactivity; Catalytic activity

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CTAB, cationic hexadecyl trimethyl ammonium bromide; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetic acid; FMD, formamidase; GST, glutathione S-transferase; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; IPTG, isopropyl β-D-thiogalactopyranoside; M, mycelium; NBT, nitrobluetetrazolium; NP40, Nonidet P-40; *Pb*, *Paracoccidioides brasiliensis*; PCM, paracoccidioidomycosis; PCMB, 4-chloromercuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; PVP, polyvinylpolypyrrolidone; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethylketone; Y, yeast.

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1. Introduction

Paracoccidioides brasiliensis is a dimorphic and thermoregulated fungal pathogen that infects the host by inhalation of conidia and small mycelial fragments. Those inhaled elements convert to yeasts, which are responsible for the pathogenesis of paracoccidioidomycosis (PCM). Although the majority of individuals infected with fungus do not manifest symptoms, severe and progressive infection does occur, with both pulmonary and extrapulmonary involvement [1,2]. The disease is endemic, primarily in Latin America, where up to 10 million individuals are infected. The disease may present a prolonged asymptomatic latency period, followed by reactivation, which suggests that the fungus is a facultative intracellular pathogen which can persist dormant and clinically silent in tissues. Acute and subacute PCM, also known as the juvenile form of the disease, progresses rapidly and with dissemination through the lymphatic system. The chronic form evolves gradually in the lungs and chiefly affects adult individuals [3,4].

Delineating the role of fungal antigens should facilitate the elucidation of the pathogenesis of *P. brasiliensis*. Experimental and clinical investigations have indicated the importance of humoral and cellular immune responses in the pathogenesis of PCM [5,6]. Some antigens of *P. brasiliensis* have already been identified: the glycoprotein gp43 [7,8], a 27-kDa antigen [9], an 87-kDa antigen, characterized as a member of the HSP70 family [10,11], and antigens FO and FII [12]. Our laboratory has cloned and expressed the HSP60 of *P. brasiliensis*, and has proposed its utility as a serodiagnostic marker [13,14]. Also, a homologue of mannosyltransferase has been obtained, and this antigen is reactive to sera of patients with PCM [15].

Classical approaches for the study of antigens include protein separation/purification and Western blot analysis. We have previously used two-dimensional electrophoresis followed by Western blotting as an approach to study antigens of *P. brasiliensis* and identified different proteins by Edman's degradation [16]. The proteins catalase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, fructose 1,6 biphosphate aldolase and malate dehydrogenase were characterized, and they were highly reactive with sera of PCM patients [17–19].

The present report is a continuation of our work on the identification and characterization of *P. brasiliensis* molecules potentially associated with the fungus–host interaction. We obtained a highly expressed protein species that was characterized by amino acid sequencing of peptide fragments and identified the protein as a formamidase (FMD) homologue of *P. brasiliensis*.

Formamide amidohydrolase (FMD, EC 3.5.1.49) mediates the highly specific hydrolysis of formamide to produce formate and ammonia [20]. FMDs are poorly characterized proteins in fungi and in pathogenic microorganisms. In spite of this scarce knowledge, ammonia has been described as playing a central role in the pathogenesis of human pathogens such as *Helicobacter pylori*, since it contributes to epithelial cell damage and apoptosis and is required for acid resistance [21,22]. In addition, the *amiF* gene, encoding FMD of *H. pylori*, is induced upon shift to acidic pH, as detected by microarray analysis [23]. *P. brasiliensis* expresses high levels of the transcript encoding the enzyme FMD, as we have recently shown in the transcriptome of the fungus published by the "*P. brasiliensis* Functional and Differential Genome Project-Brazilian Middle West Network" [24].

In the present paper, we report the characterization of the FMD from *P. brasiliensis*. The cDNA and the genomic sequences encoding FMD (*Pbfmd*) were obtained. The deduced protein contained putative antigenic epitopes located in 17 regions of the molecule, reinforcing its potential as a useful antigen in the serodiagnosis of PCM. We also evaluated the enzymatic activity of the purified and native FMD. This work provides initial data on a catalytically active FMD of *P. brasiliensis* and supports the further analysis of this protein as an antigen candidate in studies concerning serodiagnosis and host–parasite interactions in PCM.

2. Materials and methods

2.1. Fungal isolate and growth conditions

P. brasiliensis isolate *Pb*01 (ATCC-MYA-826) has been studied in our laboratory [13–19]. It is a commonly used laboratory strain, initially isolated from a patient with the acute form of PCM. It was grown as yeast at 36 °C, in Fava–Netto'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). The mycelium was grown at 22 °C in this medium.

2.2. Antigen preparation

Yeast cells and mycelia were harvested and washed in cold homogenization buffer (20 mM Tris–HCl pH 8.8, 2 mM $CaCl_2$) containing the protease inhibitors 50 µg/ml *N*-α-*p*-tosyl-L-lysine chloromethylketone (TLCK); 1 mM 4-chloromercuribenzoic acid (PCMB); 20 mM leupeptin; 20 mM phenylmethylsulfonyl fluoride (PMSF); 5 mM iodoacetamide. The mixture was frozen in liquid nitrogen, and the cells were disrupted until a fine powder was obtained. A suspension was made with the fine powder in 25 ml of the above buffer. The insoluble debris was removed by centrifugation at 12,000 × *g*, at 4 °C for 10 min, and the supernatant was used for further analysis. The Bradford [25] method was used to determine the protein content of each preparation.

2.3. Liquid IEF of the antigens

Ten milligrams of cellular extracts in a total volume of 25 ml (obtained as above, after centrifugation) were dialyzed for 18 h at 4 °C with several changes of ammonium bicarbon-

ate buffer (5 mM ammonium bicarbonate). The sample was then increased to 60 ml by the addition of the buffer indicated above containing 1% (v/v) NP-40, 20 mM DTT, and 1% (v/v) ampholytes (pH range 4–7 and 5–8). The sample was loaded onto a liquid isoelectric focusing (IEF) system (Rotofor, BioRad, Hercules, CA), and separation was achieved with a constant power supply of 15 W. The 15 fractions were harvested after 4 h, when the voltage reading had been stabilized. The pH of the fractions was measured, and the protein content was determined [25]. Fractions were pooled and loaded onto the IEF system for further separation. The fractions were subsequently subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with human immune sera.

2.4. Western blotting analysis

SDS-PAGE was performed with 12% polyacrylamide gels according to Laemmli [26]. The proteins were electrophoresed in each lane and stained with Coomassie brilliant blue or transferred to membranes. The membranes were incubated in 0.05% (v/v) Tween-20 plus Tris-buffered saline (TBS) containing 1% (w/v) skim milk and were then incubated with sera from patients with proven PCM, tested by immunodiffusion against *P. brasiliensis* exocellular antigens and also sera from healthy individuals. The secondary antibody was anti-human IgG, coupled to alkaline phosphatase (Sigma Aldrich, Co., St. Louis, MO). The reactions were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT). In some experiments, the chemiluminescent substrate CDP-star (Perkin–Elmer, Norwalk, CT) was used, and bands were visualized by autoradiography.

2.5. Human sera

A total of 18 human serum samples collected at the time of diagnosis from patients with confirmed PCM were pooled and used in the Western blot assays with the purified IEF fractions. Some of the serum samples were further utilized individually in the Western blot assays with the recombinant FMD of *P. brasiliensis*.

2.6. Purification of antigens and sequencing of the purified protein

IEF fractions (9–12) were pooled and concentrated by ultra filtration (Centricon-10, Amicon, Inc., Beverly, MA). The concentrated samples were then separated, based on size, by gel electrophoresis, and the proteins were electrotransferred for 1 h at 400 mA to polyvinylidene difluoride membranes (PVDF; Immobilon-P, Millipore Corp., Bedford, MA). A serum reactive protein, molecular mass of 45 kDa, was manually excised from a single Coomassie-blue-stained gel (calculated as 900 pmol of the target protein) and digested in situ with lysyl endopeptidase (Lys-C). Peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) and subjected to Edman's degradation.

2.7. DNA extraction of P. brasiliensis

P. brasiliensis yeast cells were harvested, washed and frozen in liquid nitrogen. Grinding with a mortar and pestle broke the cells, and the genomic DNA was prepared by the cationic hexadecyl trimethyl ammonium bromide (CTAB) method [27]. The cell powder was suspended in 10 ml of extraction buffer (2% (w/v) polyvinylpolypyrrolidone (PVP), 1.4 M NaCl, 0.1 M Tris–HCl pH 8.0, 0.02 M ethylenediaminetetraacetic acid (EDTA), 2% (w/v) CTAB). The mixture was incubated at 65 °C for 1 h, extracted with 50% chloroform/50% isoamyl alcohol (v/v) and precipitated with 100% ethanol. After treatment with RNase I and ethanol precipitation, the DNA was resuspended in water.

2.8. Generation of DNA probe by PCR

P. brasiliensis genomic DNA was used as a template for the PCR amplification of a partial fragment encoding the FMD. Degenerate oligonucleotide primers were designed based on the amino acid sequences of the internal peptides. The degenerate sense S1 (5′-GARCCNATHAAYGTNC-AYGC-3′) and the antisense At1 (5′-ACNGGNCCNGGR-TGRAADATNGG-3′) primers were used in a PCR reaction that was conducted in a total volume of 50 µl containing 50 ng of DNA as template. The resulting 415-bp product was subcloned into pGEM-T-Easy (Promega, Madison, USA). The sequence was determined on both strands by automated DNA sequencing, applying the DNA sequencing method of Sanger et al. [28].

2.9. Cloning of the cDNA and genomic sequences encoding FMD

A cDNA library from yeast cells has been constructed in *Eco*RI and *Xho*I sites of Lambda ZapII (Stratagene Inc., LaJolla, CA, USA). Approximately 50,000 recombinant phage plaques from the cDNA library were plated, according to standard procedures [29]. Duplicate filters of the plates were hybridized overnight with the 415-bp probe radiolabeled with [α-^{32}P]-dCTP at 45 °C, in solution containing 50% formamide. The filters were extensively washed in 1× standard saline citrate (0.015 M sodium citrate, 0.15 M NaCl), 0.2% (w/v) SDS at 65 °C. Phages showing homology with the probe were plaque purified. The in vivo excision of pBluescript SK$^+$ phagemids in *Escherichia coli* XL1-Blue MRF' was performed by using the exassist/SOLR protocol from Stratagene (Stratagene).

The complete genomic sequence encoding FMD was obtained by PCR amplification of the total DNA of *P. brasiliensis*. Primers were constructed based on the cDNA sequence. A 1622-bp PCR product was obtained by using sense primer S2 (5′-ATGGGTCTCAAGGGAATTC-3′) and antisense, At2 (5′-CATCCCCTACTTCATTC-3′). The PCR reaction was performed with 50 ng of total DNA of *P. brasiliensis*, and the amplification conditions were an initial

denaturation step at 94 °C for 1 min, 30 cycles at 94 °C for 1 min, annealing at 55 °C for 1 min and 45 s, and extension at 72 °C for 2 min. An amplified PCR product of 1622 bp was gel purified, subcloned into pGEM-T-Easy vector (Promega) and sequenced on both strands.

2.10. DNA sequencing and sequence analysis

DNA sequencing was performed by the double-strand dideoxy-chain termination method by using a MegaBACE 1000 sequencer (Amersham Biosciences, Little Chalfont, UK) for automated sequence analysis. Sequence homologies to genes in the GenBank database were determined by using the BLAST algorithm of the National Center for Biotechnology Information at the National Library of Medicine (http://www.ncbi.nlm.nih.gov) [30]. The PROSITE (http://us.expasy.org/prosite) [31] and Pfam (http://www.sanger.ac.uk/softwer/pfam/index.shtml) [32] databases were used for the analysis of the deduced protein. The antigenic determinants of the deduced protein were predicted by using the algorithm described by Kolaskar and Tongaonkar [33] at (http://www.bioweb.pasteur.fr/seganal/interfaces/antigenic.html).

2.11. Southern blot analysis

Twenty micrograms of total DNA was digested with the restriction enzymes *Dra*I, *Xba*I and *Hind*III. The DNA was electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane (Amersham Biosciences). The blot was probed for the 1248-bp nucleotide sequence, including the complete *Pbfmd* ORF, labeled with [α-^{32}P] dCTP, by using the random primers DNA labeling Kit RPN1604 (Amersham Biosciences).

2.12. RNA isolation and Northern blot analysis

Total RNA was obtained from mycelium and yeast cells grown in vitro at 22 and 36 °C, respectively, by using the Trizol method (GIBCO™, Invitrogen, Carlsbad, CA). Northern hybridization was performed with 10 µg of total RNA fractionated on a 1.2% (w/v) agarose–formaldehyde gel. The RNA was transferred to a nylon membrane. The cDNA-labeled probe was hybridized to the blot under high-stringency conditions.

2.13. Expression of the recombinant FMD by E. coli and purification of the recombinant protein

Oligonucleotide primers were designed to amplify the 1248-bp DNA containing the complete coding region of FMD. The nucleotide sequences of the sense (S2) and antisense (At2) primers were (5′-TCCT$\underline{\text{GTCGAC}}$ATGGGTCTCAAGGGAATTC-3′) and (5′-CCC$\underline{\text{GCGGCCGC}}$ATCCCCTACTTCATTC-3′), which contained engineered *Sal*I and *Not*I restriction sites (underlined), respectively. Fifty micrograms of the cDNA (*Pbfmd*) was used as template. The amplification parameters were as follows: an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and 45 s, and extension at 72 °C for 2 min. The PCR product was digested with *Sal*I and *Not*I, separated by agarose gel electrophoresis, gel excised and subcloned into the *Sal*I/*Not*I sites of pGEX-4T-3 (Amersham Biosciences). The recombinant plasmid was used to transform the *E. coli* XL1 Blue MRF' competent cells using the heat shock method [29]. Complete sequencing of the DNA was performed to confirm that it had been cloned into pGEX-4T-3 and could produce an in-frame molecule fused to glutathione S-transferase (GST).

2.14. Bacterial cell growth and induction conditions

All the cultures were grown in 1-liter batches of Luria–Bertani (LB) broth containing 100 µg/ml of ampicillin for plasmid maintenance. The cultures were incubated at 37 °C until the A_{600} reached between 0.7 and 0.8, at which point isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The cells were incubated at 30 °C for 3 h and then harvested by centrifugation at 5000 × *g* for 10 min. Preparation and purification of the protein was performed at 4 °C. The crude recombinant protein was obtained by sonicating the bacterial pellet in 20 mM Tris–HCl buffer (pH 7.6) containing 200 mM NaCl and 5 mM EDTA, followed by centrifugation at 12,000 × *g* for 10 min. Protein concentrations were determined [25]. The recombinant FMD was expressed in the soluble form by the bacteria, and the protein was purified by affinity chromatography under nondenaturing conditions, as previously reported [14]. The soluble fraction was applied to a Glutathione Sepharose™ 4B Resin column (Amersham Biosciences) (bed volume, 1.0 ml). After the resin was washed three times with PBS 1× (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), the protein was cleaved with thrombin protease (50 U/ml) and collected after overnight incubation. The purity and size of the recombinant protein were evaluated by running the molecule on a 12% SDS-PAGE followed by Coomassie blue staining.

2.15. Measurement of FMD activity

FMD activity was measured by monitoring the appearance of the ammonia, as described [34]. Samples of 50 µl (0.48–0.7 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 FMD activity was defined as the amount of enzyme required to hydrolyze 1 μmol of formamide (corresponding to the formation of 1 μmol of ammonia) per min per mg of total protein. Protein concentration was determined [25].

2.16. Nucleotide sequence accession numbers

The *P. brasiliensis* nucleotide sequences reported have been submitted to the GenBank database under accession numbers AY163575 (cDNA) and AY600303 (genomic).

3. Results

3.1. Separation of P. brasiliensis antigenic proteins by IEF and identification/characterization of the 45-kDa antigen

The *P. brasiliensis* yeast extracts were separated into 15 fractions with the Rotofor liquid IEF system. Four fractions were pooled (pH from 4.0 to 7.0) and further fractionated into 15 samples, as shown in Fig. 1A. Fractions 9–12 contained the most detectable serum reactive protein with apparent molecular mass of 45 kDa. SDS-PAGE and Western blotting analysis of the individual fractions are depicted in Fig. 1B, C, respectively. Based on the pH of the fractions 9–12 obtained with the Rotofor system, the 45-kDa protein appeared to have an isoelectric point (p*I*) of approximately 6.0. After the last step of IEF separation, the samples (9–12) were subjected to preparative electrophoresis, and the proteins were transferred to PVDF membranes for further sequencing of their amino acid residues.

Four endoproteinase Lys-C-digested peptides from the 45-kDa antigen were selected for sequencing by Edman degradation (Fig. 1D). They were PASEQKGLHNRWHPDIPPCASIK, VVAKPPEPINVHAGSASDAIK, ARTIPGRPEHGGNCD and KSPIFHPGPVEPQFSPGRYLTFEGFSVD. Comparative analysis was performed with the four peptides and sequences at the database exhibited homology (identity values of 100–66%) to FMDs from several fungi.

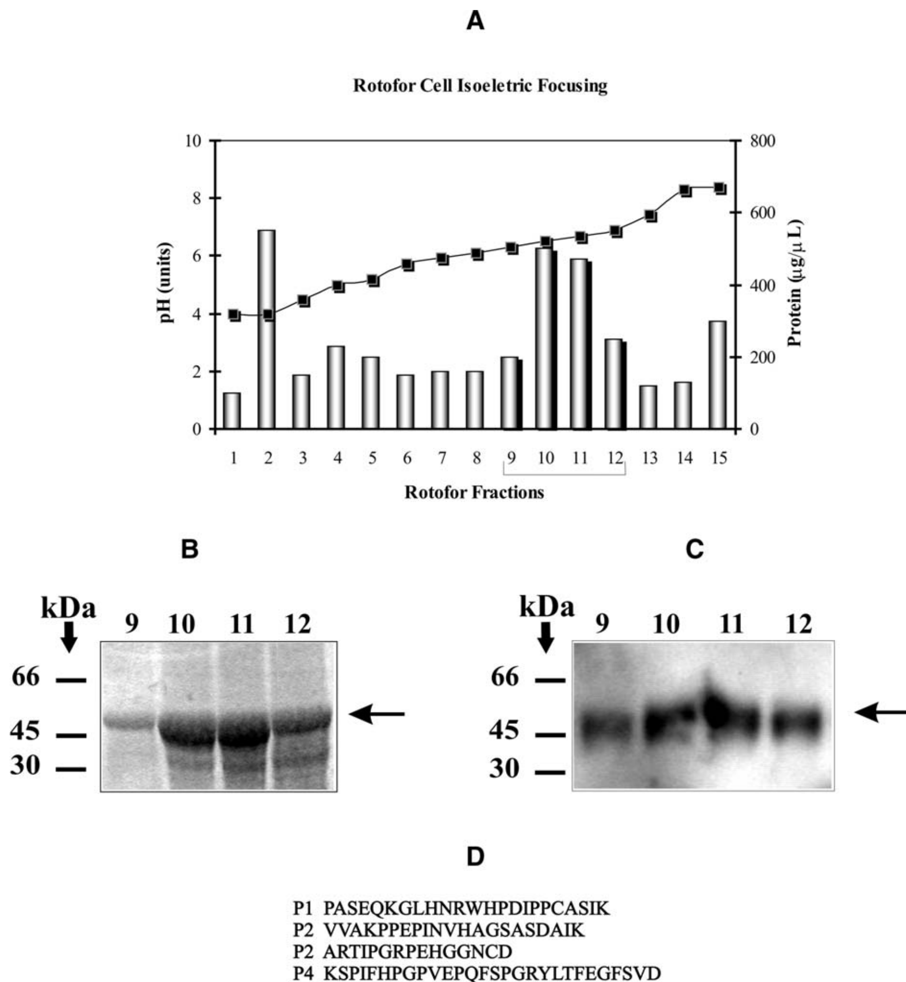

Fig. 1. Identification and characterization of the *P. brasiliensis* FMD. A- Separation of *P. brasiliensis* proteins by liquid IEF. Bars indicate protein concentration (in micrograms per microliter); black squares represent pH units. B- SDS-PAGE of four fractions (9–12). C- Western blot analysis of fractions 9–12 by using sera of patients with proven PCM. The reaction was developed by using the chemiluminescent substrate CDP-star. D- Characterization of the 45-kDa antigenic protein. Sequence of the amino acids obtained from 04 endoproteinase Lys-C-digested peptides (P1–P4) of the native FMD.

3.2. Identification of the cDNA and the gene encoding FMD

In order to isolate the complete gene and cDNA encoding the 45-kDa FMD of *P. brasiliensis*, we obtained a 415-bp PCR product using the primers S1 and AT1 (Fig. 2). This PCR product manifested identity with sequences of FMDs present in the database. The entire cDNA encoding FMD (*Pbfmd*) was obtained by screening the cDNA library of the yeast phase of *P. brasiliensis*. The cDNA sequence of 1711 bp contained an open reading frame of 1248 bp, and the deduced amino acid sequence had 415 residues with a predicted molecular mass and p*I* of 45.6 and 6.3, respectively.

The complete genomic sequence was obtained by PCR by using the S2 and At2 oligonucleotide primers and was compared to the cDNA (Fig. 2). The cloned genomic sequence exhibited five exons interrupted by four introns of 93, 103, 92, and 86 bp. The boundaries of introns/exons conformed to the basic consensus GT/AG for eukaryotic splice donor and acceptor sites [35]. The putative splice box matches the filamentous fungi consensus sequence (NNCTPuAPy), located at the 3′ terminus of the introns [36].

3.3. Characterization of the deduced amino acid sequence and comparative analysis with sequences present at databases

A FASTA search of the GenBank database revealed that the deduced amino acid sequence of the *Pbfmd* gene product displayed identity to FMDs of both prokaryotic and eukaryotic origins. Alignment of the *P. brasiliensis* predicted protein sequence with reported sequences of FMDs performed using the Clustal X program [37] is shown in Fig. 3. This analysis revealed the existence of several conserved residues. In particular, a conserved cysteine at position 229 in *Pb*FMD was identified in all sequences. This region of the gene is essential to the catalytic activity of the enzyme, as described for *H. pylori* amidases. In addition, a conserved aspartate residue at position 230 in *Pb*FMD may also be involved in the enzyme activity, since it is reported to be essential for the conformational stability of the AmiE and AmiF paralogues of *H. pylori* [34].

The sequence alignment (Fig. 3) revealed a high degree of homology. The highest sequence similarity and identity were between the FMD of *P. brasiliensis*, *Emericella nidulans*, *Magnaporthe grisea* and *Podospora anserina* (Table 1) and were most apparent in the region encompassing approximately 350 amino acids of the protein. In addition, a search for antigenic determinants was performed. The search delineated 17 putative antigenic determinants in the FMD of *P. brasiliensis*. Several of the putative B cell epitopes were located in unconserved regions of *Pb*FMD (Fig. 3). Those epitopes were present in hydrophilic regions of the deduced protein. The profile defined on the DAS program [38] yielded an overall hydropathy index of 0.94, indicative of a strongly hydrophilic protein. We observed, on examination of the secondary predicted structure performed by using the PHD program (http://www.predictprotein.org) [39], that approximately 20% of the amino acid residues of the *Pb*FMD were in alpha helix conformation, and these residues were located in the middle region of the primary structure. β Sheets were not observed in this simple prediction (data not shown).

3.4. Hybridization analysis

Southern blot analysis using the complete cDNA *Pbfmd* as a probe detected a single DNA fragment in the *P. brasiliensis* DNA digested with the restriction enzymes *Dra*I, *Xba*I or *Hind*III (Fig. 4A). Northern blot analysis detected the presence of one mRNA species of 1.8 kb. Expression was more pronounced in the mycelial phase than the yeast phase (Fig. 4B). These data indicate the presence of a single *fmd* gene in the *P. brasiliensis* genome.

3.5. Expression and purification of the recombinant PbFMD and Western blotting analysis

The *Pb*FMD protein was produced and purified from crude extracts of *E. coli*. The expression of the pGEX-4T-3-FMD produced about 5 mg of fusion protein in one liter of *E. coli* culture. The predicted molecular size of the recombinant protein was 71 kDa, which included the vector-encoded fusion peptide of 26 kDa at its N-terminus. SDS-PAGE was used to verify the composition of the cell lysates obtained from *E. coli* XL1-Blue, which had been transformed with either the empty vector (lane 2) or the plasmid construct (lane 3), as shown in Fig. 5A. The fusion protein was purified using glutathione-sepharose 4B (lane 4) and was subsequently cleaved by the addition of thrombin protease (lane 5). The purified protein migrated on SDS-PAGE as a single band of around 45 kDa.

Serum samples from *P. brasiliensis*-infected and control individuals were tested in immunoblot assays with the recombinant *Pb*FMD (Fig. 5B). Strong reactivity was observed with sera of patients with PCM (Fig. 5B, lanes 1–4). No cross-reactivity was observed with control sera (Fig. 5B, lanes 5–8).

3.6. FMD activity of P. brasiliensis cells and of the recombinant protein

We investigated the FMD activity using extracts of yeast and mycelium cells, as shown in Table 2. FMD activity, as detected by ammonia production, was higher in mycelium when compared with the yeast extracts. The activity of the recombinant protein was around 2.2-fold that detected for yeast and 1.5-fold that measured in the mycelium extracts.

4. Discussion

A new antigen, identified as a FMD, was purified from the fungus yeast extracts. We cloned the *fmd* cDNA and gene

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-109 gtttttttttttgctggttggctggttatacttgacagttacctcccatatcatattcaaacgcttcggaatagaacta
 -29 ccagcgagcgacaccatatcc tccaatcATGGGTCTCAAGGGAATTC GCACGGCCATGAAGGTCGATCTTCATAAGCCAG
                         S2 --> M  G  L  K  G  I  R  T  A  M  K  V  D  L  H  K  P
  53 CATCGGAGCAGAAGGGCTTGCAT gtatgctgctgatctcatttgaacatgccccagctactgtgctattaagatcacaat
  18 A  S  E  Q  K  G  L  H
 133 gcctggctaaccactcaaaacccctcctgtat ctagAACCGCTGGCATCCAGACA gtacgtaaacgtcaaactgatgctc
  26                                      N  R  W  H  P  D
 213 cggttatcatccataaggattgatgtactctactgtgtatatatatatatatg agctaatagacgtatgagctcagTT
  32                                                                              I
 293 CCACCCTGCGCATCGATCAAGAATG GGGAAGTCGTCAAGATCGAATGCTTGGACTGGACCGGCGGACAGATTAAAAATAA
  33  P  P  C  A  S  I  K  N  G  E  V  V  K  I  E  C  L  D  W  T  G  G  Q  I  K  N  N
 373 CGATTCCGCCGACGACATGCGTGATATCGACTTGACTCGCGTGCATTATCTGACAGGGCCCTTTGAGATCGAAACTGCAG
  60  D  S  A  D  D  M  R  D  I  D  L  T  R  V  H  Y  L  T  G  P  F  E  I  E  T  A
 453 AACCAGGTGATGTGCTACTAGTTGAAATTCAGGACGTCCAGCCATTCCAGGACCAGCCATGGGGATTCACGGGCATCTTT
  86 E  P  G  D  V  L  L  V  E  I  Q  D  V  Q  P  F  Q  D  Q  P  W  G  F  T  G  I  F
 533 GATGCGAAGAATGGAGGCGGGTTTCTGGACGAGTTCTATCCCAATGC gtatgtaattgtgaatgtttcatttgcccccct
 113  D  A  K  N  G  G  G  F  L  D  E  F  Y  P  N  A
 613 ttagttacgtatgcgtttgcaaagcttcagaaacttatgtcaaaacatatgacact tagCGCAAAAGCAATTTGGGATTT
 129                                                        A  K  A  I  W  D  F
 693 TGAAGGCATATTCTGCTCGTCTCGCCACATCCCGCATGTCCGCTTTGCTGGATTGATCCACCCAGGGATCCTGGGTTGTG
 136  E  G  I  F  C  S  S  R  H  I  P  H  V  R  F  A  G  L  I  H  P  G  I  L  G  C
 773 CCCCGTCAGCCGAGGTCCTAGCTGAATGGAACAGGCGCGAAAGTGATCTCCTCTCGGCCAATGCGGGTTCAGTAAAGGTT
 162 A  P  S  A  E  V  L  A  E  W  N  R  R  E  S  D  L  L  S  A  N  A  G  S  V  K  V
 853 GTGGCTAAGCCACCT GAGCCAATCAATGTGCACGC AGGCAGTGCTAGTGATGCCATCAAAGCGAAAGTG GGCAGGGAGGG
                  S1 -->
 189  V  A  K  P  P  E  P  I  N  V  H  A  G  S  A  S  D  A  I  K  A  K  V  G  R  E  G
 933 AGCGAGGACCATTCCT gtaagattacgtctcctttcagcgtgcaaactatcacattaagagcaaccagtgcaaaaaaat t
 216   A  R  T  I  P
1013 tctaaccgccaccaacccacagGGCCGGCCCGAGCACGGTGGCAACTGCGACATCAAAAACCTCAGCAGGGGCAGTAAGG
 221                      G  R  P  E  H  G  G  N  C  D  I  K  N  L  S  R  G  S  K
1093 TGTATCTCCCCGTCCATGTGAAGGGCGCCAAATTCTCCGTGGGCGACCTTCATTTCTCGCAGGGTGACGGCGAAATCTCC
 240 V  Y  L  P  V  H  V  K  G  A  K  F  S  V  G  D  L  H  F  S  Q  G  D  G  E  I  S
1173 TTCTGCGGTGCCATCGAGATGGCTGGCATCATCACCATAAAATTCAACGTCATCAAGAATGGCATGGCGCACATGGGAAT
 267  F  C  G  A  I  E  M  A  G  I  I  T  I  K  F  N  V  I  K  N  G  M  A  H  M  G  M
1253 GAAGTCGCCCATCTTCCACCCGGGACCAGT GGAGCCACAGTTCAGCCCTGGCCGCTACTTGACCTTCGAGGGCTTCAGTG
                          <-- At1
 294  K  S  P  I  F  H  P  G  P  V  E  P  Q  F  S  P  G  R  Y  L  T  F  E  G  F  S
1333 TCGATCACCACGGCAAGCAGCATTTCCTGGATGCCACGGTTGCGTATCGTGAAACTTGCATGCGT GTCATTGAGTACCTG
 320 V  D  H  H  G  K  Q  H  F  L  D  A  T  V  A  Y  R  E  T  C  M  R  V  I  E  Y  L
1413 CGCCGCTATGGCTATGACGATTATCAGATTTATTTGCTGCTGAGTTGTGCGCCTGTGCAAGGGCATATTGCAGGCTTGGT
 347  R  R  Y  G  Y  D  D  Y  Q  I  Y  L  L  L  S  C  A  P  V  Q  G  H  I  A  G  L  V
1493 GGATATTCCAAATGCGTGCACGACACTGGGGGTGCCCATGGATATTTTTGATTTTGATATTCGGCCTGAGTCGAAGGTGG
 374  D  I  P  N  A  C  T  T  L  G  V  P  M  D  I  F  D  F  D  I  R  P  E  S  K  V
1573 TGAAGATGGACATGGGCAGCTGTGCGTATA CAAGTGATAGAATGAAGTAGgggatgttcttctgggtttggggactactt
 400 V  K  M  D  M  G  S  C  A  Y  T  S  D  R  M  K                  <-- At2
1653 gagctgttatctatcggcttaacgttgtatgcgtgtatgtctggatgcatttattc atttattt ggctattcgatgtttg
1733 aaatgctgctgttgaatgattcttgaattgagggagtttgcctcga cgagctttgcacgtatgtatttatttagttgttt
1813 cttttgatgtggttgagggagtaaatattccgtccgccactcatttcggacagagagatttatcttgacacgaattctac
1893 tgacagcagacttttcaactattaaccgacctacatccatttttttcagaaagccaaaaaaaaaaaaaaaaaaaaa
1973 aaaaa
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Fig. 2. Nucleotide sequences of the cDNA, of the gene and the deduced amino acid sequence of *Pbfmd*. Nucleotide and amino acid numbers are shown on the left. The introns are represented in lowercase. Nucleotides in bold italics represent the conserved 5′ and 3′ consensus of the introns. The putative splice boxes of the intron sequences are marked by dashed rectangles. The putative poli-A signal is marked by a double rectangle. Primers S1, S2, At1 and At2 are underlined. Numbering of the amino acid sequence begins at the methionine encoded by the first initiation codon and ends at the first termination codon, both in bold. Amino acids inside gray boxes and in bold represent sequences identified by microsequencing of the endoproteinase Lys-C-digested peptides of the 45-kDa protein purified by liquid IEF.

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P. brasiliensis   -MGLKGIRTAMKVDLHKPASEQKGLHNRWHPDIPPCASIKNGEVVKIECLDWTGGQIKNN    59
E. nidulans       -MGTKAIRTAVKVDLNKPAREQKGLHNRWHPDIPSYGTIANNEVVKIECLDWTGGQIKND    59
P. anserina       -MGKSAIRTVCEVDFDKPASEQPYLHNRWHPDIPPAATIKDGETVKIECLDWT GGQIKND   59
N. crassa         NMGKTEIRNVCKVDFDKPASEQPQLHNRWHPDIPFAGFIKDGETVKIECVDWTGGQIGNN   120
M. grisea         LYIMPEIRKVCKVSLDKPAAEQSALHNRWHPDIPFAGTIKNGETVKIECVDWTGGQIGNN    79
G. zeae           --MSRKIRTAQSVSFDIPAAEQPHLHNRWHP---------EGETVKIECLDWTGGQIGNN    49
S. pombe          ----MAPRTIVKVDLNKPAWEQPNLHNRWHPDIPFAESIDEGETVKIECLDWTGGQIKND    56
C. albicans       -MPTEPIRYVTKVDLTKPGDQQPHLHNRWHPDIPFAETIKPGETVKIECLDWTGNQIKND    59
                         *   .*.:  *. :*  *******          .*.*****:****.** *:

P. brasiliensis   DSADDMRDIDLTRVHYLTGPFEIETAEPGDVLLVEIQDVQPFQDQPWGFTGIFDAKNGGG   119
E. nidulans       DSADDIKNVDLTQIHYLSGPFDIETAEPGDVLLVEIQDVQPFEDQPWGFTGVFARENGGG   119
P. anserina       DSADDIKNVDLTRVHYLSGPFAIENAEPGD ALLVEIMDVQPFEDQPWGFTGIFDKSNGGG  119
N. crassa         DSADDMKNVDLTKIHYLSGPFEVEGAQPGDLLLVEIMDVQPFQDRPWGFTGIFDKANGGG   180
M. grisea         DSADDIRNVDLTKIHYLSGPFEVETAEPGDLLLVEIMDVQPFEDQPWGFTGVFDKSNGGG   139
G. zeae           DSADDVRDVDLTKIHYLTGPFE IKGSEPGDLLVVNITDIQPFDHSPWGFTGVFSKDNGGG  109
S. pombe          DSAEDIKNVDLTRIHYLSGPFEIKGAEPGDVLVVEIQDVQPLENQPWGYSGIFAKENGGG   116
C. albicans       DTADDILNVDLSRIHYLSGPFNIEGAEPGDALVVEIKDVQPLERQPWGYCGIFHKKNGGG   119
                  *:*:*: ::**::: ***:*** :: ::*** *:*:* *:**::  ***: *:*   ****

P. brasiliensis   FLDEFYPNAAKAIWDFEGIFCSSRHIPHVRFAGLIHPGILGCAPSAEVLAEWNRRESDLL   179
E. nidulans       FLDEIYPEPAKAIWDFEGIFCSSRHIPHVRFAGLIHPGILGCAPSAEVLAEWNRREGELI   179
P. anserina       FLDELYPSAAKAIWDFEGIYCTSRHIPHVKFAGLIHPGILGCAPSQEVLDTWNKREAELI   179
N. crassa         FLDEIYPSAAKAIWDFEGIYATSRHIPGVKFAGLIHPGIIGCAPSAEVLATWNKREGELV   240
M. grisea         FLDEIYPSAAKAIWDFEGIYCTSRHIPHVKFAGLIHPGILGCAPSAEVLAEWNKREGDLI   199
G. zeae           FLSEHYPDAAKAIWDFDGVYCSSRHIPGVRFPGLIHPGILGCAPSAEILAEWNRREGELA   169
S. pombe          FLDEHYPKAAKAVFDFEGIFCSSRHIPGVRFPGLIHPGIIGTAPSKEILAEWNRREGALV   176
C. albicans       FLDKFYPEAAKAIFDLEGIHATSRHIPHVKFTGLIHPGIMGTAPSAEVLKEWNSREIGLI   179
                  **.: **..***::*::*:..:***** *:*.*******:* *** *:*  ** **  *

P. brasiliensis   S--ANAGSVKVVAKPPEPINVHAG-SASDAIKAKVGREGARTIPGRPEHGGNCDIKNLSR   236
E. nidulans       A--ANT-LGRDVAKPPEPSNVHAG-SADGELAAKIGREGARTIPGRPEHGGNCDIKNLSR   235
P. anserina       V--ANKL-DRDVALPPQPLNVHAG-SADPNTKEKVGLQGARTIPGRPEHGGNCDIKNLSR   235
N. crassa         A--ANKLADRDVAHLPQPINTHAG-AASKEIAEKVAKEGARTVPGRPEHGGNCDIKNLSR   297
M. grisea         A--ANKL-ERDVAKPPEPVNVHAG-AADASLTARVGKEGARTIPGRPEHGGNCDIKNLSR   255
G. zeae           TSMAGSHPDKVFAQPPNEINAHGG-LAKGDVMARIAKEGARTIPGRPEHGGNCDINNISR   228
S. pombe          A--ENPHSTHVMAQLPNASYAFAGILADEKLSATVASEGARTIPGRPENGGNCDIKNLSR   234
C. albicans       N-DIQHQHETNVANGPLETNSHPG-SATGTLASKIAKEGARTIPGRPENGGNC DIKNLSR  237
                            .*  *     . *  *       :. :****:*****:******:*:**

P. brasiliensis   GSKVYLPVHVKGAKFSVGDLHFSQGDGEISFCGAIEMAGIITIKFNVIKNGMAHMGMKSP   296
E. nidulans       GSKVYLPVHVPGAKFSVGDLHFSQGDGEISFCGAIEMAGVITLK FTVIKDGMAKMAMKSP  295
P. anserina       GSKVYLPVHVPGANFSVGDLHFSQGDGEISFCGAIEMAGIITINFSVIKSGVSQLSLTSP   295
N. crassa         GSKVYLPVHVPGAKFSVGDLHFSQGDGEISFCGAIEMAGVITINFKVIKNGVEGMGLKSP   357
M. grisea         GSKVYLPVHVSGAKFSVGDLHFSQGDGEISFCGAIE MAGVITINFKVIKNGMADLGLKSP  315
G. zeae           GSKTYLPVHVPGAKFSVGDLHFSQGDGEISFCGAIEMAGIITIKFDLIKGGVKSRGLKSP   288
S. pombe          GSKVFLPVHVPGAKLSIGDLHFSQGDGEISFCGAIEMAGSITIKCKILKNGISDLAMKSP   294
C. albicans       GSKCYLPVYVDGAKLSVGDLHFSQGDGE ISFCGAIEMPGVITIKVSIIKGGIKKLSLKSP  297
                  *** :***:* **::*:*******************.* **::  ::*.*:    .:.**

P. brasiliensis   IFHPGPVEPQFSPGRYLTFEGFSVDHHGKQHFLDATVAYRETCMRVIEYLRRYGYDDYQI   356
E. nidulans       IFHPGPVEPQFGPGRYLTF EGFSVDEKGKQHYLDATVAYRQTCLRVIEYLRRYGYNDYQI  355
P. anserina       IYIPGPVEPHFGPGRYIYFEGFSVDQHGKQHYLDVAVAYRQTTLRCIEYLRRFGYSDYQI   355
N. crassa         IYIPGPVEPSFGPGRYIYFEGFSVDEHGKQHYMDVTVAYRQTVLRCIEYLRRFGYSDYQI   417
M. grisea         IYIPGPVEPHF GPGRHIYFEGFSVDQHGKQHYMDVTVAYRQTCLRVIEYLRRFGYSDYQI  375
G. zeae           VYRPGDMGPTFSPSRYLTFEGFSVDEQGKQHFMDATIAYRQSCLRAIEYLKQFGYSGEQI   348
S. pombe          MYLPGPVEPHFSPSRYLTFEGFSVDESGKQHYLCTTTAYRQTCLRVIEYFRRFGYNDYQL   354
C. albicans       IFI PGDVQNHYGPSRYLTFEGFSVDEAGKQQYLCATTAYRQACIRAIEYLRKFGYNDYQI  357
                  :: ** :    :.*.*:: *******. ***::: .: ***:: :* ***::::**.. *:

P. brasiliensis   YLLLSCAPVQGHIAGLVDIPNACTTLGVPMDIFDFDIRPESKVVK ----MDMGSCAYTSDR  412
E. nidulans       YLLLSCAPVQGHIAGLVDIPNACTTLGVPMDIFDFDIRPEADAVK ----LDMGSCAFASK-  411
P. anserina       YLLLSCAPIQGHVAGIVDIPNACTTLGLPMDIFDFDISPSAIPVK ---KLDMGRCAFETGK  412
N. crassa         YLLLSCAPIQGHVAGIVDVPNACTTLGLPMDIFDFDISPAAALKAGETKRDLGTCAFETGV  477
M. grisea         YLLMSCAPIQGHIAGIVDIPNACTTLGLPMDIFDFDISPSAVPAK ---KLDMGTCAFETGV  432
G. zeae           YLLLSCAPIRGAIAGIVDIPNACTTLGIPMDIFDFDISIESEPVV ----RNLGACPISR--  403
S. pombe          YLLLSCAPIQGHVAGIVDIPNSCTTIGVPMDIFEFDVSPNGKAKI ----IDLGSCAFANS-  410
C. albicans       YLLLSSAPIEGHIAGIVDVPNACTTLGIPMDIFDFDISPEGNFEE ----KDMKNCAFVEGA  414
                  ***:*.**:.* :**:**:***:*:*****:**:     .           ::  *.
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Table 1
Summary of calculated values for amino acid similarities and identities between *P. brasiliensis* FMD and FMD sequences of other fungi

Organism	GenBank accession number	Identity (%)	Similarity (%)
E. nidulans	XP_408714	84	91
M. grisea	EAA51938	77	89
P. anserina	CAD60770	75	85
Neurospora crassa	XP_331137	73	85
Schizosaccharomyces pombe	NP_595015	70	83
Gibberella zeae	XP_389218	69	81
Candida albicans	EAL02801	66	79

and determined its entire sequence. This antigen may be useful in detecting PCM.

Amino acid sequence is the most exclusive criterion for the identification of proteins. The isolated protein (molecular mass of 45 kDa) yielded four sequence tags which, when matched against databases, revealed the protein to be a FMD. The cloned cDNA and gene (*Pbfmd*) encoded peptides from the native protein, thus suggesting that the protein we had isolated was indeed the FMD from *P. brasiliensis*. The pattern obtained in the Southern blot analysis suggests the presence of only one *fmd* gene in *P. brasiliensis*. The Northern blot showed a single transcript migrating as an mRNA species of 1.8 kb, reinforcing the presence of only one gene in the fungal genome.

Comparison between *Pb*FMD and other members of the fungal FMDs showed a well-conserved primary structure. However, we found three regions of the deduced *Pb*FMD (amino acid residues 177–193; 204–210; 396–402) that are very divergent in the compared fungi sequences and which had been predicted as B cell epitopes, raising the possibility of exploring those regions to develop specific molecules for diagnostic purposes.

The deduced FMD is a putative cytoplasmic protein, as suggested by computational analysis. However, if FMD induces antibody production in the infected host, it could be a candidate as a diagnostic antigen. Results of theoretical epitope mapping [33] showed that there were 17 presumed regions in the molecule (60.7% of total molecule) that could be antigenic. The recognition of amidases by sera of infected patients had been previously reported. Immunoproteomic studies have shown that sera of infected patients recognize the AmiE of *H. pylori* [40,41].

We observed strong expression of the transcripts of *Pbfmd* in vitro, and this gene is also probably strongly expressed in vivo, since antibodies reacting with *Pb*FMD are present in sera of *P. brasiliensis*-infected patients. Also, the enzymatic assay suggests a high expression of *Pb*FMD, especially in mycelium. Data obtained from the fungus transcriptome confirmed the high expression of FMD in mycelium [24]. The role of the protein in vivo remains unclear. Due to its high expression in *P. brasiliensis*, *Pbfmd* is likely to be involved in the nitrogen metabolism of this fungus. A major physiological role of FMD could be the hydrolysis of formamide with the production of ammonia, which can be used as source of nitrogen, and formate. This last product can be readily oxidized to carbon dioxide by the action of an NAD^+ linked formate dehydrogenase, resulting in NADH, which can be available for oxidation by the respiratory chain, as described in the *P. brasiliensis* transcriptome [24]. The source of formamide, especially in mycelium, which expresses high levels of the transcript, remains to be determined. It could be a by-product in the decay of environmental organic matter where the saprobic phase is found. Also, the presence of FMD could account for the production of ammonia that could induce tissue damage by yeasts during colonization. Reinforcing this hypothesis is the finding that several fungi utilize formamide as a nitrogen source [42].

It is likely that *Pb*FMD is not the only enzyme capable of hydrolyzing formamide in *P. brasiliensis*. Indeed, analysis of the yeast and mycelium transcriptomes of *P. brasiliensis* (http://www.biomol.unb.br/Pb) identified some ESTs encoding acetamidase and amidase that could degrade formamide and could account for a small fraction of the FMD activity in the mycelium and yeast cellular extracts. It has been observed, however, that most of the purified acetamidase of bacteria has well-defined substrate specificity, since they present much higher activities with short-chain aliphatic amides than with formamide [43,44]. In addition, most of the amidases exhibit poor reactivity with formamide as substrate [45,46]. The high expression of the *Pbfmd* 1.8-kb transcript as well as the high level of enzymatic activity observed in the cellular extracts suggests an active role for the enzyme in the *P. brasiliensis* metabolism.

Data described herein report for the first time the purification and characterization of a catalytically active FMD antigen of *P. brasiliensis*. It is also the first description of the overexpression of this recombinant molecule in a heterologous system and the analysis of its immunological reactivity

Fig. 3. Comparison of the deduced amino acid sequence of *Pb*FMD with those of FMDs from eukaryotes. Asterisks indicate conserved amino acid residues. Double and single dots denote a decreasing order of matching similarity between each corresponding amino acid pair. The residues of the four peptides of the native FMD are in bold and marked in gray. The cysteine residue putatively related to the enzyme catalytic activity, as well as the aspartate residue described as essential to the conformational stability, are marked with arrows. Brackets indicate predicted antigenic determinants. Accession numbers were as follows: *E. nidulans* - XP_408714; *P. anserina* - CAD60770; *N. crassa* - XP_331137; *M. grisea* - EAA51938; *G. zeae* - XP_389218; *S. pombe* - NP_595015; *C. albicans* - EAL02801.

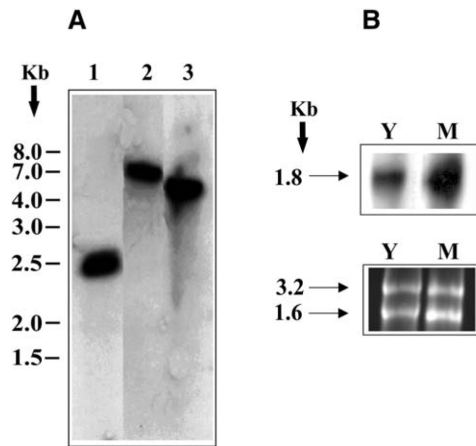

Fig. 4. Analysis of the *P. brasiliensis fmd* gene organization and of the gene transcript. A- Southern blot analysis for determination of the copy number of *P. brasiliensis fmd* gene. Twenty micrograms of total DNA was digested with restriction enzymes *Dra*I, *Xba*I and *Hind*III (lanes 1–3, respectively). The blot was hybridized to the radiolabeled cDNA probe. The position of the size markers is indicated. B- Northern blot of total RNA from yeast (Y) and mycelium (M) cells. Total RNA from yeast and mycelium was fractionated on a 1.2% formaldehyde agarose gel and hybridized to the cDNA insert encoding FMD. The RNA size was calculated by using the 0.24–9.5-kb marker RNA ladder (Invitrogen™ Life Technologies). The amount of loaded RNA was standardized by ethidium bromide staining of the agarose gels, showing the major and minor ribosomal RNAs of about 3.2 and 1.6 kb, respectively.

with sera of infected patients. The usefulness of the recombinant *Pb*FMD in future diagnosis of PCM is promising, since

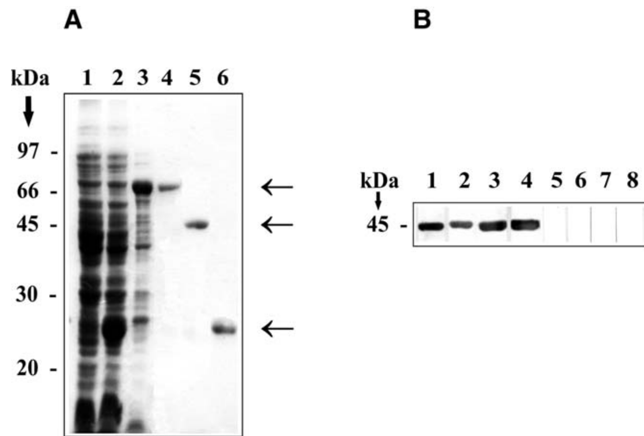

Fig. 5. A- SDS-PAGE analysis of the 45-kDa recombinant protein expressed by *E. coli* transformed with the *Pb*FMD construct. *E. coli* XL1-Blue cells harboring the PGEX-4T-3-FMD plasmid were grown at 30 °C to an A_{260} of 0.8 and subsequently incubated with 0.1 mM IPTG. The cells were lysed by extensive sonication. Lane 1- XL1-Blue; Lane 2 - XL1-Blue transformed with PGEX-4T-3 plasmid; Lane 3- XL1-Blue transformed with PGEX-4T-3-FMD construct (3 h with IPTG); Lane 4- The affinity-isolated recombinant GST-FMD; Lane 5- The recombinant fusion protein cleaved by thrombin; Lane 6- GST protein after purification of recombinant FMD. Electrophoresis was carried out on a 12% SDS-PAGE and the proteins were stained by Coomassie blue R-250. Molecular markers are indicated (Amersham Biosciences). B- Immunblotting of the recombinant protein (1 µg of the purified FMD) was reacted with sera of PCM patients, 1:100 diluted (lanes 1–4) and to control sera, 1:100 diluted (lanes 5–8). After reaction to the anti-human IgG alkaline phosphatase coupled antibody (diluted 1:1000), the reaction was developed with BCIP/NBT.

Table 2
FMD activity of protein extracts of yeast, mycelium and of the recombinant protein of *P. brasiliensis*

Protein source	Specific activity [a]
Yeast	1.24 ± 0.0586
Mycelium	1.79 ± 0.0896
Purified recombinant FMD (*Pb*FMD)	2.73 ± 0.0058

[a] Specific activity was calculated as described in Section 2. FMD activity was measured at 37 °C with formamide as substrate. Activities are means of three independent determinations.

the recombinant molecule was able to react to antibodies present in the sera of patients with PCM, and not in sera of control individuals. Testing the usefulness of the FMD as a tool for serodiagnosis will require studies with a larger number of patients and comparison with existing antigens.

Upon induction with IPTG, large amounts of a 45-kDa protein, corresponding in molecular mass to purified native FMD and also to the deduced *Pb*FMD, were produced. The recombinant protein displayed the ability to convert formamide into ammonia. Availability of large quantities of the purified recombinant protein can facilitate detailed structural analysis of the enzyme and its kinetics parameters. Future work will focus on those subjects.

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Characterization of the formamidase of *Paracoccidioides brasiliensis*: protein analysis, localization and intermolecular interactions.

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Summary

Paracoccidioides brasiliensis is a fungal human pathogen with a wide distribution in Latin America. It causes paracoccidioidomycosis, the most widespread systemic mycosis in Latin America. Formamidases (E.C. 3.5.1.49) are enzymes that hydrolyze formamide to produce formic acid and ammonia. The gene encoding formamidase in *P. brasiliensis* was previously characterized. The deduced protein presents 415 amino acids with a calculated molecular weight of 45-kDa. Also the purified molecule presented a molecular mass of 45-kDa. In this work, we identified a 180-kDa protein, reactive with the antibody produced in mice against the *P. brasiliensis* recombinant purified formamidase. The heat-denatured species of 180-kDa rendered a species of 45-kDa recognized by the polyclonal antibody anti-formamidase, indicating that the fungal formamidase has most likely a homotetrameric strucutre. The 180-kDa protein was identified as formamidase by peptide mass fingerprinting using mass spectrometry. The cellular localization of formamidase was analyzed in *P. brasiliensis* yeast cells and the protein was detected in equivalent amounts in the cytoplasm and in the fungal cell wall. The search for protein-protein interactions performed using yeast two-hybrid system demonstrated cytosolic and cell-wall membrane proteins as interacting molecules with the fungal formamidase.

Keywords: *Paracoccidioides brasiliensis*, formamidase, native protein, mass spectrometry, cellular localization, inter molecular interactions.

1. Introduction

Paracoccidioides brasiliensis, a dimorphic fungus of the phylum Ascomycota, is a major human pathogen with a broad distribution in Latin America (Restrepo *et al*., 2003). *P. brasiliensis* grows as a saprophytic mould in the environment, but undergoes phase transition to a yeast form

at the mammalian physiological temperature. The fungus which is the etiologic agent of paracoccidioidomycosis (PCM) is primarily a respiratory pathogen, infecting the host through the inhalation of airborne propagules from the mycelia phase. In the pulmonary alveolar epithelium, through differentiation, the mycelia produce the parasitic yeast form that can spread to multiple organs and tissues (Franco, 1987; Restrepo *et al*., 2003).

In a previous work we identified by liquid isoeletric focusing and sodium dodecyl sulfate electrophoresis (SDS-PAGE) a protein species with 45-kDa that was characterized by amino acid sequencing of endoproteinase Lys-C digested peptides as a formamidase of *P. brasiliensis.* We also demonstrated the presence of antibodies reacting with the formamidase in sera of *P. brasiliensis* infected patients. Additionally, were demonstrated the enzymatic activity of cellular extracts from yeast and mycelium towards the substrate formamide, as well towards the recombinant formamidase, both displaying the ability of converting formamide into ammonia (Borges *et al*., 2005).

The *P. brasiliensis* nitrogen metabolism presents the particularity of having a complete urea cycle, including an arginase catalyzing conversion of arginine to urea plus ornithine (Arraes *et al*., 2005). Additionally, the fungus overexpresses the gene encoding for formamidase, with higher amounts of transcripts and the cognate protein in the mycelia phase (Felipe *et al*., 2003; Borges *et al*., 2005). Formamide aminohydrolase (FMD, EC 3.5.1.49) catalyzes the highly specific hydrolysis of formamide to produce ammonia and formate (Skouloubris *et al*., 1997). Our previous data indicated that the *P. brasiliensis* formamidase presents high and specific affinity to formamide. These observations indicate that the *P. brasiliensis* formamidase play a role in the nitrogen metabolic pathway that may operate in this organism. Corroborating this suggestion, some species of microorganisms are able to use formamidase as a source of nitrogen for growth. In this way, the fungus *Aspergillus nidulans* possess a formamidase gene (*fmd*S), acting in nitrogen metabolism and utilizing formamide as a sole nitrogen source. The *fmd*S gene is regulated by endogenous nitrogen metabolite repression, suggesting that nitrogen starvation can promote the *fmd*S expression and that formamidase of *A. nidulans* act in nitrogen metabolism (Fraser *et al*., 2001).

The role of formamidase in *P. brasiliensis* biology is yet unknown. The protein was found to be highly expressed in *P. brasiliensis* mycelium (Borges *et al*., 2005), and analysis revealed that the cognate transcript represented a high proportion of the total sequenced ESTs of *P. brasiliensis*, isolate *Pb*01 (Felipe *et al*., 2003). Also the formamidase of *P. brasiliensis* reacts with immune sera from patients with PCM providing an association of the protein with fungal pathogenesis (Borges *et al*., 2005).

In this study we set out to perform the characterization of a 180-kDa protein present in fungal cells and reactive with a polyclonal antibody produced against the recombinant formamidase, overproduced in a heterologous system. The native formamidase was purified in two steps of column chromatography and the protein was identified by mass spectrometry of the tryptic digested products. We also determined the cellular localization of the native protein in fungal yeast cells. The fungal formamidase was located in the cytoplasm and cell wall. The function of most proteins is dependent on their interaction with other molecules, including other proteins. In this way, we performed the screening of interaction of formamidase with *P. brasiliensis* proteins using the yeast two-hybrid system and co-immunoprecipitation assays. The identified interacting proteins may be highlighting the uncommon localization in the fungus cell wall, as well as the predicted function of the *P. brasiliensis* formamidase.

2. Material and methods

2.1. Maintenance of *P. brasiliensis*

P. brasiliensis isolate 01 (ATCC MYA-826) was used and previously described (Barbosa *et al*., 2006). The yeast phase was grown at 36 ºC, in Fava-Netto´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; 4% (w/v) glucose, 1% (w/v) agar, pH 7.2] for 7 days.

2.2. Preparation of mycelia and yeast cells protein extracts

Yeast and mycelium protein crude extracts were obtained as described (Borges *et al*., 2005). For the preparation of total cell homogenate, mycelium and yeast cells were frozen and exhaustively ground with mortar and pestle in the presence of protease inhibitors: 50µg/mL *N*-α-ρ-tosyl-L-lysine chloromethylketone (TLCK); 1 mM 4-chloromercuribenzoic acid (PCMB); 20 mM leupeptin; 20 mM phenylmethylsulfonyl fluoride (PSMF) and 5 mM iodoacetamide in homogenization buffer (20mM Tris-HCl, pH 8.8; 2mM $CaCl_2$). The mixture was centrifuged at 12000 x *g* at 4 °C for 10 min, and the supernatant was used. The protein content of samples was determined by the Bradford method (Bradford, 1976).

2.3. Heterologous protein expression and generation of polyclonal antibody

The production and purification of the recombinant formamidase was performed as described (Borges *et al*., 2005). In brief, the cDNA encoding formamidase (GenBank accession number AY63575) was cloned into the *Sal*I/*Not*I restriction sites of pGEX-4T-3 (GE Healthcare ®,

Chalfont St Giles, UK). The recombinant protein was expressed in the soluble form by the bacteria and was purified by affinity chromatography under non-denaturing conditions. The soluble fraction was applied to a Gluthatione Sepharose^{TM} 4B resin column (GE Healthcare ®). The resin was washed three times in PBS 1X (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and the recombinant protein was cleaved by addition of thrombin protease (50 U/mL). The purity and size of the recombinant protein was evaluated by running the molecule on a 12% SDS-PAGE followed by Coomassie blue staining.

The recombinant formamidase was used to generate specific mice polyclonal antibody. The purified protein (300μg) was injected into mice with Freud's adjuvant, three times at 2 weeks intervals. The obtained serum, containing specific anti-formamidase polyclonal antibody, was sampled and stored at –20 °C. The mice were also bled before immunization to obtain pre-immune serum.

2.4. Protein fractionation by electrophoresis and western blot analysis

Total protein extract of *P. brasiliensis* yeast cells was submitted to isoelectric focusing, as described (O'Farrell, 1975). Gels were loaded with the proteins in sample buffer, containing 9.5 M urea, 1.6% (v/v) ampholines 5.0–8.0, 0.4% (v/v) ampholines 3.5–10.0, 2% (v/v) non-ionic detergent Nonidet P-40, 5.0% (v/v) β-mercaptoethanol. The strips were treated for 30 min with equilibration buffer [0.08M Tris–HCl pH 6.0, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) glycerol, 0.01% (w/v) bromophenol blue]. The second dimension was performed on a 5–15% gradient polyacrylamide gel, as described by Laemmli (1970). Immunoblot reactions with sera from immunized or control mice were carried out for 2 h at room temperature under shaking. The membranes were washed three times with 0.1% (vol/vol) Tween 20 in PBS and subsequently incubated for 1 h at room temperature with goat anti-mice IgG coupled to alkaline phosphatase (Sigma Aldrich, Co., St. Louis, MO). The reactions were developed with 5-bromo-4-chloro-3-indolylphosphate/ nitrobluetetrazolium (BCIP/NBT).

2.5. Measurement of the formamidase activity

The formamidase activity was measured by monitoring the appearance of ammonia, as described (Skouloubris *et al.*, 1997). Protein samples (500 ng), were added to 200 μL of formamide substrate solution at a final concentration of 100 mM in 100mM phosphate buffer pH 7.4 and 10 mM EDTA. The reaction mixture was incubated at 37 ^{o}C for 30 min. Subsequently it was added 400 μL of phenol-nitroprusside and 400 μL of alkaline hypochlorite solution (Sigma Aldrich,

Co.). The samples were incubated at 50 ^oC for 6 min and absorbance was read at 625 nm. The amount of released ammonia was determined from a standard curve. One unit (U) of formamidase was defined as the amount of enzyme required to hydrolyze 1 μmol of formamide per min per mg of total protein.

2.6. Purification of the native formamidase

Total protein extract of *P. brasiliensis* yeast cells was obtained as described above. The extract was ressuspended in buffer 25 mM Tris-HCl, pH 7.5, and applied onto a 10 mL DEAE Sepharose column (GE Healthcare®) previously equilibrated with the same buffer. The column was washed, and the proteins were eluted with a linear gradient performed for 30 min in the same buffer from 0.1 to 1 M NaCl and then with 1.0 M NaCl, for 10 min, at 0.5-mL/min flow rate. Fractions (2 ml) were collected and aliquots were tested for formamidase activity, as described above. The enzymatically active fractions, were applied onto a 10 mL Phenyl Sepharose column (GE Healthcare®), previously equilibrated with buffer 50 mM Na_2HPO_4, 0.5 M $(NH_4)_2SO_4$, pH 7.0. Protein flowing through the column was collected and assayed for formamidase activity. Fractions containing activity were pooled and stored for further analysis.

2.7. Proteolysis and mass spectrometry

Two protein bands separated by SDS-PAGE were excised from the gel and soaked in 50 μL acetonitrile (ACN). The solvent was dried under vacuum using a Speed Vac evaporator (Savant, Farmingdale, NY, USA), and incubated in 50 μL buffer containing10mM DTT in 100mM NH_4HCO_3 for 1h at 56 ºC under agitation. The DTT solution was removed and replaced by 55 mM iodoacetamide in 100mM NH_4HCO_3 for 45 min at room temperature in the dark. The gel pieces were then subjected to alternate 5 min washing cycles with NH_4HCO_3 and ACN, dried down, reswollen in 50 μL of 50 mM NH_4CO_3 containing 12.5 ng/mL sequencing grade modified porcine trypsin (Promega, Madison, WI, USA), and incubated at 37ºC overnight. The resulting tryptic peptides were extracted with 20 μL 5% (v/v) acetic acid, followed by a second extraction with 20 μL 5% (v/v) acetic acid in 50 % (v/v) in a sonicator for 20 min each. The extracts were pooled, dried under vacuum, and then solubilized in 15 μL 0.1% TFA for MS analysis. The tryptic digest sample was analyzed by using a MALDI-TOF mass spectrometer (Reflex IV, Bruker Daltonics, Karlsruhe, Germany). The obtained peptide masses list was used for protein identification through MASCOT software (http://www.matrixscience.com) search against the SwissProt database (http://expasy.org/sprot).

2.8. Immunocytochemistry of formamidase

For ultrastructural immunocytochemistry studies, yeast cells were fixed in a mixture containing 4% (wt/vol) paraformaldehyde, 0.5% (vol/vol) glutaraldehyde, and 0.2% (wt/vol) picric acid in 0.1 M sodium cacodylate buffer at pH 7.2 for 24 h at 4 °C. The cells were rinsed several times using the same buffer, and free aldehyde groups were quenched with 50 mM ammonium chloride for 1 h, followed by block staining in solution containing 2% (wt/vol) uranyl acetate in 15% (vol/vol) acetone for 2 h at 4 °C (Berryman and Rodewald, 1990). Dehydratation was performed in a series of ascending concentrations of acetone (30 to 100%) (vol/vol) and subsequently the material was embedded in LR Gold resin (Electron Microscopy Sciences, Washington, Pa.). The ultrathin sections were collected on nickel grids, preincubated in 10 mM PBS containing 1.5% (wt/vol) bovine serum albumin (BSA) and 0.05% (vol/vol) Tween 20, (PBS-BSA-T), and subsequently incubated for 1 h with the polyclonal antibody to the recombinant formamidase (diluted 1:100). Cells were washed with PBS-BSA-T, and incubated for 1 h at room temperature with the labeled secondary antibody mouse IgG, Au conjugated (10-nm average particle size; 1:20 dilution; Electron Microscopy Sciences, Washington, USA). Subsequently, the grids were washed with PBS-BSA-T, washed with distilled water, stained with 3% (wt/vol) uranyl acetate, and lead citrate, and imaged with a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan). Controls were incubated with mouse pre-immune serum at 1:100, followed by incubation with the labeled secondary antibody. The gold particles were quantified in three independent preparations of yeast cells. The particles were counted in total cell distribution, as well as in the cytoplasm and cell wall, as previously described (Barbosa *et al*., 2006). Results were expressed as the number of gold particles, represented as the means of the counts performed three times with standard deviations included.

2.9. cDNA library construction and two -hybrid assays

A cDNA library was obtained by using RNA extracted from *P. brasiliensis* yeast cells. The cDNAs were synthesized by using the SMART PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA) and were cloned into the prey vector pGADT7 in order to perform yeast two-hybrid screens using the Matchmaker Two-Hybrid System 3 (Clontech). To identify potential protein-protein interactions with formamidase, the cDNA encoding formamidase (Borges *et al*., 2005) was sub cloned into the bait vector pGBKT7. Briefly, the generation of transformants was obtained by introducing bait vector into *Saccharomyces cerevisiae* yeast strain Y187 (*MATα, trp1-901*), a tryptophan depleted strain and the prey vector into the *S. cerevisiae* strain AH109 (*MATa, leu2-3*), a leucine depleted strain. Experimental

procedures were conducted according to the Matchmaker GAL4 Two-Hybrid System 3 manual and the Yeast Protocol Handbook (Clontech). After cells mating, it was performed the selection of the *S. cerevisiae* diploids, which contains two vectors, through selection into plates with minimal media SD/–Leu/–Trp. To exclude false positive clones, the colonies were replicated to high stringency plates with minimal media SD-Ade /–His /–Leu/–Trp. The screening of positive clones included the presence of 5-Bromo-4-Chloro-3-indolyl-α-D-galactopyranoside as the substrate that employs blue/white screening directly on the plate, since adenine and histidine are reporter genes which are expressed together with *lacZ* (α-galactosidase reporter gene). The positive clones were used to PCR colony assay using AD-LD 5' and AD-LD 3' supplied oligonucleotides for pGADT7-Rec bait plasmid. The PCR products of the identified transformants were submitted to DNA sequencing performed by the double-strand dideoxy-chain termination method by using a MegaBACE 1000 sequencer (GE Healthcare ®) for automated sequence analysis. Search for sequence homologies to genes in the GenBank database were performed by using the BLAST algorithm (http://www.ncbi.nlm.nih.gov) as described by Altschul *et al* (1990).

2.10. *In vitro* translation and co-immunoprecipitation assays

The cDNA encoding formamidase and the indentified cDNAs that potentially interact with formamidase were synthesized by using the TNT® Coupled Reticulocyte Lysate System (Promega, Madison, USA). The PCR products of selected colonies were used as template for the *in vitro* transcription/translation. The proteins were *in vitro* synthesized and labeled with ^{35}S-methionine (Perkin-Elmer, Wellesley, MA), using rabbit reticulocyte lysate. The reaction was incubated at 30 °C for 2 h, and 2.5 μL of the translated samples were loaded onto a SDS-gel for the analysis of the translated products.

For the co-immunoprecipitation experiments, the translated formamidase fused to c-myc epitope (c-myc-FMD) and the translated proteins fused to hemaglutinin epitope (HA-Prey) were mixed at 25 °C for 1 h. The mixture was incubated with protein A Agarose beads and with the monoclonal c-myc antibody in PBS 1X at 25 °C for 1 h. After washing, the beads containing proteins were resuspended in SDS-loading buffer [50 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol, 2% (w/v) SDS; 0.1% (w/v) bromophenol blue; 10% (v/v) glycerol], followed by boiling at 80 °C for 5 min. The proteins were separated on a SDS-PAGE 4–12 % linear gradient. The gel was fixed with 20% (v/v) ethanol and 10 % (v/v) acetic acid for 30 min, and incubated in 20 mL of fluorographic reagent NAMP 100 (Amplify Fluorographic Reagent - GE Healthcare ®). The gels were dried at 80 °C for 90 min under vacuum and autoradiography was obtained.

Controls were performed. Each assay was repeated three times with a different batch of *in vitro* translated product to confirm the results.

3. Results

3.1. Mice immunization and western blotting

Mice immunization was performed with the purified recombinant formamidase. Total protein extracts of *P. brasiliensis* yeast cells and mycelium, total protein extract obtained from *E. coli* cells and the purified recombinant formamidase, were electrophoresed, blotted onto membranes and reacted with the polyclonal antibody (Figure 1A, lanes 1-4). A single protein species was detected in the total protein extract of yeast cells (Figure 1A, lane 1) and mycelium (Figure 1A, lane 2). Also, the recombinant purified formamidase was recognized as a single protein species by the polyclonal antibody (Figure 1A, lane 4). No reaction was observed with total protein extracts from *E. coli* (Figure 1A, lane 3). No cross-reactivity was detected when the same samples were incubated with the mouse pre-immune serum (Figure 1A, lanes 5-8). A single protein species was detected in two-dimensional western blot assays, with molecular mass of 45-kDa, p*I* of 6.3 (Figure 1B, lane 1) and no reaction was detected in sample incubated with the pre-immune serum (Figure 1B, lane 2).

3.2. Purification of the native formamidase of *P. brasiliensis*

Protein fractionation was performed of yeast cells protein extracts non-denaturated by heat, depicting a 180-kDa protein species, as shown in Figure 2A. The protein species of 180-kDa was detected in western blot assay performed with the polyclonal antibody anti-formamidase, reacted with the total yeast cells protein extract non-denaturated by heat (Figure 2B, lane1). A protein species of 45-kDa was detected in the protein extract denaturated by heat, suggesting that the 180-kDa protein could represent a tetramer of units of the 45-kDa formamidase.

In order to purify the high molecular weight protein of 180-kDa, reactive to the anti-formamidase polyclonal antibody, we performed two steps of purification, subjecting the total protein extract of yeast cells to a combination of ion exchange and hydrophobic interaction chromatography (Figure 3A; Table 1). Each step of chromatography was assayed for enzymatic activity to formamidase (Figure 3A). By use of formamide as a substrate, the formamidase activity consistently eluted as a single peak in the two chromatographic steps. By this procedure, the formamidase could be purified approximately 1.7 fold, with a 65 % yield (Table 1) which yielded a homogenous preparation, as concluded by a single protein species on SDS-PAGE

(Figure 3B and 3C). The Phenyl Sepharose rescued fraction was loaded onto a SDS-PAGE gel (Figure 3B). A protein species of 180-kDa was evidenced in the heat non-denaturated (Figure 3B, lane 1), whereas a protein of 45-kDa was obtained in the same sample denatured by heat (Figure 3B, lane 2). The results support the hypothesis that the purified 180- kDa protein is a tetrameric state of four same oligomers and that the temperature could interfere with this pattern. Western blot analysis of purified Phenyl Sepharose fraction was performed by using the polyclonal antibody anti-formamidase (Figure 3C). A single 180-kDa protein species was detected in the sample non-denatured by heat (Figure 3C, lane 1). The same sample heat-denaturated depicts the 45-kDa protein species in this assay (Figure 3C, lane 2).

3.3. Characterization of the purified native protein by peptide mass fingerprinting

In order to characterize the purified 180-kDa protein species, the purified Phenyl Sepharose fraction (Figure 3B, lane 1) was digested with trypsin and the resulting peptides were submitted to mass spectrometry analysis. The identified peaks with the respective molecular masses are shown in Figure 3 (panel D). Mass values obtained for the detected peptides were compared to those theoretically deduced from sequences deposited in database. Ten peptides showed matches to peptides obtained by theoretical digestion of the predicted formamidase of *P. brasiliensis* (Table 2; Figure 3, panel D). The analysis reveals that the purified 180-kDa protein is the *P. brasiliensis* formamidase, and supports the concept that the 180-kDa species is the native formamidase of *P. brasiliensis*.

3.4. Cellular localization of *P. brasiliensis* formamidase

We exploited the cellular localization of the formamidase by using the polyclonal antibody anti-formamidase in immunoelectron microscopy. In yeast cells processed by the post embedding method, gold particles were present in both, cytoplasm and cell wall (Figure 4, panels B and D). Control sample exposed to the pre-immune serum was free of label (Figure 4, panel C). An additional control demonstrating the integrity of the cellular components is shown in Figure 4 (panel A). The quantification of gold particles was performed and demonstrated that similar number of particles were present in the cytoplasm and cell wall (Figure 5).

3.5. Screening for protein interactions with *P. brasiliensis* formamidase

In an attempt to identify proteins putatively interacting with the *P. brasiliensis* formamidase, it was performed two-hybrid assays to screen interactions with proteins from a cDNA library constructed with RNAs obtained from *P. brasiliensis* yeast cells. The resulted mating clones

were used in PCR reactions, and the products were submitted to DNA sequencing. The obtained cDNAs encoding proteins that were putative interacting molecules with the *P. brasiliensis* formamidase were summarized in the Table 3. Some cDNAs were redundant such as those encoding for the polyubiquitin (*ubq10*) homologue of *Neurospora crassa,* the FKBP-type peptidyl-prolyl isomerase homologue of *Aspergillus clavatus* and the Protein kinase C homologue of *Aspergillus fumigatus*. Also, the cDNAs encoding for the cell wall protein glycosyl hydrolase (*dfg*5 like), for the calnexin, homologues of *P. brasiliensis*, as well as for 2-oxoglutarate dehydrogenase E1 of *Ajellomyces capsulatus* were detected. Some of the identified proteins were submitted to *in vitro* confirmation of the interactions by coupled transcription/translation followed by co-immunoprecipitation of the translated products. The Figure 6 presents the products of *dfg5*, *ubq10* and *fkbp*, confirmed by the co-immunoprecipitation assays. The co-immunoprecipitation was assayed utilizing the c-myc antibody that reacts with c-myc epitope fused to formamidase, which immunopreciptated linked with interacting proteins that were synthesized by in vitro translation. The immunopreciptated proteins species were identified in same lane. The Dfg5-like (Figure 6, lane 1), the Ubq10 (Figure 6, lane 2) and the Fkbp (Figure 6, lane 3). Negative controls were performed by using the c-myc antibody with *in vitro* synthesizes proteins Dfg5-like, Ubq10 and Fkbp, to confirm the specific binding of c-myc antibody with formamidase (Figure 6, lanes 4 to 6).

3.6. A Model of how *P. brasiliensis* formamidase could interact with other *P. brasiliensis* proteins

The most relevant proteins identified by yeast two- hybrid system were used to construct a model of how those interactions could occur in *P. brasiliensis* cells. The identified proteins that were described in Table 3, and depicted in the model presented in Figure 7, are FKBP-type peptidyl-prolyl isomerase, calnexin, 2-oxoglutarate dehydrogenase and the cell wall glycosyl hydrolase (*Pb*Dfg5-like). The unfolded cytosolic formamidase could interact with Fkbp and calnexin, both accelerating the formamidase folding. The *Pb*Dfg5, a cell wall protein could interact with formamidase on cell wall, since both are cell-wall molecules of *P. brasiliensis* (present work; Castro *et al*., 2008) and may be responsible to the localization of formamidase at the fungal cell wall. Also the putative role of the formamidase in fungal nitrogen metabolism could be reinforced by its association with a subunit of 2-oxoglutarate dehydrogenase, a key enzymatic complex in nitrogen metabolism (Figure 7).

Discussion

In a previous work we performed the characterization of the gene and cDNA encoding to the formamidase of *P. brasiliensis*. Also, it was performed the heterologous expression and purification of the recombinant protein. We also demonstrated that the *P. brasiliensis* formamidase was catalytically active and could play a role in the host-parasite relationship, since the protein was recognized by sera of patients with proven paracoccidioidomycosis (Borges *et al*., 2005).

In this study we sought to continue the characterization of the *P. brasiliensis* formamidase. The purified recombinant protein was efficiently used to produce polyclonal antibody in mice. The polyclonal antibody anti-formamidase presented high specificity, detecting only a protein species in *P. brasiliensis* total protein extracts of yeast cells and mycelium. No cross-reaction with *P. brasiliensis* proteins was detected utilizing pre-immune serum.

The formamidase belongs to amidase/nitrilase family. The *Methylophilus methylotrophus* formamidase probably have homotetrameric structure (O'Hara *et al*., 1994; Wyborn *et al*., 1996). A homohexameric structure was showed in *H. pylori* crystallized formamidase (Hung *et al*., 2007). In order to have insights in the *P. brasiliensis* formamidase structure, studies were performed. Western blot analysis in reducing conditions revealed a 180-kDa protein species in *P. brasiliensis* yeast cells. Furthermore, heat-denatured protein samples revealed a protein species with 45-kDa. The formamidase of *P. brasiliensis* could be purified from the cell extract by a combination of ion exchange and hydrophobic interaction chromatography. This purification procedure yielded a homogenous preparation of the formamidase enzyme with a subunit molecular mass of 45-kDa and a native molecular mass of 180-kDa. These results suggest that this enzyme has a homotetrameric structure.

Immunocitochemistry analysis identified the *P. brasiliensis* formamidase in the cytoplasm and at the cell wall of *P. brasiliensis*. The *P. brasiliensis* formamidase could be a cytoplasmic protein, as suggested by computational analysis (http://www.psort.org) of the deduced protein sequence, since it does not present a N-terminal signal peptide. The localization of some classic cytoplasmatic molecules lacking N-terminal signal peptide in other cellular compartments is not uncommon, as described in our laboratory to the *P. brasiliensis* glyceraldehyde-3-phosphate-dehydrogenase - GAPDH (Barbosa *et al*, 2006) and to the triosephosphate isomarase - TPI (Pereira *et al*., 2007) both, present in the cytoplasm and at the fungal cell wall. Molecules that lack an N-terminal signal peptide sequence have been described in cell wall of *S. cerevisiae*, in addition to the usual cytoplasmatic localization (Nombela *et al*. 2006). Many macromolecules have been described out of cytoplasm in extracellular vesicles secreted by *Histoplasma*

capsulatum (Albuquerque *et al*., 2008) and *Cryptococcus neoformans* (Rodrigues *et al*., 2008). Some of them are classic cytoplasmic molecules, such as GAPDH (Albuquerque *et al*., 2008; Rodrigues *et al*., 2008) TPI, as well as formamidase (Albuquerque *et al*., 2008). The described data in other fungal species support our experimental results of double localization of *P. brasiliensis* formamidase in the cytoplasm and in cell wall.

Interactions of proteins are essential for all biological processes. The *P. brasiliensis* formamidase interacts with calnexin, involved in the correct folding (Pollock *et al*., 2004) and related to increase in the secretion of proteins in organisms by *Hansenula polymorpha* (Klabunde *et al*., 2007). Additionally, the formamidase of *P. brasiliensis* interacts with FKBP-type peptidyl prolyl isomerase, related to acceleration of folding of proteins (Solscheid and Tropschug 2000). The Ubq10 related to the degradation of cytoplasmatic proteins was found to interact with formamidase. The monoubiquitylation of proteins is related to trafficking of membrane proteins between various cellular compartments, directing ubiquitin-conjugated membrane proteins mainly to endocytic machinery, like lysosome and vacuoles (Mosesson *et al*., 2006). The Dfg5 cell wall protein of *S. cerevisiae* is related to resistence to alkaline environment. The *dfg5* mutant cells grew very poorly at pH 7.8 (Serrano *et al*., 2006). The cell wall Dfg5 (Castro *et al.,* 2008) interacts with formamidase probably at the fungal surface, where both are located, probably acting in alkaline resistence. Other proteins such as protein kinase C and 2-oxoglutarate dehydrogenase could interact with the fungal formamidase. The predicted interaction of the E1 component of the 2-oxoglutarate dehydrogenase is particularly noteworthy, since the enzymatic complex activity play an important role in modulating the flux from 2-oxoglutarate into amino acid metabolism (Araujo *et al*., 2008). Furthermore, some of these proteins that interacts with *P. brasiliensis* formamidase were found in the extracellular vesicles secreted by *H. capsulatum*, such as FKBP-type peptidyl-prolyl isomerase, 40S ribosomal protein S4, 2-oxoglutarate dehydrogenase (Albuquerque *et al*., 2008), as well as the calnexin protein found in the extracellular vesicles secreted by *C. neoformans* (Rodrigues *et al*., 2008), which can be related to the localization of *P. brasiliensis* formamidase in the cell wall of the fungus. Regarding to the putative role of the antigenic formamidase in *P. brasiliensis* physiology*,* the uncommon localization of this enzyme in the cell wall could highlight some metabolic features of this enzyme in *P. brasiliensis* cells, such as its antigenic property, as well as the putative role of this enzyme in nitrogen metabolism, producing ammonia. We can speculate that ammonia could be involved in tissue damage and in acid resistence in host tissues, as well as could be a source for nitrogen assimilation. Moreover, due to its characterized enzymatic activity in fungal cytoplasmic extracts (Borges *et al*., 2005), it could be speculated that the formamidase of *P.*

brasiliensis works in the nitrogen metabolism in the fungus cytoplasm, as described to *H. pylori* (van-Vliet *et al*., 2003). The interaction of formamidase with the subunit of 2-oxoglutarate dehydrogenase could reinforce the protein role in the fungal metabolism of nitrogen (Araujo *et al*., 2008).

The role of this high expressed molecule remains unclear; although this molecule was found in *P. brasiliensis in vitro* transcriptome of yeast cells (Felipe *et al*., 2003, 2005), in yeast cells recovered from infected mouse liver (Bailão *et al.,* 2006; Costa *et al*., 2007), as well as in host mimicking conditions (Bailão *et al*., 2006, 2007). If this molecule play a role in *P. brasiliensis* cells beyond the basic role in nitrogen metabolism, such as tissue damage and acid resistance, as described to *H. pylori* (Bury-Mone *et al*., 2004), its remains to be investigated.

Acknowledgements

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FIGURES

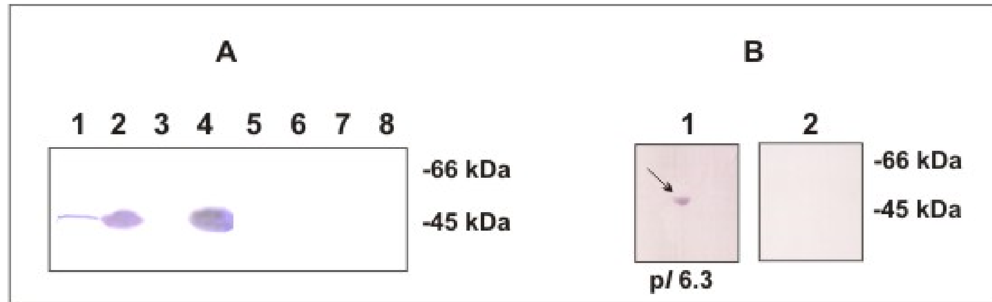


Figure 1: Production and characterization of the polyclonal antibody anti-*P. brasiliensis* formamidase. A- Protein fractionation by one dimensional gel electrophoresis and western blot analysis. Protein extracts from yeast cells (lanes 1 and 5), from mycelium (lanes 2 and 6), total extracts of *E. coli* XL1-blue cells (lanes 3 and 7) and the purified recombinant formamidase (lanes 4 and 8) were fractionated and transferred to membrane. Western blot analysis was performed with the anti-formamidase antibody,1:1000 diluted (lanes 1-4) or mouse pre-immune serum,1:1000 diluted (lanes 5-8). After reaction with the anti-mouse IgG alkaline phosphatase coupled antibody (diluted 1:2000) the reaction was developed with BCIP/NBT. Molecular size markers are indicated. B- Fractionation of *P. brasiliensis* protein extracts by two-dimensional gel electrophoresis and western-blot analysis. 1-Protein extracts from yeast cells after reaction with the polyclonal anti-formamidase antibody. 2-The same extract as in 1 reacted with the pre-immune serum.

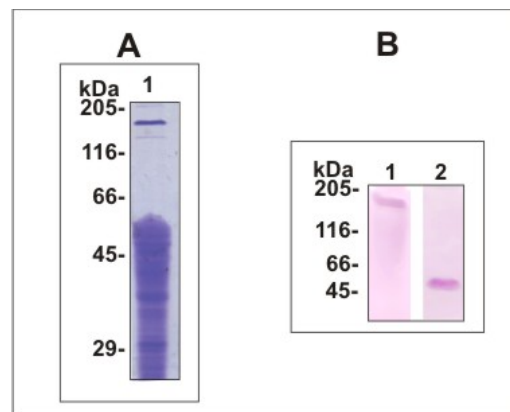


Figure 2: Fractionation of *P. brasiliensis* protein extracts and western-blot analysis. A –*P. brasiliensis* total yeast cells protein extract non-denaturated by heat. The proteins were stained by Coomassie blue R-250. B- *P. brasiliensis* total yeast cells protein extract non-denaturated (lane 1) and denaturated by heat (lane 2), was fractionated and transferred to membrane. Western blot analysis was performed with the anti-formamidase antibody, 1:1000 diluted. After reaction with the anti-mouse IgG alkaline phosphatase coupled antibody (diluted 1:2000) the reaction was developed with BCIP/NBT (lanes 1 and 2). Molecular size markers are indicated.

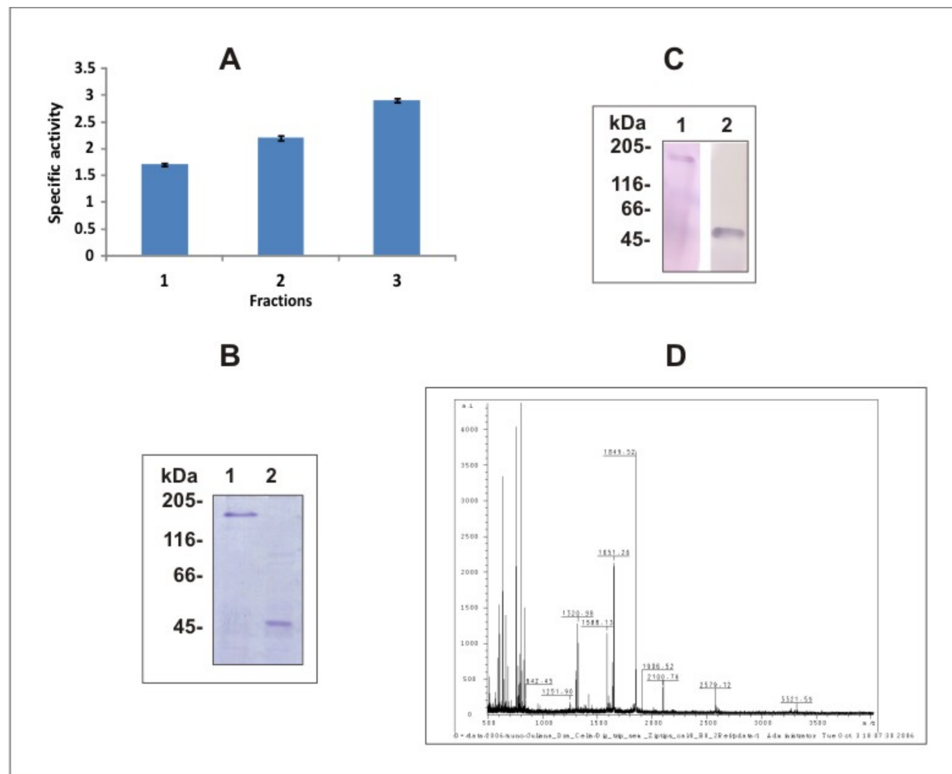


Figure 3: Purification and characterization of the *P. brasiliensis* native formamidase. A- The purification of the native formamidase was performed in two steps of chromatography – Total yeast protein extracts (lane 1) the chromatographic fractions from DEAE Sepharose (lane 2) and Phenyl Sepharose (lane 3) were assayed for formamidase activity. B- Polyacrylamide gel electrophoresis (12% SDS-PAGE) of: Phenyl Sepharose fraction non-denaturated (lane 1) or denaturated by heat (lane 2). The proteins on the gel were stained by Coomassie blue R-250. C- Reactivity of the protein fraction from Phenyl Sepharose to the polyclonal antibody, as determined by western blot analysis. The Phenyl Sepharose eluted fraction non-denatured (lane 1) or denaturated by heat (lane 2) was fractionated, transferred to membrane and reacted to the anti-formamidase antibody. After reaction with the anti-mouse IgG alkaline phosphatase coupled antibody (diluted 1:2000) the reaction was developed with BCIP/NBT. Molecular size markers are indicated. D-Peptide mass fingerprinting of trypsin digested formamidase – Ten peaks were observed and the obtained sequences were submitted to analysis in database.

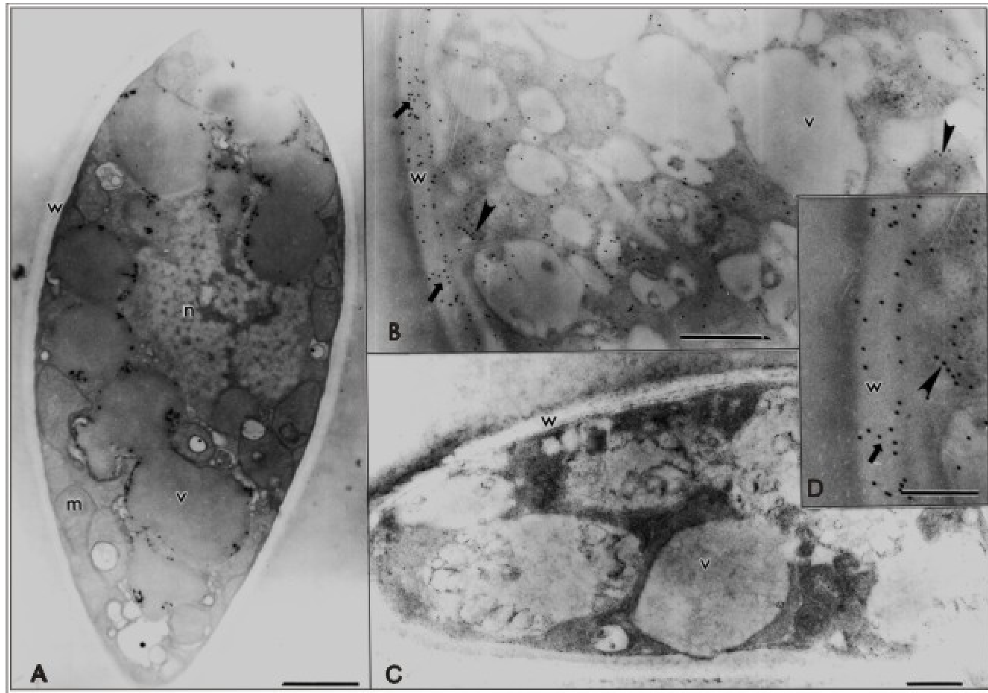


Figure 4: Immunoelectron microscopy detection of the formamidase in *P. brasiliensis* yeast cells by postembedding method. For ultrastructural immunocytochemistry studies, the ultrathin sections were incubated with the polyclonal antibody anti- formamidase (diluted 1:100). After, the cells were washed and incubated with the labeled secondary antibody anti-mouse IgG, Au conjugated, 1:20 dilution. Controls were incubated with mouse pre-immune serum at 1:100, followed by incubation with the labeled secondary antibody. A- Transmission electron microscopy of *P. brasiliensis* yeast cells showing the nucleus (n), cytoplasmic vacuoles (v), mitochondria (m) and cell wall (w). B – The gold labeled particles were observed in the cytoplasm and in the cell wall of yeast cells. The arrowheads indicate gold particles. C – Negative control is exposed to the pre-immune serum and is free of label. D – Magnification of the cell wall/membrane. Bars 1 μm (A), 0,5 μm (B and C) and 0,2 μm (D).

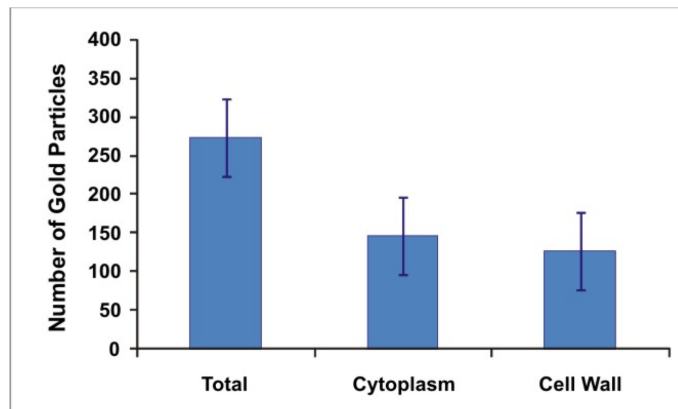


Figure 5: Quantification of gold particles in the *P. brasiliensis* yeast cells. The gold particles were quantified in three independent preparations of yeast cells. The particles were counted in total cell distribution, as well as in the cytoplasm and cell wall. Results were expressed as the number of gold particles, represented as the means of the counts performed three times with standard deviations included ($P \leq 0.05$).

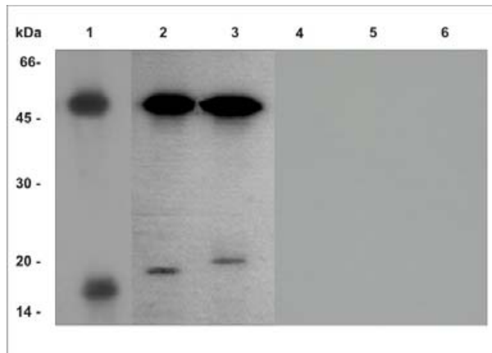


Figure 6: Co-immunopreciptation of *P. brasiliensis* proteins putatively interacting with *P. brasiliensis* formamidase. The proteins were *in vitro* synthesized and labeled with ^{35}S-methionine. The translated formamidase fused to c-myc epitope (c-myc-FMD) and the translated proteins fused to hemaglutinin epitope (HA-Prey) were mixed and the mixture was incubated with protein A Agarose beads and the monoclonal c-myc. The proteins were separated by SDS-PAGE. The gel was fixed, dried under vacuum and autoradiography was obtained. Dfg5-like protein (lane 1), Ubq10 (lane 2) and Fkbp protein (lane 3). Negative controls were performed; lanes 4-6, the same proteins as in 1-3.

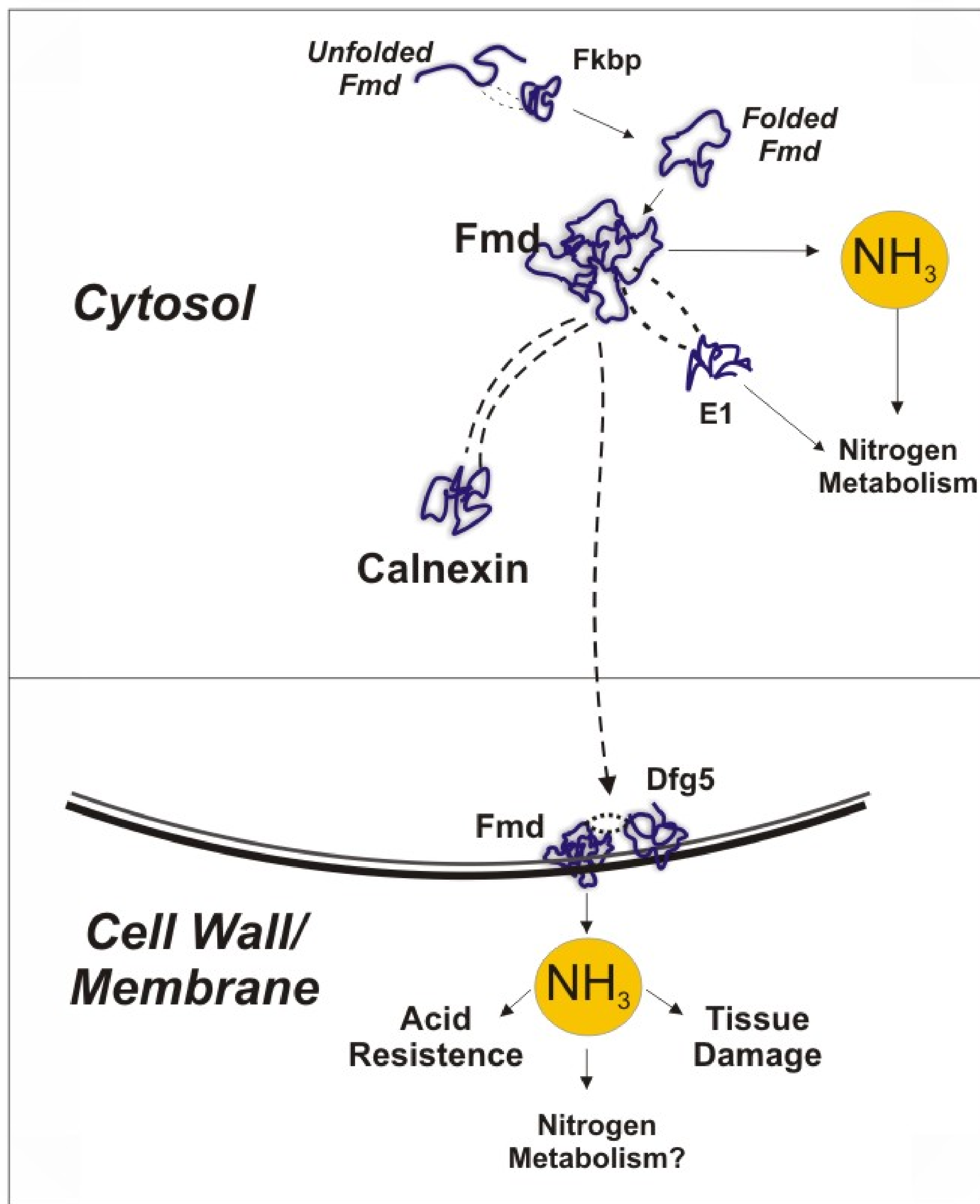


Figure 7: A model of how the *P. brasiliensis* formamidase could interact with other fungal proteins. FMD (formamidase), NH_3 produced by formamidase, E1 (2-oxoglutarate dehydrogenase E1), Fkbp (Peptidyl-prolyl isomerase), Dfg5 (glycosyl hidrolase) and calnexin are some of the proteins that putatively interact with formamidase of *P. brasiliensis* identified by yeast two -hybrid assay.

TABLES

TABLE 1 - Purification of the native formamidase of *P. brasiliensis*

Protein source	Specific activity[a]	Purification factor[b]	Yield (%)
Total protein extract of yeast cells	1.7	1	100
DEAE – Sepharose purified fraction	2.2	1.3	73
Phenyl – Sepharose purified fraction	2.9	1.7	62

[a] Specific activity: µmol of ammonia per min per mg of protein.
[b] (specific activity of purified fraction) / (specific activity of total protein of yeast extract)

TABLE 2 - Identification of *P. brasiliensis* formamidase by peptide mass fingerprinting

Tryptic peptide mass (Da)		
Experimental data (*in-gel* digestion)[a]	Expected data (*in silico* digestion)	Identified amino acid sequence
1,251.204	1,250.297	^{12}VDLHKPASEQK^{22}
1,420.000	1,418.993	^{46}IECLDWTGGQIK^{57}
1,600.123	1,599.116	^{116}NGGGFLDEFYPNAAK^{130}
1,588.132	1,587.125	^{131}AIWDFEGIFCSSR^{143}
2,579.118	2,578.110	^{150}FAGLIHPGILGCAPSAEVLAEWNR^{173}
2,100.763	2,099,755	^{188}VVAKPPEPINVHAGSASDAIKA^{208}
1,651.256	1,650.248	^{218}TIPGRPEHGGNCDIK^{232}
1,849.518	1,848.511	^{295}SPIFHPGPVEPQFSPGR^{311}
1,637.213	1,636.206	^{312}YLTFEGFSVDHHGK^{325}
1,320.977	1,319.969	^{326}QHFLDATVAYR^{336}

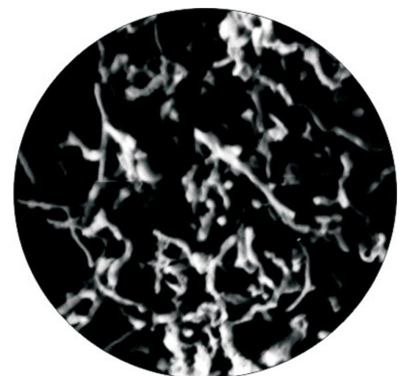
[a]The formamidase was digested with trypsin, and masses of resulting peptides were determined by mass spectrometry and compared to the theoretical ones produced by *in silico* digestion of proteins found in the SwissProt databases (http://expasy.org/sprot/). Experimental mass was obtained with accuracy of 0.1 to 0.2 Daltons.

TABLE 3–cDNAs identified in yeast two- hybrid assays for which the cognate proteins putatively interact with the *P. brasiliensis* formamidase.

Gene Product	Best blast hit/Accession number[a]	Redundancy	Putative function in other organisms
Polyubiquitin (Ubq10)	*Neurospora crassa/* XP_958803.1|	3	Protein degradation/Trafficking of membrane proteins
FKBP-type peptidyl-prolyl isomerase (Fkbp)	*Aspergillus clavatus*/ XP_001274819	5	Protein folding, assembly and trafficking.
Protein kinase C (PkC)	*Aspergillus fumigatus* /XP_753454.1	5	Signal transduction
40S ribosomal protein S4 (Rps4)	*Ajellomyces capsulatus*/ XP_001537815	2	Ribosome biogenesis / Protein synthesis
2-oxoglutarate dehydrogenase E1 component, mitochondrial precursor	*Ajellomyces capsulatus*/ XP_001544488	2	Nitrogen metabolism/ Tricarboxylic acid cycle
Calnexin (Cne1)	*Paracoccidioides brasiliensis* /ABB80132.1	1	Protein folding in the endoplasmic reticulum
Cysteine protease (atg4)	*Coccidioides immitis*/ XP_001248363	1	Protein degradation, proteolysis and autophagy
Cell wall glycosyl hydrolase Dfg5 (Dfg5)	*Paracoccidioides brasiliensis* / DQ534495	1	Protein putatively required for cell wall biogenesis

[a] GenBank accession numbers (http://www.ncbi.nlm.nih.gov)

Discussão



Caracterização da enzima formamidase de *Paracoccidioides brasiliensis*

Discussão

Estudos de genes/proteínas estágio específicos, com expressão diferencial durante a transição dimórfica do fungo (Silva *et al.* 1994) e de antígenos (Fonseca *et al.,* 2001) são alvo de estudos em *P. brasiliensis*. Uma proteína de 45-kDa isolada por meio de focalização isoelétrica líquida demonstrou-se reativa com anticorpos presentes em soros de pacientes com PCM, sugerindo um potencial papel da proteína na interação do fungo com o hospedeiro. A proteína purificada de extrato total de células leveduriformes de *P. brasiliensis*, foi digerida e os peptídicos obtidos foram seqüenciados e caracterizados. O gene e o cDNA codificantes para formamidase de *P. brasiliensis* foram clonados, caracterizados e a expressão heteróloga da proteína recombinante foi obtida (Borges *et al*., 2005).

O transcrito codificante para a enzima formamidase de *P. brasiliensis* é altamente expresso na fase miceliana do fungo (Felipe, *et al.*, 2003; Borges *et al.*, 2005), bem como a proteína apresenta expressão aumentada na fase miceliana quando comparada com a fase leveduriforme. Análises de *Southern* e *northern blot* revelaram que *P. brasiliensis* apresenta uma única copia do gene que codifica para formamidase (Borges *et al.*, 2005). Esses dados foram confirmados por análises *in silico* do genoma estrutural de três isolados de *P. brasiliensis* (*Pb*01, *Pb*03, *Pb*18), (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html).

A comparação entre a seqüência deduzida da proteína formamidase com seqüências de outros organismos depositadas em banco de dados, revelou um alto índice de conservação na estrutura primária da proteína. Contudo, três regiões da seqüência deduzida da proteína não apresentaram conservação (177-193, 204-210, 396-402), as quais poderiam ser utilizadas para análises como candidatas para desenvolvimento de ensaios para diagnóstico. Além disso, a análise da seqüência deduzida da proteína revelou 17 regiões na molécula com potencial antigênico (Kolaskar *et al*., 1990). O reconhecimento de amidases por anticorpos presentes em soros de pacientes também foi descrito, por meio de estudos imunoproteômicos em *H. pylori* (Haas *et al*., 2002; Bury-Moné *et al*., 2003).

A proteína heteróloga recombinante purificada apresentou reatividade com anticorpos presentes em soros de pacientes com PCM. A proteína recombinante não apresentou reação cruzada com anticorpos presentes em soros de indivíduos controle, sugerindo que a molécula poderia ser usada em estudos de proteínas candidatas à utilização em diagnóstico. A proteína

recombinante mostrou atividade enzimática de formamidase. A atividade de formamidase foi detectada em extrato protéico total obtido de células de leveduras e de micélio de *P. brasiliensis,* com maior atividade em micélio quando comparada com a fase leveduriforme. Essas observações sugerem que a proteína possa apresentar papel diferencial nas fases de *P. brasiliensis* (Borges, *et al.*, 2005).

A formamidase de *P. brasiliensis* não é, possivelmente, a única enzima capaz de clivar formamida, uma vez que amidases e acetamidases foram descritas no transcritoma de *P. brasiliensis* oriundo de células micelianas e leveduriformes cultivadas *in vitro* (Felipe *et al.*, 2003). Contudo, a atividade da acetamidase purificada de bactérias é altamente específica, sendo maior para amidas de cadeia curta do que para formamida (Kobayashi, *et al.*, 1993; Wyborn, *et al.*, 1996). Amidases apresentam baixa atividade para formamida, como descrito em *Pseudomonas aeruginosa* (Clarke & Dew, 1988; Maestracci, *et al.*, 1988). A atividade enzimática da formamidase de *P. brasiliensis* mostrou-se altamente específica para formamida, uma vez que a atividade é baixa quando se usa como substrato a uréia, reforçando a idéia de que a formamidase de *P. brasiliensis* deve desempenhar papel no metabolismo de nitrogênio do fungo (Borges *et al.*, 2005).

Em trabalho subseqüente, Borges e colaboradores (2008, manuscrito em anexo), realizaram estudos adicionais visando à caracterização da formamidase de *P. brasiliensis*. A proteína recombinante foi produzida, purificada (Borges *et al.*, 2005) e utilizada eficientemente para a produção de anticorpo policlonal em camundongos. Análises realizadas por meio de *immunobloting* evidenciaram que o anticorpo reconhece a proteína recombinante purificada, bem como reconhece apenas uma espécie protéica em extrato total de *P. brasiliensis* obtido de células micelianas e leveduriformes. Além disso, o soro pré-imune obtido dos camundongos, não apresentou reação cruzada com nenhuma espécie protéica utilizando-se os mesmos extratos de *P. brasiliensis* (Borges *et al.*, 2008, manuscrito em anexo). Esses dados reforçam aqueles obtidos nas análises anteriores, mostrando que a proteína formamidase de *P. brasiliensis* é antigênica e pode ser considerada uma candidata para estudos de testes de diagnóstico da paracoccidioidomicose (Borges *et al.*, 2005), especialmente se utilizada em conjunto com outras proteínas de *P. brasiliensis* (Correa *et al.*, 2007; Carvalho *et al.*, 2008).

Formamidases são descritas como enzimas da família de amidases/nitrilases como enzimas homodiméricas, homotriméricas e homotetraméricas (Wyborn *et al.*, 1996), bem como apresentando dois tetrâmeros ligados (O'Hara *et al.*1994). A cristalografia da formamidase de *H. pylori* revelou estrutura homo-hexamérica (Hung *et al.,* 2007). Devido a essas diferenças encontradas na organização entre monômeros de formamidase, foram realizados ensaios de

Western blot, utilizando-se o anticorpo policlonal anti-formamidase. Extrato protéico total de células leveduriformes de *P. brasiliensis* não desnaturado por fervura, foi submetido à immunobloting utilizando-se o anticorpo policlonal anti-formamidase. Uma espécie protéica de 180-kDa foi identificada nesta condição, assim como uma espécie protéica de 45-kDa foi identificada em extrato protéico total desnaturado por fervura. Esses resultados sugerem que a espécie protéica de 45-kDa seria a formamidase na forma monomérica, enquanto a espécie protéica de 180-kDa, a formamidase na forma homotetramérica (Borges *et al*., 2008).

Com o intuito de se identificar a espécie protéica de 180-kDa detectada no ensaio de *immunobloting*, extrato protéico total de células leveduriformes de *P. brasiliensis* foi purificado, em dois passos de cromatografia, em uma combinação de cromatografia de troca iônica e de interação hidrofóbica. A fração purificada mostrou alta atividade de formamidase e revelou, por meio de fracionamento de proteínas em gel de poliacrilamida, uma espécie de 180-kDa. A proteína de 180-kDa, foi analisada por meio de espectrometria de massas e identificada como formamidase de *P. brasiliensis*, reforçando a hipótese de que a proteína purificada de 180-kDa, trata-se da formamidase na forma tetramérica.

O anticorpo policlonal anti-formamidase foi utilizado em ensaios de imunolocalizacão da proteína em células leveduriformes. Análises computacionais da seqüência deduzida da formamidase, revelam uma proteína com localização citoplasmática. Nos ensaios de imunolocalização, por meio de microscopia eletrônica de transmissão, a proteína foi encontrada em quantidades equivalentes, tanto no citoplasma quanto na parede celular de *P. brasiliensis*. (Borges *et al*., 2008). Análise por microscopia confocal revelou a presença da formamidase de *P. brasiliensis* no citoplasma e na parede celular/membrana (Anexos: Figura 1).

A presença de proteínas com localização predita no citoplasma em outros compartimentos celulares tem sido descrita, em nosso laboratório, para a gliceraldeído-3-fosfato-desidrogenase - GAPDH (Barbosa *et al*, 2006) e para a triosefosfato isomarase - TPI (Pereira *et al*., 2007) ambas, localizadas no citoplasma e na parede celular. Em *S. cerevisae*, moléculas que não apresentam peptídeo sinal N-terminal têm sido descritas na parede celular do fungo (Nombela *et al*. 2006). Várias moléculas, possivelmente citoplasmáticas, foram identificadas em vesículas secretadas por *H. capsulatum* (Albuquerque *et al*., 2008) e por *Cryptococcus neoformans* (Rodrigues *et al*., 2008). Várias dessas moléculas presentes nas vesículas são proteínas citoplasmáticas, como as enzimas da via glicolítica frutose 1,6-bifosfato aldolase, gliceraldeído-3-fosfato desidrogenase e triose fosfato isomerase. Algumas proteínas são reativas com soros de pacientes e descritas como fatores de virulência (Albuquerque *et al*.,

2008; Rodrigues *et al*., 2008). A enzima formamidase foi encontrada em vesículas secretadas por *H. capsulatum* (Albuquerque *et al*., 2008), o que corrobora nosso resultado de dupla localização da formamidase de *P. brasiliensis* no citoplasma e na parede celular.

A enzima formamidase (EC 3.5.1.49), catalisa a hidrólise de formamida produzindo formato e amônia (Hynes, 1975; Fraser *et al.*, 2001). A amônia pode ser usada como fonte de nitrogênio e o formato pode ser oxidado e seus produtos utilizados na cadeia respiratória. A atividade de amidases na hidrólise de amidas tem sido descrita em microrganismos na obtenção de nitrogênio do solo, como já caracterizado em *A. nidulans* (Hynes, 1975) e em organismos que apresentam, pelo menos, uma etapa do seu ciclo de vida no ambiente (Bury-Moné *et al.*, 2003). A atividade de amidases é importante para a defesa de patógenos. Um dos produtos finais da catálise da enzima, a amônia, tem papel importante na patogênese de *H. pylori,* auxiliando na destruição tecidual e em sua resistência ao pH ácido estomacal (Bury-Moné *et al.*, 2004).

O papel da formamidase em *P. brasiliensis* além da provável ação no metabolismo de nitrogênio permanece elusivo. A interação entre proteínas é importante para vários processos metabólicos. Utilizando-se o sistema de duplo híbrido em *S. cerevisiae* foi possível a identificação de várias proteínas que interagem com a formamidase de *P. brasiliensis*. Foram encontradas interações com proteínas relacionadas ao processamento/dobramento de proteínas como a calnexina, envolvida no dobramento correto de proteínas (Pollock *et al*., 2004) e no aumento da secreção de proteínas em *Hansenula polymorpha* (Klabunde *et al*., 2007) e a FKBP-peptidil prolil isomerase, a qual está envolvida na aceleração do dobramento de proteínas (Solscheid & Tropschug 2000). Proteínas relacionadas à degradação protéica como a poli-ubiquitina e a cisteína protease também foram identificadas. A ubiquitinação direciona as proteínas para o sistema de degradação do proteassoma o qual pode estar acoplado à cisteína protease na degradação final da formamidase de *P. brasiliensis* (Borges *et al*., 2008). A monoubiquitinação está relacionada com o tráfego de proteínas em compartimentos celulares, principalmente promovendo endocitose de proteínas de membrana conjugadas à ubiquitina, as quais podem ser direcionadas para lisossomos ou vacúolos, onde podem ser degradadas (Mosesson *et al*., 2006). A proteína Dfg5 de parede celular de *P. brasiliensis* (Castro *et al*., 2008) interage com a formamidase e pode estar relacionada com a sua localização nesta região celular. Foi observada a interação da formamidase com a 2-oxoglutarato desidrogenase. A 2-oxoglutarato desidrogenase é uma enzima do ciclo do ácido tricarboxílico e está envolvida na modulação do fluxo de 2-oxoglutarato no metabolismo de aminoácidos e de nitrogênio (Araujo, *et al*., 2008), o que reforça o papel da formamidase no metabolismo de nitrogênio de

P. brasiliensis. A interação da formamidase com algumas proteínas cujos homólogos são secretados em vesículas de *H. capsulatum* e ou *C. neoformans*, como FKBP-peptidil-prolil isomerase, proteína ribossomal 40S S4-Rps4, 2-oxoglutarato desidrogenase e calnexina (Albuquerque *et al*., 2008; Rodrigues *et al*., 2008), corrobora os resultados de localização da formamidase na parede celular. Ressalte-se que a formamidase de *H. capsulatum* é uma proteína de secreção vesicular (Albuquerque *et al.,* 2008).

A formamidase de *P. brasiliensis* deve desempenhar funções celulares relevantes, visto a alta expressão do transcrito e da proteína (Felipe *et al*., 2003, Borges *et al*., 2005), e a expressão diferencial em condições que mimetizam infecção em nichos do hospedeiro (Bailão *et al*., 2006; Costa *et al*., 2007). A localização celular da formamidase na superfície de *P. brasiliensis* poderia estar relacionada à destruição celular e resistência à ambientes ácidos, além de seu provável papel no metabolismo de nitrogênio do fungo. Nesse sentido, a formamidase em *H. pylori*, está relacionada à destruição tecidual e à resistência do microrganismo a ambientes ácidos (Bury-Moné *et al*., 2004).

Conclusões

- Purificou-se um novo antígeno de 45-kDa de *P. brasiliensis*, o qual apresentou identidade com formamidases de diferentes organismos;
- Foram caracterizados o cDNA e o gene codificantes para formamidase de *P. brasiliensis*, sendo que uma única cópia do gene está presente no genoma do fungo;
- O cDNA que codifica para formamidase foi clonado e a proteína heteróloga de 45-kDa foi purificada, a qual foi reconhecida por anticorpos presentes em soros de pacientes com PCM;
- A proteína recombinante purificada apresentou atividade enzimática de formamidase, assim como ocorreu em extratos protéicos de levedura e micélio, sugerindo que a enzima é funcional no metabolismo de *P. brasiliensis*;
- A proteína recombinante foi utilizada eficientemente na produção de anticorpo policlonal em camundongos, reconhecendo a formamidase recombinante e uma única espécie protéica em extrato protéico total de *P. brasiliensis*;
- Uma proteína de 180-kDa, reativa com o anticorpo policlonal anti-formamidase, foi purificada e caracterizada como formamidase na forma homotetramérica;

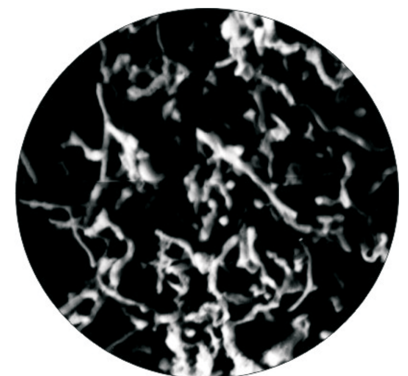
- A proteína formamidase de *P. brasiliensis* apresenta dupla localização celular, tendo sido identificada, por meio de imunolocalização, tanto no citoplasma quanto na parede celular;

- Foram realizados estudos de interações da formamidase com outras proteínas de *P. brasiliensis*. Proteínas relacionadas ao metabolismo de nitrogênio, dobramento e degradação de proteínas foram caracterizadas. Proteínas de superfície foram detectadas em experimentos de duplo-híbrido, reforçando a localização celular da formamidase.

- Os resultados sugerem que a formamidase de *P. brasiliensis* deve desempenhar funções extras às relacionadas ao metabolismo de nitrogênio do fungo, tais como interações entre o fungo e o hospedeiro, resistência a ambientes hostis e papel antigênico, uma vez que anticorpos de pacientes com PCM reconhecem a formamidase de *P. brasiliensis*.

Capítulo III

Análises transcricionais no estudo de genes diferencialmente expressos



Genes potentially relevant in the parasitic phase of the fungal pathogen *Paracoccidioides brasiliensis*

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Running title: Genes up regulated in the yeast fungal phase

Keywords: *Paracoccidioides brasiliensis*, yeast phase, cDNA subtracted libraries.

Summary

The mycelia propagules, the infective form of the fungal pathogen *Paracoccidioides brasiliensis*, are inhaled into the host lungs in which they differentiate to yield another form, the yeast fungal phase. Dimorphism results from a process termed phase transition which is regulated by temperature of incubation. At the room temperature *P. brasiliensis* grows filamentously, but at 36 ºC, the fungus develops as a multinucleated yeast form. We studied the changes in gene expression in the yeast phase by using subtractive cDNA methodological strategies. In an effort to help identify gene products associated with the yeast parasitic phase, cDNAs profiles were generated from yeast cells of isolate *Pb*01 and from isolate *Pb*4940, the last growing as mycelia at the host temperature.

In this study, we attempted to characterize the physiological response of yeast cells growing at 36 ºC in isolate *Pb*01 comparing to the *Pb*4940 isolate. Transcripts exhibiting increased expression during development of the yeast parasitic phase comprised those involved mainly in response to stress, transcriptional regulation and nitrogen metabolism. In this way, the isolate *Pb*01 increased the expression of a variety of transcripts encoding cell rescue proteins such as the heat shock protein HSP30, alpha threalose phosphate synthase and DDR48 stress protein, suggesting the relevance of the defense mechanism against oxidative/heat shock stress in the fungal yeast phase. The transcripts encoding to the protein CLPA, to the plasma membrane ATPAse H+ (PMA1) and to the cell-wall protein SED1p were reduced in isolate *Pb*01 compared to the isolate *Pb*4940. Some of the transcripts with altered expression have no alignment to known genes. A great number of genes with differential expression between the two isolates code for cell/wall membrane related proteins suggesting the relevance of the fungal surface and its remodeling to the dimorphism.

Introduction

Paracoccidioides brasiliensis is an important human pathogen causing paracoccidioidomycosis (PCM), a systemic mycosis with broad distribution in Latin America (Restrepo *et al.*, 2001). Although the area of incidence spreads non-uniformly from Mexico to Argentina, the incidence of disease is higher in Brazil, Venezuela and Colombia (Blotta *et al*., 1999). The fungus is thermo-dimorphic, that is, it grows as a yeast-like structure in the host tissue or when cultured at 35-36 ºC, and as mycelium in the saprobic condition or when cultured at room temperature (18-23 ºC). The infection is caused by inhalation of airborne propagules of the mycelial phase of the fungus, which reach the lungs where it differentiates into the yeast parasitic phase (Lacaz, 1994).

In the genome era, it has been common to use gene expression profiling of a microorganism under certain conditions to understand its biology. *P. brasiliensis* monitor the environment and alters gene expression in response to many factors, mainly temperature during the fungal conversion to the yeast phase (Felipe *et al*., 2005; Nunes *et al*., 2005; Bastos *et al*., 2007). Large, dynamic changes in expression of genes implicated in membrane/cell wall remodeling, metabolism, general adaptative responses had been reported during fungal phase transition. To enhance our understanding of the molecular events of *P. brasiliensis* to respond to temperature and establish the yeast phase, we used Representational Difference Analysis (RDA). Specifically, transcriptional profiles in the present study were generated by RDA from two fungal isolates presenting or not the ability of thermal dimorphism. Selected cDNAs were recovered to perform validation experiments by using reverse northern blot and RT-PCR analysis. We discovered that the yeast phase of *P. brasiliensis* over expresses genes related to the stress response, virulence and development. Those genes support the concept that the yeast phase can potentially respond to the attack by the host conditions. Additionally, some genes were down regulated in the yeast phase, such as those encoding for alternative oxidase, for the homologue for a structural protein Sed1p, required for compensating for cell wall instability in fungi. Key themes identified by our analysis included differential expression of genes encoding fungal surface proteins, stress related proteins, as well as proteins involved in morphogenesis and transcriptional control. The collective results of this study demonstrate the utility of RDA approaches to studying dimorphism in *P. brasiliensis*.

Materials and methods:

P. brasiliensis growth conditions:

P. brasiliensis isolate 01 (ATCC MYA-826) has been studied at our laboratory (Barbosa *et al.*, 2006, Bailão *et al*., 2006). The isolate *Pb*4940 (ATCC MYA-3044) has been previously investigated by our group (Okamoto *et al*., 2006). The isolates of *P. brasiliensis* were grown at 36 ºC, 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; 4% (w/v) glucose, 1% (w/v) agar, pH 7.2] for 7 days.

Scanning electron microscopy:

The *P. brasiliensis* cells of each isolate were fixed by immersion in a solution containing 2% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.05 M sodium cacodylate buffer, pH 7.4 for 12 h at room temperature. The material was washed with the same buffer and the cells were post-fixed in 1% osmium tetroxide (OsO_4) and 0.8% (w/v) potassium ferricyanide in sodium cacodylate buffer. The cells were washed to remove excess of OsO_4 and dehydrated in a graded acetone series from 30% to 100% (v/v). The specimens were mounted on stubs, dried by the critical point method (Lea and Ramjohn 1980) and gold-coated in a Sputter Coater Balzers SCD 050 before examination in a Jeol JEM 840A scanning electron microscope.

DNA extraction and PCR analysis:

P. brasiliensis yeast cells from isolates *Pb*01 and *Pb*4940 were harvested, washed and frozen in liquid nitrogen. Grinding with a mortar and pestle broke the cells, and the genomic DNA was prepared by the cationic hexadecyl trimethyl ammonium bromide (CTAB) method according to Del Sal *et al*. (1989). *P. brasiliensis* genomic DNA was used as a template for the PCR amplification of a partial fragment encoding the gene encoding for the 43-kDa glycoprotein *Pb*GP43 (Cisalpino *et al*., 1996). The oligonucleotide primers for the PCR reaction are described in Table 1. The PCR reaction was conducted in a total volume of 25 µL containing 20 ng of DNA as template. PCR conditions were 30 cycles at 95 ºC for 1min; annealing at 58 ºC for 2 min; 72 ºC for 1 min. Amplicons were analysed by agarose gel electrophoresis (1%). The resulting 317 bp product was subcloned into pGEM-T-Easy (Promega) and sequenced.

RNA extractions, subtractive hybridization and generation of subtracted libraries:

Total RNA of the *P. brasiliensis* cells growing at 36 ºC, was extracted by the use of Trizol (GIBCO™, Invitrogen, Carlsbard, CA) according to the manufacturer's instructions. The quality of RNA was assessed by use of the A_{260} /A_{280} and by visualization of rRNAs on 1.2% agarose gel electrophoresis. The RNAs were used to construct double-stranded cDNAs. Subtractive hybridization and cDNA libraries construction was performed as previously described (Bailão *et al*., 2006; 2007). Briefly, for the subtractive hybridization, 1.0 µg of total RNAs was used to produce double strand cDNA by using the SMART PCR cDNA synthesis kit (Clonetech Laboratories, Palo Alto, CA, USA). First-strand cDNA synthesis was performed with reverse transcriptase (RT Superscript II, Invitrogen, CA, USA) and it was used as template to synthesize the second-strand of the cDNA. The resulting cDNAs were digested with the restriction enzyme *Sau3A*I. Two subtracted cDNA libraries were made using driver and tester

cDNAs of both isolates, from 7 day-old-cultures at 36 ºC. The resulting products were purified using GFX kit (GE Healthcare, Chalfont St. Giles, UK). The tester-digested cDNA was bound to adapters (a 24-mer annealed to a 12-mer). For the 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 1:10 and 1:100 were performed to generate second differential products. Adapters were changed between cross-hybridization, and differential products were purified using the GFX kit (GE Healthcare ®). The adapters used on subtractive hybridizations were: NBam12, 5' GATCCTCCCTCG 3'; NBam24, 5'AGGCAACTGTGCTATCCGAGGGAG 3'; RBam12, 5' GATCCTCGGTGA 3'; RBam24, 5' AGCACTCTCCAGCCTCTCTCACCGAG 3'.

After the second subtractive reaction, the finally 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 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 pre-processed using the Phred (Ewing and Green, 1998) and Crossmatch programs (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm). Only sequences with at least 100 nucleotides and Phred quality greater or equal to 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). To assign functions, the valid ESTs and the assembled consensus sequences were locally compared against a nonredundant 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). Also ESTs and assembled consensus sequences were compared to the *P. brasiliensis* genomic database at http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

Sequences were classified into three categories: a) annotated, which corresponds to sequences showing significant matches with protein sequences with an identified function in databanks; b) hypothetical protein corresponds to sequences for which the E value was $\geq 10^{-5}$, or for which no match

was observed in databanks; c) conserved hypothetical corresponds to protein group sequences for which significant matches ($e \leq 10^{-5}$) and homology to a protein with no identified function was observed.

ESTs were grouped in clusters, represented by contigs and singlets. Sequences were grouped in functional categories according to the classification of MIPS functional catalogue (Munich Center for Protein Sequences; http://www.mips.gst.de/).

Reverse northern blot analysis:

Plasmid DNAs of selected clones were obtained. Serial dilutions of DNAs were performed and the material was applied, under vacuum, on Hybond-N+ nylon membranes (GE Healthcare). The DNAs were hybridized to cDNAs, which were obtained from specific conditions, labeled by using the Random Primer labeling module (GE Healthcare). Detection was performed by using the Gene Image CDP-Star detection module (GE Healthcare ®). Probes were as follows: 30 kDa heat shock protein (*hsp30*); GATA transcription factor (*nsdd*); C6 transcription factor 1 beta (*ctf1*β); predicted cell wall/membrane protein (*cwp*); alternative oxidase (*aox1*); translation elongation factor 1 alpha (*eef1a*); 40S ribosomal protein S27 (*rps27*); Plasma membrane ATPAse H^+ (*pma1*).

RT-PCR analysis:

RT-PCR experiments were performed. Cells cultured at 36 ºC from both isolates, were used to obtain total RNAs. Those RNAs were obtained from experiments independent from those used in the cDNA subtractions. The single stranded cDNAs were synthesized by reverse transcription toward total RNAs, by using the Superscript II RNAse H – reverse transcriptase, and PCR was performed using the cDNA as the template in a 30 μL reaction mixture. Specific primers were used as described in Table 1. The reaction mixture was incubated initially at 95 ºC for 1 min, followed by 25 cycles of denaturation at 95 ºC for 1 min, annealing at 55-64 ºC for 1 min, and extension at 72 ºC for 1 min. The DNA product was separated by electrophoresis in 1.0% agarose gel, stained, and photographed under ultraviolet light illumination.

Results

Analysis of fungal dimorphism in isolates *Pb*01 and *Pb*4940:

Cultures of both isolates were grown at 36 ºC by sub culturing at every 7 days. As demonstrated in Fig. 1 A, both cultures exhibited distinct morphological patterns at this temperature. The isolate *Pb*01 exhibited the characteristic yeast morphology with cells presenting multiple buds, whereas the isolate *Pb*4940 presented typical mycelia at this temperature, with no conversion to the yeast phase. Both isolates were analyzed regarding to the presence of the gene encoding the glycoprotein Gp43 (Cisalpino *et al*., 1996), the most studied molecule in the pathogen. As demonstrated in Fig. 1B in both isolates it was amplified by PCR a genomic fragment of 317 bp, encoding to the *gp*43 gene.

Description of the ESTs in the subtracted cDNA libraries:

We wished to determine the changes in gene expression that could be relevant in *P. brasiliensis* yeast parasitic phase by subtracting cDNAs from isolates *Pb*01 and *Pb*4940, the latter a non-differentiating isolate at the host temperature. The RDA approach was performed between the yeast cells of isolate *Pb*01 and the mycelia-like form of isolate *Pb*4940, both cultured at 36 ºC and used as drivers and testers in different subtraction reactions. Subtraction was performed by incubating the drivers and the testers. Selection of the cDNAs was achieved by construction of subtracted cDNA libraries in pGEM-T-Easy, as described earlier. Fig. 2 shows the RDA products of the two conditions of subtraction, using both isolates as testers and drivers. Different patterns of DNA amplification were observed after two cycles of RDA, as shown.

A total of 427 clones were successfully sequenced. From them, 258 were obtained by using the isolate *Pb*01 as the tester, and comprehend predicted over regulated genes in the yeast phase of *Pb*01, compared to the mycelial form of isolate *Pb*4940, both cultivated at 36 ºC. A total 169 clones were obtained by using the isolate *Pb*4940 as the tester and corresponded to predictable down regulated genes in isolate *Pb*01, compared to the isolate *Pb*4940.

The nature of genes differentially expressed, classified as up or down regulated in the isolate *Pb*01 , were inferred by classifying the ESTs into groups of functionally related genes (Tables 2 and 3). We interpreted these data with the perspective of seeking to understand the genes important for the establishment of the yeast phase. We analyzed the redundancy of the transcripts by determining the number of ESTs related to each transcript. The most redundant cDNAs appearing in the subtracted cDNA library using the isolate *Pb*01 as tester, and consequently designed up regulated transcripts, were those encoding for the following proteins: predicted cell wall/ membrane protein (151 ESTs); 30 kDa heat shock protein, Hsp30 (42 ESTs); C6 transcription factor 1 beta, Ctf1β (25 ESTs) and GATA transcription factor, Nsdd (18 ESTs), as shown in Table 2.

By using the isolate *Pb*4940 as tester it was possible to identify genes putatively repressed in isolate *Pb*01, such as those encoding a hypothetical protein (58 ESTs); translation elongation factor 1 alpha, Eef1α (32 ESTs); conserved hypothetical protein homologue to *Giberella Zeae* (24 ESTs); alternative oxidase, Aox1 (17 ESTs), as shown in Table 3.

Transcripts predicted to be associated to stress response and to morphogenesis/ development/virulence, up regulated in isolate *Pb*01:

Table 4 describes some candidate homologs for stress response in the isolate *Pb*01, yeast phase, at 36 ºC, when compared to isolate *Pb*4940 cultured at the same temperature. We classified 03 induced genes, comprehending 47 ESTs, by homology to other organisms, as codifying for proteins with defined role in

stress response. TPS1 was catalogued as a potential stress related protein in *P. brasiliensis,* since it appears to increase resistance to heat. Trehalose acts primarily as stress protectant for proteins and membranes during exposure to high temperature (Petzold *et al*., 2006). The *hsp*30 gene was shown to be activated by several stresses including heat shock, ethanol shock, glucose starvation and sorbate exposure (Piper *et al*., 1997). The transcript encoding the DDR48p is over expressed upon exposure of *Candida albicans* to antifungals (Liu *et al*., 2005).

Table 5 summarizes the transcripts detected with over expression in isolate *Pb*01, potential candidates to present roles in development/morphogenesis/virulence. A total of 3 genes, comprising 23 ESTs were reported here. TPS1 is associated to sporulation and pathogenesis of organisms including *C. neoformans* and *M. grisea* (Petzold *et al*., 2006; Foster *et al*., 2003; Wilson *et al*., 2007). The Ddr48p is essential for *C. albicans* filamentation (Dib *et al*., 2008). The NsdD transcriptional factor is necessary for the sexual development of *Aspergillus nidulans* (Han *et al*., 2001).

The putative cellular localization of the products of up and down regulated transcripts in isolate *Pb*01, yeast phase

Table 1, supplementary material describes the putative localization on *P. brasiliensis* of some predicted proteins whose transcripts were up or down regulated in isolate *Pb*01, by comparison to the isolate *Pb*4940. Interestingly, a high percentage of induced/repressed genes (7 in the total of 23) codified for proteins with predictable localization at the fungal surface.

Validation of the differentially expressed transcripts:

We used reverse northern blot analysis in order to confirm the expression of 8 transcripts. The selected transcripts were identified as up (Table 2) or down regulated (Table 3) in isolate *Pb*01 compared to isolate *Pb*4940 (Fig. 3A and B). The data are in general agreement with the ESTs observations. In this way, corroborating the EST analysis genes encoding the proteins Hsp30, NsdD, Ctf1β and Cwp were demonstrated to be up regulated in isolate *Pb*01 (Fig. 3A). The genes encoding for Aox1, Eef1α, Rps27 and Pma1 were demonstrated to be down regulated in isolate *Pb*01 (Fig. 3B).

A total of 7 genes were further examined with RT-PCR using RNA harvested from different cultures, from those used in the RDA analysis. As shown in Fig. 4 the expression profiles confirmed the previous data indicating the presence of up regulated and down regulated transcripts in the yeast established fungal phase.

Discussion

A limited number of studies have focused on the molecular basis of the yeast phase establishment in *P. brasiliensis*. Some studies have employed genomic screening approaches to study patterns of differential gene expression (Felipe *et al.,* 2003; 2005; Goldman *et al*., 2003). Those studies have

confirmed up regulation of genes in the yeast phase (Felipe *et al.,* 2003; 2005), as well as during fungal transition from mycelium to yeast (Nunes *et al*., 2005; Bastos *et al*., 2007).

In the present investigation we used subtraction of cDNAs approach to identify transcripts differentially expressed by *P. brasiliensis* in the yeast fungal phase. We hypothesized that there are genes and gene products in *P. brasiliensis* yeast phase that play a role in the successful execution of that fungal phase. Several of these genes that were over expressed in the yeast established form of isolate *Pb*01 compared to the mycelia phase depicted by the isolate *Pb*4940 at 36 ºC, are involved in stress response such as those encoding to Hsp30, Ddr48p,Tps1. Transcription factors such as those encoding for NsdD and Ctf1β were observed. Additionally a predicted cell wall/membrane protein encoding transcript was the most over expressed in the yeast fungal phase of isolate *Pb*01 in comparison to the cells of isolate *Pb*4940. Confirmation that the RDA data presented that reliably identified up or down regulated genes, was obtained through independent reverse northern blot and RT-PCR analysis of a subset of genes.

Among the up regulated transcripts in isolate *Pb*01, some had been functionally characterized in fungi. The Ddr48 protein was demonstrated to be essential in *C. albicans*, since only a heterozygote but not a homozygous null mutant was generated. Also the mutant was filamentation-defective evidencing that Ddr48p is essential to morphogenesis, as well to stress response in *C. albicans* (Dib *et al*., 2008). As might be expected we identified heat shock/stress related proteins that were preferentially expressed in the yeast fungal phase, including the Hsp30. Similar observations were previously noted in the establishment of the yeast phase in *Penicillium marneffei* (Chandler *et al*., 2008). Of special note, the stress responsive gene *hsp*30 encodes a plasma membrane protein that negatively regulates the plasma membrane ATPAse H+ (PMA1) in *Saccharomyces cerevisiae* (Piper *et al*, 1997); the transcript encoding Pma1 was down regulated in the isolate *Pb*01, yeast phase. Tps1 protein is a central regulator of infection-related processes in *M. grisea*. The importance of Tps1 to cellular differentiation and fungal virulence results from its wide ranging role as central regulator of both, sugar metabolism and nitrogen source utilization (Wilson *et al*., 2007). In this sense, the up regulation of genes encoding to acidic amino acid transporter simultaneously to the *tps1* transcript seems relevant. The presence of the NsdD gene is necessary for full accumulation of the transcript encoding for a predictable β-1,3-endoglucanase in *A. nidulans* at the fungal early sexual development, suggesting the NsdD relevance to fungal morphogenesis and cell wall remodeling (Choi *et al*., 2005).

Some of the down-regulated transcripts in the yeast phase of isolate *Pb*01, compared to *Pb*4940, encoded for stress-related proteins such as Aox1 and ClpA. It is interesting to note that the transcript encoding to the alternative oxidase homologue was one of the most prevalent in the subtracted cDNA library obtained by using the isolate *Pb*4940 as the tester. In a previous study Felipe *et al*., (2005) suggested that the metabolism of the mycelium form is more aerobic that that of yeast cells. Some works have focused on alternative oxidase's ability to prevent the production or damage of reactive oxygen

species (ROS), by limiting their mitochondrial formation (Akhter *et al*., 2003). In this sense the existence of an alternative oxidase in *P. brasiliensis* has been recently described (Martins *et al*., 2008). Also the transcript encoding for a cell wall Sed1p protein was found which constitutes the most abundant protein in stationary phase of *S. cerevisiae*, being putatively related to cell defense mechanisms in the stationary phase (Shimol *et al*., 1998). Of special note, the transcript encoding Sed1p is required to reconstruct cell wall stability in *S. cerevisiae* mutants defective in multiple GPI-anchored proteins (Hagen *et al*., 2004).

Interestingly, a great percentage of up/down regulated transcripts encoded for surface associated proteins. Studies on the synthesis and control of expression of those cell surface proteins are needed to elucidate the detailed mechanism of cell surface reorganization that occurs in *P. brasiliensis* dimorphic process.

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TABLES

Table 1 - Gene-specific primers used in PCR and RT-PCR reactions

Target Gene	Sequence (5'→ 3') direction[a]	Sequence (5'→ 3') direction[b]	Amplicon size (bp)
Glicoprotein 43 kDa (*gp43*)	GGAATGGCTTTTTGGCGC	GCTGAGCGGACAATTCGG	317
DDR48 stress protein (*ddr48*)	CCAGAGGAGCCGTGAGAG	GATTCATACGGAGCTTCTGG	318
GATA Transcription factor 1 beta (*nsdd*)	GAGGATCGATGAGCTTGCAC	CTGCCAGCCAACTTCCGG	379
C6 transcription factor (*ctf1*β)	CATCGCATGCCATAGTCCG	CCAACGCCTCACTCCCTG	314
Predicted cell wall/membrane protein (*cwp*)	GGGAGGATCAGGATAATAAAG	CATACAACCTTATCGTACTATG	294
Alternative oxidase (*aox1*)	CTGTGCTATCCGAGGGAGG	GCCGCAGAGAGTTCAGGTG	453
Plasma membrane ATPase (*pma1*)	GACCAGTGCCACCAGCGG	GGTGTCATTTGCGGTCTCC	421
Cell wall protein (*sed1p*)	CCGAGGGAGGATCACTCAC	GGACGGTGCCGTTGGTGTG	184
L34 ribosomal protein (*l34*)	CAAGACTCCAGGCGGCAAC	GCACCGCCATGACTGACG	750

[a] Forward; [b] Reverse

Table 2- *P. brasiliensis*, up regulated genes of the yeast phase of isolate *Pb*01, identified by cDNA-RDA, by comparison to the isolate *Pb*4940

Functional gene classification[a]	Homologous Gene Product	Best Hit/Accession number[b]	*E*-value	EST Redundancy in isolate *Pb*01
Metabolism				
	Alpha-trehalose-phosphate synthase (TPS1)	*Aspergillus clavatus*/ XP_001273234	1e-37	02
	Aromatic L-amino acid decarboxylase (DDC)	*Paracoccidioides brasiliensis*/ ABH03461	7e-113	03
	Acidic amino acid permease (DIP5)	*Ajellomyces capsulatus*/ XP_001543575	1e-13	02
Protein Synthesis				
	CAP 20 virulence factor (CAP20)	*Aspergillus fumigatus*/ XP_750384	8e-29	03
Cell Rescue, defense and virulence				
	30 kDa Heat Shock Protein (HSP30)	*Ajellomyces capsulatus*/ XP_001540271	1e-35	42
	DDR48 stress protein (DDR48)	*Ajellomyces capsulatus*/ XP_001539717	1e-25	03
Transcription				
	GATA transcription factor (NSDD)	*Aspergillus nidulans*/ XP_660756	7e-28	18
	C6 transcription factor 1 beta (CTF1β)	*Aspergillus nidulans*/ XP_747220	5e-41	25
Hypothetical proteins				
	Predicted Cell Wall/Membrane Protein (CWP)*	*Paracoccidioides brasiliensis**	3e-23	151
	PT repeat family protein	*Aspergillus fumigatus*/ XP_756037	2e-44	07
	Hypothetical protein	-		02

[a] Annotation based on *Saccharomyces cerevisiae* functional catalogue.
[b] Sequences identified on NCBI database (http://www.ncbi.nlm.nih.gov/blast)
* Identified by Psort prediction analysis tool (http://www.psort.org).

Table 3- *P. brasiliensis*, down regulated genes of the yeast phase of isolate *Pb*01, identified by cDNA-RDA, by comparison to the isolate *Pb*4940

Functional gene classification[a]	Homologous Gene Product	Best hit/Accession number[b]	*E*-value	EST Redundancy in isolate *Pb*4940
Energy				
	Alternative Oxidase (AOX1)	*Neurospora crassa*/ Q01355	2e-64	17
Protein Synthesis				
	Translation elongation factor 1 alpha (EEF1α)	*Hypocrea jecorina*/ P34825	2e-176	32
	40S Ribosomal protein S27 (RPS27)	*Neurospora crassa*/ XP_366796	2e-35	07
	24S Ribosomal protein	*Neurospora crassa*	4e-33	13
Protein Fate				
	Ubiquitin-conjugating enzyme E2 (RAD6)	*Podospora anserina*/ XP_001910347	2e-41	1
Cell Rescue, defense and virulence				
	Heat shock protein CLPA (CLPA)	*Paracoccidioides brasiliensis*/ AAO73810	4e-54	02
Transport facilitation				
	Plasma Membrane ATPase H+ (PMA1)	*Neurospora crassa/* AAA33563	6e-107	07
Control of cellular organization	Cell wall protein (SED1p)	*Saccharomyces cerevisiae*/AAU07726	2e-07	07
Hypothetical Protein				
	Hypothetical protein	-	-	58
	Conserved Hypothetical protein	*Gibberella zeae*/ XP_390400	1e-12	23
	Hypothetical protein	-	-	01
	Hypothetical protein	-	-	01

[a] Annotation based on *Saccharomyces cerevisiae* functional catalogue.
[b] Sequences identified on NCBI database (http://www.ncbi.nlm.nih.gov/blast)

Table 4: Candidate homologs for stress response factors induced in isolate *Pb*01

Gene product	Function in other fungi	Reference
Alpha- trehalose-phosphate synthase (TPS1)	Required for high temperature (37 ºC) growth of *Cryptococcus neoformans*	Petzold *et al*., 2006.
30-kDa heat shock protein (HSP30)	Activated by several stresses including heat shock	Piper *et al*., 1997.
DDR48 stress protein (DDR48p)	Required for *Candida albicans* resistance to hydrogen peroxide	Dib *et al*., 2008.

Table 5: Candidate homologs for morphogenesis/development/virulence factors induced in isolate *Pb*01

Gene product	Function in other fungi	Reference
Alpha- trehalose-phosphate synthase (TPS1)	Mutants *Δtps1* of *Magnaporthe grisea* are non-pathogenic and present poor sporulation; linked to sporulation in *Cryptococcus neoformans*	Foster *et al*., 2003; Wilson *et al*., 2007; Lin and Heitman, 2005.
DDR48 stress protein (DDR48p)	Essential for *Candida albicans* filamentation	Dib *et al*., 2008.
GATA transcription factor (NSDD)	Necessary for sexual development of *Aspergillus nidulans*	Han *et al*., 2001.

Supplementary Table 1: Possible distribution of the cognate products of the identified up and down regulated transcripts at the cell wall/membrane

Gene product	Reference[c]
Acidic amino acid permease[a] (Dip5)	Regenberg *et al*., 1998
30 kDa heat shock protein[a] (HSP30)	Piper *et al*., 1997
DDr48 stress protein[a] (Ddr48p)	Dib *et al*., 2008
Predicted cell wall protein[a] (Cwp)	As determined by Psort analysis
Translation elongation factor 1 α[b] (Tef1α)	Albuquerque *et al*., 2008; Rodrigues *et al*., 2008.
Plasma membrane ATPase[b] (PMA1)	Serrano ,1988
Cell wall membrane SED1p[b] (Sed1p)	Shimol *et al*., 1998.

[a] up regulated in isolate *Pb*01
[b] down regulated in isolate *Pb*01
[c] as determined in other fungi.

Figures

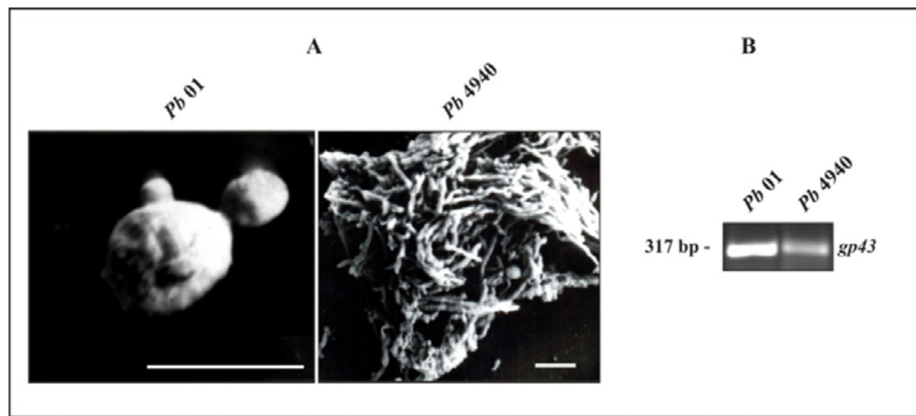


Figure 1: Morphological analysis of *P. brasiliensis* isolates and PCR amplification of the gene encoding the GP43 protein. A- The microscopic analysis was performed as described in Material and Methods. B- PCR amplification of the DNA using oligonucleotides for the gene encoding the Gp43 protein.

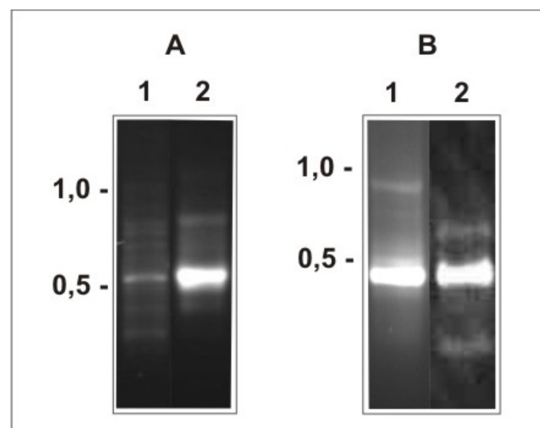


Figure 2: Agarose gel electrophoresis of subtracted differential cDNA pools derived from *P. brasiliensis*, isolates *Pb*01 and *Pb*4940, used as testers and drivers. Panel A – Products of the first and second rounds of subtraction performed using as tester the cDNA obtained from RNAs of isolate *Pb*01. Panel B - Products of the first and second rounds of subtraction performed using as tester the cDNA obtained from RNAs of isolate *Pb*4940. The numbers on the left side are molecular size markers.

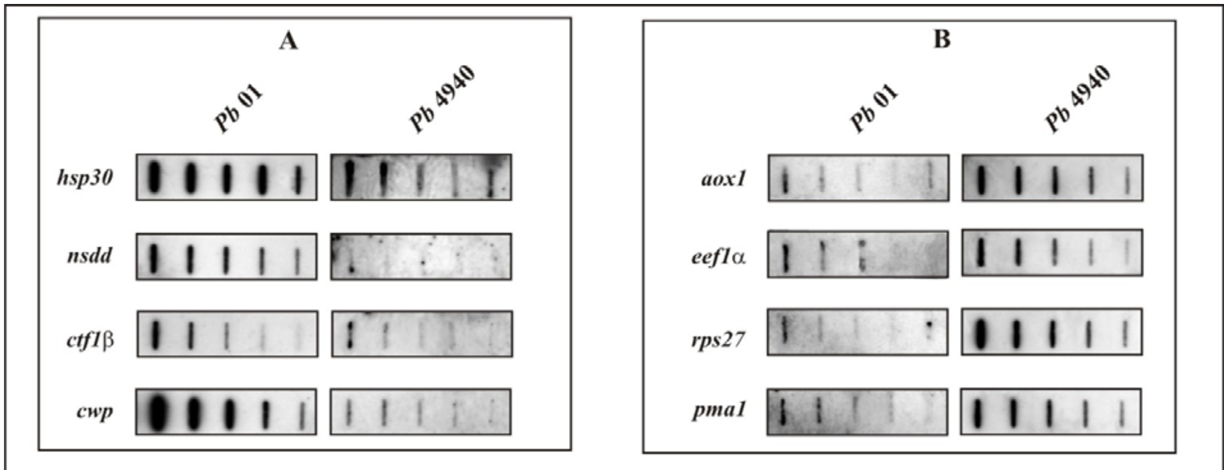


Figure 3: Validation of cDNA-RDA results. (A, B) Dot blot analysis of cDNA-RDA clones. Plasmidial DNA of individual clones was prepared and several dilutions were blotted. The individual blotted clones were hybridized to the labeled cDNAs obtained from *P. brasiliensis* isolate *Pb*01 and isolate *Pb*4940 (Panel A and B). The clones were as follows: (A) 30 kDa heat shock protein (*hsp30*), GATA transcription factor (*nsdd*), C6 transcription factor 1 beta (*ctf1*β), predicted cell wall/membrane protein (*cwp*). (B) Alternative oxidase (*aox1*), Translation elongation factor 1-alpha (*eef1-a*), 40S ribosomal protein S27 (*rps27*) and Plasma membrane ATPase H+ (*pma1*).

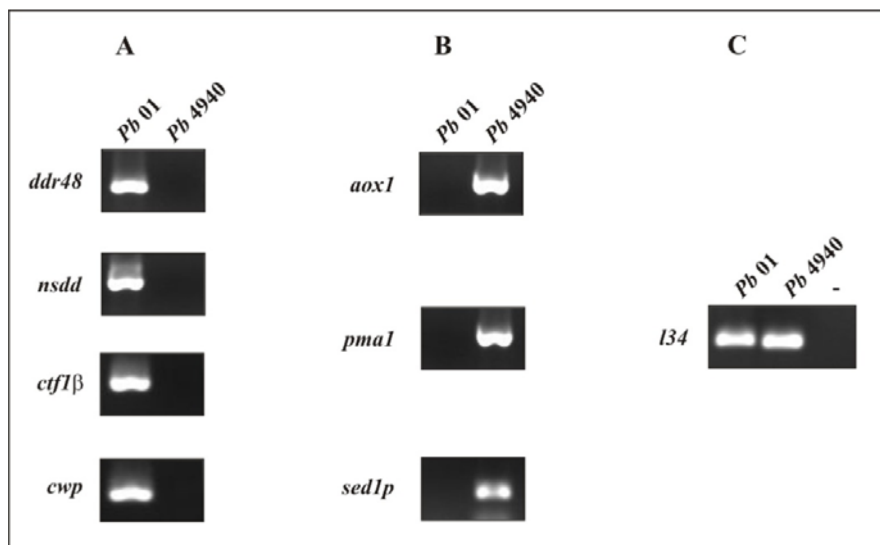
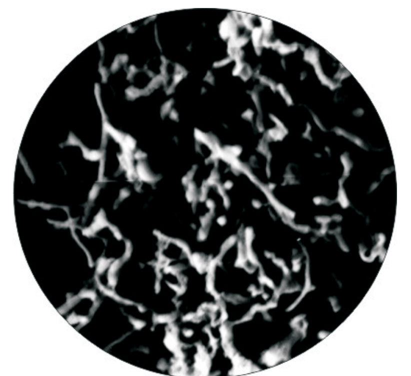


Figure 4: Validation of RDA results by RT-PCR. RT-PCR analysis was carried out with specific primers, as described. The same amounts of cDNAs were used for all PCR reactions. The RNAs used for RT-PCR were obtained from independent samples, from those used for the RDA experiments. Clones names are written on the left side of the figure, and were as follows: Ddr48 stress protein (*ddr48*); GATA transcription factor (*nsdd*), C6 transcription factor 1 beta (*ctf1*β), Predicted cell wall/membrane protein (*cwp*). (B) Alternative oxidase (*aox1*), Plasma membrane ATPase H+ (*pma1*) and Predicted cell wall protein (*sed1p*). (C) L34 ribosomal protein as a positive control and the negative control is indicated (-).

Discussão



Análises transcricionais no estudo de genes diferencialmente expressos em *P. brasiliensis*

Discussão

O fungo patogênico humano *P. brasiliensis* é um fungo termodimórfico, sendo que a transição de micélio para levedura é etapa importante para o estabelecimento da infecção (San-Blas *et al*., 2002). Genes estágio específicos e genes diferencialmente expressos durante a transição dimórfica têm sido objeto de estudo de vários grupos de pesquisadores (Salem-Izacc *et al*., 1997; Felipe *et al*., 2003; Goldman *et al.* 2003; Marques *et al.,* 2004; Nunes *et al.* 2005; Bastos *et al*., 2007). Alguns estudos têm ilustrado as bases moleculares da fase parasitária do fungo.

Neste trabalho, realizamos ensaios de RDA para a identificação de genes diferencialmente expressos em isolados que apresentam ou não a habilidade de transitar da forma miceliana para a forma leveduriforme. O nosso objetivo foi identificar genes relevantes para a transição dimórfica e para o estabelecimento/manutenção da forma parasitária de *P. brasiliensis*.

Genes relacionados à resposta a estresse foram induzidos no isolado *Pb*01 (forma leveduriforme) em relação ao isolado *Pb*4940 (forma miceliana), ambos cultivados *in vitro* a 36 ºC. Proteínas de choque térmico são induzidas em resposta à mudança de temperatura, tanto *in vitro* quanto no hospedeiro. O gene codificante da HSP30 foi induzido no isolado *Pb*01. A produção de HSPs está envolvida tanto na proteção celular quanto em reparos de danos relacionados ao estresse, além de outras funções importantes na divisão celular, transcrição, tradução de proteínas e transporte protéico (Mager & Ferreira, 1993; Liberek *et al*., 2008). No transcritoma de *P. brasiliensis* isolado *Pb*01, 51 ESTs codificantes para a proteína HSP30 foram identificadas (Felipe *et al*., 2005). O transcrito *hsp30* foi identificado com alta redundância em células leveduriformes de *P. brasiliensis* recuperadas de fígado de camundongo infectado (Bailão *et al*., 2006; Costa *et al*., 2007); a proteína foi descrita como importante no estabelecimento da fase leveduriforme em *Penicillium marneffei* (Chandler *et al*., 2008). A HSP30 se localiza na membrana plasmática e está relacionada à modulação da fluidez e do estado físico da membrana em situações de choque térmico em bactérias (Seymour & Piper, 1999; Coucheney *et al*., 2005), além de regular negativamente a proteína de membrana plasmática PMA1 em *S. cerevisiae* (Piper *et al*., 1997). Essa descrição tem

relevância, pois corrobora aos nossos resultados, uma vez que a ATPase H+ de membrana plasmática (Pma1) foi descrita como regulada negativamente no isolado *Pb*01.

O transcrito que codifica para a DDR48p, uma proteína relacionada ao estresse, foi induzido no isolado *Pb*01. A proteína DDR48 é descrita como essencial em *C. albicans*, uma vez que somente mutantes heterozigóticos são formados. Mutantes homozigóticos não são formados. Além disso, mutantes são defectivos na filamentação e na resposta ao estresse em *C. albicans* (Dib *et al*., 2008).

O transcrito que codifica para a alfa-trealose fosfato sintase (TPS1) foi induzido no isolado *Pb*01. Em *M. grisea*, TPS1 é uma proteína central reguladora dos processos de infecção do fungo, sendo importante para a diferenciação celular e desempenha um papel global na regulação do metabolismo de carboidratos e na utilização de fontes de nitrogênio (Wilson *et al*., 2007). Essa descrição parece relevante, uma vez que o transcrito que codifica para a DIP5 está induzido no isolado *Pb*01, onde ambas DIP5 e TPS1 devem participar do metabolismo de nitrogênio. Ainda com relação à DIP5, o transcrito cognato é super expresso em várias condições experimentais de infecção (Bailão *et al*., 2006; 2007), assim como durante a transição micélio-levedura no isolado *Pb*01 (Bastos *et al.,* 2007). A DIP5 está possivelmente relacionada à captação de glutamato, precursor da síntese de glutamina. A quitina é detectada em ambas as formas do fungo apresentando um maior teor em formas leveduriformes quando comparado a micélio (San-Blas *et al.* 1987, Kurokawa *et al.* 1998). A expressão maior do transcrito *dip5* no isolado *Pb*01 pode estar relacionada à maior deposição de quitina na forma parasitária do fungo.

O transcrito que codifica para a proteína decarboxilase para aminoácidos aromáticos (DDC) foi induzido no isolado *Pb*01. A síntese de melanina está implicada na patogênese de fungos (Hamilton & Gomez 2002, Taborda *et al*., 2008). O crescimento de *P. brasiliensis* com L-DOPA, resulta na melanização das células fúngicas (Gomez *et al*., 2001), bem como a melanina protege *P. brasiliensis* de fagocitose (Silva *et al*., 2006). Além disso, o transcrito que codifica para a DDC foi induzido em células de *P. brasiliensis* recuperadas de fígado de camundongo infectado, corroborando a idéia que a DDC deva ser importante na fase leveduriforme de *P. brasiliensis*, especialmente durante o processo infectivo (Bailão *et al*., 2006; Costa *et al*., 2007).

Foi descrito também no isolado *Pb*01 o transcrito que codifica para o fator de transcrição Nsdd, o qual está relacionado à formação correta de hifas e com a reprodução sexual de ascomicetos. A presença do gene que codifica para NSDD é necessária para a acumulação do transcrito que codifica para uma provável β-1,3-endoglucanase em *A. nidulans*,

sugerindo que NSDD é relevante para morfogênese e para o remodelamento da parede celular (Choi *et al*., 2005). Além disso, o fator de transcrição foi induzido nos estágios iniciais de diferenciação de micélio para levedura (Bastos *et al*., 2007), sugerindo sua importância no processo de diferenciação para a fase leveduriforme.

Foram identificados transcritos regulados negativamente em *Pb*01, quando comparado com *Pb*4940. Foi identificado o transcrito que codifica para a proteína AOX1. Na forma miceliana, *P. brasiliensis* parece apresentar metabolismo predominantemente aeróbico, quando comparado à forma leveduriforme (Felipe *et al*., 2005). Embora a função da AOX1 não tenha sido definida em *P. brasiliensis*, em *C. neoformans*, a proteína auxilia na destruição ou na prevenção da produção de espécies reativas de oxigênio (ROS), limitando sua formação mitocondrial (Akhter *et al*., 2003). Ressalte-se que função de uma oxidase alternativa foi recentemente descrita em *P. brasiliensis* (Martins *et al*., 2008)**.**

O transcrito que codifica para a SED1p, uma provável proteína de membrana, foi regulado negativamente em *Pb*01. Em *S. cerevisiae*, SED1p é a proteína mais abundante descrita na fase estacionária do fungo, possivelmente relacionada a mecanismos de defesa, importantes nessa fase (Shimol *et al*., 1998). Além disso, SED1p é requerida para a reconstrução da parede celular em linhagens mutantes para proteínas GPI-ancoradas de *S. cerevisiae* (Hagen *et al*., 2004).

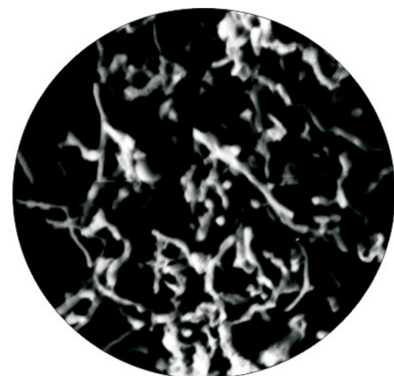
Os resultados descritos sugerem que alguns dos genes identificados, possivelmente devem estar relacionados com a transição dimórfica e/ou a manutenção de *P. brasiliensis* na fase leveduriforme. Além disso, vários dos transcritos identificados, estão entre os identificados em situações que mimetizam nichos do hospedeiro (Bailão *et al*., 2006; 2007; Costa *et al*., 2007) e durante os estágios iniciais de diferenciação de micélio para levedura (Bastos *et al*., 2007). Transcritos que possivelmente codificam para proteínas hipotéticas, foram encontrados como induzidos nos dois isolados. Em *Francisella tularensis*, uma bactéria patogênica humana, a análise diferencial de genes expressos a 36 ºC revelou uma alta expressão de proteínas hipotéticas possivelmente relacionadas com a patogênese e virulência (Horzempa *et al*., 2008). Além disso, uma grande porcentagem de transcritos induzidos positiva/negativamente codificam para proteínas associadas à superfície celular. Estudos relacionados a essas moléculas são necessários para elucidar os mecanismos de organização da parede/membrana celular de *P. brasiliensis*, possivelmente relacionados à transição dimórfica. A comprovação experimental e o melhor entendimento dos papéis desempenhados pelos transcritos identificados deverão ser objeto de estudo, o que requer o desenvolvimento de ferramentas genéticas eficientes para estudos funcionais em *P. brasiliensis*.

Conclusões

- *P. brasiliensis* apresentou expressão diferencial de genes entre os dois isolados analisados (*Pb*01 e *Pb*4940), que transitam ou não da forma miceliana para a forma leveduriforme por alteração da temperatura. Os resultados evidenciam que a técnica de RDA pode ser utilizada com eficiência na identificação de genes diferencialmente expressos;
- Transcritos possivelmente relacionados à transição dimórfica e/ou estabelecimento de fase leveduriforme foram identificados, como por exemplo: proteínas relacionadas ao estresse, à membrana/parede celular e metabolismo de nitrogênio e carboidratos;
- Transcritos não identificados em bancos de dados e altamente expressos, reforçam o conhecimento ainda incipiente sobre os mecanismos moleculares relacionados à transição dimórfica e o estabelecimento de fases em *P. brasiliensis*.

Capítulo IV

Artigo com autoria compartilhada



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Transcriptome profiling of *Paracoccidioides brasiliensis* yeast-phase cells recovered from infected mice brings new insights into fungal response upon host interaction

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Paracoccidioides brasiliensis is a fungal human pathogen with a wide distribution in Latin America. It causes paracoccidioidomycosis, the most widespread systemic mycosis in Latin America. Although gene expression in *P. brasiliensis* had been studied, little is known about the genome sequences expressed by this species during the infection process. To better understand the infection process, 4934 expressed sequence tags (ESTs) derived from a non-normalized cDNA library from *P. brasiliensis* (isolate *Pb*01) yeast-phase cells recovered from the livers of infected mice were annotated and clustered to a UniGene (clusters containing sequences that represent a unique gene) set with 1602 members. A large-scale comparative analysis was performed between the UniGene sequences of *P. brasiliensis* yeast-phase cells recovered from infected mice and a database constructed with sequences of the yeast-phase and mycelium transcriptome (isolate *Pb*01) (https://dna.biomol.unb.br/Pb/), as well as with all public ESTs available at GenBank, including sequences of the *P. brasiliensis* yeast-phase transcriptome (isolate *Pb*18) (http://www.ncbi.nlm.nih.gov/). The focus was on the overexpressed and novel genes. From the total, 3184 ESTs (64.53 %) were also present in the previously described transcriptome of yeast-form and mycelium cells obtained from *in vitro* cultures (https://dna.biomol.unb.br/Pb/) and of those, 1172 ESTs (23.75 % of the described sequences) represented transcripts overexpressed during the infection process. Comparative analysis identified 1750 ESTs (35.47 % of the total), comprising 649 UniGene sequences representing novel transcripts of *P. brasiliensis*, not previously described for this isolate or for other isolates in public databases. KEGG pathway mapping showed that the novel and overexpressed transcripts represented standard metabolic pathways, including glycolysis, amino acid biosynthesis, lipid and sterol metabolism. The unique and divergent representation of transcripts in the cDNA library of yeast cells recovered from infected mice suggests differential gene expression in response to the host milieu.

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†These authors contributed equally to this work.

Abbreviations: EST, expressed sequence tag; KEGG, Kyoto Encyclopedia of Gene and Genomes; sqRT-PCR, semiquantitative RT-PCR.

The GenBank/EMBL/DDBJ accession numbers for the ESTs of *Paracoccidioides brasiliensis* identified in this study are EST1487–EST6420.

Two supplementary tables listing the overexpressed and novel genes identified during this study and supplementary material describing the EST dataset analysed are available with the online version of this paper.

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INTRODUCTION

The dimorphic pathogenic fungus *Paracoccidioides brasiliensis*, the aetiological agent of paracoccidioidomycosis, undergoes a complex transformation; the fungus switches from the mycelial infective form, growing at environmental temperatures, to the yeast form, growing at the mammalian host temperature. The fungus is a pathogen that infects around 10 million individuals in the regions where it is endemic, distributed from Mexico to Argentina (Restrepo *et al.*, 2001). During infection, the host inhales spores from the mycelial form that convert to the budding-yeast form within hours. The disease is characterized by a chronic granulomatous inflammation, and patients may present a broad spectrum of clinical manifestations (Montenegro & Franco, 1994).

Analysis of the response of *P. brasiliensis* during infection provides a window into the alterations required for the organism to survive in the host milieu. Transcriptional profiles of fungal cells, as well as the relative expression of transcripts in each *P. brasiliensis* phase, have been examined previously (Felipe *et al.*, 2003; Goldman *et al.*, 2003; Marques *et al.*, 2004; Felipe *et al.*, 2005). Transcriptional responses to temperature, mimicking the events of differentiation upon fungal inhalation by the host, have also been studied (Nunes *et al.*, 2005; Bastos *et al.*, 2007). Regarding the isolate *Pb*01, the subject of the present work, previous *in silico* electronic subtraction and cDNA microarray studies have provided a view of the fungal metabolism, demonstrating upregulated transcripts and differential expression patterns in yeast phase and mycelium (Felipe *et al.*, 2005).

We have been studying differentially expressed genes in *P. brasiliensis* yeast-form cells upon exposure to host-like conditions. We have previously investigated, by cDNA-representational difference analysis (cDNA-RDA), the genes overexpressed by *P. brasiliensis* upon infection in a mouse model, as well as upon incubation of yeast cells with human blood (Bailão *et al.*, 2006). Genes putatively related to fungal transport, cell defence and cell wall synthesis/remodelling were particularly upregulated under the host-like conditions analysed. In the present work we sought to amplify our studies of genes potentially related to fungal–host interaction by analysing the transcriptome of yeast-phase cells recovered from livers of infected mice. We analysed 4934 expressed sequence tags (ESTs) generated from a cDNA library. Novel genes as well as upregulated genes, compared to the *in vitro* transcriptome (https://dna.biomol.unb.br/Pb/) and to the GenBank (http://www.ncbi.nlm.nih.gov/) ESTs, provided insights into metabolic adaptations performed by *P. brasiliensis* during infection. The yeast-phase cells significantly overexpress genes related to glycolysis and ethanol production, fatty acid synthesis and nitrogen metabolism, suggesting a nutrient-rich microenvironment. The overproduction of transcripts from genes represented by these pathways also indicates metabolically active fungal cells that can utilize carbohydrate, lipid and nitrogen sources to generate the necessary compounds and energy for carrying on cellular processes or responding to the surrounding microenvironment.

METHODS

Maintenance of *P. brasiliensis* and animal infection. *P. brasiliensis* (ATCC MYA-826) was grown for 7 days in BBL Mycosel Agar (Becton Dickinson), supplemented with 10 % fetal calf serum, at 36 °C for the yeast phase (control cells). Infection of mice was performed as previously described (Bailão *et al.*, 2006). *P. brasiliensis* yeast-phase cells were harvested from 7-day-old cultures, suspended in sterile PBS (7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.137 mM NaCl, 2.7 mM KCl, pH 7.4). Male B.10A mice, 8–12 weeks old, were infected intraperitoneally with 5×10^6 yeast-phase cells. Animals were sacrificed 7 days after infection; livers were removed and homogenized in 5 ml sterile PBS. The cellular suspensions were washed three times, centrifuged at 1000 ***g*** and resuspended in 1 ml PBS. Aliquots (100 μl) of the suspension were plated onto BBL Mycosel Agar, supplemented with 10 % fetal calf serum. After 14 days incubation, the cells were recovered and total RNA was extracted. Procedures involving animals and their care were conducted in conformity with the rules of the local ethics committee and international recommendations. Control yeast-phase cells and those recovered from infected tissue were used for RNA extraction.

RNA extractions. Total RNA was extracted under all experimental conditions by the use of Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNAs were used to construct double-stranded cDNAs.

Construction of the cDNA library. The *P. brasiliensis* cDNA library was constructed following the protocols of the SUPERSCRIPT plasmid system with GATEWAY technology for cDNA synthesis and cloning (Invitrogen).

DNA sequencing. The cDNA library was plated to approximately 200 colonies per plate (150 mm Petri dish). The colonies were randomly selected and transferred to a 96-well polypropylene plate containing LB medium and grown overnight. Plasmid DNA was isolated and purified. cDNA inserts were sequenced from the 5′ end by employing a standard fluorescence labelling DYEnamic ET dye terminator kit (Amersham Biosciences) with the M13/pUC flanking vector primer. Automated sequence analysis was performed in a MegaBACE 1000 DNA sequencer (GE Healthcare).

EST processing pipeline, annotation and differential expression analysis. EST sequences were 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 a Phred quality greater than or equal to 20 were considered for further analysis. ESTs were screened for vector sequences against the UniVec data. The resulting sequences were uploaded to a relational database (MySQL) on a Linux (Fedora) platform, and processed using a modified version of the PHOREST tool (Ahren *et al.*, 2004). The filtered sequences were compared against the GenBank non-redundant (nr) database from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), the Gene Ontology database (http://www.geneontology.org/GO) and InterPro's databases of protein families (http://www.ebi.ac.uk/InterProScan/). The Munich Information Center for Protein Sequences (MIPS) (http://mips.gsf.de/) database was used to assign functional categories and Kyoto Encyclopedia of Gene and Genomes (KEGG) (http://www.kegg.com/) was used to assign Enzyme Commission (EC) numbers and metabolic pathways.

The database sequence matches were considered significant at *E*-values $\leqslant 10^{-5}$. The clusters were compared to the *P. brasiliensis* transcriptome database (https://dna.biomol.unb.br/Pb/), to select novel and overexpressed genes. For the description of novel genes, sequences were also compared to sequences in GenBank (http://www.ncbi.nlm.nih.gov/) that also included the ESTs reported previously by Goldman *et al.* (2003) available in the NCBI database. BLASTX analysis (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1997) was used to find matching sequences with *E*-values $\leqslant 10^{-5}$. With CAP3 assembly (Huang & Madan, 1999) information stored in the relational database, SQL queries were performed to identify transcripts unique to a specific EST library and/or present in two or more libraries. We have constructed a database to host all the sequence data and the analysis results obtained from this study. The database can be accessed through a web interface at http://www.lbm.icb.ufg.br/phorestwww/index.php. All the ESTs were submitted to GenBank under accession numbers EST1487–EST6420.

***In silico* determination of upregulated genes.** To assign a differential expression character, the contigs formed with mycelium, control yeast-phase cells and yeast-form cells recovered from infected mice ESTs were statistically evaluated using the method of Audic & Claverie (1997). Overexpressed genes, compared to the *P. brasiliensis* transcriptome database (https://dna.biomol.unb.br/Pb/), were determined with a 95 % confidence rate.

Infection of Vero cells with *P. brasiliensis*. Cultures of Vero cells (ATCC CCL81) were maintained in Medium 199 (Sigma–Aldrich) supplemented with 10 % (v/v) heat-inactivated fetal calf serum. The cells were washed three times in 199 medium and 10^8 yeast-form cells of *P. brasiliensis* were added and incubated for 48 h at 36 °C, as described previously (Mendes-Giannini *et al.*, 2006). The cells were washed three times in PBS followed by incubation in PBS containing trypsin (0.2 %) and EDTA (0.02 %) for 30 min for total monolayer removal. The cells were centrifuged (1400 ***g***, 5 min) and the pellet was recovered for further RNA extraction.

PCR analysis of genomic DNA of *P. brasiliensis*. The presence of novel genes was initially assayed by PCR of genomic DNA of *P. brasiliensis* yeast-form cells, prepared according to standard methods. PCR of selected genes was performed with specific sense and antisense primers, as described in Table 1.

Semiquantitative RT-PCR analysis of *P. brasiliensis* regulated genes. Total RNAs were obtained from control yeast-form cells from a different batch of infected animals to those used for the construction of the cDNA library and from fungal yeast forms infecting Vero cells. Single-stranded cDNAs were synthesized. PCRs were performed using cDNAs as templates in 30 μl reaction mixtures containing specific primers (Table 1). PCR conditions were: 95 °C for 1 min, 95 °C for 30 s, annealing at 55–65 °C for 2 min, 25–35 cycles at 72 °C for 1 min, final extension at 72 °C for 7 min. The annealing temperature and the number of PCR cycles were optimized for each experimental condition to ensure exponential amplification in semiquantitative RT-PCR (sqRT-PCR) analysis. Amplicons were analysed by agarose gel electrophoresis (1 %). The analyses of relative differences were performed using Scion Image Beta 4.03 software (http://www.scioncorp.com/pages/scion_image_windows.htm).

RESULTS

Overview of ESTs from *P. brasiliensis* yeast-form cells recovered from infected mice

The purpose of this study was to identify a set of upregulated genes, as well as novel genes, expressed by *P. brasiliensis* in a mouse model of infection, as a first step towards a large-scale screen for genes associated with fungal pathogenesis. A total of 4934 high quality sequences were obtained and used to establish an EST database consisting of 1602 unique sequences from *P. brasiliensis* yeast-phase cells recovered from livers of infected mice (http://www.lbm.icb.ufg.br/phorestwww/index.php). A total of 1172 sequences (23.75 % of the total) corresponded to overexpressed sequences when compared to the transcriptome of *in vitro*-cultivated *P. brasiliensis* yeast-form cells. A total of 1750 sequences (35.47 % of the total) had no homology to sequences found previously in *P. brasiliensis,* as demonstrated by comparative analysis to the ESTs from the *P. brasiliensis* transcriptomes described elsewhere (Felipe *et al.*, 2005; Goldman *et al.* 2003). All sequences were arranged into

Table 1. Oligonucleotide primers used in PCR and or sqRT-PCR

Sequence name	Forward primer (5′→3′)	Reverse primer (5′→3′)	Size of amplified product (bp)
Indigoidine synthase A-like protein (*indA*)	ATAGCCGACCTGACTGAACT	CCCTCTCTTGAATGCCGTAT	323
Oligopeptide transporter protein (*opt*)	CAAGCGACTGGAGCAACCGA	CTGCGTTGTGTATTGAAGCCG	228
Rho guanyl nucleotide exchange factor (*gef*)	TCTCCCAAACGCTGAACACT	ATCAATCGTCCAGAGGGTAG	325
Oxidation resistance 1 protein (*oxr1*)	TCCCAGTCCGAATCTCAATC	CTGCTCGCAAATGCCTTACA	410
Glucokinase (*glk*)	GGTCTGGCGTAAATGTGCAC	GGCTGGTGAATTTGTATCGC	368
Carbonic anhydrase (*ca*)	ACACGGGACGAAAGCACTAT	AAACCTGCTGGCATTGTGGC	322
Myosin 2 isoform (*myo2*)	TGGCGAAATCATGAAAGCGG	GGCGGGCACAGCATGGTAA	291
Telomerase reverse transcriptase (*tert*)	TGGGAACATCATCGACACGT	GGCTGCCATAGTCCGAATAA	343
Poly(A) polymerase 1 (*pap1*)	TCGCGATCCCATACAACCTT	GACGAGTTGGACCTTCACCT	345
Orotate phosphoribosyltransferase (*ura5*)	CAGCTGCAGTCGTTACAACA	GGGTTGGAGGAGAGGAAAG	249
Patatin-like serine hydrolase (*pat*)	GGATCATGTGTCTGCGCTAC	GGGAAGAGATCGATTTGAGG	468
Squalene synthase (*erg9*)	GCTGACTATTGCCGAAAGG	GTTCGAGGGTTGCAATGGC	460
Ribosomal L34 protein (*l34*)	ATTCCTGCCCTCCGACCC	CCCGCCATTCTCGTCCCGC	750
Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*)	CAGACAGCTGCATCTTCT	TCTCTCTCTTCCTCTTGCG	1106

1041 contigs and 561 singletons that represented different transcripts. The complete dataset is available as supplementary material with the online version of this paper.

Functional annotation and analysis of sequences

The EST sequences were compared to the non-redundant database from NCBI using the BLASTX algorithm (Altschul *et al.*, 1997). ESTs and UniGenes (clusters containing sequences that represent a unique gene) were given a putative assignment according to the classification developed by MIPS (Fig. 1). The major MIPS categories represented included metabolism, cellular transport, energy, cell cycle and DNA processing, cell rescue and virulence, protein synthesis and protein fate. A high proportion of the ESTs (41.12 %) exhibited sequence similarity only to genes of unknown function or encoding hypothetical proteins, which may reflect the specialization of these structures in *P. brasiliensis*.

Identification of overexpressed genes by *in silico* EST subtraction and of novel transcripts in yeast-form cells recovered from infected mice

To select transcripts upregulated during the infection process of *P. brasiliensis* isolate *Pb*01, we performed comparative analysis of the ESTs with the transcriptome database generated previously with *in vitro*-grown mycelium and yeast-phase cells (Felipe *et al.*, 2005). The distribution of the overexpressed ESTs, representing 1172 sequences, is presented in Supplementary Table S1, available with the online version of this paper. Analysis of the MIPS categories showed a statistically significant difference between the *in vitro*-cultured cells and the infectious library for several biological processes. The results indicated that the overexpressed genes identified by comparative analysis encoded enzymes from several metabolic pathways, transcription factors and membrane transporters, among other protein. The data illustrate the functional diversity of these overexpressed ESTs, with particular functional categories dominating the analysis.

To identify novel transcripts expressed during the infection process of *P. brasiliensis* we performed comparative analysis of the generated ESTs (http://www.lbm.icb.ufg.br/phorestwww/index.php) with the transcriptome database (https://dna.biomol.unb.br/Pb/) and with the ESTs and complete sequences in GenBank (http://www.ncbi.nlm.nih.gov/). The distribution of the novel ESTs, representing 1750 sequences, is presented in Supplementary Table S2, available with the online version of this paper. The ESTs were classified into 16 groups of functionally related genes, with sequences encoding enzymes involved in cell metabolism

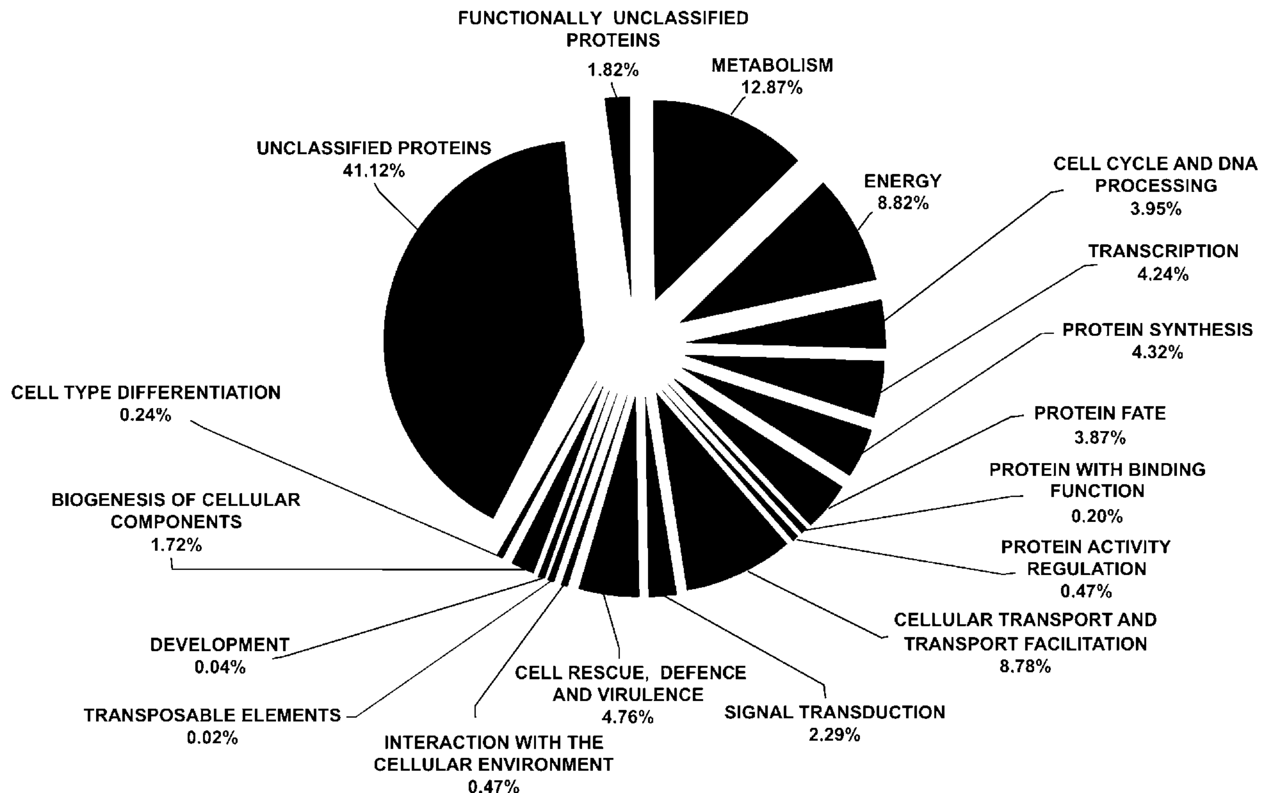

Fig. 1. Overview of ESTs from the *P. brasiliensis* transcriptome. Classification of the ESTs based on *E*-value and according to the functional categories of the MIPS functional annotation scheme.

and energy, transport facilitators and transcription factors dominating the analysis.

Highly redundant genes

Table 2 lists the 50 most abundant ORFs in the EST collection in the present transcriptome. A total of 50 contigs containing 892 ESTs were highly redundant. This accounted for 18.08 % of the total high-quality ESTs. The minimum number of ESTs that made up these most highly redundant contigs was 10. A large number of ESTs encoded membrane transporters, stress-related proteins, molecules related to nitrogen metabolism or enzymes of carbohydrate and lipid metabolism. Most of the abundant transcripts represent overexpressed genes. Included in the highly abundant transcripts are some that have been described previously as upregulated in yeast-form cells when compared to mycelia, such as those encoding alcohol dehydrogenase, aromatic-L-amino acid decarboxylase and isocitrate lyase (Felipe *et al.*, 2005). The presence of novel *P. brasiliensis* genes in the highly abundant category, such as those encoding carbonic anhydrase and glucokinase, was noted.

Overview of *P. brasiliensis* infective transcriptome: pathway analysis based on the KEGG classification

EC numbers were used to judge which sequences pertained to a specific pathway. A total of 320 unique sequences including 265 contigs and 55 singlets accounted for 19.97 % of unique sequences and matched enzymes with an EC number. The distribution of novel and upregulated ESTs was examined (Table 3). Based on the KEGG classification, it was observed that novel and overexpressed transcripts were predominantly involved in carbohydrate, amino-acid, energy and lipid metabolism.

Most relevant aspects of metabolic pathways upregulated during the infection process

Analysing the data presented in Tables 1 and 2, and supplementary material, some insights were obtained into the metabolic features of *P. brasiliensis* yeast-form cells during the infection process. The metabolic features are summarized in Table 4. Among the processes that appear to be increased in yeast-form cells during the infection process, carbohydrate, lipid and nitrogen metabolism showed the most significant changes.

Metabolism of carbohydrates

Homologues of nine genes related to carbohydrate synthesis/degradation were identified as novel or overexpressed in the UniGene set (Table 4). Transcripts encoding acylphosphatase, quinoprotein alcohol dehydrogenase, glucokinase and phosphoglycerate mutase, potentially related to the anaerobic metabolism of glucose, were identified. Glycogen phosphorylase I might be required for the mobilization of glycogen, providing glucose for energy production. D-Ribose-5-phosphate ketol isomerase would allow oxidative reactions to continue by the production of hexose phosphates. Systems for the transport of sugars, MFS1, MFS2 and PTS, are also overexpressed, putatively providing additional fuel for the oxidative reactions.

Lipid metabolism

Genes involved in lipid metabolism that were overexpressed or represented novel transcripts in *P. brasiliensis* are summarized in Table 4. The overexpressed malic enzyme is required for the transport of acetyl groups to the cytosol and provides NADPH for lipid synthesis. Carbonic anhydrase, which could provide bicarbonate for the synthesis of malonyl-CoA by acetyl-CoA carboxylase and is a key regulatory enzyme in fatty acid metabolism, is overexpressed during the infection process. Fatty acyl CoA synthase is also overexpressed in the transcriptome analysed, reinforcing the suggestion of active synthesis of lipids by yeast cells during infection.

The synthesis/remodelling of membrane components, including ergosterol, might be induced. Transcripts encoding MBOAT, a putative acetyltransferase involved in phospholipid biosynthesis/remodelling, a patatin-like protein with putative phospholipase A_2 activity and a phospholipase A1 are overexpressed under infection conditions. Delta-9 fatty acid desaturase (*Ole1*), an overexpressed gene, could introduce a double bond into saturated fatty acyl-CoA substrates, giving rise to monounsaturated fatty acids. The transcript encoding sterol *C*-methyltransferase, ERG6, which is related to the biosynthesis of ergosterol, is upregulated; a novel transcript encoding a homologue of squalene synthase, ERG 9, catalysing the first committed step in the sterol biosynthesis pathway, was also detected. The synthesis of sphingolipids could be increased by overexpression of delta-8-sphingolipid desaturase.

TCA and the glyoxylate cycle and energy production

The glyoxylate cycle could be induced in yeast-form cells during the infection process. The isocitrate lyase gene is upregulated. Genes with functions associated with the glyoxylate cycle were also induced, such as the gene encoding hydroxymethyl glutaryl-CoA lyase, which could provide acetyl-CoA. The transport of acetyl-CoA into the mitochondria might be upregulated. Carnitine acetyltransferase and carnitine/acylcarnitine translocase are required for the transport of acetyl-CoA from the peroxisomes into the mitochondria. Components of the classical pathway of oxidative phosphorylation are also induced.

Nitrogen metabolism

Comparison of our EST data with KEGG revealed that many overexpressed transcripts encode proteins that are

Table 2. Identification of the highly abundant clusters (⩾10 reads) of *P. brasiliensis* transcripts

50 ORFs representing the highest number of ESTs in the cDNA library are listed.

Gene product	Best hit/Accession no.	*E*-value	EC no.	Redundancy	Metabolic role
ADP-ribosylation factor (ARF)	*Ajellomyces capsulata*/D49993	4e–67	–	13	Protein trafficking in the Golgi apparatus
ADY2 – protein essential for the acetate permease activity	*Aspergillus nidulans*/XP_409363.1	3e–47	–	12	Acetate transmembrane transport
Coatomer zeta subunit†	*Aspergillus nidulans*/XP_410217.1	1e–67	–	14	Protein transport to Golgi
Copper transport protein*	*Aspergillus nidulans*/XP_407254.1	4e–56	–	55	Copper transport
GTP-binding protein of the Rab family (YPT1)	*Neurospora crassa*/gi|384298	1e–22	–	10	ER to Golgi secretory pathway
High-affinity methionine permease*	*Yarrowia lipolytica*/XP_505883.1	8e–52	–	11	Methionine transport
Lipocalin-1-interacting membrane receptor (LMBR1L)*	*Aspergillus nidulans*/XP_408348.1	2e–36	–	12	Transport of small hydrophobic molecules
MFS peptide transporter (PTR2)*	*Aspergillus nidulans*/XP_407545.1	3e–63	–	14	Peptide transport
Mitochondrial succinate–fumarate transporter*	*Aspergillus nidulans*/XP_411424.1	9e–28	–	15	Succinate and fumarate transport
Heat-shock protein 30 (HSP30)	*Aspergillus oryzae*/BAD02411.1	5e–47	–	18	Stress related
Heat-shock protein 70 (HSP70)	*Paracoccidioides brasiliensis*/AAK66771.1	6e–74	–	16	Stress related
Heat-shock protein 90 (HSP90)	*Paracoccidioides brasiliensis*/AAX33296.1	0.0	–	10	Stress related
Heat-shock-inducible inhibitor of cell growth (HMF1)*	*Aspergillus nidulans*/XP_413217.1	6e–46	–	14	Stress related
Rho1 GTPase*	*Paracoccidioides brasiliensis*/AAQ93069.2	2e–78	–	13	Stress related
3-Isopropylmalate dehydrogenase*	*Aspergillus nidulans*/gi|50083229	2e–80	1.1.1.85	10	Nitrogen metabolism/Leucine biosynthesis
Aromatic-L-amino-acid decarboxylase (DDC)	*Gibberella zeae*/XP_385471.1	5e–46	4.1.1.28	23	Nitrogen metabolism/Melanin biosynthesis
Cystathionine beta-synthase (CYS4)*	*Aspergillus nidulans*/XP_409957.1	9e–87	4.2.1.22	11	Nitrogen metabolism/Cysteine biosynthesis
Formamidase	*Paracoccidioides brasiliensis*/gi|47118080	3e–94	3.5.1.49	10	Nitrogen metabolism/Production of ammonia
Glutamine synthetase*	*Aspergillus nidulans*/XP_408296.1	3e–64	6.3.1.2	11	Nitrogen metabolism/Glutamine biosynthesis
Homocitrate synthase*	*Aspergillus fumigatus*/XP_751780.1	0.0	2.3.3.14	26	Lysine biosynthesis
Alcohol dehydrogenase I	*Neurospora crassa*/gi|7800883	2e–47	1.1.99.8	27	Anaerobic respiration
Glucokinase†	*Escherichia coli*/NP_288958.1	9e–82	2.7.1.2	45	Carbohydrate metabolism/Glycolysis
Phosphoglycerate mutase*	*Aspergillus nidulans*/XP_406010.1	1e–40	5.4.2.1	13	Carbohydrate metabolism/Glycolysis
Isocitrate lyase 2*	*Paracoccidioides brasiliensis*/AY350913.2	7e–51	4.1.3.1	13	Glyoxylate cycle
Chitinase family 18*	*Paracoccidioides brasiliensis*/AAQ75798	7e–55	3.2.1.14	10	Cell wall metabolism/Hydrolysis of chitin
UDP-glucose pyrophosphorylase*	*Aspergillus nidulans*/XP_413285.1	6e–70	2.7.7.9	12	Carbohydrate metabolism/Biosynthesis of cell wall components
ATP synthase F_0F_1 subunit 9*	*Aspergillus nidulans*/XP_408635.1	2e–44	3.6.3.14	88	Aerobic respiration
Flavodoxin-like protein	*Aspergillus nidulans*/XP_404434.1	3e–54	–	17	Aerobic respiration
Choline sulfatase	*Aspergillus nidulans*/XP_409586.1	1e–53	3.1.6.6	16	Sulfur metabolism
Sulfate adenylyltransferase	*Aspergillus niger*/AF538692.1	4e–105	2.7.7.4	17	Sulfur metabolism/Sulfate assimilation
Carbonic anhydrase†	*Magnaporthe grisea*/XP_364389.1	4e–36	4.2.1.1	11	Lipid biosynthesis/HCO_3^- production

Table 2. cont.

Gene product	Best hit/Accession no.	*E*-value	EC no.	Redundancy	Metabolic role
Delta-9-fatty acid desaturase (OLE1)*	*Ajellomyces capsulatus*/gi|46395695	7e–102	1.14.19.1	22	Lipid metabolism/ Monounsaturated fatty acid biosynthesis
Malic enzyme*	*Aspergillus nidulans*/XP_410305.1	2e–89	1.1.1.40	11	Related to fatty acid biosynthesis
Long-chain base-responsive inhibitor of protein kinases Pkh1p and Pkh2p (PIL1)*	*Aspergillus nidulans*/XP_409354.1	7e–45	–	11	Protein activity regulation
Ornithine decarboxylase antizyme*	*Emericella nidulans*/AF291577.1	1e–26	–	10	Proteasomal ubiquitin degradation
Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase 1*	*Aspergillus nidulans*/XP_407601.1	5e–55	2.1.1.77	14	Protein fate/Repair of β-aspartyl linkages
Peptidyl-prolyl *cis–trans* isomerase	*Neurospora crassa*/gi|38567156	1e–61	5.2.1.8	17	Protein fate/Regulation of RNA transcription and splicing
Polyubiquitin	*Schizosaccharomyces pombe*/ AAC64787.1	3e–65	–	33	Protein degradation
Probable type-III integral membrane protein (YTP1)†	*Aspergillus nidulans*/XP_406436.1	5e–25	–	10	Not defined
Serine proteinase*	*Paracoccidioides brasiliensis*/ AAP83193	1e–85	3.4.21.–	14	Protein degradation
Ubiquitin-conjugating enzyme E2	*Aspergillus nidulans*/XP_407263.1	1e–64	6.3.2.–	12	Protein degradation
3-Dimethylubiquinone-9,3-methyltransferase*	*Coccidioides immitis*/ XP_001248608.1	6e–38	2.1.1.64	20	Ubiquinone biosynthesis
Ferrochelatase*	*Aspergillus nidulans*/XP_411889.1	3e–76	4.99.1.1	10	Porphyrin metabolism/Insertion of iron into haem
Elongation factor 1-alpha	*Coccidioides immitis*/AAK54650	4e–80	–	13	Protein biosynthesis
Histone H2A*	*Aspergillus nidulans*/XP_412176.1	3e–54	–	19	Nucleosome assembly
Nucleosome assembly protein*	*Gibberella zeae*/XP_387643.1	5e–55	–	10	H2A and H2B nucleosome assembly
Small nuclear ribonucleoprotein U6 (Lsm3)*	*Aspergillus nidulans*/XP_404184.1	1e–33	–	10	RNA metabolism/RNA splicing
Transcription factor spt3 (SPT3)*	*Aspergillus fumigatus*/CAF32113	3e–48	–	16	Transcription/Assembly of RNA polymerase
Translation initiation factor subunit Sui1	*Gibberella zeae*/XP_389056.1	4e–49	–	12	Protein biosynthesis/Ribosomal recognition of the initiation codon
CAP20-virulence factor*	*Aspergillus nidulans*/XP_408358.1	3e–38	–	40	Not defined

*Overexpressed in comparison to the transcriptome of the *in vitro*-cultured yeast-phase cells (https://dna.biomol.unb.br/Pb/).
†Novel genes of *P. brasiliensis* as defined by comparison to the transcriptome of *in vitro*-cultured yeast-form cells and to the NCBI database (http://www.ncbi.nlm.nih.gov/).

probably involved in amino acid pathways (Table 4). Glutamine synthetase catalyses the ATP-dependent conversion of glutamate and ammonium to glutamine. In this context, urease in yeast-form cells could lead to the overproduction of ammonia arising from urea. Analysis of the amino acid metabolism pathways indicated that during the infection process *P. brasiliensis* could independently synthesize asparagine. Imidazole glycerol phosphate synthase catalyses the closure of the imidazole rings within the histidine biosynthesis pathway; this enzyme links amino acid and nucleotide biosynthesis, providing the substrate for *de novo* purine biosynthesis. 3-Isopropylmalate dehydrogenase catalyses the last step in leucine biosynthesis. The first and a key enzyme in the lysine biosynthesis pathway, homocitrate synthase, is also upregulated. Also lysine uptake should be increased by the overexpression of a lysine-specific permease. Cysteine synthase B could promote thiosulfate assimilation and cysteine could be overproduced by the action of the upregulated transcript encoding cystathionine β-synthase. The overexpression of the high-affinity methionine permease could promote uptake of methionine and cysteine. Aci-reductone dioxygenase could

Table 3. KEGG pathway mapping for novel and upregulated genes of *P. brasiliensis* yeast cells recovered from infected mouse liver

KEGG pathway	Number of sequences		Percentage of total	
	Novel	Upregulated	Novel*	Upregulated†
Carbohydrate metabolism				
Glycolysis/gluconeogenesis	53	–	3.03	–
Pyruvate metabolism	–	32	–	2.73
Pentose phosphate pathway	3	–	0.17	–
Glycogen metabolism	2	–	0.11	–
Citrate cycle (TCA cycle)	–	24	–	2.05
Amino sugar metabolism	–	16	–	1.36
Glyoxylate and dicarboxylate metabolism	–	13	–	1.11
Nucleotide sugar metabolism	–	12	–	1.02
Energy metabolism				
Nitrogen metabolism	8	–	0.46	–
Oxidative phosphorylation	9	99	0.51	8.45
Nucleotide metabolism				
Purine and pyrimidine metabolism	5	4	0.28	0.34
Amino acid metabolism				
Tryptophan metabolism	3	–	0.17	–
Alanine and aspartate metabolism	1	9	0.06	0.77
Glycine, serine and threonine metabolism	–	11	–	0.94
Glutamate metabolism	–	27	–	2.30
Valine, leucine and isoleucine degradation	2	4	0.11	0.34
Valine, leucine and isoleucine biosynthesis	1	10	0.05	0.85
Methionine metabolism	–	4	–	0.34
Urea cycle and metabolism of amino groups	–	7	–	0.60
Cysteine metabolism	1	–	0.06	–
Lipid metabolism				
Fatty acid metabolism	1	8	0.06	0.68
Glycerophospholipid metabolism	4	7	0.23	0.60
Glycerolipid metabolism	–	6	–	0.51
Linoleic acid metabolism	2	–	0.11	–
Biosynthesis of steroids	4	–	0.23	–
Metabolism of cofactors and vitamins				
Ubiquinone biosynthesis	–	40	–	3.41
Porphyrin and chlorophyll metabolism	–	10	–	0.85
Nicotinate and nicotinamide metabolism	1	–	0.06	–
Folate biosynthesis	–	8	–	0.68
Cell growth and death				
Cell cycle	1	–	0.06	–
Transcription				
RNA polymerase	8	5	0.46	0.43
Replication and repair				
DNA polymerase	–	4	–	0.34
Protein folding, sorting and degradation				
Ubiquitin-mediated proteolysis	–	14	–	1.19
Signal transduction				
Calcium signalling pathway	–	6	–	0.51
Phosphatidylinositol signalling system	1	–	0.06	–

*Percentage in relation to the total number of novel genes.
† Percentage in relation to the total number of overexpressed genes.

promote the methionine salvage pathway (MTA). Adenylosuccinate lyase, which encodes an enzyme involved in adenylate synthesis, and orotate phosphoribosyltransferase, involved in pyrimidine biosynthesis, are both novel genes.

Table 4. Overexpressed and novel genes involved in carbohydrate, lipid, amino acid and energy-yielding metabolism in the transcriptome of *P. brasiliensis* yeast-phase cells recovered from livers of infected mice

Pathway	Gene product	Annotated function	EC no.	Redundancy
Carbohydrate synthesis and degradation	Acylphosphatase (AcP)†	Putative regulator of the glycolytic pathway	3.6.1.7	6
	Quinoprotein alcohol dehydrogenase family protein†	Alcoholic fermentation	1.1.99.8	7
	Glucokinase (GLK)†	Carbohydrate metabolism/glycolysis	2.7.1.2	45
	Phosphoglycerate mutase (GPM1P)*	Carbohydrate metabolism/glycolysis	5.4.2.1	13
	Glycogen phosphorylase 1 (GPH1)†	Glycogen breakdown/glycogenolysis	2.4.1.1	2
	D-ribose-5-phosphate ketol-isomerase (RIP5)†	Pentose phosphate pathway	5.3.1.6	3
	Monosaccharide transporter (MFS1)*	Transport of sugars	–	9
	Glucose transporter (MFS2)†	Transport of sugars	–	3
	Phosphotransferase system, phosphocarrier HPR protein (PTS)†	Transport of sugars	2.7.3.9	2
Lipid/phospholipid synthesis and degradation	Malic enzyme (ME)*	Fatty acid biosynthesis/transfer of acetate to cytosol	1.1.1.40	11
	Carbonic anhydrase (CA)†	Biosynthesis of bicarbonate	4.2.1.1	11
	Acetyl-CoA carboxylase (ACC1)*	Fatty acid biosynthesis	6.4.1.2	6
	Fatty-acyl-CoA synthase (beta-subunit)*	Fatty acid biosynthesis	2.3.1.86	8
	Member of the MBOAT family of putative membrane-bound *O*-acyltransferases (Yor175cp)†	Acetyl transferase for phospholipid biosynthesis	2.3.–.–	1
	Patatin-like serine hydrolase (phospholipase A2 activity)†	Hydrolysis of phospholipids	–	2
	Phosphatidic acid-preferring phospholipase A1†	Hydrolysis of phospholipids	3.1.1.32	1
	Delta-9-fatty acid desaturase (OLE1)*	Biosynthesis of monounsaturated fatty acids	1.14.19.1	22
	Delta(24)-sterol *C*-methyltransferase (ERG6)*	Biosynthesis of ergosterol	2.1.1.41	6
	Squalene synthetase (ERG9)†	Biosynthesis of ergosterol	2.5.1.21	1
	Delta 8-sphingolipid desaturase†	Biosynthesis of membrane sphingolipids	1.14.99.–	1
TCA cycle and glyoxylate cycle	Isocitrate lyase (ICL)*	Glyoxylate cycle	4.1.3.1	13
	Hydroxymethylglutaryl-CoA lyase (HMGCL)*	Leucine degradation/acetyl-CoA production	4.1.3.4	4
	Carnitine acetyl transferase (CAT)*	Transport of acetylcarnitine into mitochondria	2.3.1.7	9
	Carnitine/acylcarnitine translocase (CACT)†	Transport of acetylcarnitine into mitochondria	2.3.1.–	1
Oxidation of NADH and energy generation	ATP synthase F_0F_1 J chain*	Aerobic respiration	3.6.3.14	7
	ATP synthase F_0F_1 subunit 9*	Aerobic respiration	3.6.3.14	88
	ATP synthase F_0F_1 subunit e (TIM11l)†‡	Aerobic respiration	3.6.3.14	1
	Cytochrome *c* oxidase subunit VIIa (CCO)†	Aerobic respiration	1.9.3.1	6
	Cytochrome *c* oxidase subunit I (COX1)†	Aerobic respiration	1.9.3.1	4
Nitrogen/amino acid metabolism	Glutamine synthetase (GLNA)*	Conversion of ammonia and glutamate to glutamine	6.3.1.2	11
	Urease (URE)*	Hydrolysis of urea to carbon dioxide and ammonia	3.5.1.5	7
	Asparagine synthase (AS)†	Biosynthesis of L-asparagine from L-aspartate	6.3.5.4	1
	Imidazole glycerol phosphate synthase HisHF (IGP synthase)†	Histidine biosynthesis/*de novo* purine biosynthesis	2.4.2.–	1

Table 4. cont.

Pathway	Gene product	Annotated function	EC no.	Redundancy
	3-Isopropylmalate dehydrogenase (LEU2)*	Leucine biosynthesis	1.1.1.85	10
	Homocitrate synthase (LYS21)*	Lysine biosynthesis	2.3.3.14	26
	Lysine-specific permease (LYP1)†	Uptake of lysine	–	8
	Cysteine synthase B (CYSM)†	Thiosulfate assimilation	2.5.1.47	1
	Cystathionine β-synthase (CYS4)*	Cysteine biosynthesis	4.2.1.22	11
	High-affinity methionine permease (MUP1)*	Uptake of methionine and cysteine	–	11
	Aci-reductone dioxygenase 1(ARD)†	Methionine salvage pathway	1.13.11.54	1
	Adenylsuccinate lyase (ADE13)†	*De novo* purine nucleotide biosynthetic pathway	4.3.2.2	1
	Orotate phosphoribosyltransferase (URA5)†§	*De novo* biosynthesis of pyrimidines	2.4.2.10	5
	Nitrogen metabolite repression regulator (NMRA)*	Part of a system controlling nitrogen metabolite repression in fungi	–	5

*Overexpressed genes identified in *P. brasiliensis* transcriptome of yeast-phase cells recovered from infected mouse liver.
†Novel genes identified in *P. brasiliensis* transcriptome of yeast-phase cells recovered from infected mouse liver.
‡Genes not described previously in *P. brasiliensis* isolate *Pb*01, but present in public databases.
§Novel transcripts also detected in a *P. brasiliensis* dimorphic transition transcriptome (Bastos *et al.*, 2007).

Validation of the ESTs by PCR analysis and expression of selected genes in yeast-phase cells recovered from infected mice and in an *ex vivo* model

We initially validated five novel genes by PCR analysis of *P. brasiliensis* genomic DNA, as shown in Fig. 2(a). The novel genes encoding indigoidine synthase A-like protein (*indA*), oligopeptide transporter protein (*opt*), Rho guanyl exchange factor (*gef*), oxidation resistance protein (*oxr1*) and glucokinase (*glk*) were demonstrated to be present in the genome of *P. brasiliensis*. In the next series of experiments, confirmatory data regarding the expression levels from EST redundancy analysis were provided by sqRT-PCR analysis. Transcripts encoding carbonic anhydrase (*ca)*, myosin 2 isoform (*myo2*), telomerase reverse transcriptase (*tert*), poly(A) polymerase (*pap1*), orotate phosphoribosyltransferase (*ura5*) and patatin-like serine hydrolase (*pat*) were confirmed as being present at higher levels in yeast-form cells recovered from infected mouse livers (Fig. 2b). Also, some novel transcripts were validated by sqRT-PCR of RNAs obtained from yeast forms interacting with *in vitro*-cultured Vero cells. The novel transcripts encoding *ca*, *myo2*, *tert*, *pap1*, *pat*, squalene synthetase (*erg9*), *oxr1* and *glk* were present in yeast cells in the *ex vivo* model (Fig. 3).

DISCUSSION

Here we report *in silico* analysis and comparison of ESTs from yeast-form cells of *P. brasiliensis* recovered from infected mouse livers with previously described *P. brasiliensis* transcriptomes. The expression profiles of genes encoding enzymes involved in primary metabolism show that there is a striking degree of coordinate regulation of some of the genes in the same pathway. For example, genes encoding enzymes, regulators and transporters in carbohydrate metabolism are significantly overexpressed in fungal cells recovered from infected tissue. Transcripts of genes involved in lipid synthesis are also expressed at high levels.

A great number of induced and novel genes in yeast-form cells recovered from liver were involved in carbohydrate metabolism. In a previous study, comparing mRNA expression of mycelia and yeast-phase cells, Felipe *et al.* (2005) suggested that the metabolism of yeast-form cells is more anaerobic than that of mycelium toward the production of ethanol. Our data suggest that infection of liver by *P. brasiliensis* yeast-form cells exacerbates their anaerobic behaviour, when compared to *in vitro*-cultured yeast-form cells. There is actually an increase in mRNA expression of several genes involved in glycolysis. Corroborating our data, a glucokinase gene of *Saccharomyces cerevisiae* has been shown previously to be overexpressed under conditions of ethanol induction (Herrero *et al.*, 1999). Although the physiological role of acylphosphatase is as yet unknown, the enzyme plays a part in the regulation in the glycolytic pathway, by increasing the rate of glucose fermentation in yeast (Raugei *et al.*, 1996). The predicted upregulation of glycolysis in *P. brasiliensis* described here is corroborated by a previous description of the predominance of glycolytic metabolism in *Candida albicans* colonizing mouse tissues (Barelle *et al.*, 2006). Additionally, the emphasis on the overexpression of these enzymes of carbohydrate metabolism suggests that the milieu may provide an adequate nutritional environment

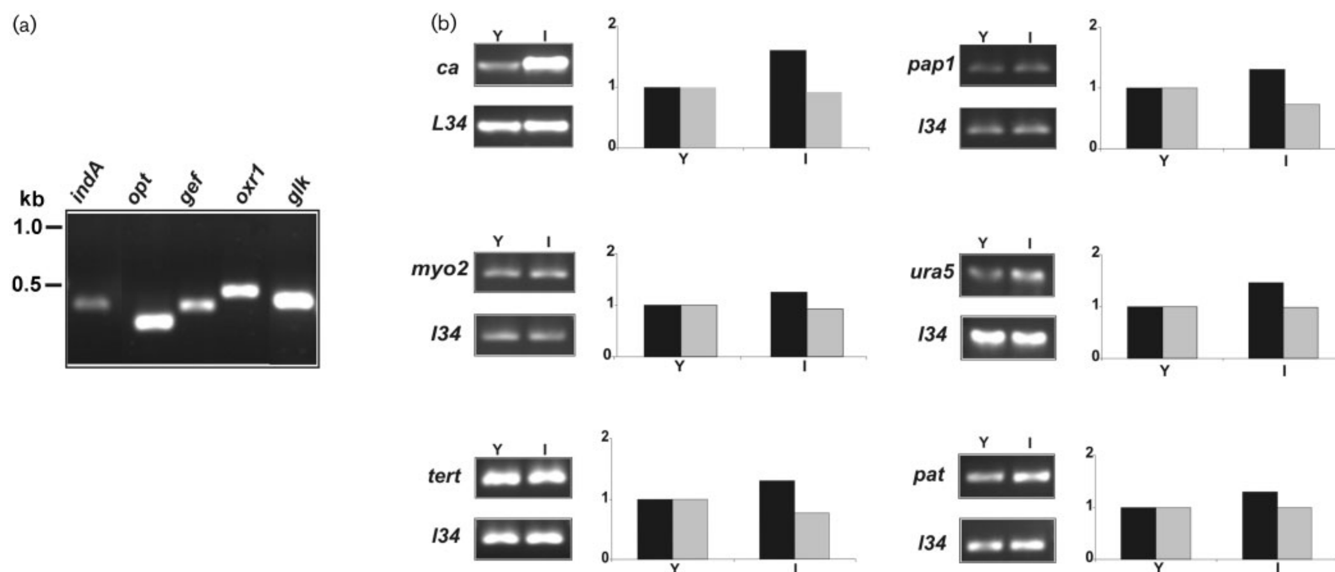

Fig. 2. Validation of the cDNA library for the presence of genes and analysis of redundancy of some transcripts. PCR and sqRT-PCR analysis were carried out with specific sense and antisense oligonucleotide primers, respectively, as described in Table 1. (a) PCR of total genomic DNA of *P. brasiliensis* with specific primers. (b) SqRT-PCR of RNAs from yeast-phase cells. The RNA samples were obtained from yeast-phase cells, *in vitro* cultured (Y); and yeast-phase cells recovered from livers of infected mice (I). The bar diagrams indicate fold differences relative to the data for the reference *in vitro*-cultured yeast cells: control reactions with the ribosomal L34 protein are indicated by grey bars; black bars indicate the reactions for the selected genes. The sizes of the amplified products are listed in Table 1.

to enable the the glycolytic pathway to be shifted toward the production of ethanol, a metabolic pathway that should be particularly important during liver infection because of abundant glucose in this host milieu.

Ethanol could become a relevant carbon source by entering the glyoxylate cycle, which has been previously described as being upregulated in fungal yeast-phase cells (Felipe *et al.*, 2005). The cycle may be more active in yeast-form cells infecting mouse liver, as demonstrated here by the overexpression of the regulatory enzyme isocitrate lyase, suggesting that some non-fermentable compounds are important for energy production during infection as described previousy for fungi such as *Candida albicans* and *Cryptococcus neoformans* (Lorenz & Fink, 2001; Ramírez & Lorenz, 2007; Rude *et al.*, 2002). Interestingly, the isocitrate lyase gene of *Penicillium marneffei* has been shown previously to be strongly induced at 37 °C, even in the presence of a repressing carbon source, such as glucose (Cánovas & Andrianopoulos, 2006), a condition occurring in liver.

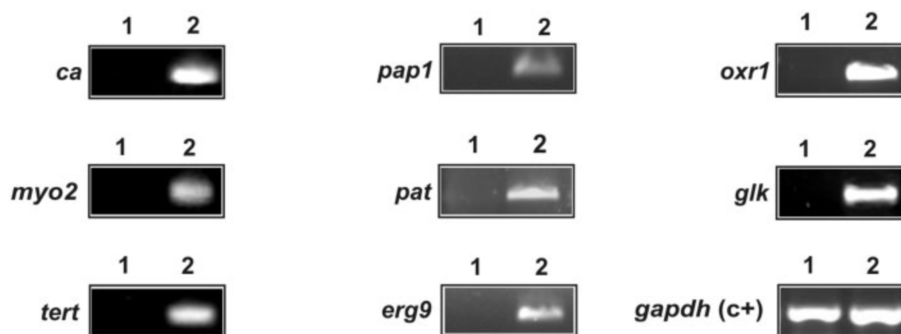

Fig. 3. Transcript analysis of the interaction of *P. brasiliensis* with Vero cells: sqRT-PCR analysis of selected transcripts in yeast-phase cells in the *ex vivo* model of infection. Lane 1, detection of transcripts in control Vero cells; lane 2, detection of transcripts in *P. brasiliensis* yeast forms infecting Vero cells.The sizes of the amplified products are given in Table 1. The gene encoding GAPDH was used as an internal control for Vero cells.

The biosynthesis of lipids may be upregulated during the infection process. The overexpression of transcripts encoding lipogenic enzymes corroborates this suggestion. Fatty acid synthesis is maximal when carbohydrate and energy are plentiful, a condition believed to occur in liver. Regarding carbonic anhydrase, previous studies have demonstrated that this enzyme's activity is required for *C. neoformans* fatty acid biosynthesis (Bahn *et al.*, 2005). It has not escaped our attention that the increase in carbonic anhydrase mRNA may reflect the high CO_2 levels in the host tissue.

Membrane composition seems to change during infection. Ergosterol is the major sterol in fungal membranes and affects their fluidity and permeability. Transcripts encoding ERG6 and ERG9 were differentially regulated. Also increased were the mRNA levels for enzymes related to the synthesis and remodelling of the cell membrane, such as OLE1, involved in regulating membrane fluidity in animal cells and micro-organisms (Gargano *et al.*, 1995), and responsible for the adjustments in the membrane composition in response to nutritional change (Vigh *et al.*, 1998).

P. brasiliensis seems to perform oxidative phosphorylation by classical pathways during infection. Of special note is the huge overexpression of the ATP synthase F_0F_1 subunit 9, the relevance of which is not clear.

Nitrogen metabolism is one aspect of basic metabolism which is still quite unknown in the field of pathogenesis. The most critical genes for *S. cerevisiae in vivo* survival were found to be those required for amino acid biosynthesis (Kingsbury *et al.*, 2006). We described here 14 novel/overexpressed genes related to the metabolism of amino acids, suggesting that this aspect of metabolism should be very relevant to fungal survival in the host liver environment. Among the genes were those encoding several metabolic steps in biosynthesis of amino acids, as well as the transcriptional regulator NMRA gene, encoding a predictable nitrogen metabolite repressor, suggesting that *P. brasiliensis* is subject to nitrogen metabolite repression under host conditions, probably reflecting ammonia and glutamine availability in liver.

It can be suggested, on the basis of the transcriptional data provided by this study, that increased glutamine, asparagine, histidine, lysine, cysteine and methionine biosynthesis are important for the survival of *P. brasiliensis* during infection. Glutamine formation plays a key role in nitrogen metabolism, ensuring the reassimilation of nitrogen released from cellular processes and providing the source of amino groups in a wide range of biosynthetic processes. Our analysis indicated that during infection *P. brasiliensis* seems to be able to synthesize asparagine, providing, in addition to glutamine, another site for transient storage of nitrogen. The novel transcript encoding aci-reductone dioxygenase suggests the presence of the methionine salvage pathway cycle (Hirano *et al.*, 2005) providing additional methionine, which could be scarce in the host environment. Overall, the presumed increase in synthesis of the amino acids listed above implies that those compounds are not present at sufficient levels in host tissue to support growth of *P. brasiliensis.*

To obtain further corroboration of the validity of our EST results, we performed RT-PCR analysis of some selected transcripts in yeast cells recovered from infected tissue in a different series of experiments from those used to construct the cDNA library, as well as in an *ex vivo* model of infection. Several novel transcripts, such as those encoding glucokinase and carbonic anhydrase, were confirmed, further corroborating the validity of our EST analysis and suggesting the relevance of those transcripts in the infectious process.

Importantly, several of the genes identified in this work had previously been implicated in pathogenesis in other organisms. The most important types of melanin in fungi are DHN-melanin and DOPA-melanin, which have been implicated in pathogenesis (Hamilton & Gomez, 2002). Transcripts encoding aromatic L-amino acid decarboxylase were abundant in yeast-phase cells under our experimental conditions, reinforcing the relevance of DOPA-melanin in infection, as suggested elsewhere (Gomez *et al.*, 2001; Silva *et al.*, 2006; Bailão *et al.*, 2006). Polyketide synthase is a novel transcript, suggesting that *P. brasiliensis* could synthesize melanin via the polyketide synthase pathway, as described previously for other fungi (Paolo *et al.*, 2006). *Candida albicans* carbonic anhydrase mutants cannot induce true hyphae in response to high CO_2, a condition of induction of filamentation (Klengel *et al.*, 2005). Tissue damage and dissemination by *Coccidioides* involve the ammonia-based alkalinization of the host environment through the activity of fungal urease (Mirbod-Donovan *et al.*, 2006). The oxidation resistance (OXR1) protein is involved in protection of cells from oxidative hydrogen peroxide damage (Elliott & Volkert, 2004). The gene encoding orotate-5-monophosphate pyrophosphorylase in *Histoplasma capsulatum* is essential for fungal virulence in a mouse infection model (Retallack *et al.*, 1999). These findings further encourage the study of the relevance of these genes to *P. brasiliensis* pathogenesis.

In summary our data suggest that *P. brasiliensis* probably uses multiple carbon sources during liver infection, including glucose and substrates of the glyoxylate cycle. In addition, the metabolism of nitrogen can be very active during the infection process, suggesting that, while some nitrogenous compounds can be preferentially acquired from the host, others must be supplemented by the pathogen. Also, the biosynthesis of lipids appears to be very active, suggesting the plentiful availability of carbohydrates and energy.

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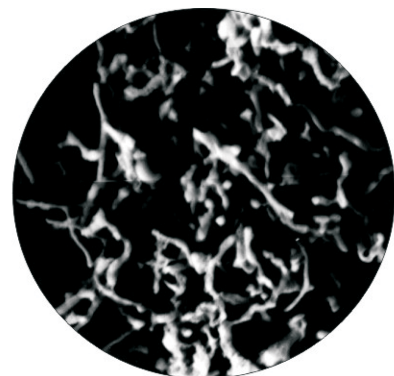
Capítulo V

Artigo 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.

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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.

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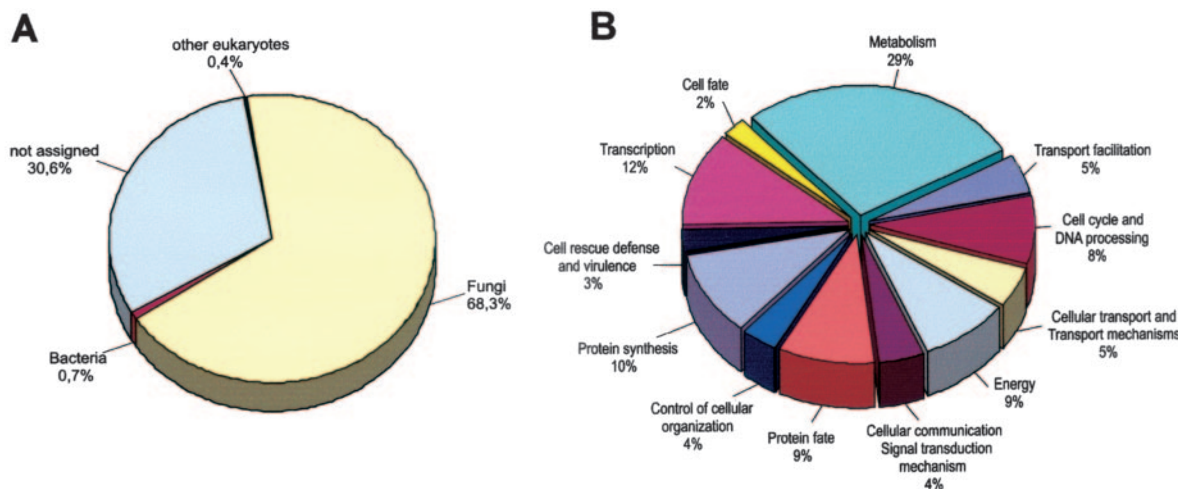

FIG. 1. ***P. brasiliensis* transcriptome characterization.** *A*, distribution of blast best hit among organisms. Each PbAEST was tested against the GenBank™ nr data base, and the best hit organism was computed. A PbAEST was considered as not assigned when the best hit exceed an *E* value of 10^{-10}. *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

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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-

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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-

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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

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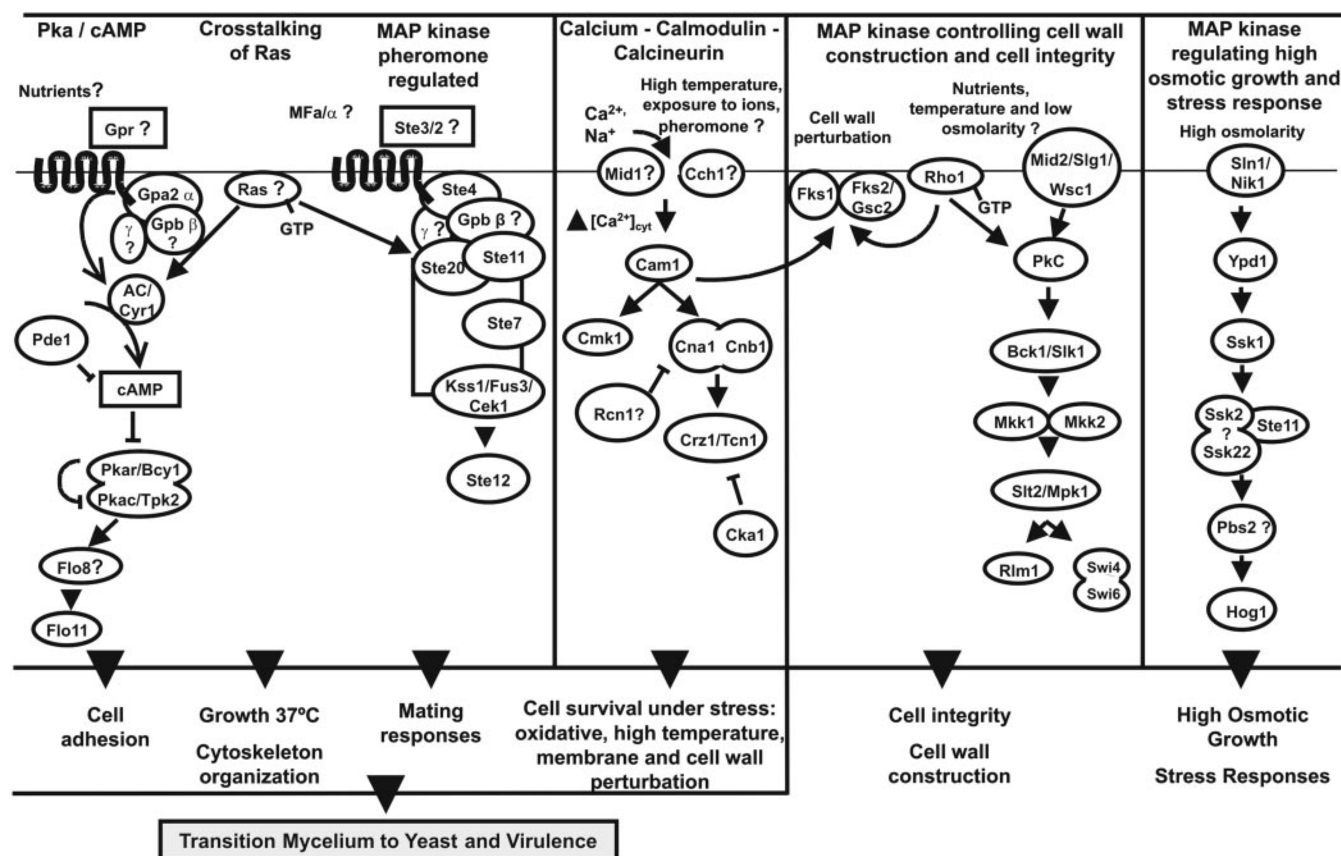

FIG. 4. **Signaling pathways in *P. brasiliensis.*** Shown are cAMP/protein kinase A regulating fungal development and virulence; MAPK signaling for cell integrity, cell wall construction, pheromone/mating, and osmoregulation; calcium-calmodulin-calcineurin controlling cell survival under stress conditions; 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

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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.

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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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Original article

Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: Representational difference analysis identifies candidate genes associated with fungal pathogenesis

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Abstract

Paracoccidioides brasiliensis causes infection by the host inhalation of airborne propagules of the mycelia phase of the fungus. These particles reach the lungs, and disseminate to virtually all organs. Here we describe the identification of differentially expressed genes in studies of host-fungus interaction. We analyzed two cDNA populations of *P. brasiliensis*, one obtained from infected animals and the other an admixture of fungus and human blood thus mimicking the hematologic events of the fungal dissemination. Our analysis identified transcripts differentially expressed. Genes related to iron acquisition, melanin synthesis and cell defense were specially upregulated in the mouse model of infection. The upregulated transcripts of yeast cells during incubation with human blood were those predominantly related to cell wall remodeling/synthesis. The expression pattern of genes was independently confirmed in host conditions, revealing their potential role in the infection process. This work can facilitate functional studies of novel regulated genes that may be important for the survival and growth strategies of *P. brasiliensis* in humans.
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Keywords: Paracoccidioides brasiliensis; Representational difference analysis; Infection

1. Introduction

Paracoccidioides brasiliensis causes paracoccidioidomycosis (PCM) the most prevalent systemic mycosis in Latin America. The infection occurs primarily in the lungs from where it can disseminate via the bloodstream and or lymphatic system to many organs systems rendering the disseminated form of PCM [1].

In order to establish a successful infection, *P. brasiliensis* that colonize within the dynamic substrate of a human host must have the ability to adapt to and modify gene expression in response to changes in the host environment. In recent years, several approaches have been developed to identify genes putatively related to the host- fungus interaction. The

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transcriptional profile of *P. brasiliensis* yeast cells and mycelium revealed genes that are potentially related to fungal virulence, in addition to a comprehensive view of fungal metabolism [2,3]. Despite those descriptions, a lack of information exists regarding *P. brasiliensis* gene expression when the organism interacts with the host.

Our aim is to uncover candidate genes that might contribute to *P. brasiliensis* adaptation to and survival in the host milieu during infection. The approach was the use of cDNA representational difference analysis (cDNA-RDA) [4] to identify *P. brasiliensis* genes induced during the infective process in a mouse model of infection and in conditions, which mimic the hematologic route of fungal dissemination. Although frequently employed in eukaryotes [5], this strategy has not been applied to the investigation of differentially expressed genes from human pathogenic fungi. Here we show the identification of candidate genes that *P. brasiliensis* may express as an adaptation to the host. Our results provide the first view of *P. brasiliensis* transcriptional response to host–pathogen interaction.

2. Materials and methods

2.1. Maintenance of P. brasiliensis

P. brasiliensis isolate 01 (ATCC MYA-826) was used in all the experiments. The yeast phase was maintained in vitro by sub culturing and grown at 36 °C, in Fava-Netto's medium for 7 days, as described [6].

2.2. Preparation of inocula and infection of mice

Mice infection was carried out, as previously described, with minor modifications [7]. Male B.10A mice (five animals) were infected intraperitoneally with 5×10^6 yeast cells and killed on the 7th day after infection; livers were removed and homogenized. Aliquots (100 μl) of the suspension were plated onto BBL Mycosel Agar (Becton-Dickinson, MD, USA), supplemented with 10% fetal calf serum. After 14 days of plating, the yeast cells were used to extract total RNA. As control, *P. brasiliensis* yeast cells from Fava-Netto cultures were transferred to the medium above and taken to prepare control cDNA samples. In additional experiments, yeast cells of *P. brasiliensis* (10^8 cells in PBS) were inoculated in the BALB/c mice by intravenous injection in the mouse tail. Blood was obtained by intracardiac puncture after 10 and 60 min of the fungus injection. Blood of control animals was obtained.

2.3. Treatment of P. brasiliensis yeast cells with human blood

Human blood from 10 healthy donors was collected by venipunctures using heparinized syringes. *P. brasiliensis* yeast cells were harvested from 7-day-old cultures and washed once with PBS. The fungal cells (5×10^6 cells/ml) were incubated in 7.5 ml of fresh human blood for 10 or 60 min at 36 °C, under shaking in the original blood collection tube. The fungal cells were washed and collected by centrifugation. As control, 7.5 ml of the same fresh whole blood were taken to prepare control cDNA samples.

2.4. Subtractive hybridization and generation of subtracted libraries

Total RNAs was extracted from all experimental conditions by the use of Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). For subtractive hybridization, 1.0 μg of total RNAs was used to produce double stranded cDNA by using the SMART PCR cDNA synthesis kit (Clonetech Laboratories, Palo Alto, CA, USA). First-strand synthesis was performed with reverse transcriptase (Invitrogen Life Technologies) and used as template to synthesize double stranded cDNA. The resulting cDNAs were digested with the restriction enzyme *Sau3*AI. A subtracted cDNA library was constructed using driver cDNA from 7 day old in vitro cultures of *P. brasiliensis* yeast cells grown in Fava-Netto's medium and tester cDNAs synthesized from RNAs extracted from *P. brasiliensis* recovered from infected animals and of yeast cells after treatment with human blood. The resulting products were purified using a GFX kit (GE Healthcare, Chalfont St. Giles, UK).The tester-digested cDNA was ligated to adapters (a 24-mer annealed to a 12-mer). For the 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 [5]. Two successive rounds of subtraction and PCR amplification using hybridization tester-driver ratios 1:10 and 1:100 were performed. Adapters were changed between cross-hybridization, and different products were purified using the GFX kit.

The amplified cDNA pools were purified from the gels and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). In order to generate the 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.

2.5. EST processing pipeline, annotation and differential expression analysis

EST sequences were pre-processed using the Phred and Crossmatch (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm) programs. Sequences with at least 100 nucleotides and Phred quality greater or equal to 20 were considered for further analysis. The resulting sequences were uploaded to a relational database (MySQL) on a Linux (Fedora) platform, and processed using a modified version of the PHOREST tool [8]. The filtered sequences were compared against the GenBank non-redundant (nr) database from National Center for Biotechnology Information (NCBI), Gene Ontology (GO) besides InterPro's databases of protein families, domains and functional sites. MIPS (http://mips.gsf.de/), GO and KEGG databases were used to

assign functional categories, EC numbers and metabolic pathways, respectively. The clusters were compared with *P. brasiliensis* transcriptome database (http://www.biomol.unb.br/Pb), using the BLAST program [9]. The ESTs have been submitted to GenBank under accession numbers EB085196 to EB086102.

2.6. Dot-blot and Northern-blot analysis

Serial dilutions of plasmid DNA were vacuum spotted in nylon membrane and hybridized to the specific cDNAs labeled by using the Random Prime labeling module (GE Healthcare). Hybridization was detected by a Gene Image CDP-Star detection module (GE Healthcare). In the Northern experiments, the RNAs were hybridized to correspondent cDNA probe in Rapid-hyb buffer (GE Healthcare). Probes were radiolabeled by using the Rediprime II Random Prime labeling System (GE Healthcare). The analyses of relative differences were performed by using the Scion Image Beta 4.03 program.

2.7. Semi-quantitative RT–PCR analysis (sqRT–PCR) and RT–PCR analysis of representative regulated genes

RNA used for sqRT–PCR was prepared from independent experiments and not from those used in the cDNA subtraction. cDNAs were synthesized by reverse transcription using the Superscript II RNAse H -reverse transcriptase (Invitrogen Life Technologies). cDNA was used for PCR in 30 μl reaction mixture containing specific primers, sense and antisense, respectively, as described in figure legends. PCR conditions were: 25–35 cycles at 95 °C for 1 min; annealing 55–65 °C for 2 min; 72 °C for 1 min. The annealing temperature and the number of PCR cycles were optimized for each experimental condition to ensure linear phase of amplification. Amplicons were analyzed by agarose gels electrophoresis (1%). For RT–PCR analysis the single stranded cDNAs were synthesized as described above. PCRs were performed using cDNAs obtained from *P. brasiliensis* contained in the blood of infected mice, as template in a 30-μl reaction mixture containing specific primers.

3. Results

3.1. Identification of P. brasiliensis genes with differential expression in host interaction conditions

The RDA experimental design included three conditions: (i) *P. brasiliensis* yeast cells from liver of B.10 A infected mice; (ii) *P. brasiliensis* yeast cells treated with human blood; and (iii) *P. brasiliensis* yeast cells grown in Fava Netto's medium. The two first conditions were used independently as tester populations and the latter as driver population. Subtraction hybridization was performed and the cDNAs libraries were constructed.

A total of 907 clones were successfully sequenced. From them, 490 originated from the *P. brasiliensis* yeast cells derived from infected mice and 417 from the fungus incubated in human blood. Using the BLASTX program, 6.4% of the ESTs corresponded to proteins of unknown function, with no matches in databases. In addition, 93.6% of the ESTs displayed significant similarity to genes in the *P. brasiliensis* database (http://www.biomol.unb.br/Pb), while 6.4% did not exhibit similarity to *P. brasiliensis* known genes.

3.2. Characterization and hypothetical roles of the subtracted cDNAs from P. brasiliensis isolated from infected mice

A broad view of the nature of the adaptations made by *P. brasiliensis* during host infection was obtained by classifying the ESTs into nine groups of functionally related genes (Table 1). The data illustrate the functional diversity of these highly expressed ESTs, denoting particular functional categories dominating the analysis. Noteworthy is the observed high redundancy of transcripts encoding 30 kDa heat shock protein (Hsp30), high-affinity zinc/iron permease (ZRT1), high-affinity copper transporter (CRT3) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the *P. brasiliensis* yeast cells recovered from infected mice. The cellular machinery for protein synthesis including eukaryotic translation initiation factor 4A and 3β (eIF-4A and eIF-3β), translation elongation factor 1γ (eEF-1γ), as well as components of the pos-translational machinery were upregulated in that condition. Transcripts encoding tyrosinase and aromatic-L-amino acid decarboxylase (DDC) were upregulated in cited condition, suggesting active melanin synthesis during the infective process. A control cDNA-RDA library was obtained from RNA of yeast cells grown in the same medium used to recover *P. brasiliensis* from infected liver, as the tester, and RNA from in vitro cultured yeast cells grown in Fava-Netto's medium as the driver. A total of 120 clones were successfully sequenced, as controls. The transcriptional profile did not manifest similarity to that described for yeast cells recovered from the livers of infected mice, thus excluding the possibility of the interference of growth conditions in the expression profiles (data not shown).

3.3. Subtracted cDNAs isolated from P. brasiliensis incubated in human blood

The nature of adaptations made by *P. brasiliensis* in this condition can be inferred by classifying the ESTs into 11 groups of functionally related genes (Table 2). The most redundant cDNAs appearing during human blood treatment for 10 min were as follows: H^+/nucleoside cotransporter (*cnt3*) and glutamine synthetase (*gln1*). After 60 min treatment the most abundant transcripts were those encoding 70 kDa heat shock protein (Hsp70), acidic amino acid permease (DIP5), eEF-1γ, GLN1, eIF-4A and pyridoxamine 5′-phosphate oxidase (PPO1), as shown in Table 2. A control cDNA-RDA library was obtained from human blood using RNA from in vitro cultured yeast cells (Fava-Netto's medium) as the driver, and a total of 100 clones were successfully sequenced. All of them corresponded to human genes, as demonstrated by BLAST search analysis (data not shown).

Table 1
ESTs with high abundance in the yeast cells recovered of liver of infected mice versus the in vitro condition

MIPS Category	Gene product	Best hit/accession number[a]	e-value	Redundancy
Metabolism	2-Methylcitrate dehydratase	*Neurospora crassa*/XP324860	3e-90	2
	Aromatic-L-amino-acid decarboxylase	*Aspergillus nidulans*/EAA64468	1e-57	1
	Delta 8-sphingolipid desaturase	*Neurospora crassa*/XP331184	2e-58	2
	Dolichol-phosphate mannose synthase	*Paracoccidioides brasiliensis*/AAR03724	9e-26	1
	Formamidase	*Paracoccidioides brasiliensis*/AAN87355	9e-40	1
	Glutamine synthetase[c]	*Aspergillus nidulans*/EAA59420	9e-86	1
	Phosphoenolpyruvate carboxykinase	*Aspergillus nidulans*/EAA65083	4e-43	1
	Pyridine nucleotide-disulphide oxidoreductase family protein	*Aspergillus fumigatus*/CAE47920	5e-09	1
	RNA lariat debranching enzyme[b]	*Cryptococcus neoformans*/EAL19833	1e-06	1
	Trehalose synthase	*Aspergillus nidulans*/EAA61099	1e-41	1
	Tyrosinase	*Magnaporthe grisea*/EAA48077	1e-42	3
Energy	Acetate-CoA ligase	*Penicillium chrysogenum*/JN0781	4e-91	2
Cell cycle and DNA processing	Septin-1	*Pyrenopeziza brassicae*/CAB52419	8e-81	1
Protein synthesis	60s ribosomal protein L20	*Gibberella zeae*/EAA68901	1e-48	1
	Eukaryotic translation initiation factor 3	*Aspergillus nidulans*/EAA65765	8e-63	3
	Eukaryotic translation initiation factor 4A[c]	*Aspergillus nidulans*/EAA63503	2e-84	6
	Eukaryotic translation elongation factor 1, gamma chain[c]	*Aspergillus nidulans*/EAA57903	1e-48	6
Transport facilitation	ATP-binding cassette ABC transporter (MDR)	*Venturia inaequalis*/AAL57243	2e-74	1
	High-affinity copper transporter[c]	*Gibberella zeae*/EAA70719	5e-27	56
	High-affinity zinc/iron permease[c]	*Candida albicans*/EAK96467	8e-57	140
	Low-affinity zinc/iron permease	*Aspergillus nidulans*/EAA60007	4e-24	3
Cellular communication/signal transduction	Ras small GTPase, Rab type	*Aspergillus niger*/CAC17832	1e-94	1
	Rho1 GDP-GTP exchange protein	*Aspergillus nidulans*/EAA61571	1e-55	1
	Serine/threonine protein phosphatase	*Aspergillus nidulans*/EAA59291	4e-10	1
Cell defense and virulence	Glyceraldehyde 3-phosphate dehydrogenase[c]	*Paracoccidioides brasiliensis*/AAP42760	1e-109	28
	30 kDa heat shock protein 30-Hsp30[c]	*Aspergillus nidulans*/EAA60998	2e-17	165
	70 kDa heat shock protein 70-Hsp70[c]	*Paracoccidioides brasiliensis*/AAK66771	7e-67	5
	90 kDa heat shock protein 90-Hsp90	*Ajellomyces capsulate*/S21764	4e-77	1
	Heat shock protein ClpA	*Paracoccidioides brasiliensis* AAO73810	2e-58	1
	Serine proteinase[c]	*Paracoccidioides brasiliensis*/AAP83193	1e-94	3
Functional unclassified proteins	NADP dependent oxidoreductase	*Aspergillus nidulans*/EAA65924	2e-36	1
Unclassified protein	Conserved hypothetical protein	*Neurospora crassa*/XP323053	8e-11	8
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA59203	2e-42	6
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA59112	8e-13	2
	Conserved hypothetical protein	*Cryptococcus neoformans*/EAL22420	5e-23	1
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA60590	2e-60	3
	Hypothetical protein	*Aspergillus fumigatus*/EAA66274	4e-06	2
	Hypothetical protein[b]	–	–	17
	Hypothetical protein[b]	–	–	1
	Hypothetical protein[b]	–	–	1
	Hypothetical protein	–	–	1
	Hypothetical protein	–	–	1
	Hypothetical protein	–	–	3
	Hypothetical protein	–	–	2
	Hypothetical protein	–	–	1

[a] Accession number at GenBank (http://www.ncbi.nlm.nih.gov).
[b] Novel genes detected in *P. brasiliensis*.
[c] Validated upregulated transcripts (see Figs. 2 and 3).

Table 2
ESTs with high abundance in the yeast cells during incubation in human blood versus the in vitro condition

MIPS Category	Gene product	Best hit/Accession number[a]	e-value	Redundancy	
				B10[b]	B60[c]
Metabolism	2-Methylcitrate dehydratase	*Neurospora crassa*/XP324860	1e-53	–	1
	Aldehyde dehydrogenase	*Aspergillus niger*/AAA87596	1e-80	3	–
	Aromatic-L-amino-acid decarboxylase	*Gibberella zeae*/EAA75531	7e-49	–	2
	C-5 sterol desaturase	*Aspergillus nidulans*/EAA57846	2e-57	–	1
	Delta-1-pyrroline-5-carboxylate dehydrogenase	*Magnaporthe grisea*/EAA48531	2e-44	–	1
	Dihydropteroate synthase	*Aspergillus nidulans*/EAA58007	3e-31	–	1
	Glutamine synthetase[e]	*Aspergillus nidulans*/EAA59420	6e-98	29	21
	Glutamine-dependent NAD synthetase	*Debaryomyces hansenii*/ EAA58847	3e-43	1	–
	Phosphoenolpyruvate carboxykinase	*Aspergillus nidulans*/EAA65083	7e-16	–	1
	Pyridoxamine 5′-Phosphate oxidase[e]	*Aspergillus nidulans*/EAA64421	1e-66	4	34
	S-Adenosylmethionine synthetase	*Ascobolus immerses*/AAB03805	5e-42	–	1
Energy	Acyl CoA dehydrogenase	*Paracoccidioides brasiliensis*/ AAQ04622	3e-98	3	4
	ATP synthase gamma subunit	*Aspergillus nidulans*/EAA66125	2e-67	3	1
	Long-chain-fatty-acid-CoA-ligase	*Aspergillus nidulans*/EAA57655	6e-62	6	2
	Medium-chain-fatty-acid-CoA ligase	*Aspergillus nidulans*/EAA59300	6e-59	–	1
	Multifunctional beta-oxidation protein	*Gibberella zeae*/EAA76166	6e-73	–	1
	NADH-ubiquinone oxidoreductase	*Aspergillus nidulans*/EAA64525	2e-87	–	1
	Transaldolase	*Aspergillus nidulans*/EAA66113	1e-66	1	–
	Transketolase	*Aspergillus nidulans*/EAA65464	7e-44	–	2
Transcription	Ap-1-like transcription factor (MeaB protein)	*Aspergillus nidulans*/EAA62122	2e-35	5	1
	Cutinase like transcription factor 1	*Aspergillus nidulans*/EAA64555	5e-72	2	1
	GATA zinc finger transcription factor	*Aspergillus nidulans*/EAA63723	5e-28	–	1
	Histone deacetylase RpdA[d]	*Aspergillus nidulans*/EAA60836	4e-18	–	2
	Related to heterogeneous nuclearribonucleoproteins	*Aspergillus nidulans*/EAA63773	2e-19	–	1
	Transcription factor HACA	*Aspergillus niger*/AAQ73495	4e-43	1	2
Cell cycle and DNA processing	Cell division cycle gene CDC48	*Aspergillus nidulans*/EAA61160	2e-27	–	1
	Septin-1	*Pyrenopeziza brassicae*/AAK14773	1e-79	1	–
	Shk1 kinase-binding protein 1	*Emericella nidulans*/AAR27792	2e-51	–	1
Protein synthesis	Eukaryotic translation release factor 1[d,e]	*Aspergillus nidulans*/BAB61041	3e-35	1	2
	Eukaryotic translation initiation factor 2 beta subunit	*Aspergillus nidulans*/EAA63563	3e-47	–	2
	Eukaryotic translation initiation factor 4A[e]	*Aspergillus nidulans*/EAA63503	1e-74	3	43
	Ribosomal protein S11	*Gibberella zeae*/EAA67332	1e-62	–	1
	Ribosomal protein S1B	*Neurospora crassa*/CAD70957	5e-32	–	1
	Serine-tRNA synthetase	*Aspergillus nidulans*/EAA60155	1e-39	1	–
	Translation elongation factor 1 alpha chain	*Yarrowia lipolytica*/CAG81931	4e-20	–	2
	Translation elongation factor 3	*Ajellomyces capsulatus*/AAC13304	4e-70	–	2
	Translation elongation factor 1, gamma chain[e]	*Aspergillus nidulans*/EAA57903	5e-24	1	20
	Translation elongation factor Tu, mitochondrial	*Aspergillus fumigatus*/CAD27297	1e-90	2	1
Protein fate	Intra-mitochondrial protein sorting (MSF1)	*Aspergillus nidulans*/EAA60030	5e-80	1	2
	Endoplasmic reticulum to Golgi transport related protein[d]	*Aspergillus nidulans*/EAA60127	4e-22	4	3
Transport facilitation	Acidic amino acid permease[e]	*Aspergillus nidulans*/EAA58093	4e-63	4	20
	ATP-binding cassette ABC transporter (MDR)	*Aspergillus nidulans*/AAB88658	2e-50	2	–
	Endoplasmic reticulum calcium-transporting ATPase	*Aspergillus nidulans*/EAA62836	6e-64	–	2
	Ferric reductase[e]	*Aspergillus nidulans*/EAA60984	2e-51	1	1
	Glucose transporter	*Aspergillus nidulans*/EAA60286	8e-61	1	–
	H^+-nucleoside cotransporter[e]	*Aspergillus nidulans*/EAA62653	8e-60	10	–
	Mitochondrial substrate carrier family protein	*Aspergillus nidulans*/EAA61338	4e-16	–	1
	Multidrug resistance protein	*Trichophyton rubrum*/AAG01549	9e-38	–	2
	P-type calcium-transporting ATPase	*Aspergillus nidulans*/EAA60998	1e-60	–	4
	Putative major facilitator protein	*Aspergillus nidulans*/EAA65241	8e-06	1	–
	Putative membrane transporter	*Gibberella zeae*/EAA78075	6e-50	2	–
	Putative transmembrane Ca^{2+} transporter-protein CCC1	*Aspergillus nidulans*/EAA59889	1e-13	1	–

Table 2 (*continued*)

MIPS Category	Gene product	Best hit/Accession number[a]	e-value	Redundancy	
				B10[b]	B60[c]
Cellular communication/ signal transduction	cAMP-dependent protein kinase	*Aspergillus nidulans*/EAA60590	2e-100	6	–
	Protein with PYP-like sensor domain (PAS domain)	*Neurospora crassa*/XP326245	3e-38	6	6
	Ras small GTPase, Rab type	*Aspergillus niger*/CAC17832	2e-44	–	1
	Serine/threonine-protein kinase SCH9	*Aspergillus nidulans*/EAA59337	6e-88	6	3
	Transmembrane osmosensor SHO1[d,e]	*Gibberella zeae*/EAA77427	4e-39	1	5
Cell defense and virulence	Glyceraldehyde-3-phosphate dehydrogenase[e]	*Paracoccidioides brasiliensis*/ AAP42760	3e-75	–	2
	30 kDa heat shock protein-Hsp30[e]	*Aspergillus nidulans*/EAA60998	7e-25	–	2
	70 kDa heat shock protein-Hsp70[e]	*Paracoccidioides brasiliensis*/ AAK66771	7e-80	–	14
	90 kDa heat shock protein-Hsp90	*Aspergillus nidulans*/EAA59007	2e-30	–	4
	Serine protease	*Paracoccidioides brasiliensis*/ AAP83193	6e-90	4	5
	Putative serine protease[d,e]	*Gibberella zeae*/EAA75433	5e-14	–	2
Functional unclassified protein	Pleckstrin-like protein	*Aspergillus nidulans*/EAA59882	5e-40	–	3
Unclassified protein	Conserved hypothetical protein	*Aspergillus nidulans*/EAA62122	4e-17	1	–
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA60590	8e-58	11	2
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA64557	5e-48	1	–
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA60610	3e-36	1	–
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA62122	3e-17	1	–
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA66204	4e-16	4	–
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA61977	6e-55	–	1
	Conserved hypothetical protein	*Aspergillus nidulans*/EAA60127	4e-10	–	1
	Conserved hypothetical protein	*Neurospora crassa*/XP323499	5e-22	–	1
	Conserved hypothetical protein	*Neurospora crassa*/XP326245	5e-11	–	4
	Hypothetical protein[d]	–	–	13	2
	Hypothetical protein	–	–	1	–
	Hypothetical protein	*Aspergillus nidulans*/EAA61232	1e-12	1	–
	Hypothetical protein	–	–	1	–
	Hypothetical protein	–	–	1	–
	Hypothetical protein	*Aspergillus nidulans*/EAA59882	1e-10	–	3
	Hypothetical protein[d]	–	–	–	2
	Hypothetical protein[d]	–	–	–	1
	Hypothetical protein	*Aspergillus nidulans*/EAA61232	5e-11	–	1
	Hypothetical protein	–	–	–	1
	Hypothetical protein	–	–	–	1
	Hypothetical protein	–	–	–	1

[a] Accession number at GenBank (http://www.ncbi.nlm.nih.gov).
[b] Blood incubation of yeast cells for 10 and 60 min, respectively.
[c] Blood incubation of yeast cells for 10 and 60 min, respectively.
[d] Novel genes detected in *P. brasiliensis*.
[e] Validated upregulated transcripts (see Figs. 2 and 3).

3.4. Analysis of the genes upregulated in the mice infection and human blood treatment

Fig. 1 depicts the classification of 131 clusters of *P. brasiliensis* ESTs according to the classification developed at MIPS. As observed, most of the ESTs in the infective process corresponded to upregulated cDNAs related to transport facilitation and to cell defense mechanisms (Fig. 1A). Incubation with human blood, for 10 min, reveals that most of the upregulated transcripts were related to the cell metabolism, followed by the ESTs representing transport facilitation (Fig. 1B). The incubation in human blood for 60 min, revealed the preferential expression of genes encoding factors related to protein synthesis.

3.5. Confirmatory differential expression of P. brasiliensis *identified sequences*

To corroborate the RDA findings, we initially performed dot blot analysis of *P. brasiliensis* cDNA-RDA clones. As shown in Fig. 2A, the transcripts encoding GAPDH, ZRT1, CTR3 and Hsp30 were confirmed to be upregulated in the yeast cells recovered from liver of infected mice (Fig. 2A, panel b). The transcripts encoding DIP5, eIF-4A, serine proteinase (PR1H) and CNT3 were confirmed to be upregulated during the 10 min of *P. brasiliensis* incubation in blood (Fig. 2A, panel c). The blots also confirmed the upregulation of the transcripts encoding GLN1, DDC, PPO1, eEF-1γ,

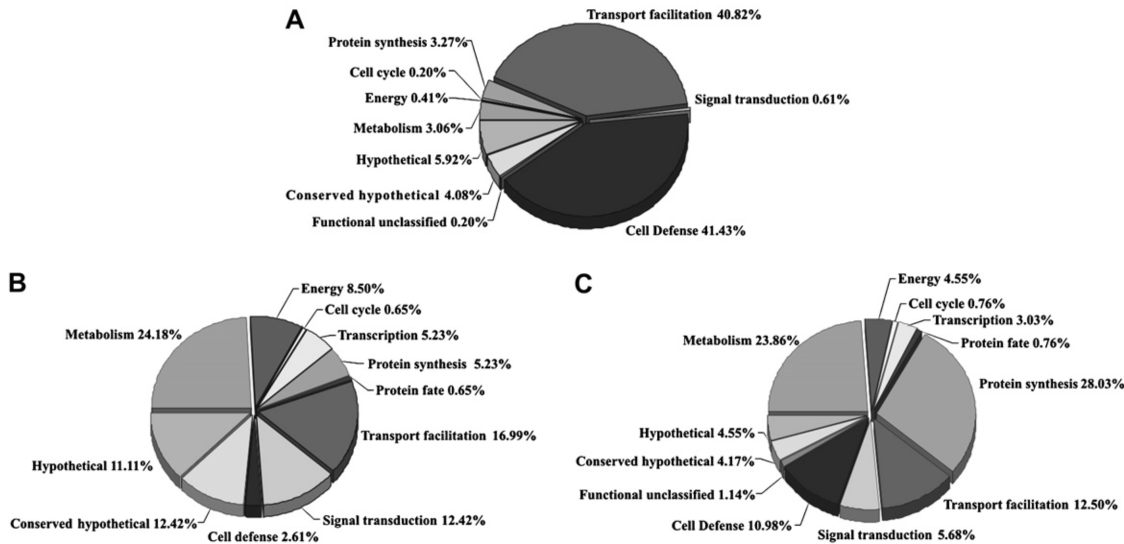

Fig. 1. Functional classification of *P. brasiliensis* cDNAs derived from RDA experiments using as testers. (A) cDNAs synthesized from the RNAs of yeast cells recovered from infected mice. (B,C) cDNAs obtained from RNA of *P. brasiliensis* yeast cells after a 10-min (B) or 60-min (C) incubation with human blood. The percentage of each functional category is shown (refer to Tables 1 and 2). Functional classification was based on BLASTX homology of each EST against the GenBank non-redundant database at a significant homology cut-off of ≤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.

Hsp70 and ferric reductase (FRE2) in the yeast cells incubated with human blood for 60 min (Fig. 2A, panel d).

Northern blot analysis was employed to evaluate the expression of some upregulated genes. The transcripts of the genes encoding GAPDH, ZRT1, Hsp30 and CTR3 were more accumulated in yeast cells recovered from liver of infected mice (Fig. 2B, panel a). The transcript encoding GLN1 was confirmed as more abundant during incubation in human blood (Fig. 2B, panel b).

3.6. *Expression profiles of genes in* P. brasiliensis *yeast cells*

Further confirmatory data about the expression level from EST redundancy analysis was provided by semi-quantitative RT–PCR (sqRT–PCR) analysis. RNA was extracted from *P. brasiliensis* yeast cells recovered from mouse liver and after incubation in human blood in a new set of experiments. The transcripts encoding Hsp30 and CTR3 were demonstrated in *P. brasiliensis* yeast cells recovered from liver of infected mice (Fig. 3A, panel a). On the other hand, the transcripts encoding eukaryotic release factor 1 (eRF1), transmembrane osmosensor (SHO1), PPO1 and serine protease (SP1) were preferentially expressed during incubation in blood (Fig. 3A, panel b). The accumulation of transcripts encoding eEF-1γ, GLN1, GAPDH and Hsp70 were detected in both conditions: *P. brasiliensis* yeast cells recovered from liver of infected mice and after incubation in human blood (Fig. 3A, panel c).

3.7. *Expression profiles of genes in* P. brasiliensis *yeast cells infecting blood of mice*

We performed RT–PCR analysis of the RNAs extracted from *P. brasiliensis* fungal yeast cells infecting blood of BALB/c mice (Fig. 3B). We detected the transcripts encoding eRF1, SP1, SHO1, PPO1, GLN1, eEF-1γ and Hsp70. Transcript encoding GAPDH was amplified as an internal control for cDNAs synthesized from RNAs obtained from mouse blood.

3.8. *A model for the* P. brasiliensis *adaptation to the host*

The most abundant ESTs listed in Tables 1 and 2 indicate a possible strategy of *P. brasiliensis* to face the host. Fig. 4A and B present a model of the adaptive changes of *P. brasiliensis* to the host milieu. See Section 4 for details.

4. Discussion

We were able to associate the assembled expressed sequences reported in the present work with different biological processes using the MIPS categories. On the basis of proven or putative gene functions we provide an interpretation and speculate on a model to interpret the upregulated transcripts.

4.1. *Transport facilitators*

During infection the level of available iron is significantly limited and therefore, microbial mechanisms to acquire iron are highly adaptive and important for successful virulence [10]. It has been shown that iron overload exacerbates meningoencephalitis in a mouse model of cerebral infection by *Cryptococcus neoformans* [11]. Ferrous ion uptake involves both high- and low-affinity transporters; the first is accompanied by a member of the copper oxidase family [12]. One of the most abundant cDNAs encodes a predictable high-affinity

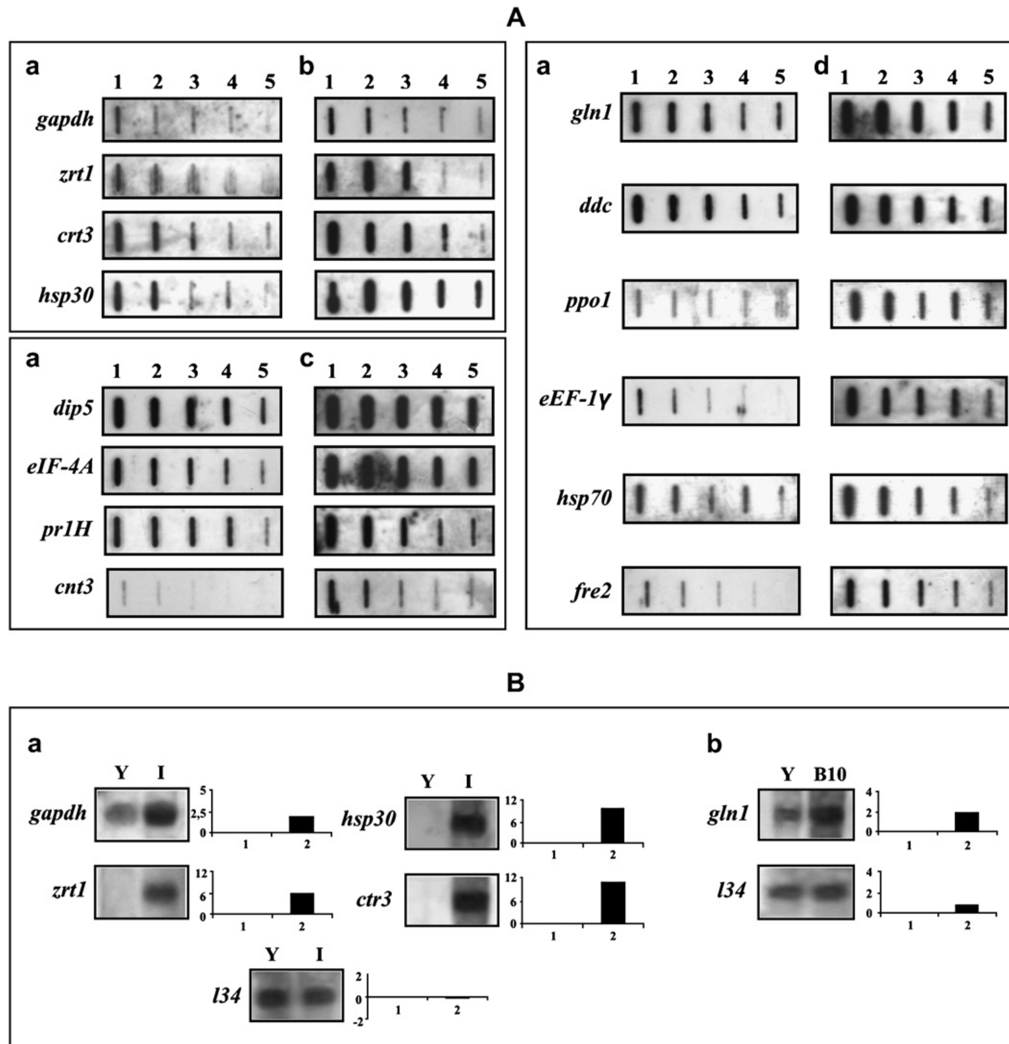

Fig. 2. Validation of the cDNA-RDA results. (A) Dot blot analysis of *P. brasiliensis* cDNA-RDA clones. DNA of individual clones was prepared and several dilutions were blotted (1−5). Individual clones were blotted and hybridized to the labeled cDNAs obtained from the control yeast cells (a); labeled cDNA obtained from *P. brasiliensis* recovered from liver of infected mice (b); labeled cDNA obtained from *P. brasiliensis* after 10 min (c) or 60 min (d) incubation with human blood. The clones were: (*gapdh*) glyceraldehyde 3-phosphate dehydrogenase; (*zrt1*) high-affinity zinc/iron permease; (*ctr3*) high-affinity copper transporter; (*hsp30*) 30 kDa heat shock protein; (*dip5*) acidic amino acid permease; (*eIF-4A*) eukaryotic initiation factor 4A; (*pr1H*) serine proteinase; (*cnt3*) H^+/nucleoside cotransporter; (*gln1*) glutamine synthetase; (*ddc*) aromatic L-amino acid decarboxylase; (*ppo1*) pyridoxamine phosphate oxidase; (*eEF-1γ*) eukaryotic elongation factor gamma subunit; (*hsp70*) 70 kDa heat shock protein; (*fre2*) ferric reductase. (B) Expression patterns of genes obtained by cDNA-RDA analyzed by Northern blot of total RNA of *P. brasiliensis*. Detection of transcripts overexpressed in yeast cells recovered from liver of infected mice (a) and of transcripts overexpressed in the blood incubation (b). Total RNA was fractionated on a 1.2% formaldehyde agarose gel and hybridized to the cDNA inserts of *gapdh*, *zrt1*, *hsp30*, *ctr3*, *gln1*. L34 ribosomal protein (*l34*) is shown as the loading control. The RNAs were obtained from in vitro cultured yeast cells (Y); yeast cells recovered from liver of infected mice (I); yeast cells after incubation with human blood for 10 min (B10). The RNA sizes were calculated using the 0.25−9.5 marker RNA ladder (GIBCO, Invitrogen). The transcript sizes were as follows: *gapdh* (2 kb), *zrt1* (1.4 kb), *hsp30* (1 kb), *ctr3* (1.4 kb), *gln1* (1.9 kb) and *l34* (0.75 kb). 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 by using Scion Image Beta 4.03 program.

zinc/iron permease. The observed redundancy of this transcript (*zrt*1) in *P. brasiliensis* was of 140 ESTs in our experimental model of infection, and of 8 ESTs in the in vitro cultured fungus [3].

The high-affinity iron uptake requires an efficient copper uptake, since maturation of the copper protein in the secretory apparatus requires acquiring of copper [13]. The redundancy of the *ctr3* transcript encoding a copper transporter of high affinity suggests the requirement for a copper−iron permease for the iron transport. In agreement, the *ctr3* transcript has been described as highly expressed in the *P. brasiliensis* yeast phase [2,3].

The *zrt*1 and *ctr*3 transcripts were not upregulated during the incubation of *P. brasiliensis* yeast cells in human blood, suggesting a no-iron-limitation condition in this treatment. Iron acquisition could also include a ferric reductase to remove iron from host-iron binding molecules such as heme. Corroborating our finding, a glutathione-dependent extracellular ferric reductase activity was recently described in *P. brasiliensis* [14].

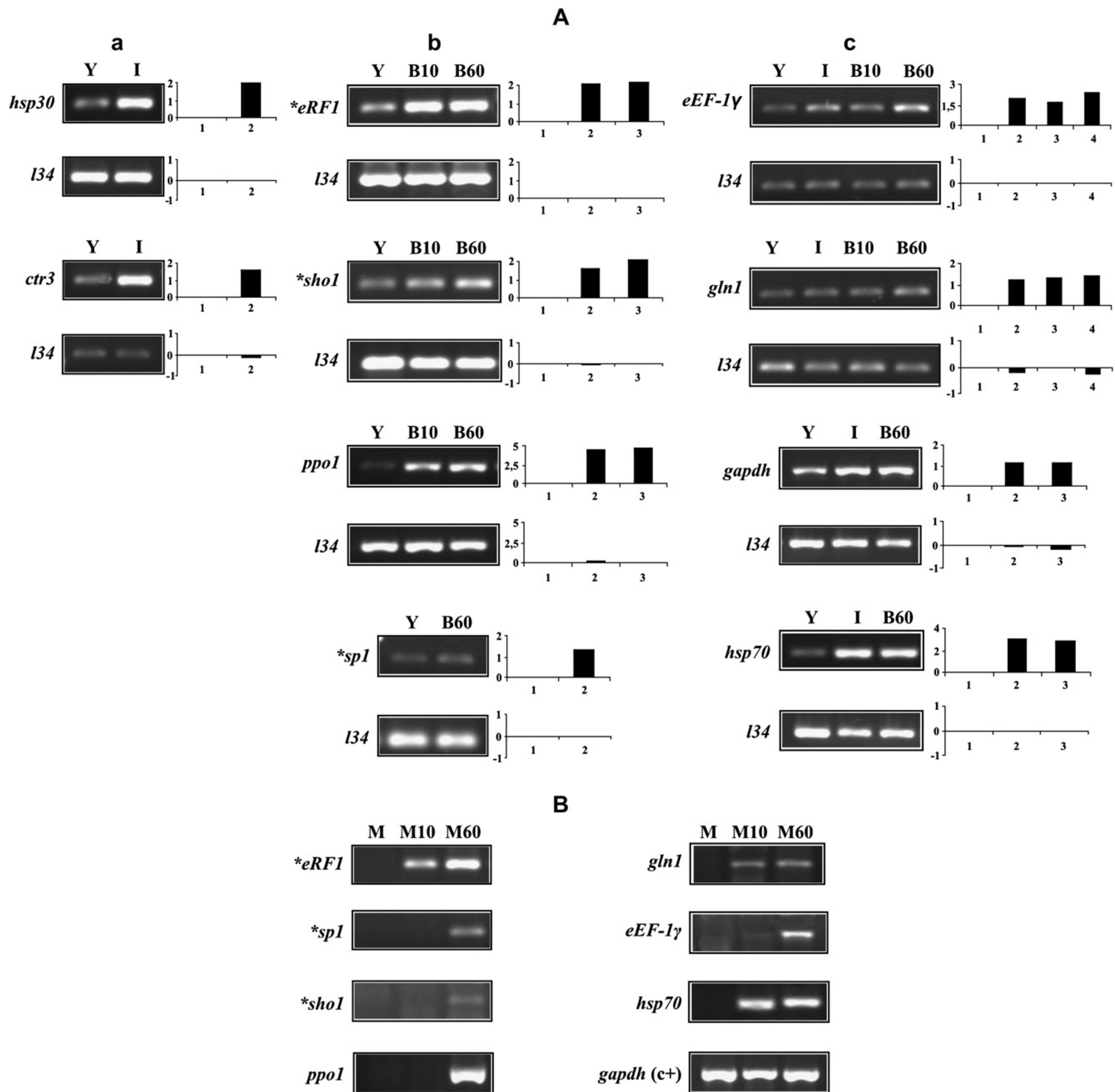

Fig. 3. In vitro and in vivo validation of the RDA results by RT−PCR analysis. (A) Validation of RDA results by semi-quantitative RT−PCR of RNAs obtained from yeast cells in host conditions. Semi-quantitative RT−PCR analysis was carried out with specific primers, as described. Numbers associated with 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 semi-quantitative analysis of the respective reactions. The same amounts of cDNAs were used for all PCRs. The RNAs used for RT−PCR were obtained from independent samples of: in vitro cultured yeast cells, yeast cells recovered from liver of infected mice and *P. brasiliensis* yeast cells incubated with human blood from those samples used for the RDA experiments. Clone names are written on the left side of the figure. The primers sense and antisense, were as following: *hsp30* 5′-GGCCTTGACAGCATTCTGG-3′ and 5′-CTGGCGATAAAGGGCA GAAG-3′; *ctr3* 5′-ATGTGAAGCAGCGAGCGG-3′ and 5′-CATGGAATGCACGGCGGC-3′; (*eRF1*) eukaryotic translation release factor 1 5′-CAACGTT GACTTTGTCATTGG-3′ and 5′-CCATGGACTTGTCATATACTG-3′; (*sho1*) transmembrane osmosensor 5′-CCACCACCGGCCACTGAC-3′ and 5′-CCCGAAACAACTGTCTCCG-3′; *ppo1* 5′-CATCGACGACTGCCTCCTC-3′ and 5′-GGACGGCTTCTGGGTGCT-3′; (*sp1*) putative serine protease 5′-CAATGGCTGCTCGTGCTGA-3′ and 5′-CCTACCAGGGGCATAAAGCT-3′; *eEF-1γ* 5′-GGCTTGGAGAGGGAGTCG-3′ and 5′-CCCTTGTTGGACGA GACCC-3′; *gln1* 5′-CGTTACCCTCACCGTAGAC-3′ and 5′-CATACGGCTGGCCCAAGG-3′; *gapdh* 5′-CAACGGATCCATGGTCGTCAAG-3′ and 5′-GCTGCGAATTCCTATTTGCCAGC-3′; *hsp70* 5′-CATATGGTGCCGCCGTCC-3′ and 5′-GGGAGGGATACCGGTTAGC-3′; *l34* 5′-CAAGACTCCAGGCGG CAAC-3′ and 5′-GCACCGCCATGACTGACG-3′. The sizes of the amplified DNA fragments are as follows: *hsp30* (221 bp); *ctr3* (373 bp); *eRF1* (392 bp); *sho1* (386 bp); *ppo1* (394 bp); *sp1* (319 bp); *eEF-1γ* (438 bp); *gln1* (494 bp); *gapdh* (1013 bp); *hsp70* (295 bp). The RNAs samples were obtained from: yeast cells, in vitro cultured (Y); yeast cells recovered from livers of infected mice (I); yeast cells treated with human blood for 10 min (B10) and 60 min (B60). Panel a: genes upregulated in yeast cells recovered from the host tissue; Panel b: genes upregulated in the human blood incubation; Panel c: genes upregulated in both conditions. L34 ribosomal protein was used as an internal control. Asterisks indicate new genes of *P. brasiliensis*. (B) Validation of the RDA-cDNA results by RT−PCR of RNAs obtained from yeast cells present in blood of infected BALB/c mice. Samples were isolated after 10 or 60 min of intravenous infection of yeast cells in BALB/c mice. RNAs from control BALB/c mice were used as references. The transcripts names listed on the left side of the panels are: *eRF1* (392 bp); *sp1* (319 bp); *sho1* (386 bp); *ppo1* (394 bp); *gln1* (494 bp); *eEF-1γ* (438 bp); *hsp70* (295 bp) and *gapdh* (1013 bp). Asterisks indicate new genes of *P. brasiliensis*. Control reactions with *Mus musculus* GAPDH are indicated by c + . The primers sense and antisense, respectively 5′-ATGTCGGCTCTCTGCTCCTC-3′ and 5′-GTAGGCCATGAGGTCCACC-3′, amplified a DNA fragment of 1062 bp in the obtained RNA.

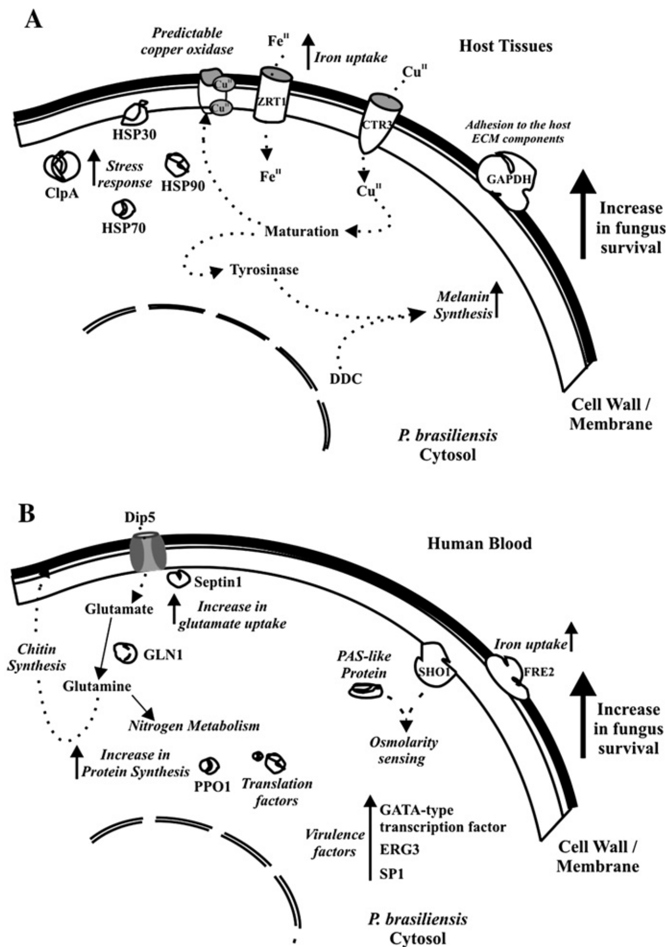

Fig. 4. Model of the adaptive changes of *P. brasiliensis* to the host milieu. (A) Model of how *P. brasiliensis* could enhance its survival in host cells. (B) Model of how *P. brasiliensis* enhance its survival during blood dissemination to organs and tissues. See Section 4 for details.

4.2. Stress response/cell wall remodeling/osmosensing/protein synthesis

The production of Hsps may contribute to the protection of cells from damage and to repair of cell following stress, which may occur during infection. Noteworthy is the high expression of the transcript encoding the Hsp30 in yeast cells recovered from liver of infected mice. This observation is particularly interesting as this Hsp is strongly induced by treatments that increase membrane fluidity, in organisms [15]. A homologue of the AP-1 like transcription factor was shown to be upregulated in the blood incubation. Homologues of the mammalian protein are found in fungi and genetic analyses indicate that those proteins can be involved in oxidative stress responses, as well as in multidrug resistance [16].

The transcript encoding glutamine synthetase (*gln1*) was strongly induced in blood incubation, suggesting that the remodeling of the cell wall/membrane may be one of the ways by which *P. brasiliensis* respond to changes in external osmolarity. Chitin synthesis has been shown to be essential in the compensatory response to cell wall stress in fungi, preventing cell death [17]. Similarly we speculate that *P. brasiliensis* increase in glutamine synthetase transcript could be related to chitin deposition in response to change in external osmolarity faced by the fungus in the blood route of dissemination. We cannot exclude the hypothesis that the increase in *gln1* could provide a mechanism for *P. brasiliensis* for ammonia reassimilation and detoxification. However, it has to be pointed out that in *Saccharomyces cerevisiae* just a small amount of ammonia is incorporated into the amide group of glutamine [18].

Acidic amino acid permease can mediate the uptake of glutamate and aspartate, resulting in chitin deposition. Glutamate could also be required to keep the cell in osmotic balance with the external medium. Septin modulates positively the activity of a glutamate transporter in mammalian astrocytes [19]. Upon incubation in blood the induction of the transcript encoding a homologue of septin was detected in *P. brasiliensis*. Also, the overexpression of the transcript encoding pyridoxamine phosphate oxidase might enhance the production of glutamate through amidotransferases, which requires the coenzyme PLP.

The HOG (high osmolarity glycerol) pathway senses osmotic stress via two membrane-bound regulators, *sln1* and *sho1*. In *Candida albicans*, *sho1* is related to the fungal morphogenesis interconnecting two pathways involved in cell wall biogenesis and oxidative stress [20]. Another class of sensor molecules comprehends PAS domain superfamily [21]. We observed preferential expression of the cited sensor transcripts in yeast cells during blood treatment and in yeast cells present in blood of infected mice, suggesting their involvement in the osmolarity sensing during fungus dissemination through the blood.

The high uptake of glutamate during *P. brasiliensis* yeast cell incubation in human blood could also provide yeast cells accordingly with the precursors to the increased rate in protein synthesis. In agreement, enzymes involved in the synthesis of the cofactors tetrahydrofolate and *S*-adenosylmethionine were upregulated in the treatment. It has not escaped our attention that the strong upregulation of genes involved in the protein synthesis machinery may reflect the *P. brasiliensis* yeast cell transfer from a nutrient-poor medium to a relatively nutrient-rich medium. Similar results were described upon incubation of *C. albicans* in human blood [22]. However, it seems that this should be the fungal condition entering the human blood when leaving host compartments in its route of dissemination.

4.3. Some putative virulence factors

Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) is upregulated mainly in fungi recovered from infected mice. We have demonstrated that the GAPDH of *P. brasiliensis* is a molecule located at the fungal cell wall. The protein is a *P. brasiliensis* adhesin, which binds components of the extracellular matrix and is capable of mediating the adherence and internalization of *P. brasiliensis* to in vitro cultured cells, suggesting its involvement in fungus pathogenesis [6].

Transcripts encoding tyrosinase and aromatic-L-amino acid decarboxylase were upregulated in yeast cells recovered from

liver of infected B.10A mice. Tyrosinase, the enzyme responsible for the first step in melanin synthesis, is a copper-containing protein [23]. Melanin is implicated in the pathogenesis of some microbial infections. Melanization of *C. neoformans* was demonstrated to be dependent of several genes, including homologues of the copper transporter and the copper chaperone [24]. *P. brasiliensis* melanin-like pigments were detected in cells growing in vitro and during infection [25]. The melanized fungal cells were more resistant than nonmelanized cells to the antifungal activity of murine macrophages [26].

GATA-factors ensure efficient utilization of available nitrogen sources by fungi and have been associated with fungal virulence [27]. A transcript encoding $\Delta^{5,6}$-desaturase (ERG3) was upregulated during incubation in blood; in *C. albicans* a homologue has been associated with virulence and the ability of the organism to undergo the morphological transition [28]. Also, a *P. brasiliensis* homologue of the histone deacetylase gene was described here. In *Cochliobolus carbonum* a homologue of this gene affects virulence as a result of reduced penetration efficiency in plant tissues [29]. Future work has to focus on those predictable virulence factors of *P. brasiliensis*.

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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

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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, AGGCAACTGTGCTATCCGAGGGAG; RBam12, GATCCTCGGTGA; 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

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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-1γ*); 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′-CATCGACGACTGCCTCCTC-3′ and 5′-GGACGGCTTCTGGGTGCT-3′; putative major facilitator protein (*ptm1*), 5′-CGATTCCTCGCAATTGGTCA-3′ and 5′-CGTTGCGCCCAATGAGTTC-3′; eukaryotic release factor 1 (*eRF-1*), 5′-CAACGTTGACTTTGTCATTGG-3′ and 5′-CCATGGACTTGTCATATACTG-3′; *eEF-1γ*, 5′-GGCTTGGAGAGGGAGTCG-3′ and 5′-CCCTTGTTGGACGAGACCC-3′; *gln1*, 5′-CGTTACCCTCACCGTAGAC-3′ and 5′-CATACGGCTGGCCCAAGG-3′; *sho1*, 5′-CCACCACCGGCCACTGAC-3′ and 5′-CCCGAAACAACTGTCTCCG-3′; and ribosomal L34 protein (*l34*), 5′-CAAGACTCCAGGCGGCAAC-3′ and 5′-GCACCGCCATGACTGACG-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%

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(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

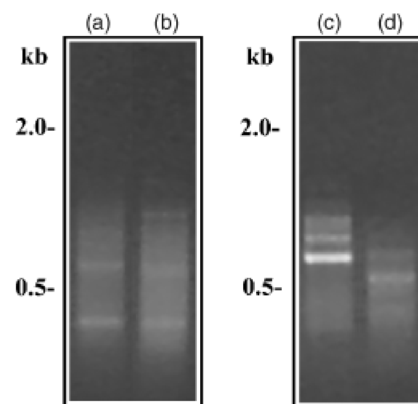

Fig. 1. Agarose gel electrophoresis of subtracted differential cDNA pools derived from *Paracoccidioides brasiliensis* yeast cells incubated with human plasma. Products of the first and second rounds of subtraction performed using as testers the cDNA obtained from RNAs of yeast cells incubated with human 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.

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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	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

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Table 1. Continued.

MIPS category	Gene product	Best hit/accession number	e-value	Redundancy	
				P10	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*.

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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).

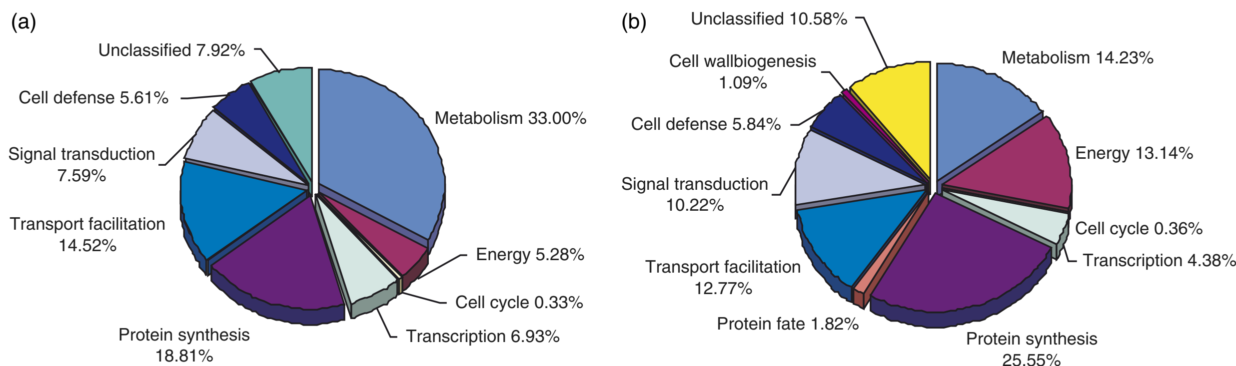

Fig. 2. Functional classification of *Paracoccidioides brasiliensis* cDNAs derived from RDA experiments using as testers the cDNAs obtained from RNA of *Paracoccidioides brasiliensis* yeast cells after incubation with human plasma for 10 min (a) or 60 min (b). The percentage of each functional category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

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

Gene product	Organism	e-value	Redundancy	
			Incubation in human plasma	
			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

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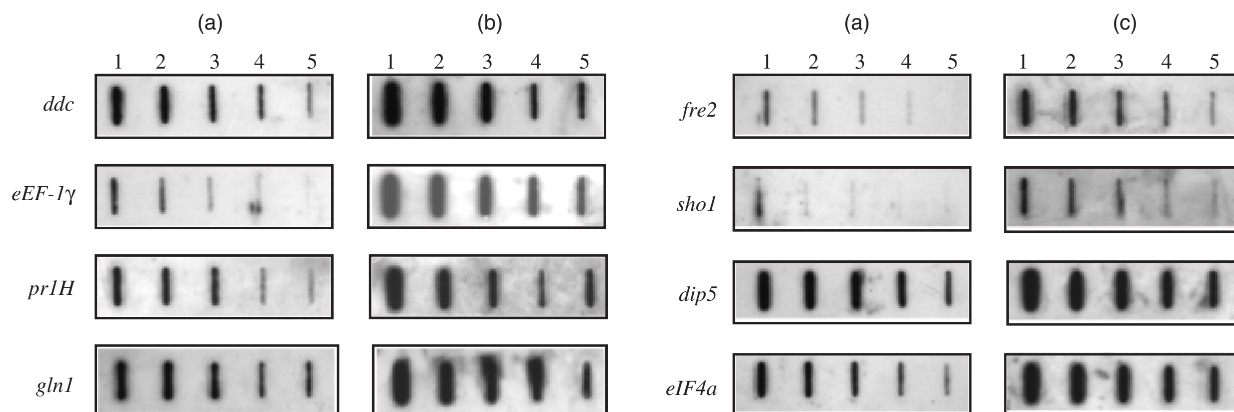

Fig. 3. Dot-blot analysis of *Paracoccidioides brasiliensis* cDNA-RDA clones. DNAs of individual clones were prepared and blotted in several dilutions (1–5). Individual clones were blotted and hybridized to the labeled cDNAs obtained from the control yeast cells (a), and labeled cDNAs obtained from *Paracoccidioides 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*).

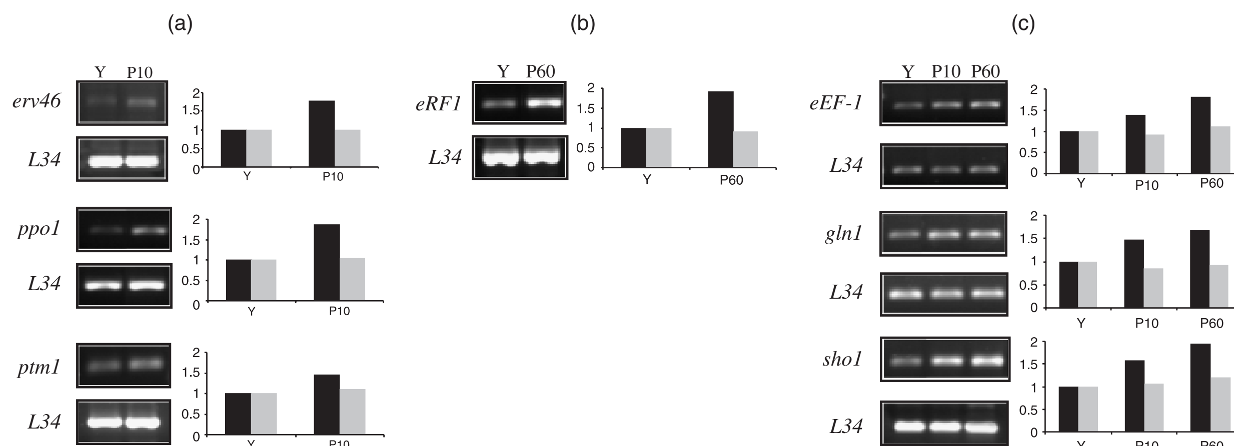

Fig. 4. Validation of RDA results by semiquantitative RT-PCR of RNAs obtained from yeast cells during incubation with human plasma. Semiquantitative RT-PCR analysis was carried out with specific primers, as described. Numbers associated with the bars indicate fold differences relative to the data for 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

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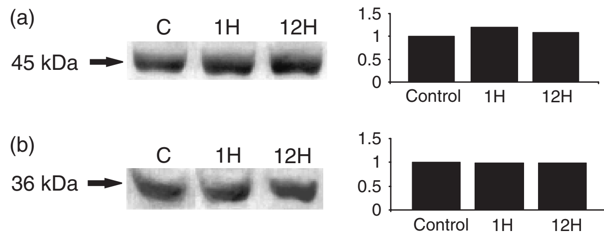

Fig. 5. Validation of the RDA results by Western blot. Total cellular extracts were obtained from yeast cells incubated with human plasma for 1 and 12 h. The proteins (25 μg) were electrophoretically transferred to a nylon membrane and checked by Ponceau S to determine equal loading. The samples were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

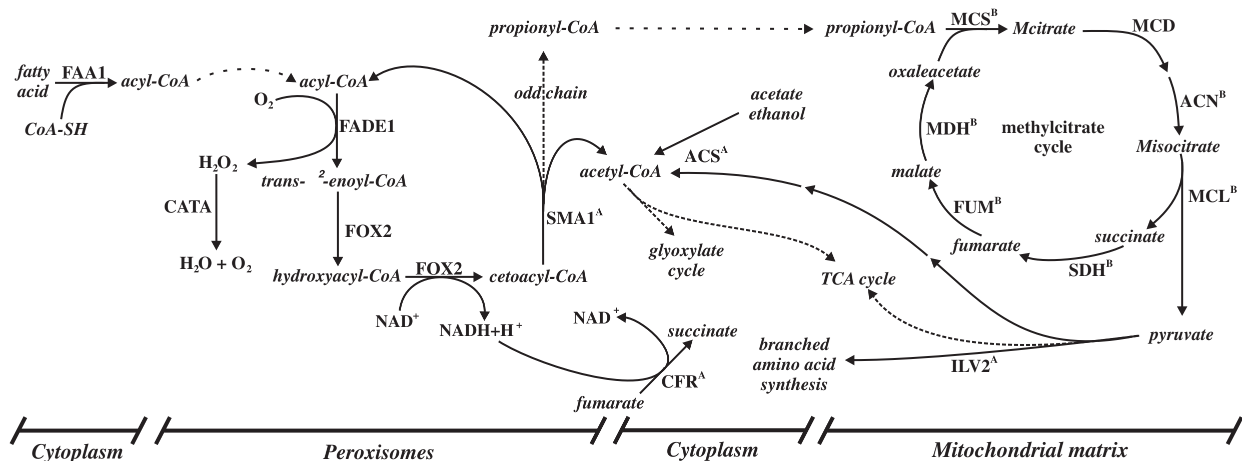

Fig. 6. Some metabolic pathways that are overexpressed during *Paracoccidioides brasiliensis* yeast cell incubation with human plasma. [(A)]Transcripts that are not overexpressed during *Paracoccidioides brasiliensis* treatment with human blood. [(B)]Transcripts present in database. FAA1, long-chain 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.

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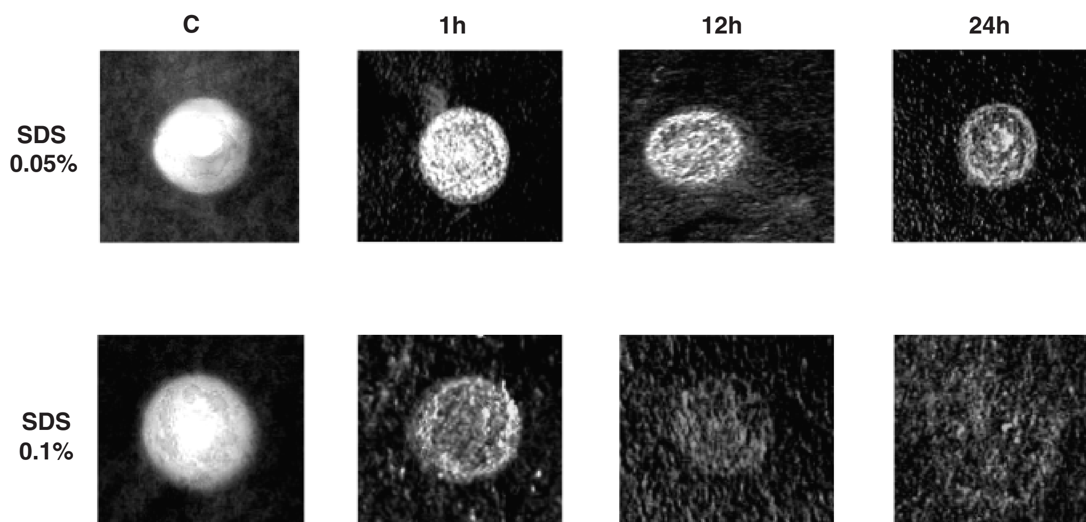

Fig. 7. Phenotypic analysis of *Paracoccidioides brasiliensis* yeast cells after incubation in human plasma for different time periods. Approximately 10^2 cells were spotted onto Fava-Neto's medium plates containing the indicated concentrations of SDS. Plates were incubated at 36 °C for 7 days. Experiments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

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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

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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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The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals novel and induced genes potentially associated to the dimorphic process

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Abstract

Background: *Paracoccidioides brasiliensis* is a human pathogen with a broad distribution in Latin America. The fungus is thermally dimorphic with two distinct forms corresponding to completely different lifestyles. Upon elevation of the temperature to that of the mammalian body, the fungus adopts a yeast-like form that is exclusively associated with its pathogenic lifestyle. We describe expressed sequence tags (ESTs) analysis to assess the expression profile of the mycelium to yeast transition. To identify *P. brasiliensis* differentially expressed sequences during conversion we performed a large-scale comparative analysis between *P. brasiliensis* ESTs identified in the transition transcriptome and databases.

Results: Our analysis was based on 1107 ESTs from a transition cDNA library of *P. brasiliensis*. A total of 639 consensus sequences were assembled. Genes of primary metabolism, energy, protein synthesis and fate, cellular transport, biogenesis of cellular components were represented in the transition cDNA library. A considerable number of genes (7.51%) had not been previously reported for *P. brasiliensis* in public databases. Gene expression analysis using in silico EST subtraction revealed that numerous genes were more expressed during the transition phase when compared to the mycelial ESTs [1]. Classes of differentially expressed sequences were selected for further analysis including: genes related to the synthesis/remodeling of the cell wall/membrane. Thirty four genes from this family were induced. Ten genes related to signal transduction were increased. Twelve genes encoding putative virulence factors manifested increased expression. The in silico approach was validated by northern blot and semi-quantitative RT-PCR.

Conclusion: The developmental program of *P. brasiliensis* is characterized by significant differential positive modulation of the cell wall/membrane related transcripts, and signal transduction proteins, suggesting the related processes important contributors to dimorphism. Also, putative virulence factors are more expressed in the transition process suggesting adaptation to the host of the yeast incoming parasitic phase. Those genes provide ideal candidates for further studies directed at understanding fungal morphogenesis and its regulation.

Background

Paracoccidioides brasiliensis is a dimorphic pathogenic ascomyceteous fungus, endemic to the Latin America that can cause primary disease in humans. In the soil the fungus grows as saprobic mycelium, resulting in the formation of propagules, which initiates the infection in humans when inhaled into the respiratory tract. Subsequently, in the lung, the mycelia propagules develop into yeast cells. The mycelium to yeast transition can be replicated in vitro by growing mycelia in conditions of elevated temperature. The ability of *P. brasiliensis* to grow in the mycelia form in the soil and shift to the yeast form in the host is important for infection and disease. Once introduced into the host, the mycelial propagules have to convert to yeasts, a condition essential for the fungus to survive and proliferate [2,3].

The morphological transition in *P. brasiliensis* is governed predominantly by the temperature and is preceded by several molecular changes. The identification of genes specifically involved in the mycelium to yeast transition in *P. brasiliensis* has been subject of great interest, since pathogenicity is intimately linked to the dimorphic transition in some fungi [4]. Approaches used in the identification of genes important for the transition process include, for example, the differential expression of *P. brasiliensis* genes in both fungal phases identified by electronic subtraction and cDNA microarray hybridization, which were employed to search for genes whose expression, displayed statistically significant modulation during the mycelium to yeast transition [5-8].

The biochemical processes that control the morphogenesis of *P. brasiliensis* are just coming to light. The dimorphic transition involves alterations in the cell wall composition and in the structure of carbohydrates polymers [9,10]. The yeast cells exhibit an energy metabolism biased towards ethanol production through fermentation, whereas mycelium metabolism tends to be more aerobic than that of yeast cells. Also the glyoxylate pathway is more active in the yeast form of *P. brasiliensis* [5]. Hyper expression of some enzymes in the sulphur metabolism pathway in the yeast phase of *P. brasiliensis*, as well as during the transition from mycelium to yeast have been reported, corroborating previous descriptions of the importance of this metabolic pathway to the dimorphic process [6,8,11].

Here, we have tested the concept that novel genes involved in *P. brasiliensis* phase transition could be described by applying a transcriptome analysis of cells undergoing mycelium to yeast transition. In this manuscript we describe EST analysis to assess the expression profile of mycelium undergoing yeast transition. This choice of approach distinguishes the present work from previous recently published papers that employed microarray hybridization, electronic subtraction and suppressive subtraction hybridization in order to assess differences using differentiated yeast and mycelium cells [5-8,12]. Using a custom analysis pipeline for sequences of *P. brasiliensis*, isolate *Pb*01, yeast and mycelium forms [1] we obtained an EST databank web interface [13].

In this study we report the in silico analyses and comparison of ESTs from mycelium undergoing the early transition to yeast with mycelium differentiated cells. Our analysis revealed 179 genes that are positively modulated during the early transition process, when compared to mycelia. Additionally 48 novel genes were described in the *P. brasiliensis* transition cDNA library. Upon categorization by known databases we have selected MIPS (Munich Center for Protein Sequences) categories for further analyses. Several ESTs were selected for semi-quantitative and quantitative analysis to examine changes in gene expression induced by the temperature induced transition of phases.

Results and Discussion

cDNA library construction, sequencing and sequence annotation

Transcriptome profiling of mycelium undergoing differentiation to yeast cells in *P. brasiliensis* has directed our studies to reveal several uncharacterized genes involved in this process. We performed in this EST-based program the sequencing 2880 randomly selected clones. Of these, 2666 gave readable sequences. 1107 sequences remained after vector and low quality sequences were removed. Of these, 166 consisted of singletons and 473 corresponded to consensus with two or more ESTs. In total, 447761 bp of assembled sequences were obtained corresponding to an average consensus sequence length of 404 bp. The 1107 sequences were annotated. A total of 828 sequences (74.8%) showed significant similarity to known protein sequences (E value $\leq 10^{-4}$) based on BLAST searches and 433 ESTs (39.1%) had unknown function and were classified as hypothetical proteins. 992 sequences (89.6%) gave significant hits to ESTs present in the *P. brasiliensis* transcriptome database [1] or in the GenBank database. In addition, 115 sequences (10.4% of the total) represented novel genes of *P. brasiliensis*.

Description of the ESTs in the transition transcriptome

An overview of the probable adaptations made by *P. brasiliensis* mycelium during morphogenesis can be obtained by analyzing the ESTs in this early stage of cellular differentiation. As shown in Fig. 1, the ESTs were mainly represented as following: a total of 22.11% of the annotated ESTs corresponded to the fungal metabolism; 17.06% of the ESTs were related to the protein synthesis machinery; 10.83% of the transcripts corresponded to homologues encoding transport facilitators; 10.24% corresponded to

ESTs related to protein fate; 7.42% to energy; 7.27% to signal transduction proteins; 7.12% were related to the transcription machinery; 6.68% corresponded to transcripts related to the biogenesis of cellular components; 6.38% corresponded to ESTs encoding cell rescue, defense and virulence factors.

Comparison of **P. brasiliensis** *ESTs present in the transition library to those described for yeast and mycelium stage specific phases: induced genes identified by in silico EST subtraction*

We attempted to determine the putative function of the set of 639 phrap unisequences by searching for homologs in the GenBank non-redundant protein database using BLAST X. We also compared the sequenced ESTs present in the transition library to those present in the mycelium transcriptome database. According to the subtractive analysis, the classification of induced genes was designed for the ESTs that were not previously described in *P. brasiliensis* in databases or that manifested increased expression in the transition library as compared to mycelia transcriptome database [1]. This classification was performed according to the statistical test described by Audic and Claverie [14], with a 99% confidence rate. The comparative analysis of all the ESTs annotated in the transition library is available in Table 1, supplementary material. From the 1107 ESTs identified in this work, 426 of the total corresponded to induced genes in the transition library. From the 426 annotated ESTs, 115 corresponded to novel ESTs, representing 48 novel classified genes. Table 2, supplementary material, summarizes the results of such comparison. As shown, the majority of transition induced genes (82.12%) was composed of unique sequences or groups of two or three ESTs. Genes with altered expression included those involved in metabolism of amino acids, nitrogen, sulfur, nucleotides, carbohydrates, vitamins and lipids. In addition genes related to energy generation, signal transduction and cell wall biogenesis, were increased. A small subset of genes with elevated expression had unknown function. The largest induced groups of sequences consisted of a total of 24 ESTs with homology to a histidine protein kinase sensor for GlnG regulator, 18 ESTs exhibiting homology to ubiquinone/menaquinone methyltransferase, 11 ESTs with homology to arylsulfatase regulatory protein, 09 ESTs with homology to acidic amino acid permease, 06 ESTs with homology to a HSP 90 and 07 ESTs with homology to aspartyl protease.

Genes involved in sulfur assimilation, have been described as induced in *P. brasiliensis* transition from mycelium to yeast and in yeast differentiated cells [6,8]. Here, we described in the transition transcriptome the induction of a set of genes related to sulphur metabolism, such as, the transcript encoding sulfite reductase (E.C. 1.8.1.2) an enzyme of the sulfur assimilation pathway, leading to cysteine biosynthesis. Sulfite reductase contains a special acidic heme group called siroheme. One of the novel genes detected in the transiton library encodes for an urophorphyrinogen III methylase (E.C 2.1.1.107) homologue to the Met1p of *Saccharomyces cerevisiae*, related to the sirohaem and cobalamin biosynthesis [15,16]. Also, the transcript encoding sulfate permease was induced compared to the mycelia transcriptome. Sulfate is co-transported into the cells in an energy dependent process catalyzed by specific plasma membrane permeases [17]. An arylsufatase regulatory protein probably involved in the regulation of sulfatase genes was described in the transition transcriptome. The transcript in *P. brasiliensis* has sequence identity to bacterial and fungal arylsulfatases regulatory proteins. Sulfatases catalyze hydrolytic cleavage of sulfate ester bonds, liberating sulfate and the corresponding alcohol [18]. In *Neurospora crassa* arylsulfatase is up regulated by sulfur starvation and appears to function as a mechanism for sulfur scavenging [19]. Also, a thiosulfate sulphurtransferase (TST) (E.C. 2.8.1.1) putatively, a mitochondrial matrix protein that plays roles in formation of iron sulfur proteins, as well as in modification of iron-sulfur proteins [20] was induced in the transition transcriptome. The increase in the expression of genes related to the sulphur metabolism, including the description of novel transcripts corroborates the previous descriptions of the involvement of sulphur metabolism in the transition process of *P. brasiliensis* [6,8,11].

The list of induced genes also includes several ESTs encoding proteins related to lipid metabolism, to signal transduction and to carbohydrate metabolism that will be referred below. Also proteases, such the Lon protease putatively related to degradation of damaged or nonnative proteins in the mitochondrial matrix are induced [21]. An aspartyl protease and a zinc metalloprotease were among the transcripts with increased expression. Of special note molecules related to protein fate, such as to glycoslylation and degradation, are abundant in the transition transcriptome, as shown in Table 2, supplementary material.

An overview of genes related to the membrane/cell wall remodeling presenting increased expression in the transition library

We catalogued the ESTs potentially associated with fungal cell wall/membrane synthesis/remodeling described during the mycelium to yeast transition. Table 1 depicts the ESTs predominantly related to the synthesis of those components. The transcripts with increased expression include those encoding enzymes related to the cell wall carbohydrates biosynthesis and degradation, the transporters of the precursors for the synthesis of such mole-

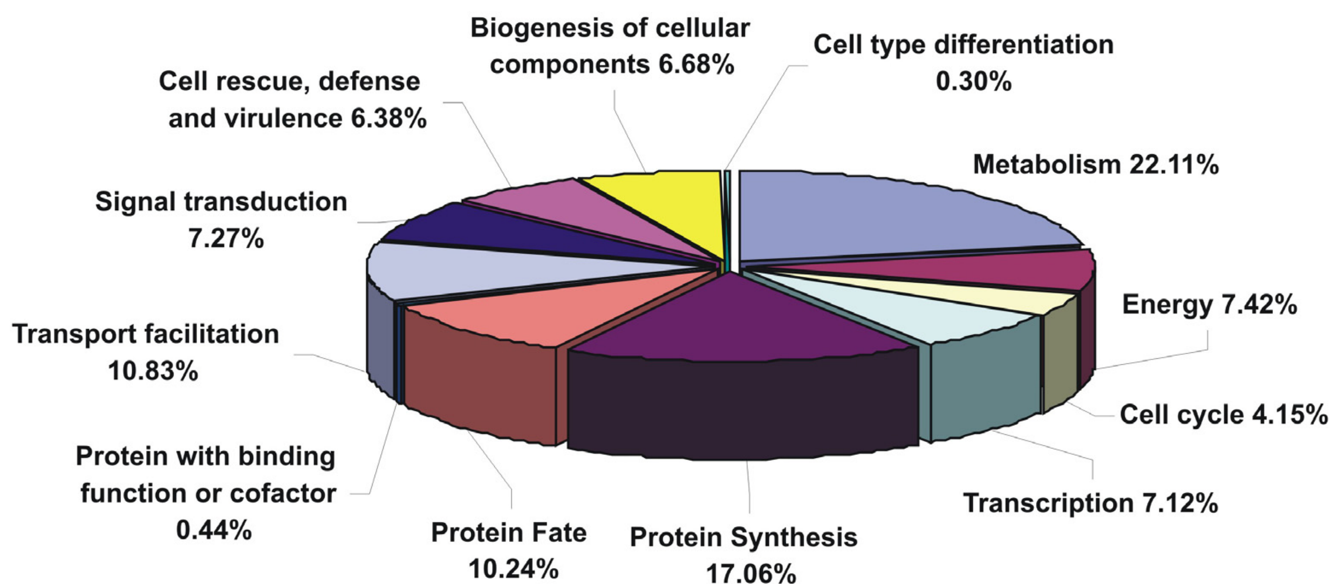

Figure 1
Classification of ESTs from the transition cDNA library of *P. brasiliensis*. The classification was based on *E* value and performed according to the functional categories developed on the MIPS functional annotation scheme.

cules, enzymes related to protein glycosylation and to the synthesis of membrane lipids.

It is presumed that the dimorphic transition occurs simultaneously with changes in the fungal cell wall composition of such compounds as phospholipids and carbohydrate polymers [3,10,22]. In *P. brasiliensis*, lipids, chitin, glucans and proteins are the main constituents of the cell wall in mycelium and yeast cells. The transition transcriptome data suggest that *P. brasiliensis* favors the membrane and cell wall remodeling in the early stages of transition, from mycelium to yeast. Transcription of 34 cell wall/membrane related genes were induced upon temperature shift (Table 1).

In Table 1 and Fig. 2A, an overview of the induced enzymes and transporters putatively related to the biosynthesis of the carbohydrate compounds of the cell wall, is shown.

Many cell wall-related proteins were found among the presently identified ESTs, including molecules related to the chitin synthesis, alpha glucan synthesis and chitin degradation. The main polysaccharide of the yeast cell wall is alpha-glucan, whereas the mycelium contains predominantly beta-glucan [23]. Several genes related to the synthesis of the carbohydrate components of the cell wall were induced in the transition library, in comparison to the mycelium transcriptome database [1]. Those genes include phosphoglucomutase (*pgm*) UDP-Glucose pyrophosphorylase (*ugp1*), and alpha -1,3 glucan synthase (*ags1*), (Table 1, Fig. 2A), putatively enabling the increase in the synthesis of alpha-1,3 glucan in the yeast incoming cell wall [10]. A novel transcript encoding an alpha glucosidase 1 (GLCase I) was described. It has been suggested that glucosidases are directly involved in the synthesis or processing of beta-1,6 glucan in *S. cerevisiae* [24].

Chitin is the major component of yeast cells in which it comprises (37% to 48%) of the total cell wall components. Of special note is the detection of a novel transcript encoding an UDP-N-acetyl glucosamine transporter (MNN2), which has been described in *S. cerevisiae*. The cytoplasm is the sole site of sugar nucleotide synthesis and sugar nucleotides must be transported into various organelles in which they are utilized as a donor substrate for sugar chain synthesis. It has been demonstrated that UDP-N-acetyl glucosamine transporter encoded by the YEA4 gene in *S. cerevisiae* is located in the endoplasmic reticulum and is involved in cell wall chitin synthesis in this fungi [25]. GDA1 (guanosine diphosphatase) generates both GMP and UMP required as antiporters for guanosine and uridine sugar transport into the Golgi lumen. Deleted strains of *Kluveromyces lactis* for *gda1* present altered cell wall stability and composition [26]. Chitinase 1 (CTS1) and 3 (CTS3), the latter a novel gene, were induced in the transition library suggesting their role in the remodeling of the cell wall and providing N-acetyl glucosamine for the synthesis of chitin. The DIP5 encoding transcript (acidic amino acid permease) was increased in

Table 1: Induced *P. brasiliensis* transcripts potentially related to membrane and cell wall synthesis/remodeling.

Gene Product	E.C. number	Annotated function	Predicted redundancy‡	
			M	T
Alpha-glucosidase I* (glcase 1)	3.2.1.106	Single glucose residues remotion from oligossaccharides	-	1
Phosphoglucomutase (pgm)	5.4.2.8	Synthesis of glucose	-	1
UDP-glucose pyrophosphorylase (ugp1)	2.7.7.9	Synthesis of UDP-Glucose	-	2
Alpha-1,3 glucan synthase (ags1)	2.4.1.183	Synthesis of α1–3-glucan	-	1
Mannitol-1-phosphate dehydrogenase (mtld)	1.1.1.17	Synthesis of fructose 6-phosphate	2	3
Monosaccharide transport protein (mstE)	-	Low affinity glucose uptake	-	1
Sugar transporter protein (stl1)	-	Uptake of hexoses	3	5
Chitinase 1(cts1)	3.2.1.14	Hydrolysis of chitin	1	2
Chitinase 3* (cts3)	3.2.1.14	Hydrolysis of chitin	-	1
Acidic amino acid permease (dip5)	-	Acidic amino acid uptake	9	9
Histidinol phosphate aminotransferase (hpat)	2.6.1.9	Synthesis of L-histidinol phosphate/glutamate	-	1
Malate permease (mael)	-	Uptake of Malate	-	2
UDP-N-acetylglucosamine transporter* (mnn2)	-	Required for transport of the chitin precursor to Golgi and Endoplasmic reticulum	-	1
Glucanosyltransferase family protein (gel)	2.4.1.-	Transglucosidase activity	1	3
Rho GTPAse activating protein* (bem3)	-	Regulation of the beta(1,3)-glucan synthase	-	1
Mannosyltransferase (mnt1)	2.4.1.131	Mannosylation of proteins/lipids	-	1
Alpha-1,2-mannosyltransferase (mnn5)	2.4.1.131	Mannosylation of proteins/lipids	3	3
Guanosine diphosphatase* (gdA1)	3.6.1.42	Synthesis of GMP	-	1
Alpha-1,2 galactosyltransferase* (gma12)	2.4.1.-	Galactose incorporation in N- and O-linked mannoproteins	-	1
Lysophospholipase (lpb1b)	3.1.1.5	Hydrolysis of phospholipids	-	1
Phospholipase A2 (plaA)	3.1.1.4	Hydrolysis of phospholipids	-	1
Glycerol-3-phosphate dehydrogenase* (NADP) (gfdA)	1.1.1.94	Synthesis of Glycerol-3-phosphate.	-	1
Glycerophosphodiester phosphodiesterase (gpdp)	3.1.4.46	Synthesis of choline and ethanolamine	1	4
Acyl-coenzyme A synthetase (acs)	6.2.1.3	Convertion of the fatty acid to acyl-coA for subsequent beta oxidation	-	1
Phosphatidylserine synthase* (pssA)	2.7.8.8	Glycerophospholipid metabolism/Phosphatidylserine synthesis	-	1
Myo-inositol-1-phosphate synthase (ino1)	5.5.1.4	Synthesis of myo-inositol 1 phosphate	-	1
Phosphatidylinositol transfer protein (pdr16)	-	Transport of phospholipids from their site of synthesis to cell membranes/Regulator of phospholipid biosynthesis	-	1
Lanosterol 14-alpha-demethylase (erg11)	1.14.13.70	Synthesis of ergosterol	3	4
Sterol delta 5,6-desaturase (erg3)	1.3.3.-	Regulation of ergosterol biosynthesis	-	1
Serine esterase (net1)	-	Catalysis of the cleavage of fatty acids from membrane lipids	-	3
Peroxisomal hydratase dehydrogenase epimerase (hde)	4.2.1.-	Beta oxidation	-	4
Fatty acid desaturase (desA)	1.14.99.-	Insaturation of acyl group of lipids	1	2
Carnitine dehydratase (caiB)	4.2.1.89	Transport of long-chain fatty acids	-	1
Suppressor of anucleate metulae B protein* (samB)	-	Morphogenesis regulation	-	1

‡ The predicted redundancy was obtained from the transition cDNA library in comparison to mycelia transcriptome database [1].
* Novel genes detected in *P. brasiliensis*.

the transition library and could provide the uptake of glutamate, a precursor required for the synthesis of chitin. We recently described that this transcript is up regulated in *P. brasiliensis* yeast cells during incubation in human blood and is hypothetically related to the cell wall remodeling supposed to occur during osmotic stress [27]. In addition, the induced enzyme HPAT (histidinol phosphate aminotransferase) could also provide glutamate for the synthesis of chitin precursors.

Sugar transporters MSTE (monosaccharide transport protein), STL (sugar transport protein), GTT (glucose transporter) were present in the transition transcriptome; the first two genes were present as increased transcripts. The increased expression may permit the fungus to increase uptake of carbohydrates, thus accelerating the synthesis of glucan and chitin (Table 1, Fig. 2A). The *mael* (malate permease) cDNA encoding the transporter for malate is an induced gene in the transition library and could provide the precursor for gluconeogenesis furnishing carbohydrate precursors to the cell wall components biosynthesis. Also the availability of compounds to the glyoxalate cycle seems to be favored during transition. The MAEL (malate transporter) could provide malate for the glyoxylate cycle. The enzymes (CITA) citrate synthase (E.C.2.3.3.1), (ACO) aconitase (E.C.4.2.1.3), (ICL) isocitrate lyase (E.C.4.1.3.1), and (MDH) malate dehydrogenase (E.C.1.1.1.37) were present in the transition library, indicating that the glyoxalate cycle is functional during the transition from mycelium to yeast. Of note the transcriptome analysis in *P. brasiliensis* showed several pathways that provide substrates for the glyoxalate cycle that is up regulated in the yeast cell, as described previously [5].

Induced transcripts in the transition library also involve those related to the phospholipids synthesis, as well as to ergosterol, as shown in Table 1 and Fig. 2B. The enzyme GFDA (glycerol 3P dehydrogenase) converts DHCP (dihy-

Table 2: List of novel genes detected in the *P. brasiliensis* transition library.

Functional categories	Gene Product	Best hit/Accession number	e-value	E.C. number
Amino acid metabolism	Diphthine synthase#	*Aspergillus fumigatus*/CAF32112	1e-38	2.1.1.98
	Acetylornithine deacetylase	*Arabidopsis thaliana*/BP845946.1	1e-31	3.5.1.16
	Histidine ammonia-lyase	*Dictyostelium discoideum*/XP_636944.1	1e-16	4.3.1.3
	Glutamate dehydrogenase (NADP(+))	*Emericella nidulans*/S04904	5e-06	1.4.1.4
Nucleotide metabolism	Nudix hydrolase family protein	*Aspergillus nidulans*/XP_409279.1	1e-19	-
	Adenosine deaminase	*Aspergillus oryzae*/BAE60718	2e-34	3.5.4.4
	Orotate phosphoribosyltransferase	*Mortierella alpina*/BAD29963.1	3e-45	2.4.2.10
Phosphate metabolism	phnO protein	*Rhizopus oryzae*/EE002192.1	4e-116	-
C-compound and carbohydrate metabolism	Chitinase 3#	*Coccidioides immitis*/AAO88269	7e-40	3.2.1.14
	Alpha-glucosidase I#	*Aspergillus fumigatus*/AAR23808	3e-46	3.2.1.106
	Glycerol-3-phosphate dehydrogenase (NAD(P)+)	*Cryptococcus neoformans*/AAM26266.1	2e-14	1.1.1.94
Lipid metabolism	Phosphatidylserine synthase#	*Neurospora crassa*/EAA30566.1	6e-38	2.7.8.8
Metabolism of vitamins, cofactors and prosthetic groups	Uroporphyrinogen III methylase	*Rhizopus oryzae*/EE010378.1	6e-109	2.1.1.107
Energy	Xanthine dehydrogenase	*Gibberella zeae*/XP_381737.1	9e-07	1.17.1.4
	Acetyl CoA hydrolase	*Aspergillus nidulans*/XP_405684.1	5e-42	3.1.2.1
Cell cycle and DNA processing	Rad21 region protein	*Neurospora crassa*/EAA34981.1	6e-17	-
	Proliferating Cell Nuclear Antigen (PCNA)	*Aspergillus nidulans*/XP_404552.1	3e-36	-
	Uracil-DNA glycosylase	*Aspergillus fumigatus*/XP_749743	3e-24	3.2.2.-
	Chromosome segregation ATPase	*Coccidioides immitis*/EAS30662	6e-52	-
Transcription	DEAD-like helicases superfamily protein#	*Aspergillus nidulans*/XP_410144.1	3e-55	-
	Transcription factor, bromodomain	*Aspergillus nidulans*/EAA60972	2e-55	-
	GatB/YqeY domain protein	*Aspergillus nidulans*/XP_410874.1	1e-22	-
	Ring type Zinc finger protein	*Aspergillus nidulans*/XP_411042.1	1e-12	-
	Zinc finger domain protein	*Aspergillus nidulans*/XP_405585.1	3e-14	-
	Arylsulfatase regulatory protein	*Blastocladiella emersonii*/CO964913.1	1e-138	-
	Transcriptional activator protein	*Coccidioides immitis*/EAS34609	8e-26	-
Protein Synthesis	14 kDa mitochondrial ribosomal protein	*Aspergillus nidulans*/XP_408748.1	4e-46	-
	Translation initiation factor 3 subunit 2	*Aspergillus nidulans*/XP_660601	6e-80	-
Protein fate	Rab geranylgeranyl transferase	*Aspergillus nidulans*/XP_412816.1	8e-13	2.5.1.60
	Guanosine diphosphatase#	*Aspergillus nidulans*/XP_405219.1	2e-15	3.6.1.42
	Ubiquitin thiolesterase otubain-like protein	*Aspergillus nidulans*/EAA60354	1e-28	3.4.-.-
	Non-ATPase regulatory subunit of the 26S proteasome	*Aspergillus nidulans*/XP_408912.1	2e-68	-
	Peptidase M28 domain protein	*Coccidioides immitis*/EAS33583	1e-22	3.4.11.15
	Alpha -1, 2-galactosyltransferase#	*Aspergillus nidulans*/XP_406106.1	3e-14	2.4.1.-
Transport Facilitation	Uridine diphosphate N-Acetylglucosamine transporter#	*Neurospora crassa*/T50997	9e-30	-
	Nuclear pore protein 84/107	*Coccidioides immitis*/EAS31445.1|	2e-13	-
	Regulator of V-ATPase in vacuolar membrane protein	*Aspergillus nidulans*/XP_404840.1	9e-59	-
	Tctex-1 family protein	*Aspergillus nidulans*/XP_405470.1	6e-25	-
	Importin-beta N-terminal domain	*Aspergillus nidulans*/XP_410143.1	1e-44	-
Signal Transduction	Two-component sensor kinase	*Anopheles gambiae*/EAA02130.2	2e-38	-
	Histidine protein kinase sensor for GlnG regulator#	*Tetrahymena thermophila*/EAR83219.1	2e-04	2.7.3.13-
	UVSB Phosphatidylinositol – 3 kinase#	*Aspergillus nidulans*/XP_411112.1	1e-29	-
	Rho GTPase activating protein	*Aspergillus nidulans*/XP_407883.1	3e-49	-
	Calcineurin subunit b	*Neurospora crassa*/P87072	1e-77	-
	Forkhead associated (FHA) protein	*Gibberella zeae*/XP_389397.1	4e-10	-
Cell Rescue, Defense and Virulence	Hemolysin like protein#	*Aspergillus nidulans*/XP_406013.1	2e-70	-
Cell type differentiation	Suppressor of anucleate metulae B protein#	*Aspergillus nidulans*/XP_404215.1	6e-46	-
Unclassified	Complex I protein (LYR family)	*Aspergillus nidulans*/XP_408902.1	8e-32	-

#Transcripts confirmed by semi-quantitative RT-PCR.

droxycetona phosphate) in G3P (glycerol 3P). The *gfdA* null mutant of *Aspergillus nidulans* displays reduced G3P levels and an osmoremediable growth defect, which is associated with abnormal hyphal morphology [28]. G3P can be produced by the action of the enzyme GDPD (glycerophosphodiester phosphodiesterase) which promotes the hydrolysis of phosphatidylethanolamine (G3PEtn). Both enzymes are induced in the transition from mycelium to yeast cells, as shown in Table 1 and Fig. 2B. The ACT (acyltransferase) promotes the addition of acyl groups to G3P generating DG3P (diacylglycerol 3P); this enzyme is described in *P. brasiliensis* in the public databases. The acyl CoA required for the synthesis of DG3P is produced by ACS (acyl-CoA synthetase) which can utilize an acyl group that can be liberated by the action of phospholipases A and B (PLAA LPB1B and respectively); all the ESTs encoding those enzymes are induced in the transition from mycelium to yeast, as described in Fig. 2B and Table 1. Also, DG3P can be produced by GDE1 (diacylglycerol pyrophosphate phosphatase). CDP-diacylglycerol (CDP-DG) produced from DG3P is the precursor of phospholipids. PSSA (phosphatidylserine synthase) produce

Figure 2

The synthesis of the cell wall components from glucose and lipids. Induced transcripts (*), novel transcripts (+), transcripts detected in the transition transcriptome without induction (#) and transcripts present at public databases (o). A – Some steps in the synthesis of glucan and chitin. GLCase 1: Alpha-glucosidase 1; HXK1: hexokinase; PGM: phosphoglucomutase; UGP1: uridine diphosphate glucose pyrophosphorylase; AGS1: alpha glucan synthase; MTLD: mannitol-1-phosphate dehydrogenase; MSTE: monosaccharide transport protein; GTT: glucose transporter protein; STL: sugar transporter protein; CTS 1: chitinase 1; CTS 3: chitinase 3; DIP 5: acidic amino acid permease; MAEL: malate permease; MDH: malate dehydrogenase; CITA: citrate synthase; ACO: aconitase; ICL: isocytrate lyase; MLS: malate synthase; UDPNAG: uridine diphosphate N acetylglucosamine; MNN2: UDPNAG transporter. B – The synthesis of some lipids from the cell membrane. LPL1B: Lysophospholipase; PLAA: phospholipase A2; DHCP: dihydroxycetone phosphate; GFDA: glycerol 3 phosphate dehydrogenase; G3P: glycerol 3 phosphate; G3PEtn: Phosphatidyl ethanolamine; GDPD: glycerophosphodiester phosphodiesterase; ACT: acyltransferase; ACS: acyl-coenzyme A synthethase; Acyl-CoA: acyl-coenzyme A; DGPP: diacylglycerol pyrophosphate; GDE1: diacylglycerol pyrophosphate phosphatase; DG3P: diacylglycerol 3 phosphate; CTP: cytidine triphosphate; PPi: pyrophosphate; CDP-DG: cytidine diphosphate diacylglycerol; PSSA: phosphatidylserine synthase; PtdSer: phosphatidylserine; PSS2: phosphatidylethanolamine serine transferase; PSD: phosphatidylserine decarboxylase; PtdEtn: phosphatidylethanolamine; PEMT: phosphatidylethanolamine metyltransferase; PtdCho: phosphatidylcholine; INO1: myo-inositol 1 phosphate synthase; Myo-Inol1P: myo-inositol 1phosphate; PtdIns: phosphatidylinositol; PDR16: phosphatidylinositol transfer protein; ERG 11: Lanosterol 14-alpha demetylase; ERG 3: sterol delta 5,6-desaturase.

Table 3: Candidate homologs for virulence factors induced in the cDNA transition library.

Virulence determinant	Function in other fungi	Reference number
Alpha -1,3 glucan synthase (ags1)	Reduction of AGS1 activity reduces the lung colonization by *Histoplasma capuslatum*	[40]
Glucanosyltransferase family protein (gel)	Required for both morphogenesis and virulence in *Aspergillus fumigatus*	[41]
Calcineurin subunit B (canB)	Required for *Candida albicans* virulence and stress resistance	[42]
Para-aminobenzoic acid synthetase (paba)	Essential for *Aspergillus fumigatus* growth in lung tissue	[43]
Peroxisomal catalase (cat P)	Putatively related to the *P. brasiliensis* protection against peroxides	[44]
Aspartyl protease (asp)	Facilitation of pathogenesis in *Candida albicans*	[45]
Zinc metalloprotease (mp)	A elastolytic metalloprotease of *Aspergillus fumigatus* is secreted during fungal invasion of murine lung	[46]
Phosholipase A2 (plaA)	Gene inactivation attenuates virulence in *Candida albicans*	[47]
Glyceraldehyde 3 phosphate dehydrogenase (gapdh)	Recombinant GAPDH and antibodies to GAPDH diminish *P. brasiliensis* yeast binding to and infection of A549 pneumocytes	[49]
Alpha-1,2 mannosyltransferase (mnn5)	Important for virulence of *Candida albicans*	[50]
Hemolysin like protein (hlp)	Phase specific gene regulated by phenotypic switching in *Candida glabrata*	[51]
Urease (ure)	Required for *Coccidioides posadasii* virulence	[52]

phosphatidylserine from CDP-DG, and is a novel transcript described in the present work. The induced transcript of INO1 (myo-inositol-1-phoshate synthase), produces myo-inositol 1P the precursor for the synthesis of phosphatidylinositol. The PDR16 (phosphatidylinositol transfer protein), also induced, transports phospholipids from their site of synthesis in the endoplasmic reticulum to the plasma membrane [29].

Polyunsaturated fatty acids (UFA) are major components of the membranes and are produced from monounsaturated fatty acids by several fatty acid desaturases in many fungi. DESA (fatty acid desaturase) was demonstrated to be induced in the transition library suggesting active membrane remodeling during the morphogenetic event in *P. brasiliensis*. The synthesis of ergosterol seems also to be induced during the transition process. ERG 11 (lanosterol 14-alpha demetylase) and ERG 3 (sterol delta 5, 6-desaturase) present transcripts induced in the transiton library (Fig. 2B, Table 1).

An overview of induced genes putatively related to signal transduction

We also identified a variety of signal transduction systems in *P. brasiliensis* ongoing differentiation to yeast cells, such as MAPK, serine/threonine protein kinases, signal histidine kinases and two component sensor kinases. The most increased transcript encodes for a histidine protein kinase sensor for GlnG regulator, which presented 24 ESTs in the transition library (Table 2, supplementary material and Table 3, supplementary material). Novel genes were also those encoding for a two-component sensor kinase (06 ESTs), calcineurin subunit b (02 ESTs), UVSB phosphatidylinositol-3-kinase (01 EST), forkhead associated protein (01 EST), Rho GTPAse activating protein (01 EST).

Histidine kinases are signaling transduction proteins that organisms in all three domains of life use to respond to environmental signals and control developmental process [30,31]. *S. cerevisiae* has a single hybrid histidine kinase, sln1p, which regulates an osmosensing mitogen-activated protein kinase (MAPK) cascade, an oxidative stress-response pathway, and cell wall biosynthesis [32,33]. *Blastomyces dermatitidis* DRK1 (for dimorphism-regulating histidine kinase) is a conserved hybrid histidine kinase that is indispensable for dimorphism, virulence and pathogenicity [34]. The ESTs encoding the putative histidine kinase induced in the transition library presents some structure domains and sequence of histidine kinase, such as the histidine-containing H-box and an aspartate-containing D-box (data not shown).

The fungal cell wall is an essential cellular boundary that controls many cellular processes. It allows cells to withstand turgor pressure preventing cell lysis. In *S. cerevisiae* a MAPK cascade which is essential in transducing signals to adapt cell wall biosynthesis under a variety of environmental conditions, is activated by the protein kinase C, constituting the PKC cell integrity pathway [35]. A MAPK and PKC proteins were induced in the transition library suggesting their involvement in the cell wall biosynthesis. In addition, calcineurin has been proposed as essential for survival during membrane stress in *Candida albicans* [36]. Also a FHA (forkhead associated) protein and an UVSB phosphatidylinositol-3-kinase were increased in the transition library suggesting the requirement of DNA damage checkpoint kinases in the dimorphic transition of *P. brasiliensis* [37,38].

In *P. brasiliensis* transition transcriptome it was detected 53 ESTs (4.78% of the total ESTS) encoding for potential

signal transduction proteins (see Table 3, supplementary material). From those, 10 are induced transcripts comprehending 06 novel genes, suggesting that the morphological transition in *P. brasiliensis* is mediated by a series of signal transduction systems that control the adaptation to the environment to the fungus survive and proliferate within the host.

Novel genes of **P. brasiliensis** *detected in the transition library*

Table 2 summarizes the transcripts detected in the transition library that were not present in the *P. brasiliensis* transcriptome [1] or in public databases. A total of 48 novel genes are reported here. Several enzymes related to the general metabolism were described as novel genes. As examples, the orotate phosphoribosyltransferase (URA5) (E.C.2.4.2.10) was present in the transition library. Also a phosphatidylserine synthase (E.C.2.7.8.8) putatively related to the metabolism of phospholipids, as cited above. Enzymes related to protein modification, transport facilitators and signal transduction were also detected as novel genes in the transition library and were discussed before.

A novel transcript encodes for a homologue of SamB, related to morphogenesis in ascomycetous fungi [39]. We exploited sequence data to examine the presence of the conserved Zn-finger like domain in the deduced homolog of *P. brasiliensis* (data not shown). It was observed the high conservation of the Zn finger-like domain in SamB, crucial for fungal morphogenesis, as described [39].

Putative virulence factors

Expression analysis can be a valuable first step in virulence genes discovery. Putative virulence factors were selected on basis with homology in other pathogenic microrganisms. With these criteria, we classified 12 induced genes as putative virulence factors of *P. brasiliensis*. Table 3 presents some induced genes, potential virulence factors in *P. brasiliensis*. AGS1 was catalogued as a potential virulence factor, since in *Histoplasma capsulatum* the reduction of its activity by RNA interference or allelic replacement leads to reduction in the fungal ability to colonize lung [40]. Mutants of *Aspergillus fumigatus* in glucanosyltransferases 1 and 2 (gel 1 and 2) have abnormal cell wall compositon and conidiogenesis and reduced virulence in a murine model of invasive aspergillosis, suggesting that beta(1–3) glucanosyltransfease activity is required for both morphogenesis and virulence in this fungal pathogen [41]. Calcineurin plays a global role in stress responses necessary for fungal cell survival and in this sense can be defined as a virulence factor [42]. Deleted para-aminobenzoic acid synthetase (paba) strains of *A. fumigatus* present complete inability in causing lethal infection in mice [43]. We previously described that the catalase P (CAT P) presents canonical motifs of monofunctional typical catalases, as well as the peroxisome PTS-1 targeting signal and its expression was induced in cells treated with H_2O_2, suggesting its involvement in protecting *P. brasiliensis* yeast cells against exogenously produced peroxides [44]. Secreted products are a common means by which fungi can promote virulence [45,46]. The aspartyl proteinase (ASP) described in Table 3 is putatively a secreted protease that may facilitate tissue invasion; the same could be hypothesized to the transcript encoding a zinc metalloprotease [46]. Phospholipases are critical for modification and redistribution of lipid substrates, membrane remodeling and microbial virulence. The null mutants and revertant strains for a phospholipase B gene of *C. albicans* present reduced phospolipase A2 activity and attenuated virulence [47]. In addition an inositol phosphosphingolipid phospholipase C (PLC) gene of *C. neoformans* promotes neurotropism of *C. neoformans* depending on the immune status of the host by protecting the fungus from the hostile intracellular environment of phagocytes [48].

Specific adhesins can enable fungal cells to adhere to host cells or the ECM components. We previously demonstrated that he fungal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a potential virulence factor of *P. brasiliensis*, since it can diminish the fungus yeast cells ability to adhere and invade in vitro cultured pneumocytes [49]. Also the mannosyltation of proteins can be related to virulence. The mnn5 mutant of *C. albicans* exhibited attenuated virulence in mice [50]. The transcripts encoding for a hemolysin like protein of *Candida glabrata* (HLP) and for urease (URE), are possible virulence factors (Table 3). Switching in *C. glabrata* which may provide colonizing populations for rapid response to the changing physiology of the host regulates the hlp expression [51]. Urease which catalyzes the conversion of urea into ammonia is described to contribute to alkalinity at the sites of fungal infection, causing a great damage to the host tissues [52]. Of special note, the up regulation of those potential virulence factors in the transition of mycelium to yeast cells suggests the fungal adaptation to the new conditions to be faced in the host milieu.

Expression profile

We validated the classification of induced transcripts by northern blot analysis, as shown in Figure 3A. The transcripts encoding aspartyl proteinase and sugar transporter protein, were classified as induced in the transition library by electronic northern and according to our experimental northern blot data, were accumulated in mycelium during transition to yeast cells. It has to be emphasized that the in silico analysis of the ESTs redundancy revealed for the transcripts encoding aspartyl protease and sugar transporter protein, 3 ESTs in the mycelium transcriptome database for both; 7 and 5 in the present transition library,

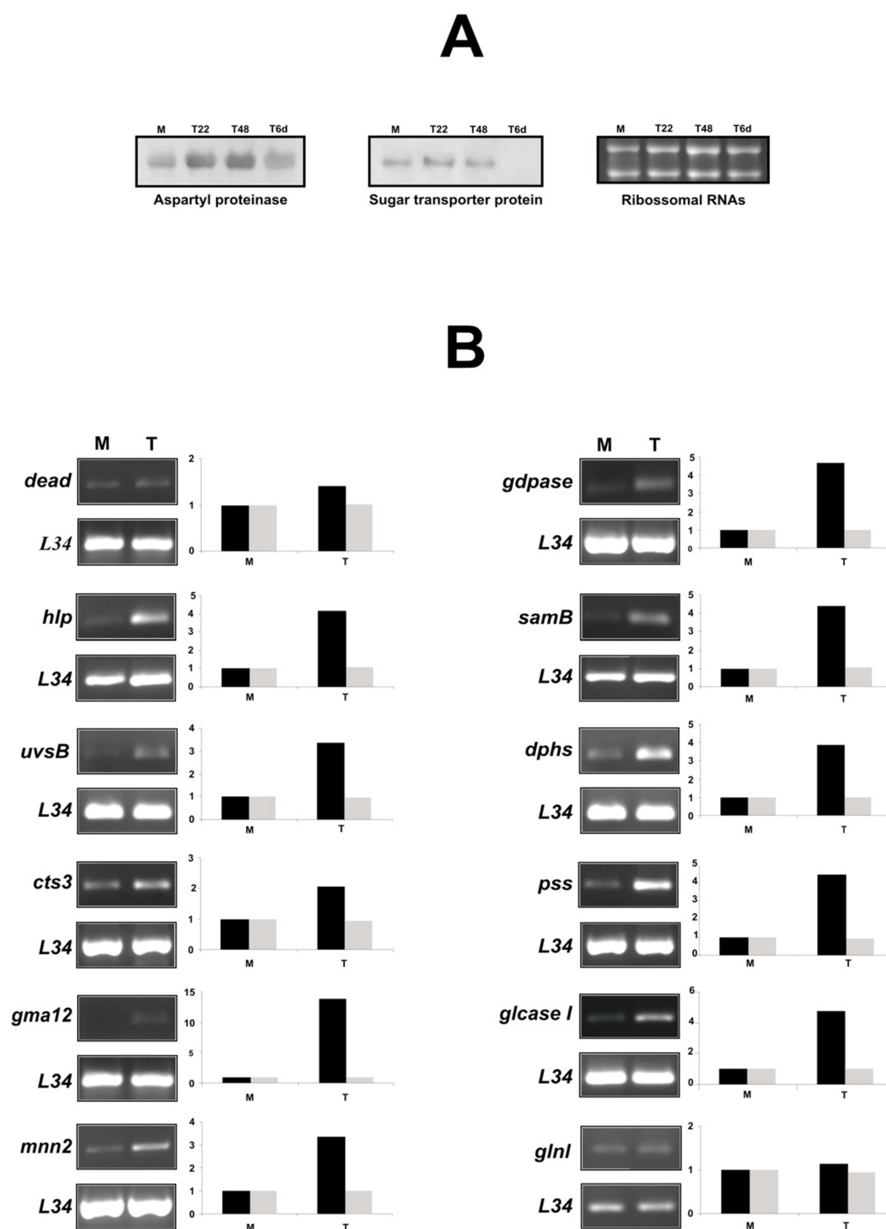

Figure 3

Validation of the classification of induced transcripts in the transition library. A – Analysis by northern blot was carried out with RNA from mycelium during transition to yeast colleted at 22 h, 48 h and 6 days after the temperature shift. Total RNA was fractionated on a 1.2% formaldehyde agarose gel and hybridized to the cDNA inserts Aspartyl proteinase (*asp*) and Sugar transporter protein (*stl*). Ribosomal RNAs are shown as the loading control. The sizes of the transcripts are as follows: *asp* 1.7 kb; *stl* 2.65 kb. B – Validation of some novel genes of *P. brasiliensis*. Semi-quantitative RT-PCR of RNAs obtained from mycelium in transition to yeast. Semi-quantitative RT-PCR analysis was carried out with specific primers, as described. Gray bars indicate the transcript level for the L34 ribosomal protein and black bars refers to the described new transcript. Numbers associated with the bars indicate fold differences relative to the data for the reference mycelium, which were established by densitometry analysis. Using varied number of cycle numbers, the exponential phase of each primer was determined and used to allow semi-quantitative analysis of the respective reactions. The same amount of cDNA was used for all PCRs. The RNAs used for RT-PCR were obtained from samples of: mycelium (M) and mycelium in transition to yeast after 22 h of the temperature shift (T). Genes and sizes of the respective amplified fragments are as follows in bp: *dead*: 408; *hlp*: 274; *uvsB*: 318; *cts3*: 268; *gma12*: 152; *mnn2*: 363; *gdpase*: 126; *samB*: 114; *dphs*: 284; *pss*: 281; *glcaseI*: 359; *glnI*: 368.

respectively and 3 for both, ESTs in the yeast transcriptome database. We also validated 12 novel genes identified in the transition cDNA library, by semi-quantitative RT-PCR, and their expression profiles are shown in Figure 3B. All transcripts were induced upon transition, as demonstrated.

Conclusion

The 1107 ESTs identified in this study represent the first effort to define the *P. brasiliensis* genes present in a cDNA library of the fungal RNA obtained during the transition from mycelium to yeast. These data increase the number of identified *P. brasiliensis* genes induced during the transition. Annotation of the unisequences revealed that 992 (89.6%) had homologues in the *P. brasiliensis* public databases, and therefore about 115 (10.4%) represent novel genes. Annotation of the ESTs revealed a great repertoire of genes that could function in cell wall/membrane remodeling during the transition process. Also, putative virulence factors, novel transduction signal proteins, novel enzymes related to sulphur metabolism, among others, had been described. Overall these data can help in accelerating research on this important human fungal pathogen.

Methods

Fungal isolate, growth conditions and induction of mycelium to yeast transition

P. brasiliensis, isolate *Pb*01 (ATCC-MYA-826), has been studied at our laboratory. It was grown in Fava-Netto'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; 4% (w/v) agar, pH 7.2], at 22°C, as mycelium. The differentiation was performed in liquid medium (Fava-Netto's medium) by changing the culture temperature from 22°C to 36°C for the mycelium to yeast transition, as we previously described [44]. The cells were previously grown in liquid medium for 18 h before changing the incubation temperature, which was maintained for 22 h.

RNA extraction and preparation of the cDNA library

Total RNA was purified from *P. brasiliensis* mycelium in transition to yeast cells (see above) using TRIZOL (GIBCO™, Invitrogen, Carlsbard, CA). The mRNA was purified by using the Poly (A) Quick R mRNA isolation kit (Stratagene, La Jola, CA). The cDNA library was constructed in the unidirectional pCMV.SPORT 6 (Invitrogen) according to the manufacturer's instructions, exploiting the *Not* I and *Sal* I restriction sites. The cDNA library was not normalized, i.e., no attempt was made to reduce the redundancy of highly expressed transcripts.

Plasmid isolation and DNA sequencing of the cDNA library

Plasmids constructs were transformed into *Escherichia coli* ElectroMAX™ DH10B cells (Invitrogen). The cDNA library was plated to approximately 200 colonies per plate (150 mm Petri dish). The colonies were randomly selected and transferred to a 96-well polypropylene plate containing LB medium and grown overnight. Plasmid DNA was isolated and purified using Millipore filters (MilliPore®). cDNA inserts were sequenced from the 5' end by employing standard fluorescence labeling DYE namic™ ET dye terminator kit with the M13 flanking vector primer. Samples were loaded onto a MegaBACE 1000 DNA sequencer (GE Healthcare, Amersham Biosciences), for automated sequence analysis.

EST Processing Pipeline, Annotation and Sequence Analysis

The resulting electropherograms were transferred to the server where the pre-processing took place. ESTs were screened for vector sequences against the UniVec data. The sequences were assembled by using the PHRED/PHRAP/CONSED [53]. EST sequences were pre-processed using the Phred [54] and Crossmatch [55] programs. Only sequences with at least 100 nucleotides and Phred quality greater or equal to 20 were considered for further analysis. A total of 1107 ESTs were selected by these inclusion criteria. The resulting sequences were uploaded to a relational database (MySQL) on a Linux (Fedora Core 3) platform, and processed using a modified version of the PHOREST tool [56]. We modified PHOREST to the assembling of the sequences using the CAP [57] and store the BLAST results of many databases including GenBank non-redundant (nr) database, Cluster of Orthologus Groups (COG), Gene Ontology (GO), MIPS [58], KEGG [59] and some fungi specific databases. In addition, an option to automatically translate EST sequences and compare their frames against the InterPro database [60] was implemented. These modifications allowed easy identification of homolog sequences, as well as the identification of domains and functional sites, which improved the manual annotation process. Similarities with E-values $\leq 10^{-4}$ were considered significant. For comparative analysis the ESTs were grouped in 639 clusters, represented by 166 contigs and 473 singlets. The clusters were compared with *P. brasiliensis* transcriptome database [1] and public databases to identify new transcripts, by using the BLAST program [61]. The ESTs had been submitted to GenBank, under accession numbers EH040628 to EH041734.

In silico determination of induced genes in the mycelium to yeast transition by electronic northern

To assign a differential expression character, the contigs formed with mycelium and the transition ESTs were statistically evaluated using the Audic and Claverie's method [14]. It were considered induced genes in the transition

Table 4: Oligonucleotides primers related to new genes selected for sqRT-PCR analysis.

Sequence name	Forward primer (5' → 3')	Reverse primer (5' → 3')
DEAD-like helicases superfamily protein (dead)	GGCCTTCTGAAACGGGGG	GAGCTTCGCAATAGGCCAAG
Hemolysin like protein (hlp)	GGCCTTCTGAAACGGGGG	GAGCTTCGCAATAGGCCAAG
UVSB Phosphatidylinosytol-3-kinase (uvsB)	CTAGCGAATGGCAATATCACT	GATAATGAGGGCATGGTCTC
Chitinase 3 (cts3)	GGAGGAGGATATGTCTCTTG	CTGCTGCCCATCCCTCAG
Alpha 1,2 galactosyltransferase (gma12)	GCTATGTCAACTTCTTCGCG	GAGAGCATGGGCCGACAG
UDP-N-Acetylglucosamine transporter (mnn2)	GCCCTCATTACGTTAACGCA	CATGGATTTTCCTTTGGCACT
Guanosine diphosphatase (gdpase)	GATCTTCCGCTTTCTCGCCA	CTCCTTGACACGGCACTGC
Suppressor of anucleate metulae B protein (samB)	CCAGTGCGCCTACTATAAATG	CAGGCATTCTTCTGGCACTC
Diphitine synthase (dphs)	CTGTTTCGCAGTGTGCCAG	CGTTCCGTAATTGCTTTTCCA
Phosphatidylserine synthase (pss)	GCTGCTCTCGGCGGACTC	CGAAGGAGACCAGATCAGC
Alpha glucosidase I (glcaseI)	CCAGCTGATAGTCCACGGC	CTTGTCCATCCTGTGAAATGC
Histidine protein kinase sensor for GlnG regulator (glnL)	CGTCTGTTGGGGCCGCAG	CATCGGGTAAAACAGCGTATC

library those that were not previously described in the mycelium transcriptome database [1], as well as those more expressed as determined with a 99% confidence rate. A web site [62] was used to compute the probability of differential regulation.

Northern blot

Northern hybridization was performed with 10 μg of total RNA fractioned on a 1.2% agarose-formaldehyde denaturing gel and transferred to a Hybond-N+ nylon membrane (GE Healthcare). The RNAs, corresponding to different times of cellular differentiation, were hybridized to the correspondent cDNA probes in Rapid-hyb buffer (GE Healthcare) and washed according to the manufacturer's instructions. Probes were radiolabeled by using Rediprime II Random Prime labeling System (GE Healthcare).

Semi-quantitative RT-PCR analysis (sqRT-PCR)

Semi-quantitative RT-PCR was performed for 12 genes to confirm the presence of new transcripts. Total RNA was extracted from *P. brasiliensis* mycelium in transition to yeast after 22 h of the temperature shift from 22°C to 36°C, as described. RNAs used for sqRT-PCR were from independent experiments from those used in the cDNA library construction. cDNAs were synthesized by reverse transcription using the Superscript II RNAse H-reverse transcriptase (Invitrogen™, Life Technologies). cDNAs were used for PCR in 30 μl reaction mixture containing specific primers, sense and antisense, as described in Table 4. PCR conditions were: 25–35 cycles at 95°C for 1 min; annealing at 55–65°C for 2 min; 72°C for 1 min. The annealing temperature and the number of PCR cycles were optimized for each experimental condition to ensure linear phase of amplification. Amplicons were analyzed by agarose gels electrophoresis (1%). The analyses of relative differences were performed by using Scion Image Beta 4.03 program [63].

Authors' contributions

KPB prepared the cDNA library, performed the DNA sequencing, the validation experiments, contributed to gene ontology classification and supported the preparation of the figures and tables. AMB contributed to the construction of the cDNA library, to the classification of gene ontology terms, to the data analysis and to the preparation of the manuscript. CLB contributed to the culture of the fungus, to the construction of the cDNA library, to the classification of gene ontology terms and to the manuscript edition. FPF contributed to the construction of the cDNA library. MSSF contributed to the results discussion and to the manuscript preparation. MGS contributed to the DNA sequencing and to the classification of gene ontology terms. WSM and RBF analyzed the raw sequences and contributed to the construction of the EST database. MP contributed to the analysis of the raw sequences and to the preparation of the manuscript. CMAS designed the project and the database, contributed to the data analysis and to the preparation of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

P. brasiliensis ***clusters annotated in the cDNA library***. *Table representing the annotated clusters that were generated by sequencing of the cDNA clones. For each cluster the table includes: the function as assigned by BLAST-based similarity, the BLAST subject species, the GenBank ID for the BLAST subject used for functional assignment and the Expect value obtained with each unisequence, the redundancy in the transition library and in the mycelium transcriptome database.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-7-29-S1.doc]

Additional File 2

***Induced* P. brasiliensis *ESTs and novel genes generated from the transition library*.** *Table representing the annotated clusters that were generated by sequencing of the cDNA clones. For each cluster the table includes: the function as assigned by BLAST-based similarity, the BLAST subject species, the GenBank ID for the BLAST subject used for functional assignment and the Expect value obtained with each unisequence, the redundancy in the transition library and in the mycelium transcriptome database.*
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Additional File 3

P. brasiliensis *induced transcripts potentially related to signal transduction*. *Table representing the annotated clusters that were generated by sequencing of the cDNA clones of the transition library. For each cluster the table includes: the function as assigned by BLAST-based similarity, the redundancy in the transition library and in the mycelium transcriptome database.*
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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.

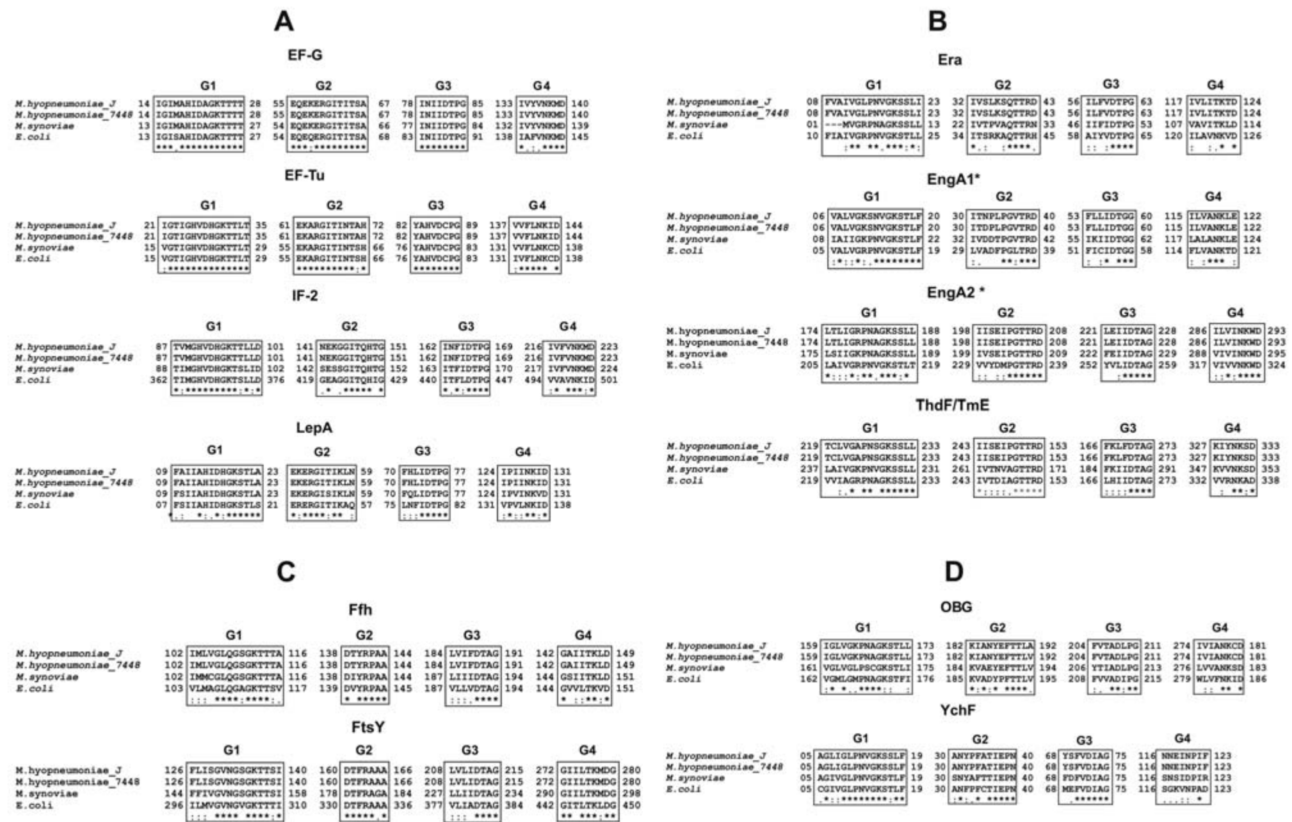

Figure 1 - Alignment of G1, G2, G3 and G4 motifs of the GTPase subfamilies. Panel A: Elongation factor subfamily. Panel B: Era subfamily. Panel C: FtsY/Ffh subfamily. Panel D: OBG YchF subfamily. The sequences used in the alignment are listed in Table 1 and were obtained from: *M. hyopneumoniae J* (*Mycoplasma 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-

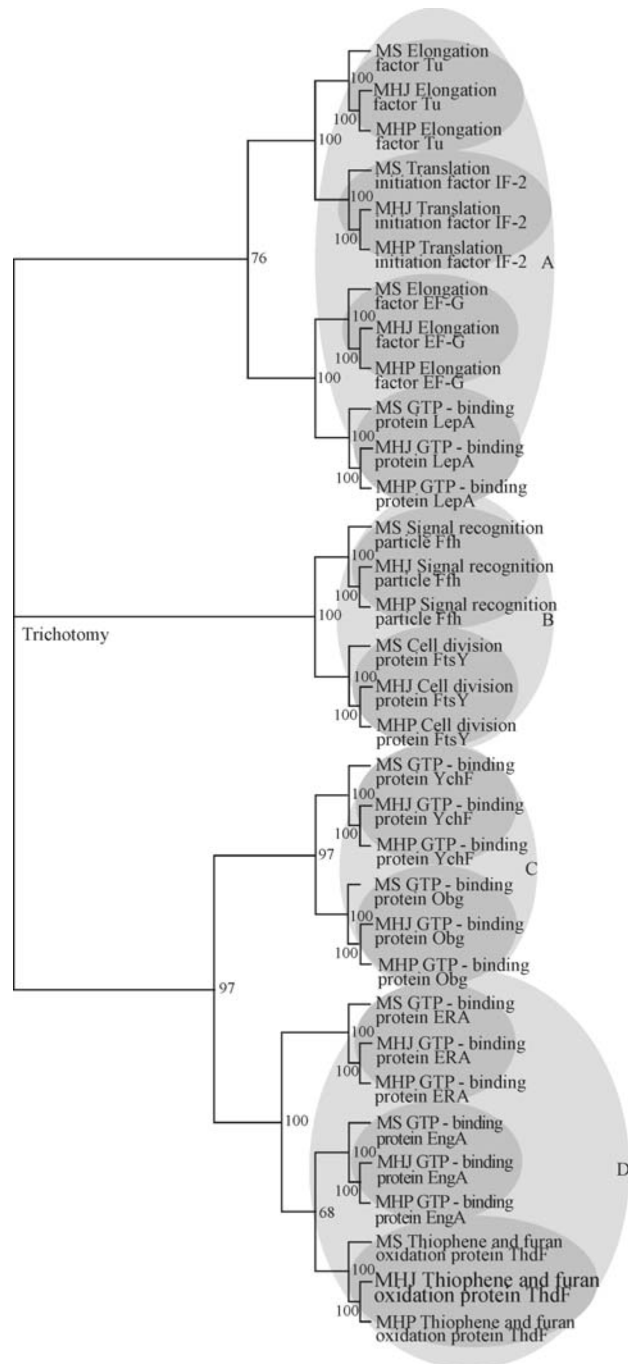

Figure 2 - Amino acid sequence relationship of the GTPase superfamily. (A) Elongation factor subfamily. (B) FtsY/Ffh subfamily. (C) OBG/YchF subfamily. (D) Era subfamily. The numbers on the branches are bootstrap values obtained with 100 replications. Members of each family are described as MS for *M.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

Acknowledgments

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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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Original article

cDNA representational difference analysis used in the identification of genes expressed by *Trichophyton rubrum* during contact with keratin

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Abstract

Dermatophytes are adapted to infect skin, hair and nails by their ability to utilize keratin as a nutrient source. *Trichophyton rubrum* is an anthropophilic fungus, causing up to 90% of chronic cases of dermatophytosis. The understanding of the complex interactions between the fungus and its host should include the identification of genes expressed during infection. To identify the genes involved in the infection process, representational difference analysis (RDA) was applied to two cDNA populations from *T. rubrum*, one transcribed from the RNA of fungus cultured in the presence of keratin and the other from RNA generated during fungal growth in minimal medium. The analysis identified differentially expressed transcripts. Genes related to signal transduction, membrane protein, oxidative stress response, and some putative virulence factors were up-regulated during the contact of the fungus with keratin. The expression patterns of these genes were also verified by real-time PCR, in conidia of *T. rubrum* infecting primarily cultured human keratinocytes *in vitro*, revealing their potential role in the infective process. A better understanding of this interaction will contribute significantly to our knowledge of the process of dermatophyte infection.
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Keywords: Trichophyton rubrum; Representational difference analysis; Infection; Dermathophytoses

1. Introduction

Dermatophytoses are among the few fungal diseases that are directly communicable from person to person. Dermatophytes infect mainly healthy individuals, causing infections of keratinized structures, including the skin, hair, and nails [1]. Dermatophytes are not part of the normal human microbial flora. They are, however, particularly well adapted to infecting these tissues because, unlike most other microbial pathogens, they can use keratin as a source of nutrients [2]. *Trichophyton rubrum* is the most frequently isolated agent of dermatophytosis worldwide, accounting for approximately 80% of reported cases of onychomycosis [3]. Since 90% of the chronic dermatophyte infections are caused mainly by *T. rubrum*, this pathogen must have evolved mechanisms that evade or suppress cell-mediated immunity [4].

Despite its prevalence, little is known about the molecular basis of dermatophyte pathogenesis. Studies regarding the structure, expression, and regulation of the genes of *T. rubrum* have been relatively limited because of its unaggressive and non-life-threatening nature. In host–pathogen interactions, the gene expression of the pathogen is modulated by signals from the host, and knowing the pattern of expression may

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provide insights into the disease mechanisms [5]. Few potential *T. rubrum* virulence factors have been examined in detail, and most of them are keratinolytic proteases.

The understanding of the complex interactions between fungus and host must include the identification of genes expressed during infection. An efficient approach to the identification of differentially expressed genes in *T. rubrum* involves rapid series of subtractive hybridizations of cDNA prepared from two cell populations. Representational difference analysis (RDA) is a powerful and sensitive tool for the identification of differentially expressed genes and enables the isolation of both up- and down-regulated genes expressed in two different cDNA populations [6]. Recently, this strategy was applied to the differentially expressed genes of the human pathogenic fungus *Paracoccidioides brasiliensis* during the host interaction, revealing a set of candidate genes that *P. brasiliensis* may express to adapt to the host conditions [7].

The aim of the present study was to identify genes differentially expressed in *T. rubrum*, cultured in the presence and absence of keratin to simulate the host infection. The role of these genes was corroborated by confirming their induction during the infective process in a primary keratinocyte cell culture. Our studies provide the first view of the *T. rubrum* transcriptional response to host–pathogen interaction.

2. Materials and methods

2.1. Strain and culture conditions

T. rubrum isolate ATCC 52021 (American Type Culture Collection) was cultured for 10 days at 25–28 °C in Sabouraud's liquid medium and transferred to two different culture media: (i) a culture referred to as "tester" in liquid Cove's medium [8] supplemented with keratin (Sigma) 100 μg/mL, and (ii) a culture named "driver" in Cove's minimal medium, both cultivated for 24 h at room temperature. As a control, a reverse experiment was conducted in which the driver RNA was extracted from keratin culture and the tester RNA from minimal medium.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from *T. rubrum* cultured under each experimental condition by using the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). First strand cDNA synthesis was performed with reverse transcriptase (RT Superscript III, Invitrogen, Life Technologies) using 1 μg of total RNA. The first strand of cDNA was used as template to synthesize the second strand, by using the SMART PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA).

2.3. Subtractive hybridization and generation of subtracted libraries

The cDNA fragments were digested with *Sau*3AI restriction enzyme (Promega, Madison, USA). A subtracted cDNA library was constructed using driver cDNA synthesized from RNA of *T. rubrum* cultured in minimal medium and tester cDNA from RNA extracted from fungus cultured in the presence of keratin. The resulting products were purified using a GFX kit (GE Healthcare, Chalfont St. Giles, UK). The digested tester cDNA was ligated to adapters (a 24-mer annealed to a 12-mer). To generate 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. Two successive rounds of subtraction and PCR amplification were performed with hybridization tester–driver ratios of 1:10 and 1:100, respectively. Adapters were changed between these cross-hybridizations, and different products were purified using the GFX kit [9,10].

After the second subtractive reaction, the finally amplified cDNA pools 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 deep-well plates. Plasmid DNA was prepared from clones using standard protocols. In order to generate the EST (expressed sequence tags) sequences, 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.

2.4. EST processing pipeline and differential expression analysis

EST sequences were pre-processed using the Phred and Crossmatch (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm) programs. Sequences with at least 100 nucleotides and Phred quality greater than or equal to 20 were considered for further analysis. ESTs were screened for vector sequences against the UniVec data, and assembled with the CAP3 program [11]. The filtered sequences were compared against the GenBank (http://www.ncbi.nlm.nih.gov) non-redundant (nr) database from the National Center for Biotechnology Information (NCBI) using the BLASTX program [12], Cluster of Orthologous Groups (COG) and Gene Ontology (GO). MIPS (http://mips.gsf.de/) and InterPro databases of protein families, domains and functional sites were used to assign functional categories.

2.5. Assay of T. rubrum–keratinocytes interaction

Cultures of keratinocytes were isolated from human breast skin obtained from routine plastic surgery, processed and kindly supplied by the Tissue Bank of the Plastic Surgery Department of the College of Medicine at the University of São Paulo (USP), São Paulo, Brazil. The cells were maintained in DMEM–F12 (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Cult lab, Brazil), incubated at 37 °C with 5% CO_2.

For adherence assays, cells were seeded (in the absence of feeder fibroblasts, antibiotics, antimycotics and fetal calf serum) into six-well plates at a density of 1.0×10^6 cells/well and grown to confluence in DMEM–F12 medium.

Next, 1.0×10^7 conidia/mL of *T. rubrum* was added to the keratinocytes and incubated for 3 h, 8 h, 24 h and 48 h at 37 °C, to follow the processes of adhesion. After the period of incubation, the medium was discarded, the cells washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and stained by the May–Grünwald (Giemsa) procedure. The plates were examined by conventional microscopy to evaluate the kinetics of interaction of *T. rubrum* with keratinocytes.

2.6. RNA extraction from keratinocytes

Keratinocytes were plated in 25-cm^2 tissue culture flasks. The conditions of cell culture and infection were as described above, 24 h of infection being chosen for the RNA extraction. After this period, the cells were washed three times in PBS, and then incubated in PBS containing trypsin (0.2%) and EDTA (0.02%) for total monolayer removal. The cells were centrifuged at 5000*g* and the pellet was recovered for RNA extraction, as described previously. RNA samples for experiments of dot blot, northern blot and real-time PCR were obtained from two independent extractions. Controls were obtained from the uninfected *in vitro* cultured keratinocytes.

2.7. Dot blot and northern blot analysis

Serial dilutions of plasmid DNA were vacuum-spotted on nylon membrane and hybridized to the specific cDNAs probes, labeled with the Random-Prime DNA Labeling Kit (GE Healthcare). Hybridization was detected by the Gene Images CDP-Star Detection Kit (GE Healthcare). In the northern blot experiments, the RNAs (20 μg) were fractionated by electrophoresis in 1.2% agarose–formaldehyde gels and transferred to nylon membrane. RNAs were hybridized to the corresponding cDNA probe (Gene Images CDP-Star Detection Kit, GE Healthcare). Probes were labeled with the Random-Prime Labeling Kit (GE Healthcare).

2.8. Real-time PCR analysis of representative regulated genes in T. rubrum

The reaction mixtures contained 2 μL of cDNA, 12.5 μL of SYBR green ROX mixture (Applied Biosystems), and 400 nM of each primer, and the volume was brought to 25 μL with nuclease-free water. The reaction program was 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and the annealing and synthesis at 60 °C for 1 min. Following the PCR, melting-curve analysis was performed, which confirmed that the signal corresponded to a single PCR product. Reactions were performed in three PCR repeats with an Applied Biosystems 7500 cycler. Data were analyzed by the $2^{-\Delta\Delta CT}$ method [13]. The cycle threshold values for the duplicate PCRs for each RNA sample were averaged, and then $2^{-\Delta\Delta CT}$ values were calculated (*chs1* − chitin synthase 1 was used as the reference). This was followed by normalization to the value for RNA samples from *T. rubrum* cultured in the absence of keratin. A negative-control sample was used that contained all reagents except *T. rubrum* cDNA and cDNA obtained from the keratinocyte culture. After 40 rounds of amplification, no PCR products were detected in either reaction.

3. Results

3.1. Identification of T. rubrum genes with differential expression

RDA was performed on the fungus cultured in the absence of keratin (driver) and the presence of keratin (tester). Different patterns of DNA amplification were observed after two rounds of subtractive hybridization, as shown in Fig. 1.

A total of 344 clones were successfully sequenced (Table 1). The mean size of ESTs was 364 nucleotides. Using the BLASTX program, 6.98% of the ESTs corresponded to proteins of unknown function, with no matches in databases. In addition, 94.7% of the ESTs had not been described in *T. rubrum* while 5.3% had been.

3.2. Characterization of the subtracted cDNAs from T. rubrum cultured in the presence of keratin

The ESTs were classified into seven groups of functionally related genes (Table 1). The data illustrated the functional diversity of these highly expressed ESTs, denoting particular

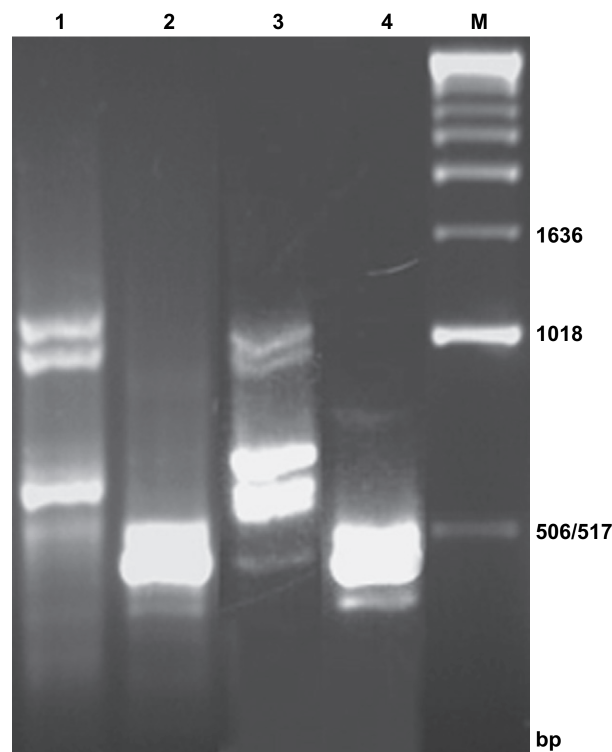

Fig. 1. RDA products analyzed by gel electrophoresis. Lanes 1 and 3: products of the first and second rounds of subtraction, respectively, performed by using tester the cDNA obtained from RNA of *T. rubrum* cultured in the presence of keratin. Lanes 2 and 4: products of the first and second rounds of subtraction, respectively, performed by using tester the cDNA obtained from RNA of *T. rubrum* cultured in Cove's medium without keratin. M: molecular markers 1 kb (Invitrogen, CA, USA). Numbers on the right indicate size in bp.

Table 1
ESTs with high abundance in *T. rubrum* cultured in the presence of keratin

MIPS category	Gene product	Organism best hit/accession number[a]	E-value	Redundancy
Transcription	Zinc finger protein[c]	*Aspergillus fumigatus*/CAD29608	3E−10	19
	Transcription factor bZIP[b]	*Aspergillus fumigatus*/XP747348	3E−47	2
	Transcription factor homeobox[b,c]	*Aspergillus fumigatus*/XP752424	9E−30	48
Cell rescue, defense and virulence	Catalase isozyme P[b,c]	*Ajellomyces capsulatus*/AAN28380	1E−100	43
	30 kDa heat shock protein	*Trichophyton rubrum*/AAV33735	6E−14	3
Cellular communication/signal transduction	G-protein subunit alpha[b,c]	*Penicillium chrysogenum*/ABH10690	3E−95	112
Metabolism	Probable ATP-dependent RNA helicase DED1[b,c]	*Neurospora crassa*/CAB88635	1E−84	51
	Formate dehydrogenase[b]	*Coccidioides immitis*/EAS37296	1E−72	7
Cellular organization	Membrane protein[b,c]	*Cryptococcus neoformans*/AAW43081	1E−12	16
Protein synthesis	Nonribosomal peptide synthetase[b]	*Aspergillus fumigatus*/XP752404	3E−33	2
Unclassified protein	Conserved hypothetical protein[b]	*Aspergillus nidulans*/XP661692	8E−39	10
	Conserved hypothetical protein[b]	*Aspergillus nidulans*/XP680743	1E−30	2
	Conserved hypothetical protein[b]	*Aspergillus nidulans*/XP662070	2E−07	5
	Hypothetical protein[b]	–	–	1
	Hypothetical protein[b]	–	–	3
	Hypothetical protein[b]	–	–	4
	Hypothetical protein[b]	–	–	3
	Hypothetical protein[b]	–	–	12
	Hypothetical protein[b]	–	–	1

[a] Accession number at GenBank (http://www.ncbi.nlm.nih.gov).
[b] Novel genes detected in *T. rubrum*.
[c] Validated up-regulated transcripts.

functional categories. The most redundant cDNAs appearing during the contact with keratin were as follows: G-protein subunit alpha (*gpa*), ATP-dependent RNA helicase DED1 (*ded1*), homeobox transcription factor (*hxf*), catalase isozyme P (*catP*), zinc finger protein (*zfp*) and membrane protein (*memb*), as shown in Table 1. A reverse cDNA–RDA experiment was conducted in which the driver was RNA from keratin culture and the tester was RNA from minimal medium culture. A total of 33 clones were sequenced, as control. The transcriptional profile did not display any similarity with that described for *T. rubrum* cultured in the presence of keratin (data not shown).

Fig. 2 depicts the classification of 19 clusters of *T. rubrum* ESTs according to the classification developed at MIPS.

3.3. *Confirmatory differential expression of* T. rubrum *identified sequences*

To corroborate the RDA findings, we initially performed dot blot analysis of *T. rubrum* cDNA–RDA clones. Dot blots displayed a differential hybridization pattern when individual clones were hybridized to labeled cDNAs obtained from the microorganism cultured in the presence and absence of keratin. The level of transcripts corresponding to cDNA clones was altered in the presence of keratin, as shown in Fig. 3A.

Northern blot analysis was employed to evaluate the expression of some up-regulated genes. The transcripts of the genes encoding GPA, CATP, ZFP and MEMB were found to have accumulated more in the fungus cultured in the presence of keratin (Fig. 3B).

3.4. *Quantitative analysis of genes in* T. rubrum *by real-time PCR*

The fungus showed high adhesion to the cell after all the periods of time and conidial germination was observed in 24 h. This time of infection was thus chosen for RNA extraction during the infection (Fig. 4). To estimate the relative transcript levels of the differentially expressed products, a real-time PCR assay was performed. Fig. 5 shows quantification of the transcript levels of several differentially expressed genes. Among the six genes evaluated, the *catP* gene was induced 3.8-fold in *T. rubrum* grown in the presence of keratin

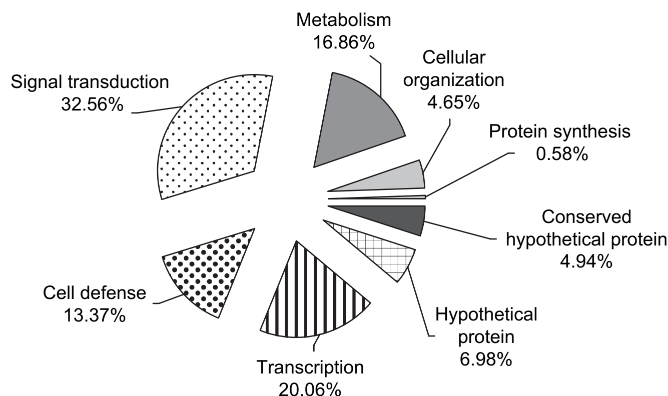

Fig. 2. Functional classification of *T. rubrum* ESTs derived from RDA experiment. This classification was based on BLASTX homology of each EST against the GenBank nr database at a significant homology cut-off of ≤1E−05 and MIPS functional annotation scheme. Each functional class is represented as a color-coded segment and expressed as a percentage of the total number of ESTs in each library.

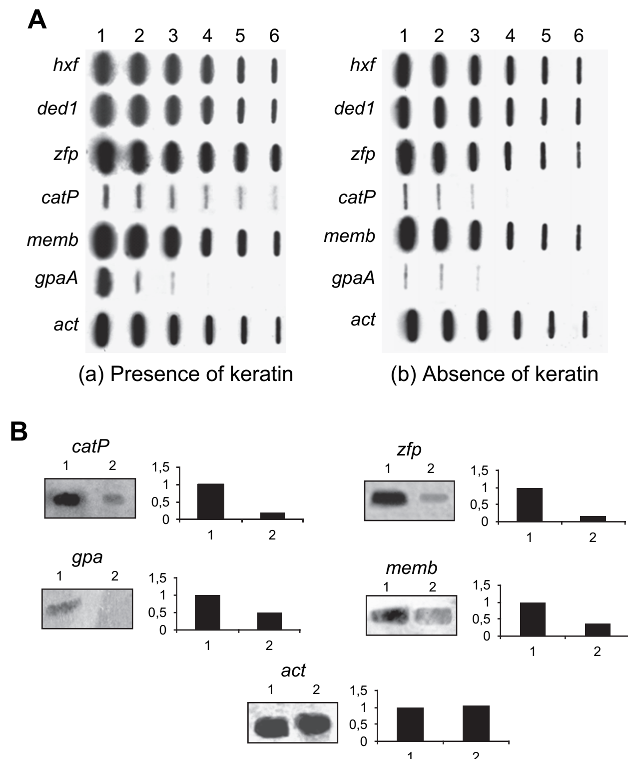

Fig. 3. Validation of the cDNA−RDA results. (A) Dot blot analysis of *T. rubrum*. DNAs of individual clones were prepared and dilutions (1:2000−1:64,000) were blotted (1−6). Panel a: individual clones hybridized to the labeled cDNA obtained from *T. rubrum* cultured in the presence of keratin. Panel b: individual clones hybridized to the labeled cDNA obtained from *T. rubrum* cultured in the absence of keratin. The clones were as follows: G-protein subunit alpha (*gpa*), ATP-dependent RNA helicase DED1 (*ded1*), homeobox transcription factor (*hxf*), catalase isozyme P (*catP*), zinc finger protein (*zfp*), membrane protein (*memb*), and actin (*act*) as control. (B) Expression patterns of genes obtained by cDNA−RDA analyzed by northern blot of total RNA of *T. rubrum* extracted after culture in the presence (1) and absence (2) of keratin. Total RNA was fractionated on 1.2% formaldehyde−agarose gel and hybridized to the cDNA inserts of *gpa*, *catP*, *zfp*, *memb* and actin (*act*) as the loading control.

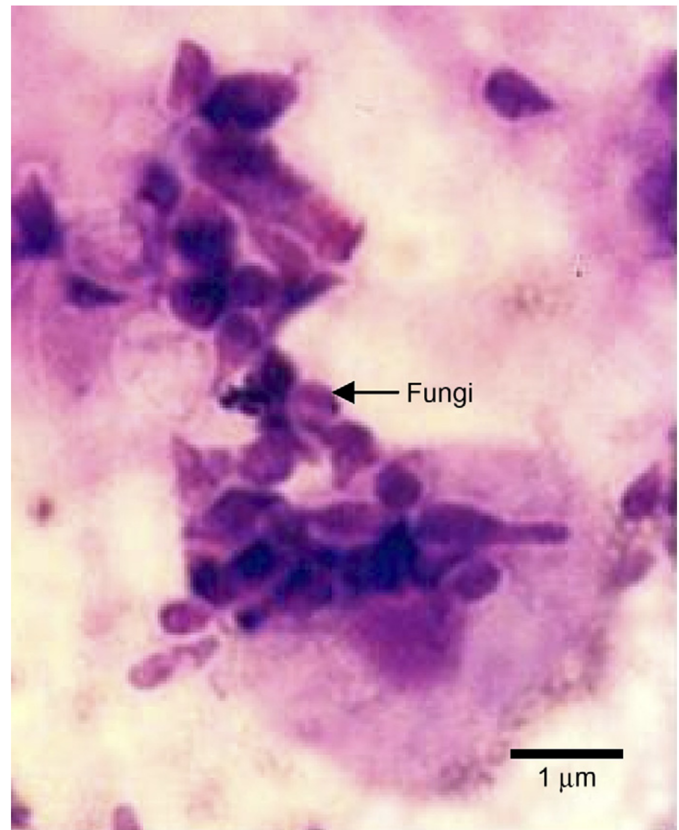

Fig. 4. Interaction between *T. rubrum* and keratinocytes. Cells were seeded into six-well plates and incubated with conidia for 24 h. The wells were washed and stained with the May−Grünwald (Giemsa) for micrographs.

and 16.3-fold after infection of keratinocytes, while, the *ded1* gene was induced 1.3- and 8.2-fold, when the fungus was grown in the presence of keratin and after infection of keratinocytes, respectively.

4. Discussion

The initial steps in the pathogenesis of cutaneous infections involve the capacity of the infecting microorganism to overcome physical and innate resistance factors, allowing initial adherence, followed by competition with the normal microbial flora and subsequent colonization of the cell surfaces [2]. This study is the first to use RDA analysis to characterize changes in gene expression after contact of *T. rubrum* with keratin, mimicking the infection in the host. The ability of RDA to identify sets of differentially expressed genes allows the gene expression to be compared in different culture conditions. Our data could have a great impact in the establishment of the role of the genes that are highly expressed in response to host conditions. Many of the genes found here have already been described in orthologous systems and some of them have functional roles during the infection process.

In our study, we sequenced 344 clones, of which 181 were ESTs identifying genes encoding proteins involved in transcription processes and signal transduction. This abundance of ESTs for transcription and signal transduction proteins may be related to fungal growth in the keratin medium. We also identified some virulence factors (catalase, 30 kDa heat shock protein). Kaufman et al. [5] constructed a suppression−subtractive hybridization (SSH) cDNA library for *T. mentagrophytes* cultivated on minimal medium with keratin; the major up-regulated transcript was thioredoxin, consistent with up-regulation of a catalase here.

On the other hand, Wang et al. [14] constructed 10 different *T. rubrum* cDNA libraries and obtained 11,085 ESTs. The identified ESTs encoded putative proteins implicated in primary metabolism, gene expression, post-translation processes and cell structure. A significant proportion of the identified ESTs were matched to genes involved in transcription and signal transduction, as found in any eukaryote.

The *G-protein subunit alpha (gpa)* transcript was the most abundant (112 ESTs), in our experimental conditions, and it was up-regulated both during the fungal contact with keratin and during the interaction with keratinocytes. The overexpression of *gpa* in *T. rubrum* in the host-like conditions described here strongly suggests that GPA may play an

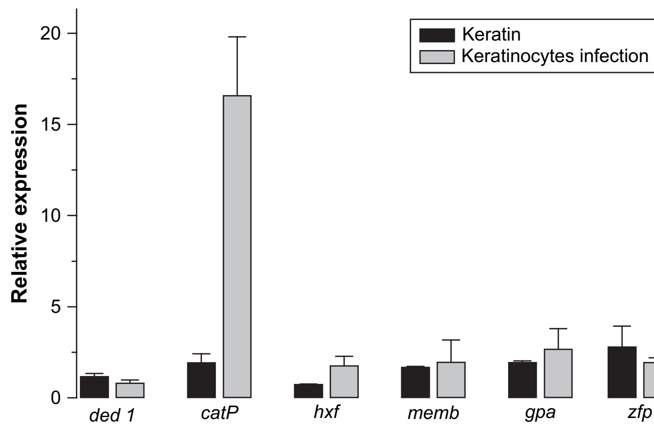

Fig. 5. Quantification of the transcript levels of differentially expressed genes from *T. rubrum* grown in the presence of keratin and after infection in culture of keratinocytes, relative to the fungus cultured in the absence of keratin (control). The results are averages of three repetitions. The values represent the levels of expression of genes normalized to the control grown in the absence of keratin (set at 1.00). The genes were as follows: G-protein subunit alpha (*gpa*), ATP-dependent RNA helicase DED1 (*ded1*), homeobox transcription factor (*hxf*), catalase isozyme P (*catP*), zinc finger protein (*zfp*), and membrane protein (*memb*).

important role in the modulation of virulence of this fungus. Segers and Nuss [15] demonstrated that *CPG-1* of *Cryphonectria parasitica* (one of the three genes encoding G alpha subunits) was essential for regulated growth, pigmentation, sporulation and virulence, indicating that tightly-controlled expression of *CPG-1* mediated by signaling is required to control these processes. Previous studies have shown that the G alpha subunit GPA-1 in *Cryptococcus neoformans* regulates a variety of cellular functions, including specialized processes such as the production of the antioxidant melanin pigment and the antiphagocytic capsule, and two well-established virulence factors in this pathogen [16].

In particular, a zinc finger protein was up-regulated in the analyzed conditions, presumably reflecting its role in fungal infection. Most zinc-containing proteins are transcription factors capable of binding DNA and function in a wide range of processes, including regulation of genes involved in the stress response, as demonstrated in human fungal pathogens [17]. In some fungi the ambient pH regulation is performed by zinc-finger transcription factors [18].

Knowledge of the metabolic responses that govern homeostatic pH and extracellular pH sensing is rather important, since these mechanisms are possibly involved in the installation, development, and survival of dermatophytes in humans.

The over-expression of catalase *isozyme P (catP)* produced by *T. rubrum* in the "tester" conditions strongly suggests its role in the fungal infection. Catalase, which is a good scavenger of H_2O_2, is considered a putative virulence factor of various fungi that could counteract the oxidative defense reactions of the host phagocytes [19]. Catalases are widespread in aerobic organisms such as *Candida albicans*, *P. brasiliensis*, *Histoplasma capsulatum*, and *Aspergillus fumigatus*.

Catalases take part in *C. albicans* survival from neutrophil attack and within the host. Disruption of the *C. albicans* catalase gene results in higher sensitivity to damage by neutrophils and to exogenous hydrogen peroxide. The *C. albicans cat1* gene, which encodes a protein with catalase activity, is involved in oxidant susceptibility; its deletion generates cells that are less virulent in the mouse model of acute systemic infection [20]. Moreira et al. [21] isolated a complete cDNA encoding a peroxisomal catalase of *P. brasiliensis* (Pb*catP*). Pb*catP* expression was induced in cells treated with H_2O_2, and the authors speculated that the activity of this enzyme was protective against endogenously produced oxygen radicals and exogenous H_2O_2. The authors observed that the protein and its transcript were regulated during *P. brasiliensis* development, increasing during the mycelium-to-yeast transition (to the pathogenic form). *H. capsulatum* yeast cells synthesize catalases during exposure to the respiratory burst of neutrophils and macrophages [22]. Paris et al. [23] examined the role of all of the conidial and mycelial catalases of *A. fumigatus* in the pathogenicity of the fungus and observed the expression of three active catalases, one in conidia and two in mycelium. The conidial catalase does not protect conidia against the oxidative burst of macrophages, but it protects against H_2O_2 *in vitro*.

To our knowledge, this study is the first to use cDNA–RDA analysis to characterize changes in gene expression by comparing two populations of *T. rubrum* cDNA, one obtained from fungus cultured in the presence of keratin and other generated during fungal growth in minimal medium. This work can facilitate functional studies of novel regulated genes that may be important during the infective process of dermatophytes.

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Comparison of transcription of multiple genes during mycelia transition to yeast cells of *Paracoccidioides brasiliensis* reveals insights to fungal differentiation and pathogenesis

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Abstract The ascomycete *Paracoccidioides brasiliensis* is a human pathogen with a broad distribution in Latin America. The infection process of *P. brasiliensis* is initiated by aerially dispersed mycelia propagules, which differentiate into the yeast parasitic phase in human lungs. Therefore, the transition to yeast is an initial and fundamental step in the infective process. In order to identify and characterize genes involved in *P. brasiliensis* transition to yeast, which could be potentially associated to early fungal adaptation to the host, expressed sequence tags (ESTs) were examined from a cDNA library, prepared from mycelia ongoing differentiation to yeast cells. In this study, it is presented a screen for a set of genes related to protein synthesis and to protein folding/modification/destination expressed during morphogenesis from mycelium to yeast. Our analysis revealed 43 genes that are induced during the early transition process, when compared to mycelia. In addition, eight novel genes related to those processes were described in the *P. brasiliensis* transition cDNA library. The types of induced and novel genes in the transition cDNA library highlight some metabolic aspects, such as putative increase in protein synthesis, in protein glycosylation, and in the control of protein folding that seem to be relevant to the fungal transition to the parasitic phase.

Keywords Dimorphic transition · Induced transcripts · *Paracoccidioides brasiliensis* · Protein synthesis · Protein folding/modification/destination

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Introduction

Paracoccidioides brasiliensis is a human pathogen with a broad distribution in Latin America. The fungus is thermally dimorphic. In the soil, the fungus grows as saprobic mycelium and upon elevation of the temperature to that of the mammalian body, the fungus adopts a yeast-like phase [1]. A human host through inhalation acquires the fungal pathogen. The disease, paracoccidioidomycosis, is characterized by a chronic granulomatous inflammation, and patients might present a broad spectrum of clinical manifestations ranging from a localized and benign disease to a progressive and potentially lethal systemic mycosis.

The severe nature of the disease and occurrence of sequelae, frequently causing pulmonary dysfunction or other disabilities, render it a pathogen of considerable medical importance [2].

P. brasiliensis can successfully establish and cause disease, highlighting the need to a better understanding of the molecular mechanisms of pathogenesis. Pathogenicity can be related to the factors associated to the transition from the saprophytic phase to the yeast parasitic phase, since fungal strains that are unable to differentiate into yeast cells are not virulent [3]. In this way, the characterization of genes/proteins related to the differentiation to the parasitic phase may bring insights to the fungal pathogenesis.

The morphological transition in *P. brasiliensis* is governed predominantly by the temperature and is preceded by several molecular changes. Some biochemical processes related to the dimorphic transition had been elucidated. In this way, the dimorphic transition involves alterations in the cell wall composition and in the structure of carbohydrates polymers [4]. Hyper expression of the enzymes of the sulfur metabolism in the yeast phase during the fungal transition to yeast reinforces previous findings that this metabolic pathway could be important for this differentiation process [5, 6]. Transcriptional analysis of genes highly expressed during the mycelia to yeast conversion identified the product 4-hydroxylphenyl pyruvate dioxygenase (4-HPPD), predicted to function in the catabolism of aromatic amino acids. Inhibition of 4-HPPD by specific compounds impairs the in vitro differentiation of mycelium to the yeast phase [7]. Also, data indicate that *P. brasiliensis* transition from mycelium to yeast is controlled by changing cAMP levels, with the onset of transition correlating with a transient increase in cAMP, suggesting activation of the cAMP-signaling pathway [8, 9].

A number of proteins and genes had been described as potentially associated to the fungal transition and putatively to the host invasion and host fungal survival. Proteomics-based discovery approaches have successfully identified potential candidates to the dimorphic process. Proteomic analysis from mycelium ongoing differentiation to yeast cells was performed allowing the characterization of proteins that could be relevant to the fungal differentiation. In this sense, the proteins HSP70, HSP60, glyceraldehyde-3-phosphate dehydrogenase, catalase P and actin were accumulated during the transition from mycelium to yeast [10–15].

A great amount of transcriptional data has been obtained from *P. brasiliensis* [16–21]. Approaches used in this fungus to identify phase-specific genes and or genes important for the dimorphic process, included microarray hybridization approaches [7, 16] and in silico EST subtraction [16]. Transcriptional profiling of microarrays built with ESTs of *P. brasiliensis* has identified 328 genes that are differentially expressed upon the phase transition [16]. In addition, constructed microarrays based on yeast-phase genes and hybridized to RNAs isolated from fungal cells at time points during the switch to the yeast phase enabled the identification of transcripts potentially associated to the fungal morphogenesis [7].

In a previous work, we have tested the concept that novel genes involved in *P. brasiliensis* phase transition could be described by applying a transcriptome analysis of cells undergoing mycelium to yeast transition. We reported the in silico analyses and comparison of ESTs from mycelium undergoing the early transition to yeast with mycelium differentiated cells. According to our data, the developmental program of *P. brasiliensis* is characterized by significant differential positive modulation of transcripts related to cellular processes, predominantly to the cell wall/membrane synthesis/remodeling, suggesting their importance in dimorphism [21].

In this study, in order to advance our understanding on the molecular mechanisms of dimorphic transition and of the initial steps of the fungal adaptation to the host, we sought to examine the profile of transcripts related to protein synthesis/processing/regulation/degradation in the ESTs generated from the cDNA library of mycelium undergoing transition to yeast cells. Using comparative sequence analyses, we could identify sequences, which were absent in the *P. brasiliensis* yeast and mycelium transcriptome and in public databases, as well as sequences induced during the early fungal transition. Through these approaches, it was found: (1) 54 possible homologues, including 18 induced/novel homologues of genes previously described as related to protein synthesis; and (2) 44 possible homologues, including 25 induced/novel homologues to genes related to protein folding/modification/destination. Those novel/induced genes provide ideal candidates

for further studies directed at understanding fungal morphogenesis and its regulation.

Materials and methods

RNA extraction and preparation of the cDNA library

The cDNA library was constructed, as previously reported [21]. Briefly, *P. brasiliensis*, isolate *Pb*01 (ATCC-MYA-826), was grown in Fava-Netto'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; 4% (w/v) agar, pH 7.2], at 22°C, as mycelium. The differentiation was performed in liquid medium (Fava-Netto's medium) by changing the culture temperature from 22 to 36°C for the mycelium to yeast transition, as we previously described [13]. The cells were previously grown in liquid medium for 18 h before changing the incubation temperature, which was maintained for 22 h.

Total RNA was purified from *P. brasiliensis* mycelium in transition to yeast cells using TRIZOL (GIBCO™, Invitrogen, Carlsbard, CA). The mRNA was purified by using the Poly (A) Quick® mRNA isolation kit (Stratagene, La Jola, CA). The cDNA library was constructed in the unidirectional pCMV.SPORT 6 (Invitrogen) according to the manufacturer's instructions, exploiting the *Not*I and *Sal*I restriction sites. The cDNA library was not normalized, i.e., no attempt was made to reduce the redundancy of highly expressed transcripts.

EST processing pipeline and annotation

The nucleotide sequences were uploaded to a relational database (MySQL) on a Linux (Fedora Core 3) platform, and processed using a modified version of the PHOREST tool [22]. The sequences generated during dimorphic transition of *P. brasiliensis* [21] were compared to sequences generated from yeast and mycelium [16]. Transcripts classification was performed by using the MIPS categorization (http://www.mips.gsf.de/). Similarities with *E*-values $\leq 10^{-4}$ were considered significant.

In silico determination of induced genes in the mycelium to yeast transition

In order to assign a differential expression character, the contigs formed with mycelium and the transition ESTs were statistically evaluated using the Audic and Claverie's method [23]. It were considered induced genes in the transition library those that were not previously described in the mycelium transcriptome database (http://www.dna.biomol.unb.br/Pb), and those more expressed as determined with a 99% confidence rate. A web site (http://www.igs.cnrs-mrs.fr/Winflat/winflat.cgi) was used to compute the probability of differential regulation. The *P. brasiliensis* transcriptome database at (http://www.dna.biomol.unb.br/Pb/) and public databases (http://www.ncbi.nlm.nih.gov) were used to identify novel transcripts, by using the BLAST program [24], as described [21].

Results and discussion

cDNA library sequence annotation

The cDNA library was constructed in a non-normalized primary library without amplification, so the clone abundance presents the relative mRNA population. The quality of the cDNA library was checked by evaluating the presence of well-characterized transcripts in the MIPS category, such as, those encoding for energy and metabolism. The results of computational homology search of the genes related to protein synthesis/folding/modification/destination obtained from the *P. brasiliensis* mycelium undergoing differentiation to yeast cells are shown in the supplementary material, Tables 1 and 2, respectively. A total of 200 ESTs (27.3% of the total transition transcriptome) showed significant similarity to sequences related to protein synthesis/folding/modification/destination (*E*-value $\leq 10^{-4}$) based on BLAST searches. A total of 184 ESTs (25.12% of the total transition transcriptome) gave significant hits to ESTs present in the *P. brasiliensis* transcriptome database or in the GenBank database. In addition, 16 ESTs (2.18% of the total transition transcriptome) represented novel genes of *P. brasiliensis* regarding to the above processes.

A broad view of the nature of the adaptations made by *P. brasiliensis* concerning to protein synthesis during early transition to yeast was obtained by classifying the ESTs into 6 groups of functionally related genes (Table 1, supplementary material). Among the transcripts classified in the transition cDNA library and related to protein synthesis, it was found predominantly ESTs coding for ribosomal proteins, comprehending 39 unigenes.

Regarding to protein folding/modification/destination, the ESTs were classified into 6 groups of functionally related genes, as demonstrated in Table 2, supplementary material. Most of the ESTs represent unigenes related to the MIPS classification of posttranslational modification of amino acids (18 unigenes), followed by transcripts related to protein modification by ubiquitination (8 unigenes), proteasomal degradation (8 unigenes), protein processing (6 unigenes), and protein folding (3 unigenes).

Description of the ESTs related to protein synthesis and fate in the transition transcriptome

As shown in Fig. 1a, the ESTs related to protein synthesis were mainly represented as following: a total of 40.68% of the annotated ESTs corresponded to the ribosomal proteins of the ribosome large subunit; 24.58% in that category were related to the ribosomal proteins of the ribosome small subunit; 15.24% of the transcripts corresponded to homologues encoding translational initiation factors; 9.32% corresponded to ESTs related to the translation elongation machinery. Other ESTs were related to aminoacyl tRNA synthetases (1.69%), ribosome biogenesis (7.64%) and translation termination (0.85%). The Fig. 1b catalogues the ESTs related to protein fate according to the MIPS categories. Most of the transcripts were related to posttranslational modifications of proteins (38.27%) and protein ubiquitination (27.16%). Transcripts related to protein processing and proteasomal degradation of proteins represented 14.81 and 11.12 %, respectively. Classes with lower number of transcripts comprehended those related to protein folding (6.17%) and protein targeting (2.47%).

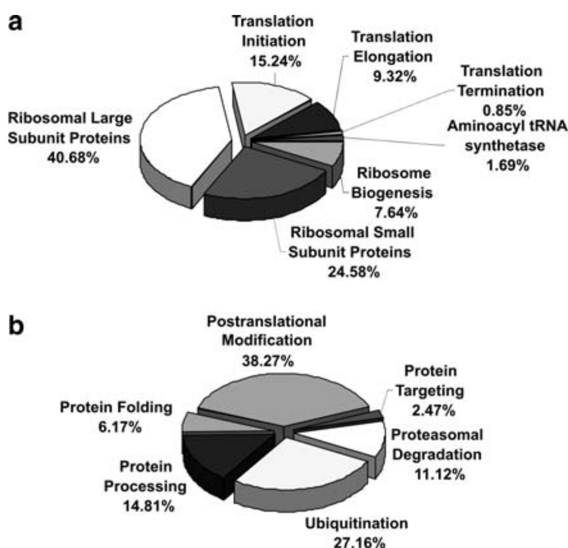

Fig. 1 Distribution of ESTs from *P.brasiliensis* mycelia ongoing transition to yeast according to their cDNA products. (**a**) Protein synthesis; (**b**) protein fate. The classification was based on *E*-value and performed according to the functional categories developed on the MIPS functional annotation scheme. The percentage of ESTs classification is indicated

High abundant ESTs related to protein synthesis and fate in the transition transcriptome

Table 1 shows the 10 most abundant ESTs related to protein synthesis and folding/modification/destination in the transition transcriptome. The minimum number of ESTs that made up these most highly redundant contigs was 5. Eight out of the ten most abundant ESTs were identified as induced sequences according to the Audic and Claverie's method and one EST represented a novel transcript. Included among the most abundant transcripts were ESTs encoding for proteins related to ribosome assembly/biogenesis [25–27] and translation [28–30]. Moreover, a transcript encoding a 14 kDa mitochondrial ribosomal protein (*mrps14*) was detected as a novel transcript. In the *P. brasiliensis* mitochondrial genome, the *mrps14* gene *was* not found [31], suggesting, as described in *Arabdopsis thaliana* [32] its possible transference to the nucleus.

Also, among the highly redundant transcripts, it was detected homologues of proteins related to the acceleration of the protein folding and ubiquitination in many organisms [33, 34]. Transcript encoding proteins related to stress conditions, such as the homologue of the L-isoaspartate O-methyltransferase (*pcmt*) that specifically recognizes and methylates

Table 1 The most abundant transcripts related to protein synthesis and protein fate expressed during transition from mycelium to yeast

MIPS category	Gene name	Gene product	Best hit/accession number	*E*-value	Enzyme commission	Redundancy in the transition cDNA lybrary	Function in organisms
Protein synthesis							
	ubi/crp-6	Ubiquitin fused to S27a protein[a]	*Aspergillus nidulans*/ XP_409009	2e–60	–	7	Required for ribosome biogenesis serving to aid in the assembly of S27a into the ribosome in *Saccharomyces cerevisiae* [25]
	mrps14	14 kDa mitochondrial ribosomal protein[c]	*Aspergillus nidulans*/ XP_408748	4e–46	–	7	Not available
	rps5	40S ribosomal protein S5[a]	*Aspergillus nidulans*/ XP_404980	8e–22	–	7	Required for the folding of 16S ribosomal RNA and translational fidelity in *E. coli* [26]
	rpl20	60S ribosomal protein L20[a]	*Magnaporthe grisea*/ XP_361110	3e–16	–	6	Required for ribosome assembly in *E. coli* [27].
	sui1	Translation initiation factor eIF1 subunit Sui1[a]	*Gibberella zeae*/ XP_389056	2e–36	–	5	Required for the recognition of the AUG codon during translation initiation and for activation of the nonsense-mediated mRNA decay pathway in *S. cerevisiae* [28, 29]
	tef1	Translational elongation factor EF-1 alpha	*Aspergillus nidulans*/ XP_405299	4e–31	–	8	Essential for the delivery of aminoacyl-tRNAs in eukaryotes [30]
Protein fate							
	ppil4	Peptidyl-prolyl cis–trans isomerase-like 4 (Cyclophilin RRM)[a]	*Coccidioides immitis*/ EAS29016	1e–46	5.2.1.8	5	Required for acceleration of proteins folding in organisms [33]
	ubc–6	Ubiquitin conjugating enzyme E2[a]	*Gibberella zeae*/ XP_388490	1e–29	6.3.2.19	7	Catalysis the covalent attachment of ubiquitin to proteolytic substrates in organisms [34]

Table 1 continued

MIPS category	Gene name	Gene product	Best hit/accession number	*E*-value	Enzyme commission	Redundancy in the transition cDNA lybrary	Function in organisms
	pcmt	Protein-L-isoaspartate (D-aspartate) O-methyltransferase[a]	*Aspergillus nidulans*/XP_407601	5e–55	2.1.1.77	5	Required for metabolization of isoaspartyl residues preventing protein damage under physiological conditions in organisms [35]
	pep	Aspartyl proteinase[a]	*Paracoccidioides brasiliensis*/AAP32823	3e–72	3.4.23.24	7	Required for protein processing and for degradation of peptides. Promotes preferential cleavage in hydrophobic amino acids of proteins in eukaryotes [36]

[a] Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

[b] Novel genes detected in *P. brasiliensis*

isoaspartyl residues in a variety of proteins [35], thus preventing the accumulation of deamidated proteins under stressing conditions, was high abundant in the morphological transition. Regarding to protein processing, the transcript encoding aspartyl proteinase (*pep*) was detected. Aspartic proteases are widely distributed in all domains of life and are related to cleavage of peptides in regions of hydrophobic amino acids under acidic conditions. The *S. cerevisiae* vacuolar Pep4p homologue to the *P. brasiliensis* *pep* product has been described as required for the turnover of damaged molecules during stress conditions [36].

Induced genes related to protein synthesis identified by in silico EST subtraction

We attempted to determine the putative function of the set of 119 phrap unisequences by searching for homologues in the GenBank non-redundant protein database using BLAST X and by comparing the ESTs in the transition library to those present in the mycelium transcriptome database. The Fig. 2 presents the percentage of induced and novel genes related to protein synthesis in comparison to the total number of ESTs in the transition transcriptome, as described [21]. As observed, from the 119 ESTs related to protein synthesis (10.66% of the total), 3.97% were induced in the transition library and 0.9% was described as novel genes in *P. brasiliensis*.

The comparative analysis of all the induced ESTs related to protein synthesis in the transition library is available; Table 2 summarizes the results of such comparison. In *P. brasiliensis*, induced transcripts, putatively playing role in ribosomal biogenesis and maturation were detected during dimorphic transition, such as 60S ribosome subunit biogenesis protein (*nip7*), GTP-binding GTP1/OBG (*ygr210*) family protein and ubiquitin fused to S27a protein (*ubi/crp-6*). Proteins composing the small and large ribosomal subunits, as well as translational initiation factors, from both cytoplasmic and mitochondrial ribosomes were over expressed in the transition library; some represent novel genes (Table 2). Similar results were described suggesting that the fungal transition is likely to involve intense synthesis of new ribosome particles, affecting the rate of protein

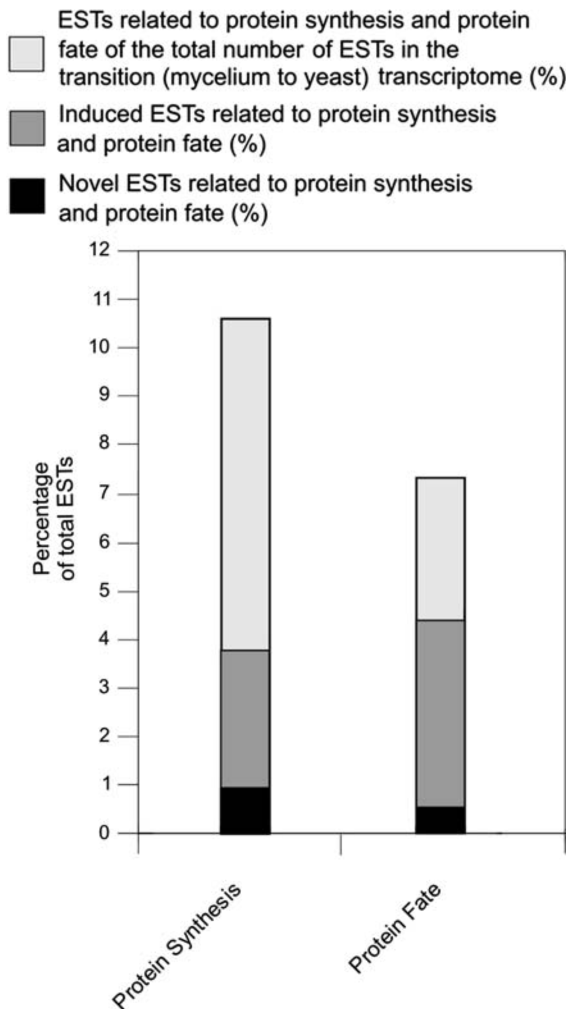

Fig. 2 Prevalence distribution of ESTs from *P. brasiliensis* mycelia ongoing transition to yeast. The percentage of ESTs related to protein synthesis and fate in the total ESTs are represented along with the percentage of over expressed and novel genes of *P. brasiliensis* in the transition library (http://192.168.0.5/phorestwww)

synthesis [7]. In addition, some of the transcripts encode for ribosomal proteins whose orthologues are differentially regulated in organisms. In this sense, the *rps26* product which has no homologue among prokaryotic ribosomal proteins [37] is differentially expressed during environmental stress in plants [38]. Also, the developmental program of organisms seems to include the differential expression of ribosomal proteins; ribosomal protein *rpl5* product was specifically identified in schizonts and was undetectable in oocysts in the organism *Eimeria tenella* [39], suggesting its regulation under different life-cycle stages.

Induced genes related to protein fate identified by in silico EST subtraction: ESTs relevant to protein processing:

We also attempted to determine the putative function of the set of 81 phrap unisequences by searching for homologues in the GenBank non-redundant protein database using BLAST X and by comparing the ESTs in the transition library to those present in the mycelium transcriptome database. The classification of induced genes was designed as described. The Fig. 2 presents the percentage of induced and novel genes related to protein folding/modification/destination in comparison to the total number of ESTs in the transition transcriptome as described [21]. As observed, from the 81 ESTs (7.32% of the transition transcriptome), 48 (4.43% of the total transition transcriptome) were induced in the transition library and 6 (0.54%) were described as novel genes in *P. brasiliensis*.

The comparative analysis of the ESTs related to protein folding/modification/destination is available; Table 3 summarizes the results of such comparison. A cyclophilin seven suppressor 1 (*cns1*) (HSP90 chaperone complex component) was detected. The Hsp90 complex is one of the most abundant and highly conserved chaperone preventing the aggregation of proteins in a folding-competent state and is essential for cell viability in *S. cerevisiae* [40]. A tailless complex polypeptide 1 chaperonin, subunit epsilon (*tcp-1*) was also detected. The *tcp-1* is localized in the cytosol of higher eukaryotes and is similar to prokaryotes GroEL. The *tcp-1* product has been related to protein folding in *S. cerevisiae* playing role in cell development and cytoskeletal organization [41]. The two ORFs encoding homologues to the above proteins, presumably reflect the heat shock condition experienced by mycelia in transition to yeast cells.

Glycosyltransferases play vital roles in the biological function of native proteins, as well as, in the biosynthesis of numerous molecules within fungi, including cell wall components and its induced expression putatively reflect the cell wall remodeling that occurs during *P. brasiliensis* morphological transition [4, 21]. The novel/induced genes encode glycosyltransferases that could be related to galactosylation of N-and O-glycans, as described in *S. cerevisiae* [42]. Mannosyltransferases (*och*1 and

Table 2 Novel and over expressed transcripts related to protein synthesis detected during dimorphic transition in *P. brasiliensis*

MIPS category	Gene name	Gene product	Best hit/accession number	*E*-value	Enzyme commission	Redundancy[d]	
						M	T
Protein synthesis							
Ribosome biogenesis							
	nip7	60S ribosome subunit biogenesis protein NIP7[b]	*Aspergillus fumigatus*/ AAM08680	3e–14	–	–	1
	ygr210	GTP-binding GTP1/OBG family protein[b]	*Aspergillus nidulans*/ XP_404829	1e–70	–	–	1
	ubi/crp–6	Ubiquitin fused to S27a protein[a]	*Aspergillus nidulans*/ XP_409009	2e–60	–	7	7
Ribosomal proteins							
Small subunit							
	mrps14	14 kDa mitochondrial ribosomal protein[c]	*Aspergillus nidulans*/ XP_408748	4e–46	–	–	7
	rps13	40S ribosomal protein S13[b]	*Neurospora crassa*/EAA34807	2e–37	–	–	1
	rps26	40S ribosomal protein S26[b]	*Neurospora crassa*/CAA39162	3e–52	–	–	1
	rps5	40S ribosomal protein S5[a]	*Aspergillus nidulans*/ XP_404980	8e–22	–	8	7
	mrps19	Mitochondrial ribosomal protein S19[b]	*Aspergillus nidulans*/ XP_404292	5e–19	–	–	1
Large subunit							
	rpl20	60S ribosomal protein L20[a]	*Magnaporthe grisea*/ XP_361110	3e–16	–	3	6
	rpl27	60S ribosomal protein L27[a]	*Aspergillus nidulans*/ XP_408359	4e–63	–	1	3
	rpl5	60S ribosomal protein L2[b]	*Coccidioides immitis*/ EAS30555	9e–54	–	–	1
	rpl3	60S ribosomal protein L3[a]	*Aspergillus fumigatus*/ AAM43909	5e–85	–	1	2
	rpl43	60S ribosomal protein L43B[b]	*Ustilago maydis*/XP_400133	1e–30	–	–	1
Translation initiation							
	eif3	Translation initiation factor 3 subunit 2[c]	*Aspergillus nidulans* / XP_660601	6e–80	–	–	3
	eif–5A	Translation initiation factor eIF–5A[a]	*Neurospora crassa*/P38672	6e–06	–	4	4
	–	Translational machinery component protein[b]	*Aspergillus nidulans*/ XP_405417	1e–19	–	–	1
	sui1	Translation initiation factor eIF1 subunit Sui1[a]	*Gibberella zeae*/XP_389056	2e–36	–	2	5
Aminoacyl-tRNA synthetase							
	ils1	Isoleucyl-tRNA synthetase[b]	*Aspergillus nidulans*/ XP_407499	1e–52	6.1.1.5	–	2

[a] Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

[b] Transcripts non detected in the mycelia transcriptome (http://www.dna.biomol.unb.br/Pb)

[c] Novel genes detected in *P. brasiliensis*

[d] M: Redundancy in *P. brasiliensis* mycelia transcriptome (http://www.dna.biomol.unb.br/Pb); T: Redundancy in *P. brasiliensis* transition library (http://192.168.0.5/phorestwww/)

Table 3 Novel and over expressed transcripts related to protein fate detected during dimorphic transition in *P. brasiliensis*

MIPS category	Gene name	Gene product	Best hit/accession number	*E*-value	Enzyme commission	Redundancy[e]	
						M	T
Protein fate							
Protein folding							
	cns1	Cyclophilin seven suppressor 1 (HSP90 chaperone complex component)[b]	*Aspergillus nidulans*/ XP_409575	8e–12	–	–	2
	tcp–1	Tailless complex polypeptide 1 chaperonin, subunit epsilon[b]	*Schizosaccharomyces pombe*/EAA65069	6e–16	–	–	2
Posttranslational modification of amino acids							
	gma12	Alpha–1, 2-galactosyltransferase[c]	*Aspergillus nidulans*/ XP_406106	3e–14	2.4.1.-	–	1
	mnt1	Alpha–1, 2-mannosyltransferase[a]	*Neurospora crassa/ CAC18268*	1e–29	2.4.1.131	3	3
	och1	Mannosyltransferase[b]	*Paracoccidioides brasiliensis*/AAK54761	3e–70	2.4.1.130	–	1
	swp1	Oligosaccharyltransferase subunit ribophorin II[d]	*Coccidioides immitis*/ EAS29547	9e–37	2.4.1.119	–	1
	rabggt	Rab geranylgeranyl transferase[c]	*Aspergillus nidulans*/ XP_412816	8e–13	2.5.1.60	–	1
	cypb	Peptidyl prolyl cis–trans isomerase[b]	*Neurospora crassa*/ CAD21421	8e–39	5.2.1.8	–	1
	ppil1	Peptidyl-prolyl cis–trans isomerase-like 4 (Cyclophilin RRM)[a]	*Coccidioides immitis*/ EAS29016	1e–46	5.2.1.8	1	5
	pcmt	Protein-L-isoaspartate (D-aspartate) O-methyltransferase[a]	*Aspergillus nidulans*/ XP_407601	5e–55	2.1.1.77	4	5
	gmd1	Guanosine diphosphatase[c]	*Aspergillus nidulans*/ XP_405219	2e–15	3.6.1.42	–	1
Proteasomal degradation							
	rpt6	26S proteasome regulatory subunit protein[b]	*Aspergillus nidulans*/ XP_411125	4e–23	–	–	1
	rpn12	26s proteasome regulatory subunit rpn12[b]	*Aspergillus nidulans*/ XP_407156	5e–30	–	–	1
	rpn5; rpne	26S proteasome regulatory subunit Non-ATPase[c]	*Aspergillus nidulans*/ XP_408912	2e–68	–	–	1
	csn5	COP9 signalosome complex subunit 5[a]	*Aspergillus nidulans*/ XP_406266	1e–35	–	1	2
Modification by ubiquitination							
	ubp1	Ubiquitin-specific protease (C19)[b]	*Aspergillus nidulans*/ XP_412211	7e–08	3.1.2.15	–	3
	ubc–6	Ubiquitin conjugating enzyme E2[a]	*Gibberella zeae*/ XP_388490	1e–29	6.3.2.19	6	7
	ubq/rpl40	Ubiquitin fusion protein[a]	*Schizosaccharomyces pombe*/NP_593923	8e–67	–	3	3

Table 3 continued

MIPS category	Gene name	Gene product	Best hit/accession number	*E*-value	Enzyme commission	Redundancy[e]	
						M	T
	ubp1; otub1	Ubiquitin thiolesterase otubain like protein[c]	*Aspergillus nidulans*/EAA60354	1e–28	3.4.-	–	1
	rfn167	Ring (really interesting new gene) type zinc finger (C3HC4) protein (E3 complex)[b]	*Schizosaccharomyces pombe*/CAB08748	5e–10	–	–	1
	fbl7	F-box/LRR-repeat protein 7 (E3 complex)[b]	*Aspergillus nidulans*/XP_408647	8e–28	–	–	3
Protein Processing							
	pep	Aspartyl proteinase[a]	*Paracoccidioides brasiliensis*/AAP32823	3e–72	3.4.23.24	3	7
	lon	Lon protease[b]	*Pseudomonas fluorescens*/AF250140_1	1e–05	3.4.21.53	–	1
	lap	Peptidase M28 domain protein[c]	*Coccidioides immitis*/EAS33583	1e–22	3.4.11.15	–	1
	mde10	Zinc metalloprotease (M12)[b]	*Neurospora crassa*/CAD21161	3e–47	3.4.24.-	–	1

[a] Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

[b] Transcripts non detected in the mycelia transcriptome (http://www.dna.biomol.unb.br/Pb)

[c] Novel genes detected in *P. brasiliensis*

[d] Genes not described previously in *P. brasiliensis* isolate *Pb*01, but present in public databases

[e] M: Redundancy in *P. brasiliensis m*ycelia transcriptome (http://www.dna.biomol.unb.br/Pb); T: Redundancy in *P. brasiliensis* transition library (http://192.168.0.5/phorestwww/)

*mnt*1) could be putatively related to the *O*-linked mannosylation of proteins, as observed in *C. albicans*. *C. albicans* mutants to either *mnt1* or *och1* showed hypersensitivity to cell wall perturbing agents, suggesting the proteins role in the cell wall maintenance [43, 44]. Moreover, a novel transcript encoding to guanosine diphosphatase (*gmd*1) was detected during the dimorphic transition, whose product is known to regulate mannosylation of N- and O-linked oligosaccharides in Golgi complex [45].

Peptidyl-prolyl cis/trans isomerases (cyclophilins) catalyze cis/trans isomerization of a prolyl bond and this isomerization is a time limiting step in folding of certain proteins [46]. Transcripts encoding to two-peptidyl prolyl cis–trans isomerases (*cypb* and *ppil*) were induced in *P. brasiliensis* during the transition from mycelium to yeast. Aside from their roles in cellular biochemistry, cyclophylins of microorganisms are particularly interesting since those proteins are found to have a key role in pathogenicity [47]. The *P. brasiliensis* CypB deduced amino acid sequence presents four conserved amino acids: Arg, Phe, Trp and His (RFWH motif, data not shown) described as involved in peptidyl-prolyl cis–trans isomerase activity and related to the activity of the protein in the folding process as described [48].

Protein processing MIPS category is represented by four unigenes induced in *P. brasiliensis* transition library; some presents orthologues with function in stress response and differentiation. The aspartic protease (*pep*) with seven ESTs, was also included with the most abundant transcripts (see Table 1). The *pep* product belongs to family A1 of aspartic protease, related to pepsin and synthesized as a propeptide with signal peptide. This peptidase family is related to stress response in *S. cerevisiae* [36]. The deduced Lon protease (*lon*) shows homology with family S16, class 001 in MEROPS database (http://www.merops.sanger.ac.uk) and is induced in the transition transcriptome sharing identity with its counterparts in bacteria. The *lon* product was first identified in *E. coli*

Table 4 Homologues for protein synthesis and fate ESTs putatively related to fungal differentiation/virulence or stress tolerance

Gene product	Described role	Redundancy[d]		Reference
		M	T	
Cyclophilin seven suppressor 1 (*cns1*)[b]	Promotes increase in heat shock response in *Saccharomyces cerevisiae.*	–	2	[40]
Alpha-1, 2-mannosyltransferase (*mnt1*)[a]	Required for adhesion and virulence in *Candida albicans*	3	3	[43]
Mannosyltransferase (*och1p*)[b]	Required for cell wall integrity and virulence in *Candida albicans*	–	1	[44]
Zinc metalloprotease (*mde10)*[b]	Required for spore development in *Schizosaccharomyces pombe*	–	1	[52]
GTP-binding GTP1/OBG family protein (*ygr210*)[b]	Involved in regulation of differentiation in *Streptomyces coelicolor.*	–	1	[53]
Peptidyl prolyl cis–trans isomerase (*cypb*)[b]	Induced in heat shock response in *Aspergillus nidulans.*	–	1	[54]
Peptidyl-prolyl cis–trans isomerase-like 4 (*ppil1*)[a]	Related to thermoresistance in *Paramecium sp*	1	5	[55]
Peptidyl-prolyl cis/trans isomerase (*ess1*)	Required for *Cryptococcus neoformans* virulence	6	1	[56]
Peptidyl-prolyl cis–trans isomerase (*mip*)	Required for *Legionella pneumophila* survival into macrophages	2	2	[57]
Protein-L-isoaspartate (D-aspartate) O-methyltransferase (*pcmt*)[a,c]	Promotes increase in heat shock survival in *Escherichia coli.*	4	5	[58]
Ubiquitin conjugating enzyme E2 (*ubc6*)[a]	Promotes enhanced in growth of *Saccharomyces cerevisiae* at high temperature.	6	7	[59]
Aspartyl proteinase[a,c] (*pep*)	Secreted by *Aspergillus fumigatus* during invasion of the host lung.	3	7	[60]
Lon protease (*lon*)[b]	Required for cellular morphology and virulence in *Agrobacterium tumefaciens*	–	1	[61]

[a] Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

[b] Transcripts non detected in the mycelia transcriptome (http://www.dna.biomol.unb.br/Pb)

[c] Also over expressed in yeast cells recovered from liver of infected mice (Costa et al. unpublished)

[d] M: Redundancy in *P. brasiliensis m*ycelia transcriptome (http://www.dna.biomol.unb.br/Pb); T: Redundancy in *P. brasiliensis* transition library (http://192.168.0.5/phorestwww/)

and its homologues were further discovered in many organisms sometimes in multiple copies, playing essential roles in protein quality control by destroying unfolded proteins [49]. In *P. brasiliensis*, a gene homologue encoding for a Lon protein of the S16 family, class 002 in MEROPS database (http://www.merops.sanger.ac.uk), was described previously [50], suggesting more than one Lon species in the fungal pathogen. A metalloprotease M28 domain protein (*lap*) was found as a novel gene in *P. brasiliensis*, encoding for a leucyl aminopeptidase. In *Thichoderma harzianum*, the M28 peptidase is induced during nitrogen starvation suggesting its importance in the amino acid acquisition [51]. Other metalloprotease induced in *P. brasiliensis* dimorphic transition is the zinc metalloprotease belonging to M12 family (*mde10*), whose members were described in fungi [52].

Putative differentiation, virulence and stress tolerance factors

Factors putatively related to the differentiation process, fungal virulence and stress tolerance were

selected on basis with homology to other microorganisms in which defined functions are available. With these criteria, we classified some transcripts as shown in Table 4. The *cns1* product is an essential component of the HSP90 complex, which is induced in heat shock response [40]. Mannosyltransferases (*mnt*1 and *och*1) orthologues are required for cell wall integrity/virulence and adhesion/virulence, respectively, in *C. albicans* [43, 44]. In *S. pombe*, the *mde10* product is essential for development of spore envelopes [52] evidencing its importance during differentiation process in the cell. The GTP-binding GTP1/OBG family product (*ygr210*) related to ribosome biogenesis has been described as a regulator of differentiation in *Streptomyces coelicolor*, playing a role in the onset of aerial mycelium formation and sporulation [53]. In *Aspergillus nidulans*, CypB is induced in response to heat shock indicating a possible role of this protein during growth in stress environments [54]. *P. tetraurelia* KIN241 homologue to peptidyl-prolyl cis–trans isomerase-like 4 (*ppil1*) is related to the organism thermoresistance [55]. A parvulin type Ess1 of *Cryptococcus neoformans* homologue to *P. brasiliensis ppil1* product is required for virulence, since Ess1 depleted strains are unable to cause experimental infection [56]. The Mip protein (macrophage infectivity potentiator) of *Legionella pneumophila* is a cyclophilin FKBP-type homolog which is related to bacterial virulence in intracellular infection in guinea pig [57]. *Escherichia coli* transformants over expressing L-isoaspartate (D-aspartate) O-methyltransferase presented increase in the heat shock survival rates [58]. Yeast strains over expressing ubiquitin conjugating enzyme E2 are more tolerant to various stresses conditions, such as high temperature [59]. The *A. fumigatus* aspartyl protease (*pep*) is highly secreted during fungal invasion of host lung [60]. The Lon protease of *Agrobacterium tumefaciens* is required for normal growth, cellular morphology and full virulence [61].

Concluding remarks

Molecular strategies relying on ESTs has proved to be an efficient approach to identify genes expressed under a variety of conditions. This study presents a screen for genes related to protein synthesis/folding/modification/destination expressed during mycelium to yeast differentiation of *P. brasiliensis* through EST analysis. By analysis of the induced and or novel genes it was possible to infer some metabolic adaptations of *P. brasiliensis* during early dimorphic transition that could include the increased control in the ribosome biogenesis and translation fidelity, increase in protein glycosylation and in the control of protein folding. In addition, the amino acids capture from the medium could be favored during the transition to the parasitic phase.

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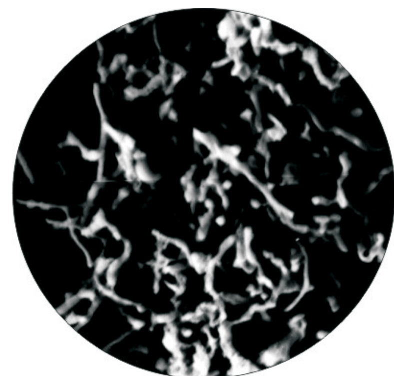
Capítulo VI

Perspectivas



IV. Perspectivas

1. Análise da expressão gênica da formamidase em meios de cultura com indução e limitação de nutrientes;

2. Estudo de vias metabólicas envolvidas no metabolismo de nitrogênio em *P. brasiliensis*;

3. Análises proteômicas de *P. brasiliensis* em meios de cultura com indução e limitação de nutrientes;

4. Avaliação do potencial uso da proteína formamidase recombinante no diagnóstico da PCM;

5. Identificação, por meio de espectrometria de massas, de proteínas diferencialmente expressas nos isolados com e sem transição dimórfica em resposta à mudança de temperatura;

7. Desenvolvimento e utilização de ferramentas genéticas para análise do papel funcional de genes diferencialmente expressos.

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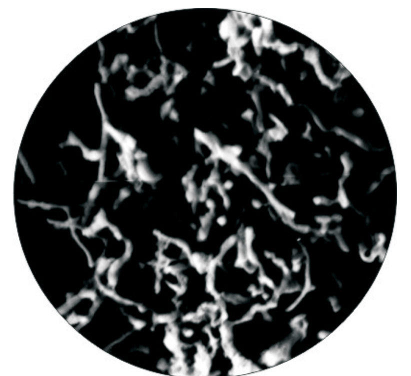

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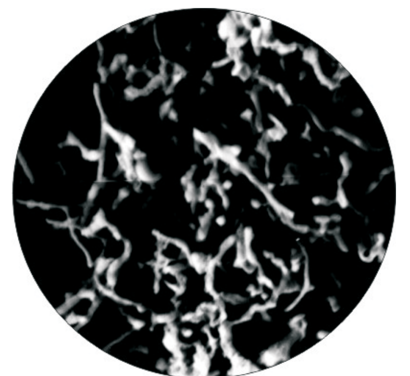
Anexos



VI. Anexos

VI.1. Figura

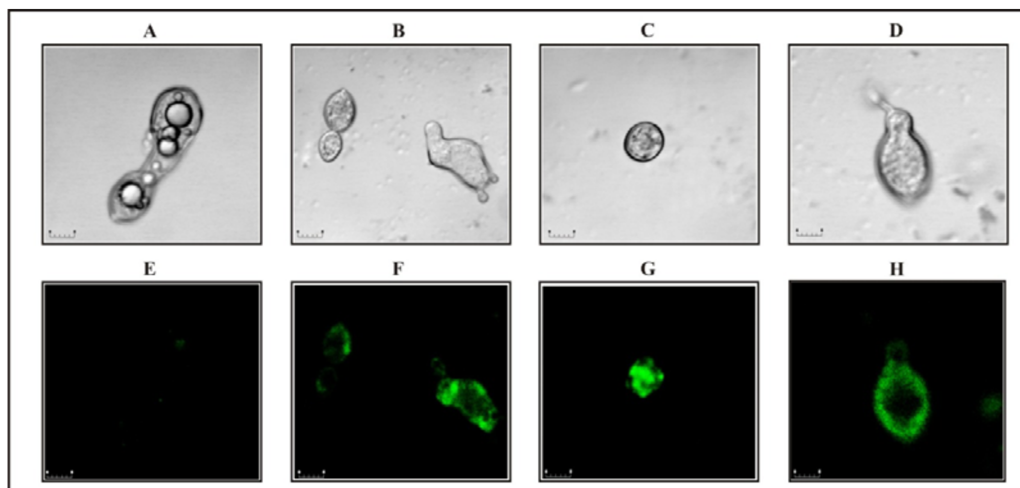


Figura 1: Microscopia confocal de células leveduriformes de *P. brasiliensis*. (A-D) mostrando células leveduriformes de *P. brasiliensis* visualizadas por microscopia óptica. (E) controle com soro pré-imune; (F e H) mostrando localização celular da formamidase de *P. brasiliensis* na membrana/parede celular e em G no citoplasma, visualizadas por microscopia confocal. Barras 5 μm.

VI.2. Produção científica durante o doutorado

VI.2.1 Artigos completos publicados em periódicos

1. PARENTE, J.A., **BORGES, C.L.**, BAILÃO, A.M., FELIPE, M.S.S., PEREIRA, M., SOARES, C.M.A. Comparison of transcription of multiple genes during mycelia transition to yeast cells of *Paracoccidioides brasiliensis* reveals insights to fungal differentiation and pathogenesis. Mycopathologia, 165: 259-274*,* 2008.

2. COSTA, M*., **BORGES, C. L*.**, BAILÃO, A. M., MEIRELLES, G. V., MENDONÇA, Y. A., MOREIRA, S. F. I., FARIA, F. P., FELIPE, M. S. S., MADLUM, E. E. W. I. M., Mendes-Giannini, M. J. S., FIÚZA, R. B., MARTINS, W. S., PEREIRA, M., SOARES, C. M. A. Transcriptome profiling of *Paracoccidioides brasiliensis* yeast cells recovered from infected mice. Microbiology. 153, 4194-4207, 2007.

3. **BORGES, C. L.**, PARENTE, J. A., PEREIRA, M., SOARES, C. M. A. Identification of the GTPAse superfamily in *Mycoplasma synoviae* and *Mycoplasma hyopneumoniae*. Genet Mol Biol. 30, 212 - 218, 2007.

4. BASTOS, K. P., BAILÃO, A. M., **BORGES, C. L.**, FARIA, F.P., FELIPE, M. S. S., SILVA, M. G., MARTINS, W. S., FIÚZA, R. B., PEREIRA, M., SOARES, C. M. A. The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals novel and induced genes potentially associated to the dimorphic process. BMC Microbiol., 10, 7 - 29, 2007.

5. BAEZA, L. C., BAILÃO, A. M., **BORGES, C. L.**, PEREIRA, M., SOARES, C. M. A., MENDES-GIANNINI, M.J. cDNA representational difference analysis used in the identification of genes expressed by *Trichophyton rubrum* during contact with keratin. Microbes Infect., 9, 1415-21, 2007.

6. BAILAO, A. M., SHRANK, A., **BORGES, C. L.**, PARENTE, J. A., DUTRA, V., FELIPE, M. S., FIUZA, R. B., PEREIRA, M., SOARES, C. M. A. The transcriptional profile of *Paracoccidioides brasiliensis* yeast cells is influenced by human plasma. FEMS Immunol Med Microbiol. 2007. 51:43-57.

7. BAILÃO, A. M., SCHRANK, A., **BORGES, C. L.**; DUTRA, V., MADLUN, E. E. W. I. M., GIANNINI, M. J. M., FELIPE, M. S. S., MARTINS, W. S., PEREIRA, M., SOARES, C. M. A. Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: Representational difference analysis identifies genes associated to fungal pathogenesis. Microbes Infect., 8, 2686-2697, 2006.

8. **BORGES, C. L.**, PEREIRA, M., FELIPE, M. S. S., FARIA, F. P., GOMEZ, F. J., DEEPE G. S., SOARES, C. M. A. The antigenic and catalytically active formamidase of *Paracoccidoiodes brasiliensis*: Protein characterization, cDNA and gene cloning, heterologous expression and functional analysis of the recombinant protein. Microbes Infect., 7, 66-77, 2005.

9. FELIPE, M. S. S., ANDRADE, R. V., ARRAES, F. B. M., NÍCOLA, A. M., MARANHÃO, A. Q., TORRES, F. A. G., PEREIRA, I. S., POÇAS-FONSECA, M. J., CAMPOS, É. G., MORAES, L. M. P.; ANDRADE, P. A., TAVARES, A. H. F. P., SILVA, S. S., KYAW, C. M., SOUZA, D. P., **BORGES, C. L.**, NETWORK, Pbgenome; PEREIRA, M., SOARES, C. M. A., BRIGIDO, M. M. Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. J Biol Chem., 280, 24706-24714, 2005.

10. FELIPE, M. S. S., GROUP Pb Genoma, **BORGES, C. L.**, PARENTE, J. A., SOARES, R. B. A., ANDRADE, E. V., PEREIRA, M., SOARES, C. M. A., BRÍGIDO, M. M. Transcriptome characterization of the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis* by EST analysis. Yeast, 20, 263-271, 2003.

VI.2.2 Artigo em revisão

01. PEREIRA, M., BAILÃO, A. M., PARENTE, J. A., **BORGES, C. L**., SALEM-IZACC, S. M., SOARES, C. M. A. Preferential transcription of *Paracoccidioides brasiliensis* genes: host niche and time dependent expression. Mem Inst Oswaldo Cruz, 2008, em revisão.

VI.2.3 Artigos em redação final

01. **BORGES, C. L**., PARENTE, J. A., BARBOSA, M. S., SANTANA, J. M., BAO, S. N., SOUSA, M. V., SOARES, C. M. A. Characterization of the formamidase of *Paracoccidioides brasiliensis*: protein analysis, localization and intermolecular interactions.

02. **BORGES, C. L**., BAILAO, A. M., BÁO, S. N., PEREIRA, M., SOARES, C. M. A. Genes potentially relevant in the parasitic phase of the fungal pathogen *Paracoccidioides brasiliensis.*

VI.2.4 Trabalhos apresentados em eventos

Resumos em anais de eventos:	21
Trabalhos completos em anais de eventos:	1
Prêmios em congressos internacionais:	2

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