UNIVERSIDADE DE BRASÍLIA

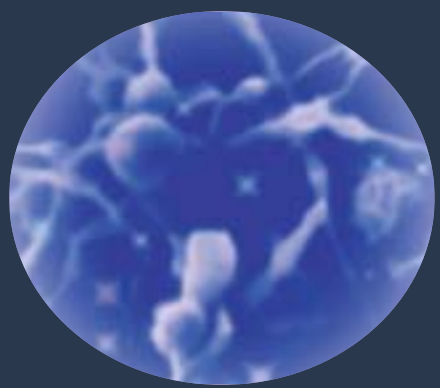
FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM

PATOLOGIA MOLECULAR

Análises transcricionais no estudo da expressão gênica de *Paracoccidioides brasiliensis* em condições que mimetizam nichos do hospedeiro

TESE DE DOUTORADO



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UNIVERSIDADE DE BRASÍLIA
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ANÁLISES TRANSCRICIONAIS NO ESTUDO DA EXPRESSÃO GÊNICA DE *Paracoccidioides brasiliensis* EM CONDIÇÕES QUE MIMETIZAM NICHOS DO HOSPEDEIRO.

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

Brasília-DF-Brasil
-2008-

TRABALHO REALIZADO NO LABORATÓRIO DE BIOLOGIA MOLECULAR, DO DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR, DO INSTITUTO DE CIÊNCIAS BIOLÓGICAS DA UNIVERSIDADE FEDERAL DE GOIÁS

APOIO FINANCEIRO: CNPq/ FINEP/SECTEC-GO

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.....Nunca deixe que lhe digam
Que não vale a pena Acreditar no sonho que se tem
Ou que seus planos nunca vão dar certo
Ou que você nunca vai ser alguém
Tem gente que machuca os outros
Tem gente que não sabe amar
Mas eu sei que um dia a gente aprende
Se você quiser alguém em quem confiar
Confie em si mesmo
Quem acredita sempre alcança

...Nunca, Nunca, Nunca deixe alguém te
dizer que aquilo que você acredita é
babaquice, que de repente o teu sonho não
vai dar certo...

Renato Russo

....Por que escutar? Somente prestam atenção nas opiniões dos outros, diferentes da própria, aqueles que não estão convictos de serem possuidores da verdade. Quem não está convicto está pronto a escutar - é um permanente aprendiz. Quem está convicto não tem o que aprender - é um permanente (eu ia dizer "professor". Peço perdão aos professores. O professor verdadeiro, acima de todas as coisas que ensina, ensina a arte de desconfiar de si mesmo...) mestre de catecismo..... Dizia Nietzsche que "as convicções são piores inimigos da verdade que as mentiras." Estranho isso? Não. Absolutamente certo. Porque quem mente sabe que está mentindo, sabe que aquilo que está dizendo é um engano. Mas quem está convicto não se dá conta da própria bobeira. O convicto sempre pensa que a sua bobeira é sabedoria.....

"Existe um mundo que acontece pelo desenrolar lógico da história, em toda a sua crueza e insensibilidade. Mas há um mundo igualmente concreto
que nasce dos sonhos: a Pietà, de Michelangelo, o Beijo, de Rodin, as
telas de Van Gogh, as músicas de Tom Jobim, os livros de Guimarães Rosa e de Saramago, as casas, os jardins, as comidas: eles existiram primeiro como um sonho, antes de existirem como fatos. Quando os sonhos assumem forma concreta, surge a beleza"

Rubem Alves

Dedico este trabalho as pessoas mais importantes da minha vida....

... ao meu pai Arlindo que nunca mediu esforços para garantir a minha formação e por sempre me ensinar os reais valores da vida.

... à .minha mãe Marilene pelo amor incondicional e dedicação aos filhos.

...à minha mãe, grande ser humano, cujo próprio comportamento diante da vida é meu maior exemplo dos verdadeiros valores que engrandecem o homem, o respeito às pessoas indistintamente e a disponibilidade em ajudar qualquer pessoa sem nunca esperar nada em troca.

...aos meus amados irmãos Rogério e Aline pelo cuidado, amor, apoio e por sempre me desejarem o melhor.

...ao meu sobrinho e xará Alexandre e minha cunhada Karla, os mais novos membros da família, que trouxeram um novo e grande sentimento de felicidade para minha família.

.. só cheguei até aqui porque vocês me apoiaram.

Amo muito todos vocês!

Muito obrigado!

AGRADECIMENTOS

Agradeço a energia especial, denominada por vários como Deus, pela benção, oportunidade e por nunca deixar que eu desistisse frente aos grandes obstáculos da vida.

Um agradecimento especial e sincero à minha orientadora Célia pela orientação inquestionável, empenho e pelo exemplo como pesquisadora. Célia, tenho a certeza que seus alunos ficam admirados com seu profissionalismo, competência e compromisso com o bem público. Apesar de todas as adversidades do meio científico e das condições do país você nunca desistiu de buscar e conseguir fundos para garantir a qualidade dos trabalhos de seus alunos. Célia parabéns você faz as coisas acontecerem. E por tudo isso e muitas outras coisas, para mim, você é um ícone do meio científico mundial. Sou muito grato pela confiança depositada em mim por todos estes 8 anos de convivência. Sou grato e honrado por ter sido seu aluno iniciação científica e no doutorado e terei sempre, ao longo da minha jornada profissional, o cuidado e a responsabilidade de ter a minha formação vinculada ao seu nome.

Agradeço ao meu GRANDE AMIGO Claytinho, meu mano... Clayton você é uma pessoa incrível e sua amizade foi fundamental para que este trabalho fosse concretizado. Além disso, a nossa convivência foi essencial para meu crescimento e amadurecimento. Obrigado por me escutar e enxugar minhas lágrimas nos momentos difíceis. Sem sua ajuda a concretização deste trabalho seria um processo infinitamente mais difícil. Tenho orgulho de ser seu amigo. Sou eternamente grato!

Ao amigo Rafael Balestra pela amizade verdadeira, apoio incondicional e por sempre me ajudar nos momentos difíceis. A minha convivência com você foi essencial para meu engrandecimento como ser humano... você é um irmão que eu pude escolher...

Aos amigos Mônica (MOCzinha) e Rodriguinho pelo companheirismo e por transformarem os árduos dias de trabalho em momentos descontraídos e agradáveis...

As minha amigas Ju Parente e Sabrina pelo apoio e ajuda na execução de etapas deste trabalho.

Sou muito grato aos meus amigos Luiz (Dindin) e Wesley (Tchatchá) pelos inesquecíveis momentos compartilhados.

Agradeço de forma especial Carol, Renata, Zeni, César, Paulo, Brício, Rhaylla, Nilma, e toda a família Cabral pelos vários momentos de felicidade que me proporcionaram e pelo apoio durante todo meu doutorado.

Sou imensamente grato ao meu amigo Michael Winters (Mike Duuuuude) por ser meu irmão durante meu estágio nos EUA . Obrigado pelo suporte emocional e pelos ensinamentos que contribuíram muito com minha formação. E é claro, muito obrigado pelos momentos de diversão no "Crazy Fox, The Best Pub in América".

Agradecimento especial a Danielle, Doug, Cindy, Jessie e toda a família Kroetz por serem minha família nos EUA e por não deixarem a solidão me atingir. Obrigado pelo "Thanksgiving Day" e "Christmas" inesquecíveis que me proporcionaram.

Aos meus orientadores no estágio SWE George Deepe e Smulian por me receberem de braços abertos e pelos ensinamentos imensuráveis que me proporcionaram. A convivência com vocês foi crucial pra meu engrandecimento como pesquisador.

Aos colegas Francisco Gómez, Heiko Tanaka, Jeff, Christine, Megan, Wendy, Jeremy, Simon, Wendy Lemen, Melanie Cushion, Reta, Holly, Rebecca e Debroski pela ajuda e pelos agradáveis momentos de convivência.

A professora Maristela pela colaboração e conversas agradáveis

Aos amigos do Laboratório de Enzimologia Val, Faustinho e Roberto pelos ótimos momentos.

A banca examinadora pela disponibilidade e contribuição.

Ao professor Jaime e funcionários da Pós-Graduação pela boa vontade em ajudar os alunos de Goiânia.

Aos professores Augusto Schrank e Maria José Soares Mendes-Giannini pela colaboração neste trabalho e pela receptividade em seus laboratórios.

Aos amigos do Rio Grande do Sul: Valéria, Luciano, Leonardo Broetto, Charley, Josy e Juliano pela ajuda e hospitalidade.

Ao meu grande amigo gaúcho Marciano companheiro das disciplinas em Brasília. Você conseguia transformar a angustia das disciplinas em momentos de muitas gargalhadas.

Ao meu amigo Ernane Bastos que me acompanhou nos meus primeiros passos no LBM. Obrigado Betão!!

Agradeço de forma sincera e muito especial a minha namorada Mirelle uma das pessoas mais especiais que já conheci. A sua presença e seus sentimentos foram cruciais para finalização deste trabalho. Você é um grande ser humano e uma namorada admirável, alguém que sabe o verdadeiro sentido da vida. Mi você é a luz que iluminou a escuridão da minha vida. Meus sentimentos por você são os mais puros e sinceros. Te amo!

Aos queridos amigos do LBM: Tereza Cristina, Nathalie, Ronney, Milce, Karinne, Bernadette, Patrícia Lima, Elisa Fávia, Fafá, Mariana, Nadya, Bruno, Kelly, Ivian, Yuri, Gabi, Moniquinha, Leandro, Daiane, Hellen, Ademar pela colaboração e boa convivência.

Aos amigos do grupo Maris: Heiko, Pati Kott, Rogério, Renata, Regilda, Aline, Kesser, Patrícia Zambuzzi, Amanda, Raquel, Neto, Christielly, Hérika, João Guilherme e Lidiane pela colaboração, apoio e os bons momentos compartilhados.

Aos professores da graduação e pós-graduação pelos ensinamentos valiosos.

A todos que de alguma maneira contribuíram para realização deste trabalho!

Muito obrigado!

SUMÁRIO

LISTA DE ABREVIATURAS

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

APS1 - 5'adenilil sulfato quinase

ATP – adenosina trifosfato

atx1 -chaperona de cobre

cAMP - adenosina monofosfato cíclico

ccc2 - transportador de cobre

CDI1 – cisteína dioxigenase

cDNA – DNA complementar

CEK1 - proteína quinase ativada por mitose)

CFS – fluido cerebroespinhal

CHS1 – colina sulfatase

Cir 1 – fator de trasnição ferro-dependente

ClpB – chaperona ClpB

CPP1 – fosfatase relacionada com formação de hifa

CST20 - quinase relacionada com formação de hifa

CTR3 – transportador de cobre de alta afinidade

dbr1 - 2'-5'-fosfodiesterase

DD – apresentação diferencial

DDC - descarboxilase de aminoácidos aromáticos

DHN - 1,8-dihidroxinaftaleno

DIP5 - permease de aminoácido ácido

DNA – ácido desoxirribonucléico

DOPA - L-3,4-dihidroxifenialanina

EBP – proteína que liga-se do estradiol

eEIF-1γ - fator de elongação da tradução, subunidade 1-gama

eIF-4A - ator de iniciação da tradução, subunidade 4A

eRF1 - fator de liberação da tradução 1

ERG3 - delta-5,6-desaturase

ERG5 – C-4-esterol metil oxidase

EST – etiqueta de seqüência expressa

Fe – íon ferro

FOX2 - 2-enoil-CoA-hidratase/3-hidroacil-CoA-desidrogenase

FRE2 – ferro redutase

Fur – regulador de captação de ferro

GAPDH – gliceraldeído 3-fosfato desidrogenase

gfaA - glutamina:frutose-6-fosfato amidotransferase

GFP – proteína fluorescente verde

GLN1 – glutamina sintase

HOG – alta osmolaridade do glicerol

Hog1 – MAPK Hog1

HSP – proteína de choque térmico

ITS – seqüência espaçadora interna – "internally transcribed sequence"

MAPK – proteína quinase ativada por mitose

Mb – megabases

MEP1 - metionina permease

metG - cistationa β-liase

NADH - nicotinamida adenina dinucleotídeo reduzido

NADPH - nicotinamida adenina dinucleótido fosfato reduzido

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

OLE1 – delta-9-desaturase

PAS - Per-Arnt-Sim

Pb Y20 – proteína homóloga à flavodoxina

PCM – paracoccidioidomicose

PCR – reação em cadeia da polimerase

PFGE - gel em eletroforese de pulso alternado

Pir - proteína com repetições internas presentes na parede celular

PLP - pirodoxal-5-fosfato

PPO1 - piridoxamina fosfato oxidase

*pr*1C – protease homóloga a subtilisina

PYP - proteínas fotoativas em amarelo

RAP-PCR – PCR de oligonucletídeo-ligado arbitrário

RAS GTPse – proteína G ligante de GTP do tipo RAS

RDA – análise de diferença representacional

rDNA – DNA ribossomal

RNA – ácido ribonucleico

mRNA – RNA mensageiro

rRNA – RNA ribossomal

RT-PCRsq – PCR semiquantitativo acoplado a transcrição reversa

SAGE – análise serial de expressão gênica

SCOTS – captura seletiva de seqüências transcritas

SH – hibridização subtrativa

sho1 – osmosensor transmembrana

sln1 – quinase sensor

snoRNA – RNA nuclear pequeno

spn1 – septina

SUR1 - sulfito redutase

TGAse – transglutaminase

UDP – uridina difosfato

zrt1 - transportador de zinco/ferro de alta afinidade

RESUMO

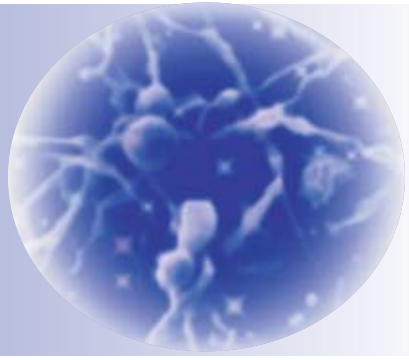
O fungo *Paracoccidioides brasiliensis* é um patógeno humano com ampla distribuição na América Latina. Este microrganismo causa a paracoccidioidomicose, a micose sistêmica mais prevalente na América Latina. O fungo causa infecção quando o hospedeiro inala propágulos da fase miceliana do organismo. Estas partículas atingem o pulmão e podem disseminar para outros órgãos e tecidos. Embora o perfil de expressão gênica em *P. brasiliensis* tem sido estudado, pouco se conhece sobre o padrão de expressão de genes desta espécie durante o processo infeccioso. O presente trabalho descreve a análise dos genes diferencialmente expressos em células leveduriformes de *P. brasiliensis* recuperado de animais infectados e durante incubação com sangue humano, que mimetiza a rota hematogênica de disseminação do fungo. Adicionalmente, também foram identificados os genes preferencialmente expressos durante incubação do fungo com plasma humano, mimetizando os sítios de infecção com inflamação. Os dados revelaram que genes relacionados com captação de ferro, síntese de melanina e defesa celular foram superexpressos no modelo de infecção animal. Os transcritos induzidos durante a incubação de células leveduriformes com sangue humano foram aqueles predominantemente relacionados com síntese/remodelamento da parede celular. Genes relacionados com degradação de ácidos graxos, síntese protéica, estresse osmótico, remodelamento da parede celular e defesa celular foram superexpressos no tratamento com plasma humano. A expressão diferencial dos genes identificados foi confirmada por ensaios de dot blot, northern blot e RT-PCRsq. Os dados gerados podem facilitar estudos funcionais de novos genes induzidos os quais podem ser importantes para estratégias de sobrevivência e crescimento do *P. brasiliensis* em diferentes nichos do hospedeiro.

ABSTRACT

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. The fungus causes infection through host inhalation of airborne propagules of the mycelial phase of the organism. These particles reach the lungs, and disseminate to other organs and tissues. Although gene expression in *P. brasiliensis* had been studied, little is known about the genome sequences expressed by this species during the infection process. Here we describe the analysis of differentially expressed genes of *P. brasiliensis* yeast cells obtained from infected animals and during incubation of the fungus with human blood thus mimicking the hematogenic route of the fungal dissemination. Additionally, we also identified the differentially expressed genes of the fungus treated with human plasma, mimicking infection sites with inflammation. The data revealed that 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. Genes related to fatty acid degradation, protein synthesis, sensing of osmolarity changes, cell wall remodeling and cell defense were upregulated in yeast cells treated with human plasma. The differential expression was confirmed by dot blot, northern blot and sqRT-PCR assays. The generated data can facilitate functional studies of novel regulated genes that may be important for the survival and growth strategies of *P. brasiliensis* in different host niches.

Capítulo I

Introdução



1- INTRODUÇÃO

1.1 – Aspectos gerais

O fungo termodimórfico *Paracoccidioides brasiliensis* é um patógeno humano agente etiológico da Paracoccidioidomicose (PCM), uma micose sistêmica restrita a América Latina (Restrepo and Tobon, 2005). A maioria dos casos da PCM são registrados no Brasil, seguido por Colômbia e Venezuela (Coutinho *et al.,* 2002). Os estados brasileiros das regiões sul, sudeste e centro-oeste são os locais onde esta moléstia é mais frequentemente diagnosticada (Paniago *et al.,* 2003).

Este microrganismo se desenvolve como leveduras nos tecidos infectados ou quando cultivado *in vitro* a 36°C. A forma infectiva miceliana é encontrada em condições saprobióticas no ambiente, ou quando cultivada em temperaturas inferiores a 28°C (Bagagli *et al.,* 2006). A forma miceliana do fungo pode ser caracterizada por micélios septados com conídios terminais ou intercalares. As leveduras são caracterizadas por apresentarem brotamentos múltiplos originados por evaginacões da célula-mãe, onde uma célula central grande é circundada por células periféricas menores, apresentado um aspecto de roda de leme de navio (Queiroz-Telles, 1994; Restrepo-Moreno, 2003).

1.2 – Paracoccidioidomicose

A PCM é uma micose humana sistêmica granulomatosa. O fungo *P. brasiliensis* infecta hospedeiros humanos usualmente através das vias respiratórias, por inalação de propágulos do micélio, como artroconídeos (Bagagli *et al.,* 2006). A primeira interação parasito-hospedeiro ocorre nos alvéolos pulmonares; a partir dos pulmões, o fungo pode disseminar-se por vias hematogênica ou linfática acometendo outros órgãos e sistemas como fígado, baço, ossos e sistema nervoso central (San-Blas, 1993; Camargo and Franco, 2000; Valera *et al.*, 2008).

A PCM apresenta duas formas clínicas principais: forma aguda ou subaguda (juvenil) e forma crônica (adulta). O progresso da patologia e a diversidade das formas

clínicas dependem dos fatores imunológicos do hospedeiro (Franco, 1987) e dos diferentes níveis de virulência dos diversos tipos de isolados do fungo (San-Blas and Nino-Vega, 2001; Panunto-Castelo *et al.*, 2003).

A distribuição geográfica da PCM apresenta-se restrita a países da América Latina, onde um grande número de habitantes é acometido (Lacaz *et al.,* 1991; Brummer *et al.,* 1993). No Brasil, a moléstia é responsável por 200 mortes ao ano, sendo o país considerado o maior centro endêmico desta micose (Coutinho *et al.,* 2002). Nos países onde a doença é endêmica, os casos não são distribuídos homogeneamente ao longo do território, pois tende a se concentrar em torno das florestas úmidas (Restrepo, 1985).

Alguns casos autóctones já foram diagnosticados na Europa, nos Estados Unidos da América e na Ásia (Joseph *et al.*, 1966; Chikamori *et al.*, 1984; Ajello and Polonelli, 1985). Estes relatos possibilitaram a determinação de um período assintomático da PCM, o qual é em média 15,3 anos, que ocorre após o contato do paciente com a área endêmica até a manifestação da doença (Brummer *et al.,* 1993). O longo período de latência, característico da PCM, dificulta o processo de determinação precisa do sítio onde a infecção ocorreu (Borelli, 1972).

Nas áreas endêmicas da doença a incidência estimada é de, aproximadamente, 1 a 3 casos clínicos para cada 100.000 habitantes por ano (Coutinho *et al.,* 2002). A PCM atinge preferencialmente trabalhadores rurais do sexo masculino, entre 30 a 60 anos de idade (Svidzinski *et al.,* 1999; Villa *et al.,* 2000). O trabalho com solo e plantações em área rural é fator ocupacional predisponente para a aquisição da PCM (Franco, 1987). A incidência da doença até à puberdade é a mesma em ambos os sexos, porém na fase adulta, mais de 80% dos pacientes são do sexo masculino (Martinez, 1997). Acredita-se que esse fato seja explicado pela ação protetora que os hormônios estrógenos conferem ao sexo feminino (Sano *et al.,* 1999), e pela ausência ou menor contato do mesmo com as fontes de infecção (Marques *et al.,* 1983).

1.3 - Classificação taxonômica

Estudos filogenéticos moleculares em fungos vêm sendo focados particularmente no RNA ribossomal (rRNA) e no DNA ribossomal (rDNA), sendo importantes

ferramentas na distinção de espécies (James *et al.,* 1996). A comparação filogenética de dermatófitos e de fungos dimórficos, com base na subunidade ribossomal 18S, classificou *P. brasiliensis*, mais precisamente, como pertencente ao filo Ascomycota, a ordem Onygenales e a família Onygenaceae, juntamente com *Blastomyces dermattidis* e *Histoplasma capsulatum* (Leclerc *et al.,* 1994; Bialek *et al.,* 2000).

Embora a forma sexuada permaneça desconhecida com base em análises filogenéticas moleculares, o fungo *P. brasiliensis* atualmente é descrito como pertencente a 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). Recentemente, estudos de dados de polimorfismo genético identificaram três linhagens distintas de *P. brasiliensis* S1, PS2 e PS3. A espécie filogenética PS3 encontra-se restrita à Colômbia, enquanto S1, com distribuição mais ampla e que tem como representante o isolado *Pb*18, é encontrada no Brasil, Venezuela, Argentina, Paraguai e Peru. Isolados da espécie PS2 também foram encontrados no Brasil e Venezuela. Todas as três espécies foram capazes de induzir a doença em hospedeiros humanos e animais, no entanto PS2 apresentou menor virulência (Matute *et al.,* 2006). Com o objetivo de analisar as relações filogenéticas entre os isolados de *P. brasiliensis*, análises foram realizadas com base na comparação de seqüências de regiões codantes, não codantes e ITS (seqüência espaçadora interna - "internally transcribed sequence") de 7 novos isolados e 14 isolados conhecidos. Todos os isolados se agruparam nos três grupos filogenéticos já descritos, com exceção do isolado *Pb*01. Este isolado claramente separa-se de todos os outros, sugerindo que possa ser uma nova espécie no gênero *Paracoccidioides* (Carrero *et al.,* 2008).

1.4 – Biologia do fungo *P. brasiliensis*

Assim como *P. brasiliensis*, outros fungos dimórficos tendem a ser restritos a regiões geográficas específicas, com microambiente de características peculiares, com climas e solos, os quais provavelmente auxiliam nas adaptações do fungo ao habitat

natural (Rappleye and Goldman, 2006). Estas regiões de características ambientais em comum são denominadas de reservárias (Restrepo-Moreno, 1994).

O nicho ecológico do *P. brasiliensis* ainda não está completamente esclarecido. Entretanto algumas hipóteses são descritas. Alguns estudos sugerem que o fungo viva saprobioticamente na natureza, pois o mesmo já foi isolado de solo, água e plantas (Restrepo *et al.,* 2001). Devido a presença de poucos isolados de *P. brasiliensis* no solo, acredita-se que este ambiente não seja o habitat permanente do patógeno, sugerindo que o fungo reside transitavelmente ou por tempo variável nestes locais (Conti-Diaz, 2007). Alguns tipo de solos devem ser mais favoráveis à produção de conídios, um fator importante por aumentar a eficiência de sobrevivência no meio ambiente, bem como a capacidade infectante (Theodoro *et al.,* 2005).

Discute-se que o fungo *P. brasiliensis* ocorra normalmente em ambientes úmidos, próximos a rios, onde possa ser protegido por representantes de espécies aquáticas heterotérmicas como moluscos, anfíbios, peixes e artrópodes. Estes organismos estariam fornecendo ao parasito nutrientes, umidade, competição biológica limitada e temperatura apropriada para sobrevivência do micélio no meio ambiente (Conti-Diaz, 2007). A infecção natural do fungo em alguns animais silvestres (Corredor *et al.,* 2005) e domésticos, também tem sido observada; entretanto estes organismos devem ser considerados hospedeiros acidentais e não reservas naturais do fungo, pois os mesmos podem ser acometidos com a doença (Conti-Diaz, 2007). Recentemente trabalhos de detecção molecular de *P. brasiliensis* em animais selvagens mortos em estradas e rodovias, foram realizados. Por meio da técnica de Nested-PCR (reação em cadeia da polimerase) com oligonucleotídeos ITS específicos para *P. brasiliensis*, detectou-se o fungo em tatu (*Dasypus sp*.), porco-da-índia (*Cavia aperea*), porco espinho (*Sphigurrus spinosus*), guaxinim (*Procyon cancrivorus*) e furão (*Gallictis vittata*). Os dados revelam que a infecção em animais silvestres de áreas endêmicas é mais alta do que inicialmente postulado, e demonstram a importância do uso de animais mortos em estradas no estudo eco-epidemiológico do *P. brasiliensis* (Richini-Pereira *et al.,* 2007).

A organização genômica do *P. brasiliensis* ainda não está totalmente esclarecida e isso se deve principalmente ao fato de não se conhecer a fase sexual ou telemórfica deste microrganismo. Nos últimos 20 anos, com o avanço das técnicas de biologia molecular,

vários aspectos genéticos foram elucidados; entretanto conclusões definitivas a respeito da composição genética de *P. brasiliensis* ainda estão longe de serem obtidas. Nesse sentido, vários estudos foram realizados, utilizando-se diferentes metodologias. Utilizando-se a técnica de gel em eletroforese de pulso alternado (PFGE), foi possível identificar-se 4 ou 5 cromossomos com 2-10 Mb tanto de fungos isolados do meio ambiente, quanto de isolados clínicos. Desta maneira, estima-se que *P. brasiliensis* apresente um genoma variando entre 23-31 Mb (Feitosa *et al.*, 2003). Por meio do uso da técnica de citometria de fluxo, estudos com 10 isolados de *P. brasiliensis*, incluindo representantes das três espécies recentemente identificadas, demonstraram que o fungo apresenta um genoma variando de 26,3 a 35,5 Mb por célula de leveduras uninucleadas. O genoma dos conídios apresentou um tamanho de 30,2 a 30,9 Mb, não havendo, portanto, nenhuma diferença significativa com a forma de levedura (Almeida *et al.*, 2007). O genoma estrutural de três isolados do *P. brasiliensis* foi obtido. Este projeto comparativo designado "Genômica Comparativa de *Coccidioides* e outros Fungos Dimórficos", tem como meta examinar a variabilidade genética entre três isolados de *Paracoccidioides sp.* e determinar características comuns e específicas no grupo de fungos patógenos dimórficos (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html).

1.5 – Dimorfismo

P. brasiliensis é um fungo caracterizado por apresentar dimorfismo térmico, aspecto que parece ser importante como mecanismo de virulência e de patogenicidade. A transição entre os morfotipos de micélio e levedura constitui-se em uma etapa essencial para o sucesso no estabelecimento da infecção e para a fase inicial da interação do fungo com o hospedeiro (San-Blas *et al.*, 2002; Nemecek *et al.*, 2006; Rappleye and Goldman, 2006). Desta maneira, o dimorfismo torna-se um mecanismo de defesa importante para a adaptação dos fungos ao ambiente hostil encontrado nos tecidos do hospedeiro (Kurokawa *et al.,* 1998; San-Blas *et al.,* 2002).

Um dos estímulos ambientais mais notórios no dimorfismo do *P. brasiliensis* é a temperatura. O fungo se apresenta como micélio a 22°C-25°C e como levedura a 35°C-37°C (San-Blas *et al.,* 2002). Além da temperatura, fatores nutricionais também podem interferir no processo dimórfico do patógeno. A adição de soro fetal de bezerro tanto à meio de cultura complexo quanto à meio quimicamente definido permitiu preservar a expressão fenotípica de leveduras, a 25° C (Villar *et al.,* 1988).

A conversão morfogenética em *P. brasiliensis* está correlacionada com mudanças na composição da parede celular. Estudos citoquímicos e estruturais da parede celular do fungo em suas duas formas foram realizados, confirmando um acúmulo preferencial de polímeros de α-1,3-glicana em leveduras, e um alto teor de β-1,3-glicana em micélio (Paris *et al.,* 1986). Quitina é encontrada em ambas as formas do fungo (San-Blas *et al.,* 1987; Kurokawa *et al.,* 1998) apresentando um maior teor em levedura quando comparado a micélio. Durante a transição de micélio para levedura ocorre uma substituição gradual do polímero de β-1,3-glicana para α-1,3-glicana (Kanetsuna *et al.,* 1969). Ainda neste sentido, estudos realizados com isolados de *P. brasiliensis* sugerem que a α-1,3-glicana, na fase leveduriforme, protege o fungo contra enzimas do sistema de defesa do hospedeiro (San-Blas, 1985). Foi sugerido que fagócitos humanos produzam β-1,3-glicanase capaz de digerir somente β-1,3-glicana presente na parede celular da forma miceliana do fungo. Entretanto, a transformação de micélio para levedura logo no início da infecção, devido ao maior teor α-1,3-glicana, deve proteger o patógeno contra a ação das enzimas β-glicanases fagocitárias, permitindo a instalação do fungo na forma leveduriforme e o estabelecimento da infecção (San-Blas, 1982).

Um dos aspectos da relação entre o parasito e o hospedeiro é o efeito de hormônios no dimorfismo do fungo. A alta incidência de PCM em adultos masculinos sugere que fatores hormonais possam desempenhar algum papel na patogênese da doença (Sano *et al.,* 1999). O hormônio feminino 17-β-estradiol tem sido relacionado à diferenciação de *P. brasiliensis*. Estudos prévios mostraram que o hormônio 17-β-estradiol é capaz de inibir a transição de micélio para levedura de maneira dose-dependente, in *vitro* (Restrepo, 1985), e *in vivo* (Sano *et al.,* 1999). Aristizabal *et al*., (2002) observaram, *in vivo*, a participação do hormônio feminino na resistência de fêmeas de rato ao desenvolvimento inicial da PCM. Estudos de transcriptomas (Felipe *et*

al., 2005) descreveram um gene preferencialmente expresso na fase leveduriforme de *P. brasiliensis* que potencialmente codifica uma proteína de ligação ao 17-β-estradiol, previamente caracterizada (Loose *et al.,* 1983). A EBP ("Estradiol Binding Protein") possui interação seletiva a estrogênios ligando-se ao estradiol no citoplasma (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 baixa incidência da PCM em mulheres.

O perfil morfoprotéico da transição *in vitro* entre as formas miceliana e leveduriforme do isolado *Pb* 01 (ATCC-MYA-826), objeto de estudo deste trabalho, foi caracterizado (Silva *et al.,* 1994). Estudos utilizando técnicas de proteômica evidenciaram alterações no perfil protéico durante a transição de fases deste isolado (Silva *et al.,* 1994; Salem-Izacc *et al.,* 1997). Além disto a expressão de proteínas durante o processo de diferenciação apresenta um padrão específico para diferentes isolados do fungo (Salem-Izacc *et al.,* 1997). Alguns genes de *P. brasiliensis*, já caracterizados, apresentam expressão diferencial durante a transição dimórfica do fungo. Os genes codificantes para as proteínas: proteína de choque térmico de 70kDa (HSP70) (da Silva *et al.,* 1999), proteína homologa à flavodoxina *Pb* Y20 (Cunha *et al.*, 1999; Daher *et al.*, 2005), proteína de choque térmico de 60 kDa (HSP60) (Izacc *et al.,* 2001; Cunha *et al.,* 2002), a chaperona Cl*Pb* (Jesuino *et al.,* 2002), manosiltransferase (Costa *et al.,* 2002), catalase peroxissomal (Moreira *et al.,* 2004) e gliceraldeído 3-fosfato desidrogenase (Barbosa *et al.,* 2004), entre outros, apresentam baixos níveis de expressão na forma miceliana quando comparados com a forma leveduriforme, sugerindo que estas proteínas possivelmente seriam fatores na composição de estratégias que *P. brasiliensis* utiliza para morfo-adaptação e sobrevivência no hospedeiro.

O processo de transição dimórfica está intimamente associado à habilidade de infecção em fungos patogênicos (Borges-Walmsley *et al.*, 2002; Morris-Jones, 2002; Vanittanakom *et al.*, 2006; Kauffman, 2007). Entretanto, o conhecimento de como estes organismos detectam sinais externos e de como respondem a eles, é, ainda, rudimentar. Estudos sobre vias de sinalização celular evidenciaram a estreita relação entre vias sinalizadoras com a expressão de genes responsáveis pela virulência e diferenciação de organismos patogênicos (Borges-Walmsley and Walmsley, 2000; Morris-Jones, 2002;

DiCaudo, 2006; Kauffman, 2007). No entanto, as vias de sinalização que controlam a transição morfológica em *P. brasiliensis* ainda são pouco conhecidas. A via de sinalização através do cAMP (adenosina monofosfato cíclico) parece ser importante, pois a adição exógena do composto inibe a transição de levedura para micélio, mantendo a forma patogênica do fungo (Paris *et al.,* 1985). Além disso, estudos utilizando estratégias combinadas de análise de expressão acopladas com ensaios de interação proteína-proteína evidenciaram relação entre os níveis de cAMP e o controle da ativação de estágios seqüenciais na mudança morfológica de *P. brasiliensis* (Chen *et al.,* 2007). A via sinalizadora da Ca^{+2}/Calmodulina também parece desempenhar papel na diferenciação celular de *P. brasiliensis*. Drogas que bloqueiam as quinases dependentes de Ca^{+2}/Calmodulina inibem a diferenciação de micélio para levedura (Carvalho *et al.*, 2003). Entretanto, a complexidade da regulação da patogênese e do processo de diferenciação celular em *P. brasiliensis* requer estudos futuros para cada elemento da rede de sinalização celular.

1.6 - Perfis de expressão gênica durante o processo infeccioso

A capacidade de microrganismos causarem infecção está relacionada com a complexa interação entre patógeno e hospedeiro. O desequilíbrio no balanço entre a resposta do hospedeiro e mecanismos de virulência pode desencadear os processos de instalação, invasão e multiplicação do microrganismo, que caracterizam a infecção. A patogenicidade destes organismos estabelece uma íntima relação com as estratégias que estes utilizam para infectar e sobreviver aos mecanismos de defesa do hospedeiro. Estas estratégias de infecção são mantidas pela expressão de um repertório de genes requeridos frente às condições encontradas nos diferentes sítios de infecção. Portanto, o estudo do perfil transcricional vem sendo amplamente utilizado na determinação dos mecanismos de sobrevivência/virulência desencadeados pelo patógeno durante o processo infeccioso.

O fungo patogênico *Cryptococcus neoformans* possui a habilidade de disseminar-se na corrente sanguínea e então cruzar a barreira sangue-cérebro causando a meningoencefalite. Utilizando-se a estratégia de análise serial de expressão gênica (SAGE) em células de *C. neoformans* isoladas de fluído cerebroespinhal (CSF) de

coelhos, o perfil de abundância de transcritos foi caracterizado, visando o entendimento das estratégias de sobrevivência/virulência do parasito na infecção *in vivo*. Os dados revelaram que os transcritos mais expressos são aqueles relacionados com resposta a estresse, sinalização celular, transporte celular, metabolismo de lipídios e carboidratos. O alto nível de expressão de genes destas categorias sugere que estas funções são requeridas para sobrevivência e proliferação do *C. neoformans* na meningoencefalite (Steen *et al.,* 2003).

O fungo patogênico *Candida albicans* comumente causa infecções superficiais em mucosas. Em pacientes imunocomprometidos este fungo pode penetrar em tecidos mais profundos, entrar na corrente sanguínea e se disseminar para outros tecidos causando infecções sistêmicas com altos índices de mortalidade. Como este processo de disseminação é um passo essencial na candidíase sistêmica, o entendimento de como o fungo pode sobreviver e escapar da corrente sanguínea pode não só elucidar a complexa interação hospedeiro-fungo no sangue, mas também identificar novos potenciais alvos para drogas, prevenindo esta forma da doença. É provável que este fungo expresse um grupo diferente de genes que habilitaria a adaptação das células a este novo ambiente hostil e permitiria a adesão e penetração das células através da camada endotelial para invadir novos tecidos. Visando a elucidação de como *C. albicans* responde aos desafios do ambiente da corrente sanguínea o perfil transcricional deste fungo exposto a sangue humano foi analisado por meio de microarranjos e ensaios de subtração de cDNA. A combinação dos dados obtidos por ambos os métodos permitiram a identificação de diferentes grupos de genes especificamente expressos em diferentes estágios deste modelo que mimetiza a infecção sanguínea. Dentre os genes diferencialmente expressos estão aqueles envolvidos em estresse, resposta antioxidativa, glicólise, ciclo do glioxalato e virulência. Ensaios com plasma humano demonstraram que a expressão de alguns genes depende da presença de células sangüíneas. Adicionalmente experimentos de confirmação revelaram que os genes identificados como sendo altamente expressos no sangue foram, também, expressos durante infecção intravenosa de camundongos (Fradin *et al.,* 2003). Trabalhos posteriores demonstraram que o padrão de expressão de genes de *C. albicans* em sangue humano é governado pelos neutrófilos do sangue (Fradin *et al.,* 2005). Estas análises fornecem introspecções nos mecanismos pelos quais *C. albicans*

pode sobreviver e escapar do ambiente hostil do sangue como um passo essencial da candidíase disseminada (Fradin *et al.,* 2003; Fradin *et al.,* 2005).

O padrão de expressão gênica de *C. albicans* foi avaliado para conhecimento da natureza da interação parasito-hospedeiro em diferentes estágios da infecção. Ensaios de microarranjos foram realizados utilizando-se populações de cDNA de *C. albicans* isoladas de rins de camundongos em diferentes etapas da infecção. A adaptação de *C. albicans* ao sítio de infecção renal resultou na expressão diferencial de 19% dos 6737 genes presentes nos microarranjos. A maioria dos genes diferencialmente expressos foi reprimida (85%). Cinqüenta por cento dos fatores regulados correspondiam a proteínas de função desconhecida em *C. albicans* e *Saccharomyces cerevisiae*. Após infecção em rins de camundongos, um grande grupo de genes reprimidos em resposta a este ambiente *in vivo* desempenham papel nas vias de utilização de glicose (Lorenz and Fink, 2001; Fradin *et al.*, 2003). Genes associados com regulação transcricional foi o principal grupo funcional induzido durante infecção *in vivo*. De forma interessante vários genes superexpressos previamente foram descritos como importantes fatores na patogênese deste organismo. Dentre os genes altamente expressos estão aqueles relacionados com crescimento na forma de hifa. A indução de fatores associados com esta via nos tecidos do hospedeiro durante o crescimento invasivo de *C. albicans* não é surpreendente, dada a natureza invasiva proposta para o morfotipo hifa (Andes *et al.,* 2005).

Para estabelecer a infecção o patógeno *C. albicans* assimila carbono e cresce no hospedeiro mamífero. Este fungo assimila compostos de seis carbonos pela via glicolítica, e de dois carbonos via gliconeogênese e ciclo do glioxalato. Conhecimentos acumulados sobre as vias metabólicas centrais na patogênese de *C. albicans* levantam o seguinte paradoxo: o ciclo do glioxalato é aparentemente requerido para virulência embora a expressão de genes deste ciclo, tal como isocitrato liase, apresenta-se reprimida por glicose em concentração fisiológica. Utilizando o sistema de sentinela GFP-fusionada ("green fluorescent protein"), foi demonstrado que genes do ciclo do glioxalato e da gliconeogênese são reprimidos em concentração fisiológica de glicose. Observou-se que estes genes são inativos na maioria das células fúngicas infectando rins de camundongos. Entretanto tais vias são induzidas na fagocitose de macrófagos e neutrófilos. Além disso, genes da via glicolítica não foram induzidos em fagossomos e são expressos no modelo

de infecção renal. Estes resultados sugerem que *C. albicans* apresenta um programa metabólico nicho-específico, onde o ciclo do glioxalato e gliconeogênese são ativados inicialmente, quando o patógeno é fagocitado por células do hospedeiro, enquanto a progressão sistêmica da doença é dependente da glicólise (Barelle *et al.,* 2006).

A patogenicidade de *C. albicans* depende de sua habilidade em responder efetivamente aos mecanismos de defesa do hospedeiro, especialmente à explosão oxidativa das células fagocitárias. A ativação da resposta ao estresse oxidativo neste fungo foi avaliada em modelos de infecção *ex vivo* e durante a infecção sistêmica do hospedeiro mamífero. Utilizando-se genes repórteres sob controle de promotores de genes responsivos ao estresse oxidativo, observou-se que as células de *C. albicans* ativam uma resposta ao estresse oxidativo em conseqüência da fagocitose de neutrófilos, embora a fagocitose por macrófagos não ative esta resposta. Significantemente, somente uma pequena porção de células do patógeno (4%) ativou uma resposta ao estresse oxidativo durante infecção em rins de camundongos. Desta maneira, a maioria das células do fungo são expostas a uma condição de estresse oxidativo, quando elas entram em contato com neutrófilos na corrente sanguínea do hospedeiro, mas a morte oxidativa não aparece como uma significante ameaça quando a infecção é estabelecida nos rins. Portanto, uma resposta ao estresse oxidativo é um fenômeno nicho-específico durante o estabelecimento e progressão da infecção sistêmica de *C. albicans* (Enjalbert *et al.,* 2007).

A interação entre *C. albicans* e macrófagos é considerado um passo essencial no desenvolvimento de uma resposta imune adequada na candidíase sistêmica. Análises integradas de genômica e proteômica foram utilizadas na compreensão das bases moleculares da interação entre *C. albicans*-macrófago. A clara repressão de genes relacionados com o metabolismo de compostos de carbono, somado com a indução de genes envolvidos com metabolismo de lipídeos, ácido graxos e ciclos do glioxalato e do ácido tricarboxílico sugere que este organismo está mudando para o modo de estarvação. Adicionalmente, o estudo da rede metabólica geral deste fungo permite sugerir que as células de *C. albicans* possivelmente disparam diferentes vias de morte celular após o contato com macrófagos. Esta técnica integrada levou ao estabelecimento de correlações de vias celulares específicas as quais refletem uma visão global do fenótipo molecular do fungo neste contexto biológico (Fernandez-Arenas *et al.,* 2007).

O estudo de expressão real de genes de bactéria durante a infecção de células ou tecidos, sem influência de processos de isolamento do patógeno, é limitado devido a escassez de material inicial e a instabilidade do RNA bacteriano (Dozois *et al.,* 2003). Existem também dificuldades envolvendo a separação de transcritos da bactéria dos RNAs abundantes do hospedeiro (Daigle *et al.,* 2001; Dozois *et al.,* 2003). Recentemente, a captura seletiva de seqüências transcritas (SCOTS) vem sendo amplamente utilizada para identificar genes de bactérias expressos durante a infecção (Hou *et al.,* 2002). A tecnologia de SCOTS permite a captura seletiva de cDNAs bacterianos de uma população complexa de cDNAs (hospedeiro e bactéria), preparada de cultura de células (Graham and Clark-Curtiss, 1999; Morrow *et al.*, 1999; Hou *et al.*, 2002; Faucher *et al.*, 2006; Fittipaldi *et al.*, 2007) ou tecidos infectados (Graham *et al.,* 2002; Dozois *et al.,* 2003; Baltes *et al.,* 2007), utilizando hibridização com DNA genômico bacteriano previamente sonicado e biotinilado. Em seqüência, as misturas de cDNAs obtidas são enriquecidas para seqüências preferencialmente expressas durante crescimento no hospedeiro, utilizando hibridizações adicionais com DNA de bactéria na presença de cDNA preparado durante o crescimento *in vitro* (Graham and Clark-Curtiss, 1999). A utilização de SCOTS tem permitido a identificação de fatores essenciais para patogênese de bactérias causadoras de doenças de impacto mundial. Estudos com *Mycobacterium tuberculosis* demonstraram que genes relacionados com reparo de DNA, aquisição de nutrientes, metabolismo de parede celular e fatores de virulência previamente conhecidos, apresentaram expressão induzida durante infecção de macrófagos humanos (Graham and Clark-Curtiss, 1999). Trabalhos com uma linhagem de *Escherichia coli* causadora de infecção de trato urinário, mostraram que a expressão do fator de virulência Iha, que desempenha funções de adesina e receptor para sideróforos, foi aumentada na infecção de trato urinário de camundongos (Leveille *et al.,* 2006). Os produtos diferencias obtidos por SCOTS foram usados em conjunto com microarranjos para determinar a expressão global de *Salmonella enterica* serovar Typhi internalizada por macrófago humano. Tal estratégia identificou um total de 628 genes superexpressos durante os diferentes tempos de infecção. Destes 117 foram induzidos em todos os tempos analisados. Este fato indica que os genes superexpressos em um tempo específico podem ser requeridos em diferentes estágios de internalização. Dentre os genes induzidos no fagossomo de macrófago estão

aqueles envolvidos no sistema de secreção do tipo III, relacionados com choque osmótico, bem como fatores envolvidos na virulência deste patógeno (Faucher *et al.,* 2006).

A tuberculose crônica é um dos principais problemas de saúde da atualidade (Cegielski *et al.,* 2002). Uma questão chave relevante para a tuberculose crônica envolve o estado fisiológico da *M. tuberculosis* durante este importante estágio da infecção. Para examinar as bases moleculares da tuberculose crônica e o papel da imunidade no crescimento da micobactéria, análises de perfis transcricionais do patógeno foram determinadas durante as fases crônicas e de reativação da tuberculose em murinos usando microarranjos de DNA *in vivo*. Dentre as vias metabólicas mais enriquecidas estão aquelas envolvidas no metabolismo de carboidratos, lipídios e produção de energia. A transcrição ativa de genes representados por estas vias indica que o patógeno está metabolicamente ativo na fase crônica da tuberculose, podendo utilizar carboidratos e lipídios como fontes na produção da energia necessária para manutenção dos processos celulares e para adaptação ao microambiente dos tecidos do hospedeiro. Por outro lado o perfil transcricional na etapa de reativação da doença evidenciou a indução de genes relacionados com transdução de sinais, divisão celular bem como no metabolismo e captação de ferro e enxofre. As análises dos perfis transcricionais durante diferentes etapas da tuberculose crônica forneceram uma visão dinâmica das mudanças adaptativas da bactéria durante os estágios crônicos e de reativação da infecção (Talaat *et al.,* 2007).

1.7 - Perfis de expressão gênica do fungo *P. brasiliensis*

Estudos dos perfis de expressão de patógenos têm sido amplamente utilizados para o desvendamento das estratégias de adaptação, sobrevivência e virulência que estes microrganismos utilizam durante o processo infeccioso. Com o objetivo de estudar a biologia de *P. brasiliensis*, suas estratégias de infecção e conseqüentemente aumentar o número de genes descritos para este fungo, diferentes abordagens no estudo de perfis de expressão têm sido aplicadas. Um Projeto Genoma Funcional de *P. brasiliensis*, desenvolvido por um consórcio de laboratórios da região Centro–Oeste do Brasil (Projeto Genoma Funcional e Diferencial de *Paracoccidioides brasiliensis*, isolado *Pb*01),

resultou no seqüenciamento de 25.511 ESTs obtidas de bibliotecas de cDNA de levedura e micélio, abrangendo aproximadamente 6.022 genes que representam cerca de 80% do número total de genes estimados para o microrganismo (Felipe *et al.,* 2003; Felipe *et al.,* 2005). Tal estratégia possibilitou a detecção de genes diferencialmente expressos nas duas fases de *P. brasiliensis*. Análises comparativas realizadas com outros fungos patogênicos humanos tais como *C. albicans*, *C. neoformans* e *Aspergilus fumigatus* identificaram genes de virulência e possíveis alvos para drogas. Além disso, estudos comparativos entre micélio e levedura identificaram um perfil metabólico diferencial exibido nas fases de *P. brasiliensis*. Em linhas gerais, a forma miceliana apresenta metabolismo aeróbio uma vez que durante a fase saprobiótica genes que codificam enzimas que participam da fosforilação oxidativa e do ciclo do ácido cítrico (isocitrato desidrogenase e succinil coenzima-A sintase) estão altamente expressos. De forma contrária, a fase leveduriforme apresenta metabolismo anaeróbio. Nesta fase, os altos níveis de expressão da álcool desidrogenase I favorecem a fermentação alcoólica e conseqüente produção de etanol. Além disso, nesse transcriptoma identificou-se 48 transcritos codificantes para proteínas de resposta a estresse chamadas de HSPs, sendo que o número destes transcritos foi 38% maior na biblioteca de levedura do que na biblioteca de micélio. Estas proteínas estão envolvidas em processos como transição dimórfica e imunopatogenicidade de organismos patógenos. Este fato é compatível com a maior temperatura (36°C) de crescimento de células leveduriformes em relação ao micélio (23°C). A coleção de seqüências codificantes para chaperonas de *P. brasiliensis* foi consideravelmente aumentada, visto que até aquele momento, somente 8 HSPs haviam sido previamente descritas para este patógeno (Felipe *et al.,* 2005).

Um grupo de pesquisadores do estado de São Paulo realizou outro projeto Genoma Funcional identificando 4.692 genes do isolado *Pb*18. No banco de ESTs gerado foram identificados vários fatores de virulência através de comparações com homólogos de *C. albicans*. Os genes de vias de transdução de sinais têm sido implicados na transição dimórfica e consequentemente na patogenicidade de fungos dimórficos. A identificação de alguns genes de *P. brasiliensis* homólogos à via de transdução relacionados à virulência de *C. albicans*, como CST20 (quinase relacionada com formação de hifa), CPP1 (fosfatase relacionada com formação de hifa) e CEK1 (proteína quinase ativada por

mitose) encontrados neste estudo, sugere que estas vias possam estar atuando em *P. brasiliensis*, provavelmente controlando a mudança morfológica. No entanto, o papel funcional destes genes na transição dimórfica e/ou durante a infecção requerem demonstração experimental apropriada. O perfil transcricional de alguns genes foi avaliado durante a transição dimórfica e foi revelado que transcritos de três proteínas de choque térmico (HSP-70, HSP-82 e HSP-104), de um homólogo da delta-9-desaturase (OLE1) e da oxidase alternativa, possuem picos de expressão durante a transição de micélio para levedura, sugerindo que a estabilização de proteínas e mudanças na organização da membrana são fatores importantes na transição morfológica de micélio para a forma de levedura (Goldman *et al.,* 2003).

Utilizando as tecnologias de microarranjos e bibliotecas subtraídas Marques *et al*., (2004) identificaram genes superexpressos na fase leveduriforme do isolado *Pb*18 de *P. brasiliensis*, proporcionando maiores informações acerca da patobiologia deste microrganismo. Foi verificada uma regulação positiva para gene CDI1 que codifica a cisteína dioxigenase em *P. brasiliensis*. Previamente, foi mostrado que células na fase miceliana de *P. brasiliensis* podem crescer na presença tanto de enxofre orgânico quanto inorgânico, enquanto que as células de levedura podem crescer somente em presença de aminoácidos contendo enxofre orgânico (Paris *et al.,* 1985). Marques *et al*., (2004) confirmam em seus experimentos a auxotrofia do isolado *Pb*18 para o enxofre orgânico. Esta auxotrofia sugere que além da temperatura, o enxofre orgânico é um importante estímulo para manter a fase leveduriforme patogênica de *P. brasiliensis*. Desta forma, verifica-se que diversos genes envolvidos na assimilação de aminoácidos que contêm enxofre, tais como metionina permease, são mais expressos na fase de levedura do que na de micélio, indicando um possível papel, como em *H. capsulatum*, para o metabolismo do enxofre na manutenção do estado de levedura (Marques *et al.,* 2004). Relatos da transição de micélio para levedura no *H. capsulatum* atribuíram um importante papel aos grupos sulfidrílicos, principalmente na forma de cisteína (Maresca and Kobayashi, 2000). Essas observações foram ampliadas mostrando que o gene que codifica a enzima cisteína dioxigenase é mais expressa em levedura do que no micélio (Hwang *et al.,* 2003).

De forma interessante também foi observado níveis aumentados de expressão do gene *ERG25* na fase de levedura de *P. brasiliensis*. O gene *ERG25* codifica uma C-4-

esterol metil oxidase que participa do primeiro passo enzimático da síntese de ergosterol em fungos. É provável que o aumento na expressão de *ERG25* esteja correlacionado ao aumento da sua reutilização na membrana celular. Sugere-se que mudanças na membrana são importantes fatores na transição morfológica a 36°C (Goldman *et al.,* 2003) e a alta reutilização de lipídios na membrana celular também pode contribuir para isso. Adicionalmente, já fora demonstrado através de análise global da expressão gênica em *S. cerevisiae,* que os genes envolvidos na biossíntese de ergosterol têm a sua expressão aumentada em condições de estresse, incluindo o estresse oxidativo, e na presença de antifúngicos que têm como alvo a biossíntese de ergosterol (Bammert and Fostel, 2000; Higgins *et al.*, 2003). Permanece a ser determinado em *P. brasiliensis* se os genes de biossíntese de ergosterol têm a sua expressão de RNAm aumentada a 36°C, ou se é um efeito específico do gene ERG25. A mudança morfológica do *P. brasiliensis* é acompanhada pela mudança na composição da parede celular onde monômeros de glucana passam de β-1,3-glicana para α-1,3-glicana, assim que o fungo adota a forma leveduriforme (San-Blas and Nino-Vega, 2001). De maneira complementar observou-se uma expressão muito maior do gene que codifica a α-1,3- glicana sintase na fase de levedura do que na fase de micélio (Marques *et al.,* 2004).

O perfil transcricional de *P. brasiliensis* durante a transição dimórfica tem sido alvo de estudos de pesquisadores devido sua íntima relação com a patogênese deste fungo. Microarranjos de DNA foram utilizados para análise de expressão de diferentes etapas da diferenciação (Nunes *et al.,* 2005). Nesse estudo foram identificados vários genes diferencialmente expressos durante a transição morfológica. As análises revelaram várias enzimas reguladas que participam do metabolismo de aminoácidos, vias de transdução de sinais, síntese protéica, metabolismo da parede celular, estrutura do genoma, resposta ao estresse oxidativo e controle de crescimento e desenvolvimento celulares. O gene codificante para a enzima 4-hidroxil-fenil-piruvato dioxigenase (4-HPPD), envolvida na degradação de aminoácidos aromáticos, foi superexpresso durante a diferenciação de micélio para levedura, e o uso de NTBC [2-(2-nitro-4-trifluorometilbenzoil)-ciclohexano-1,3-diona], um inibidor específico da atividade de 4-HPPD, foi capaz de bloquear o crescimento e a diferenciação deste organismo *in vitro* (Nunes *et al.,* 2005).

A estratégia de transcriptoma também foi utilizada para o estudo da resposta do fungo nos estágios iniciais da transição micélio-levedura (Bastos *et al.,* 2007). Este trabalho, desenvolvido pelo nosso grupo, mostrou que muitos genes envolvidos na síntese da membrana e parede celulares após 22 horas do início da diferenciação são superexpressos, sugerindo que o fungo remodela estas estruturas no estágio inicial da mudança morfológica. Neste estudo, genes envolvidos na via de assimilação do enxofre como a sulfito redutase, mostraram-se super expressos durante a transição, sugerindo o envolvimento do metabolismo do enxofre durante o processo de diferenciação em *P. brasiliensis*, como descrito anteriormente. Durante a transição também foi verificada a presença de enzimas que participam do ciclo do glioxalato, como a isocitrato liase, malato desidrogenase, citrato sintase e aconitase. A presença destes transcritos durante a diferenciação indica que esta via é funcional durante esse processo. Também foram identificados genes envolvidos em vias de transdução de sinais tais como MAPK, serina/treonina quinase e histidina quinase, sugerindo o papel de tais vias no controle e manutenção da transição morfológica em *P. brasiliensis* (Bastos *et al.,* 2007).

A via de assimilação de enxofre tem sido amplamente estudada em fungos (Marzluf, 1997; Thomas and Surdin-Kerjan, 1997; Piotrowska *et al.*, 2000). Como já descrito anteriormente, a fase leveduriforme de *P. brasilensis* apresenta auxotrofia ao enxofre orgânico (Paris *et al.,* 1985). O enxofre orgânico desempenha papel na manutenção da fase leveduriforme em *H. capsulatum* refletindo a importância do metabolismo do nutriente no ciclo de vida dos fungos patogênicos (Hwang *et al.,* 2003). Análises de expressão de genes envolvidos na utilização de enxofre foram realizadas em *P. brasiliensis* (Andrade *et al.,* 2006). O trabalho demonstrou que o enxofre inorgânico é desnecessário durante a diferenciação de micélio para levedura e vice-versa em *P. brasiliensis*. Este estudo também confirmou a super-expressão da colina sulfatase (*CHS1*) na fase leveduriforme de *P. brasiliensis*. O gene que codifica a colina sulfatase está relacionado com a reserva de enxofre para a célula (Marzluf, 1997). Interessantemente, outros patógenos intracelulares humanos como o *M. tuberculosis* dependem do enxofre para virulência e sobrevivência dentro de macrófagos (Sun *et al*. 2005).

Com o objetivo de se investigar a assimilação de enxofre inorgânico em *P. brasiliensis* a expressão de cinco genes envolvidos nas vias orgânica e inorgânica de

captação de enxofre (*CDI1*-cisteína dioxigenase, *MEP1*-metionina permease, *CHS1*-colina sulfatase, *APS1*-5'adenilil sulfato quinase, *SUR1*-sulfito redutase) foi inicialmente avaliada durante a transição de micélio para levedura e durante o crescimento da fase leveduriforme. Os genes *CDI1* e *MEP1* são envolvidos com a via de assimilação orgânica; o *CHS1* está envolvido com a mobilização e armazenamento de enxofre e *APS1* e *SUR1* são genes envolvidos com a via de assimilação inorgânica. Todos os cincos genes avaliados neste estudo apresentaram um alto acúmulo de RNAm durante a transição dimórfica e durante o crescimento da fase leveduriforme, sugerindo que nestas situações estão ocorrendo mobilização e armazenamento de enxofre, além da ativação da via de assimilação inorgânica. Estes resultados evidenciam que embora *P. brasiliensis* não use enxofre inorgânico como única fonte para iniciar a transição e o crescimento da fase leveduriforme, este fungo pode, de algum modo, utilizar ambas as vias orgânica e inorgânica durante o processo de crescimento (Ferreira *et al.,* 2006). Este estudo fornece novas informações sobre o comportamento transcricional de vários genes envolvidos no metabolismo do enxofre. Entretanto, a transformação e a inativação de fatores ainda devem ser desenvolvidas, em *P. brasiliensis*, para definir o envolvimento destes genes na transição dimórfica e/ou virulência.

Almejando avaliar as estratégias de infecção utilizadas por *P. brasiliensis,* foram desenvolvidos trabalhos nos quais foi possível definir o perfil dos transcritos do fungo, isolado *Pb*01, em leveduras pós-infecção em fígado de animais experimentais. Recentemente, o transcriptoma de *P. brasiliensis*, fase leveduriforme, recuperado de fígado de animais experimentais (camundongos B10A) foi descrito (Costa *et al.,* 2007). Foram seqüenciados clones, gerando 4.932, sendo 37,47% relacionadas a novos genes e 23,75% pertencentes a genes superexpressos. Os genes identificados foram categorizados em processos metabólicos, transporte celular e energia, entre outras categorias. Do total de ESTs geradas neste estudo, 65,53% das seqüências identificadas, também estavam presentes no transcriptoma de levedura e micélio de células obtidas de cultura *in vitro* (Felipe *et al.*, 2005). Um total de 50 transcritos representados por 892 ESTs foram superexpressos na infecção de fígado quando comparado com leveduras cultivadas *in vitro*. Um grande número dos genes altamente expressos codifica para transportadores de membrana, proteínas relacionadas com estresse, moléculas envolvidas no metabolismo de

nitrogênio e enzimas que participam do metabolismo de carboidratos e lipídios. Dentre os transcritos mais expressos estão alguns que foram previamente descritos como mais expressos em células leveduriformes quando comparadas com micélio, tais como os genes da álcool desidrogenase I, descarboxilase de aminoácido acido e isocitrato liase (Felipe *et al.,* 2005). Por outro lado notaram-se novos genes na categoria de altamente abundantes como anidrase carbônica e glicoquinase. O estudo das características metabólicas do *P. brasiliensis* indicou que em tecidos do hospedeiro os processos significativamente favorecidos são: metabolismo de nitrogênio, carboidratos e lipídios. A interpretação geral dos processos que ocorrem durante a infecção sugere que o fungo utiliza múltiplas fontes de carbono durante a colonização do fígado, incluindo glicose e substratos do ciclo do glioxalato. Complementando, o metabolismo de nitrogênio pode ser muito ativo durante o processo infeccioso, mostrando que alguns compostos nitrogenados podem ser preferencialmente adquiridos dos tecidos do hospedeiro, enquanto outros devem ser suplementados pelo patógeno. Adicionalmente, o favorecimento da biossíntese de lipídios sugere uma grande disponibilidade de carboidratos e energia. A demonstração do perfil gênico das células leveduriformes de *P. brasiliensis* recuperadas de animais infectados é um requisito essencial para o estudo do genoma funcional de modo a esclarecer os mecanismos de patogenicidade e virulência fúngica (Costa *et al.,* 2007).

O fungo *P. brasiliensis* possui inúmeros processos adaptativos ao hospedeiro em resposta à fagocitose como demonstrado por Tavares *et al*., (2007). Após a internalização, o patógeno promove adaptação metabólica induzindo a expressão de genes da biossíntese de aminoácidos, especificamente genes envolvidos na biossíntese de metionina. A cistationa β-liase de *P. brasiliensis* codificada pelo gene metG, associado à produção de metionina, foi induzida após fagocitose de macrófagos de murinos. Estudos de ruptura gênica de genes de *Salmonella enterica* envolvidos na produção de metionina mostraram que a síntese deste aminoácido é essencial durante o processo infeccioso (Ejim *et al.,* 2004). Neste sentido, a super regulação do gene metG pode conferir uma importante resposta adaptativa para sobrevivência de *P. brasiliensis* dentro dos macrófagos onde a concentração de aminoácidos é baixa (Tavares *et al.,* 2007). Neste estudo, também se observou a superexpressão do gene da HSP60 em *P. brasiliensis*. A

HSP60 é uma chaperona mitocondrial, homóloga a chaperona GroEL de *Escherichia coli* (Bukau and Horwich, 1998), que está envolvida na resposta celular ao estresse oxidativo (Cabiscol *et al.,* 2002). Tem sido observado que células que apresentam altos níveis de HSP60 são mais resistentes a agentes oxidativos como H_2O_2 e menadiona. A indução do gene da hsp60 em *P. brasiliensis* exposto a radicais oxidativos produzidos por macrófagos, pode ter função protetora similar (Tavares *et al.*, 2007).

Nos últimos anos, tem sido desenvolvidas varias técnicas que fornecem aos pesquisadores a habilidade do estudo de expressão gênica diferencial em um organismo ou célula, identificando gene(s) envolvidos em um determinado processo ou tratamento (Liang and Pardee, 1992; Diatchenko *et al.*, 1996). Estas tecnologias permitem a detecção de mudanças na expressão de RNAm através de um enriquecimento seletivo sem qualquer conhecimento prévio de seqüência dos genes específicos em questão (Pastorian *et al.,* 2000). Os métodos mais utilizados são: Análise de diferença representacional (RDA) (Hubank and Schatz, 1994), apresentação diferencial (DD) (Liang and Pardee, 1992), PCR de oligonucleotídeo-ligado arbitrário (RAP-PCR) (Mathieu-Daude *et al.,* 1999) e hibridização subtrativa (SH) (Diatchenko *et al.,* 1996). Basicamente, todos estes métodos são desenhados para amplificar seqüências de ácidos nucléicos presentes em uma amostra e ausentes em outra. Embora cada um destes procedimentos tenham sido utilizados para clonar RNAm raros e novos, uma considerável atenção deve ser dada para cada método com o objetivo de se determinar qual o mais adequado para um dado experimento (Wan *et al.,* 1996). Dentre os fatores que influenciam a escolha de um método específico estão a habilidade de amplificação de RNAs potencialmente raros, os níveis de diferença detectável, a habilidade de comparação de múltiplas amostras simultaneamente e o número de falsos positivos. Experimentos de expressão diferencial genética podem ser laboriosos e consumir muito tempo. Desta maneira a velocidade e a facilidade de uso de cada técnica são considerações importantes visto que a maioria do trabalho concentra-se em etapas posteriores de seqüenciamento, identificação, clonagem e obtenção de cDNAs completos bem como análise de função.

O RDA é uma ferramenta poderosa e sensível na identificação de genes diferencialmente expressos. Esta técnica, que em sua forma original foi projetada para identificar diferenças entre populações de DNA genômico (Lisitsyn *et al.*, 1993; Lisitsyn

et al., 1993; Lisitsyn, 1995), subsequentemente sofreu modificações que permitem comparar diferenças entre populações de RNAm (Hubank and Schatz, 1994). O RDA é baseado em etapas sucessivas de hibridização subtrativa seguida de PCR que enriquece e isola os transcritos diferencialmente expressos, enquanto simultaneamente remove RNAm com expressão não diferencial (Figura 1). O procedimento de RDA torna-se mais vantajoso em relação às outras metodologias porque é relativamente rápido e barato; ao final do processo somente os produtos diferenciais são obtidos; o número de falsos positivos é pequeno e os produtos finais são compostos de regiões codificantes (Pastorian *et al.,* 2000).

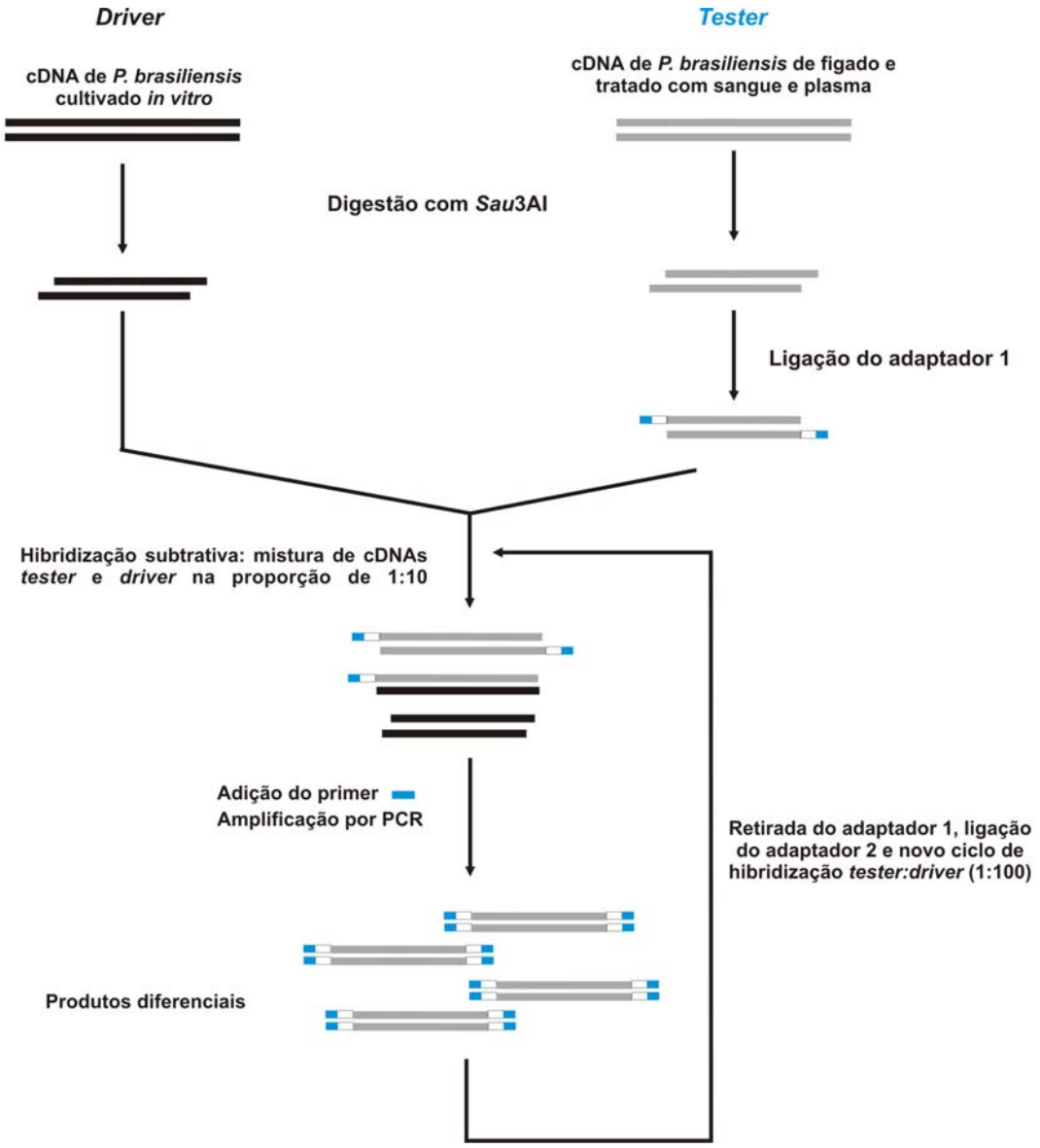


Figura 1 – Modelo das etapas da metodologia do RDA. Etapas das estratégias de análise de expressão diferencial de genes de *P. brasiliensis* em condições que mimetizam diferentes nichos do hospedeiro. Foram realizadas dois ciclos de hibridização subtrativa com taxas de cDNAs *tester*:*driver* de 1:10 e 1:100.

Recentemente a tecnologia de RDA vem sendo utilizada para estudos de perfis de expressão em fungos. *Metarhizium anisopliae* é um agente de biocontrole, bem caracterizado, de um amplo espectro de pragas como insetos e ácaros. Como o objetivo de identificar genes envolvidos no processo de infecção, o RDA foi empregado utilizando-se cDNAs gerados de conídios de *M. anisopliae* germinados em exoesqueleto de carrapato, hospedeiro do fungo. O seqüenciamento de produtos diferenciais gerados

identificou 34 genes de *M. anisopliae* superexpressos no modelo de infecção utilizado. O cultivo na presença de exoesqueleto induziu a expressão de genes relacionados com resposta a estresse, desintoxicação celular, transporte transmembrana, formação de apressório, metabolismo de parede e virulência (Dutra *et al.,* 2004). Dentre estes genes, os pesquisadores encontraram o gene *pr*1C de *M. anisopliae*, uma protease homóloga à subtilisina, que degrada a cutícula protéica do hospedeiro, assistindo a penetração da hifa do fungo além de fornecer nutrientes para posterior crescimento (Freimoser *et al.,* 2003). Esta diversidade de genes superexpressos reflete a participação de vários fatores na composição da estratégia de infecção no estágio inicial do parasitismo do carrapato (Dutra *et al.,* 2004).

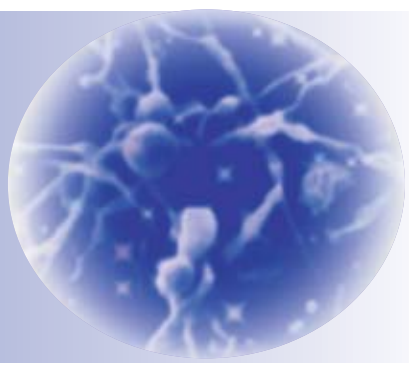
A habilidade de dermatófitos infectar pele, cabelo e unhas está relacionada com a capacidade de utilização da queratina como fonte nutricional. *Trichophyton rubrum* é um fungo antropofílico responsável por 90% dos casos de dermatofitoses. Para o estudo identificação de genes envolvidos com o processo infeccioso, o RDA foi utilizado na análise do padrão transcricional do *T. rubrum* cultivado na presença de queratina. A classificação funcional dos produtos diferenciais revelou que genes relacionados com resposta a estresse, controle da transcrição e transdução de sinal dominam a resposta adaptativa do organismo neste contexto celular. O gene codificante para catalase P, descrita como fator de proteção contra estresse oxidativo (Moreira *et al.,* 2004), foi altamente expresso na presença de queratina, sugerindo o papel desta enzima na proteção do *T. rubrum* no processo infeccioso (Baeza *et al.,* 2007).

A metodologia de RDA também foi utilizada com sucesso no estudo de expressão gênica de *C. neoformans*. Foram realizados ensaios comparativos entre os perfis transcricionais do fungo cultivado nas temperaturas de 25°C e 37°C, uma vez que a habilidade do fungo sobreviver e proliferar na temperatura do hospedeiro humano é um atributo essencial de virulência deste microrganismo. Fatores envolvidos com integridade da parede celular, resposta a estresse, metabolismo oxidativo e metabolismo de ácidos graxos foram induzidos à 37°C, temperatura do corpo humano. Um dos genes induzidos, na temperatura de 37°C foi aquele codificante para quitina sintase 2, com função no remodelamento da parede celular bem como na produção de leveduras viáveis a 30°C. Adicionalmente o transcrito codificante para diacil-glicerol-colina-fosfotransferase

apresentou-se induzido na temperatura de 37°C. Estes resultados sugerem que a parede e membrana celulares necessitam de remodelamento para viabilidade do fungo na temperatura do corpo humano (Rosa e Silva *et al.*, 2008).

No presente trabalho RDA foi a estratégia utilizada no estudo de perfis de expressão de *P. brasiliensis* durante condições que mimetizam algumas encontradas pelo fungo *P. brasiliensis* durante o processo infeccioso.

Justificativa



2 – JUSTIFICATIVA

A capacidade de *P. brasiliensis* provocar micose com grande variedade de manifestações clínicas depende da complexidade de interações com 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 a identificação, caracterização e estudo funcional de moléculas potencialmente associadas à interação do fungo *P. brasiliensis* com o hospedeiro humano. Neste contexto, projetos de seqüenciamento de ESTs em condições que mimetizam a infecção em nichos do hospedeiro destacam-se como estratégias interessantes para o entendimento da relação parasito-hospedeiro, bem como da biologia do fungo.

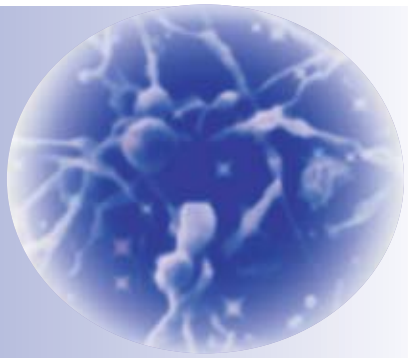
Trabalhos demonstram que o estudo de perfis transcricionais de *P. brasiliensis* é uma ferramenta rápida e eficiente na compreensão dos mecanismos utilizados por este organismo na patogênese da PCM. Desta maneira o estudo do perfil de expressão de genes em condições possivelmente encontradas por este organismo durante a infecção ajudaria na compreensão do complexo contexto molecular entre hospedeiro e parasito.

Análises do perfil transcricional de *C. albicans* em diferentes condições, as quais mimetizam diferentes ambientes encontrados pelo patógeno durante o processo infeccioso, demonstram que este fungo apresenta uma resposta transcricional nicho-específica. Os mecanismos e estratégias de sobrevivência/virulência apresentam padrões específicos que respondem a diferentes condições encontradas nos diferentes nichos do hospedeiro. Desta maneira o estudo dos perfis trasncricionais do *P. brasiliensis* em diferentes nichos do hospedeiro ajudaria na compreensão das diferentes estratégias adaptativas utilizadas pelo fungo nos diferentes ambientes que o microrganismo encontra no hospedeiro.

O fungo *P. brasiliensis* atinge os pulmões do hospedeiro através das vias respiratórias superiores. No pulmão o fungo pode entrar em contato com plasma em decorrência do extravasamento de vasos causado pela resposta inflamatória aguda neste sítio inicial da infecção. A partir dos pulmões o patógeno pode disseminar-se por vias hematogênica e linfática atingindo outros órgãos e sistemas. Portanto, a avaliação do perfil transcricional do *P. brasiliensis* durante contato com sangue humano, plasma

humano e durante a infecção em fígado de animais infectados permitiria a compreensão dos diferentes mecanismos nicho-específicos de sobrevivência do fungo em diferentes etapas da infecção.

Objetivos



3 – OBJETIVOS

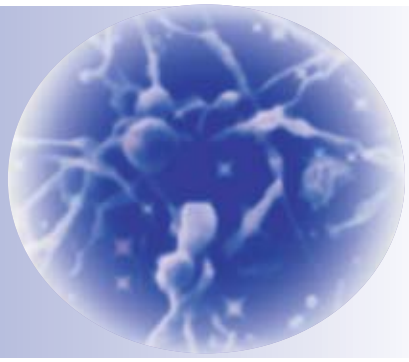
Visando o estudo da complexa interação *P. brasiliensis*-hospedeiro o presente trabalho objetivou o estudo do perfil de expressão gênica deste fungo em diferentes condições experimentais que potencialmente mimetizam o ambiente encontrado pelo microrganismo no hospedeiro humano.

Visando esse objetivo o presente estudo utilizou as seguintes estratégias:

- Obtenção de bibliotecas subtraídas de cDNAs de *P. brasiliensis*, através da técnica de cDNA-RDA, em diferentes condições experimentais
- Caracterização e categorização das seqüências obtidas através de anotação utilizando o programa Phorest (www.lbm.icb.ufg.br/phorestwww/index.php.).
- Comparação dos bancos com o transcriptoma do fungo durante o cultivo *in vitro* (https://dna.biomol.unb.br/*Pb*/) e consequente identificação de genes novos e com expressão diferencial.
- Confirmação da expressão diferencial dos genes identificados por cDNA-RDA
- Detecção da expressão dos genes selecionados durante a infecção sanguínea de camundongos
- Interpretação das análises e conseqüente compreensão das possíveis estratégias moleculares utilizadas pelo fungo *P. brasiliensis* nos diferentes ambientes encontrados no hospedeiro.

Capítulo II

Artigos Publicados



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ScienceDirect
Microbes and Infection 8 (2006) 2686–2697
Microbes and Infection
www.elsevier.com/locate/micinf

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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Received 30 May 2006; accepted 27 July 2006
Available online 24 August 2006

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.

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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doi:10.1016/j.micinf.2006.07.019

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,

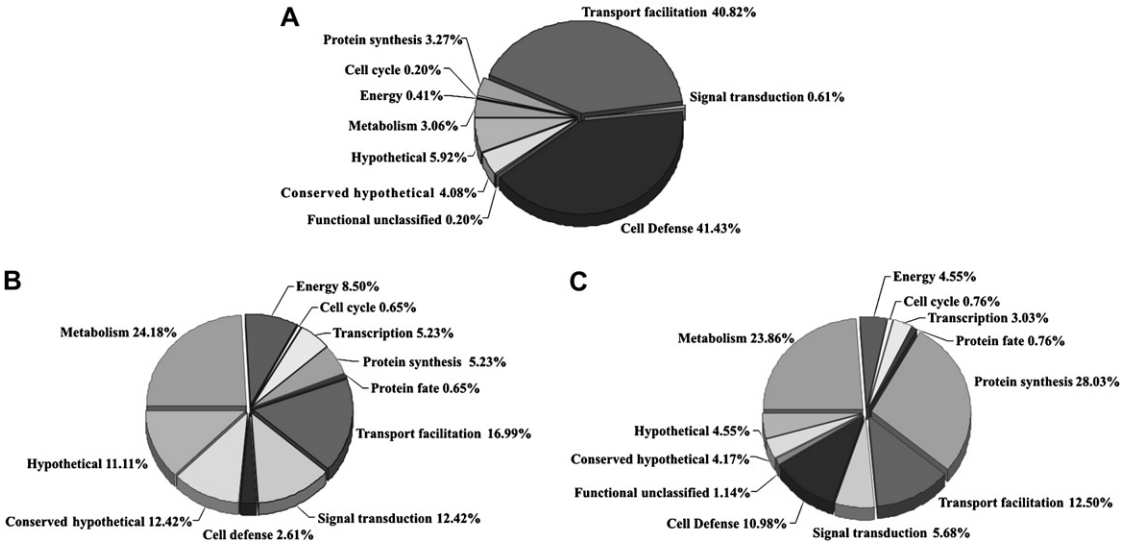

Fig. 1. Functional classification of *P. brasiliensis* cDNAs derived from RDA experiments using as testers. (A) cDNAs synthesized from the RNAs of yeast cells recovered from infected mice. (B,C) cDNAs obtained from RNA of *P. brasiliensis* yeast cells after a 10-min (B) or 60-min (C) incubation with 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

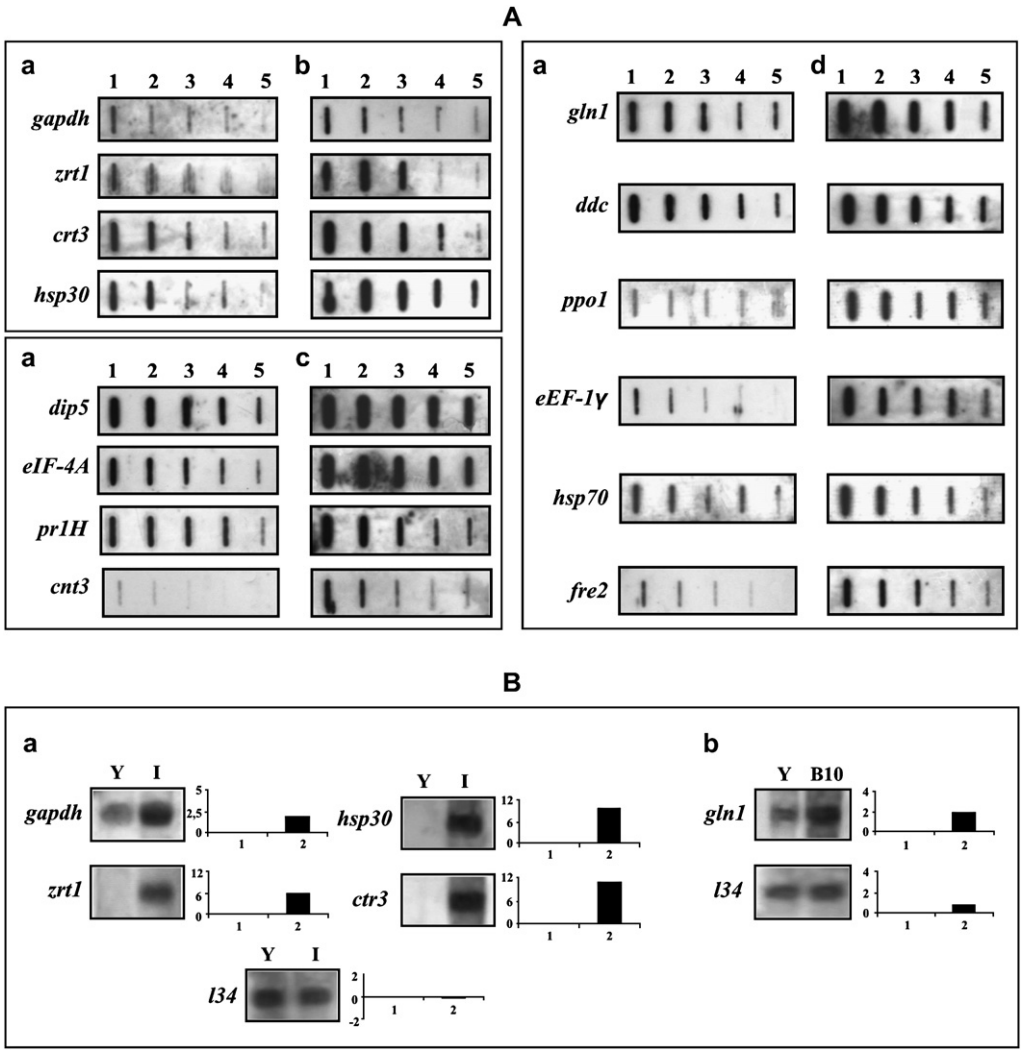

Fig. 2. Validation of the cDNA-RDA results. (A) Dot blot analysis of *P. brasiliensis* cDNA-RDA clones. DNA of individual clones was prepared and several dilutions were blotted (1–5). Individual clones were blotted and hybridized to the labeled cDNAs obtained from the control yeast cells (a); labeled 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].

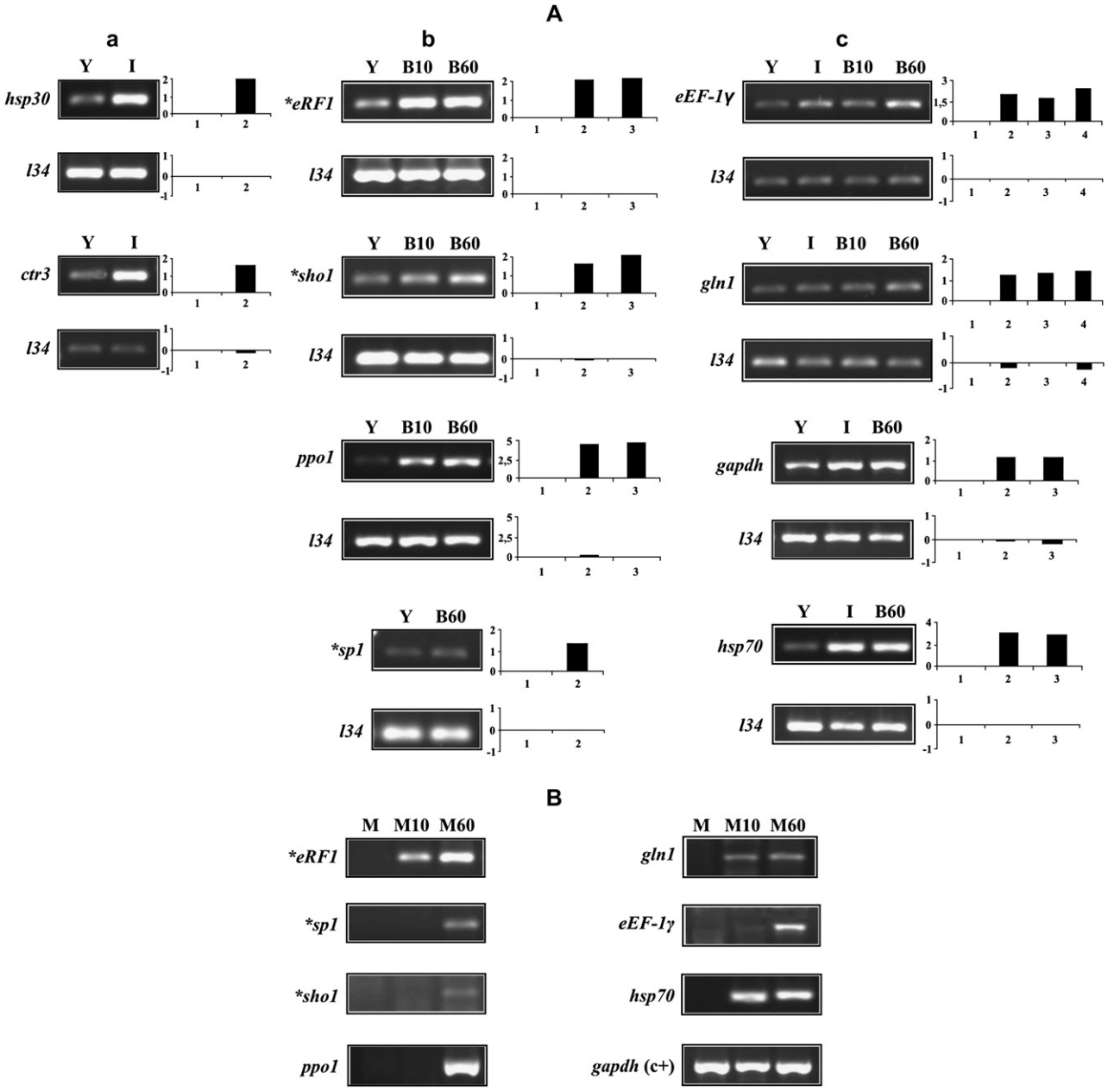

Fig. 3. In vitro and in vivo validation of the RDA results by RT−PCR analysis. (A) Validation of RDA results by semi-quantitative RT−PCR of RNAs obtained from yeast cells in host conditions. Semi-quantitative RT−PCR analysis was carried out with specific primers, as described. Numbers associated with 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.

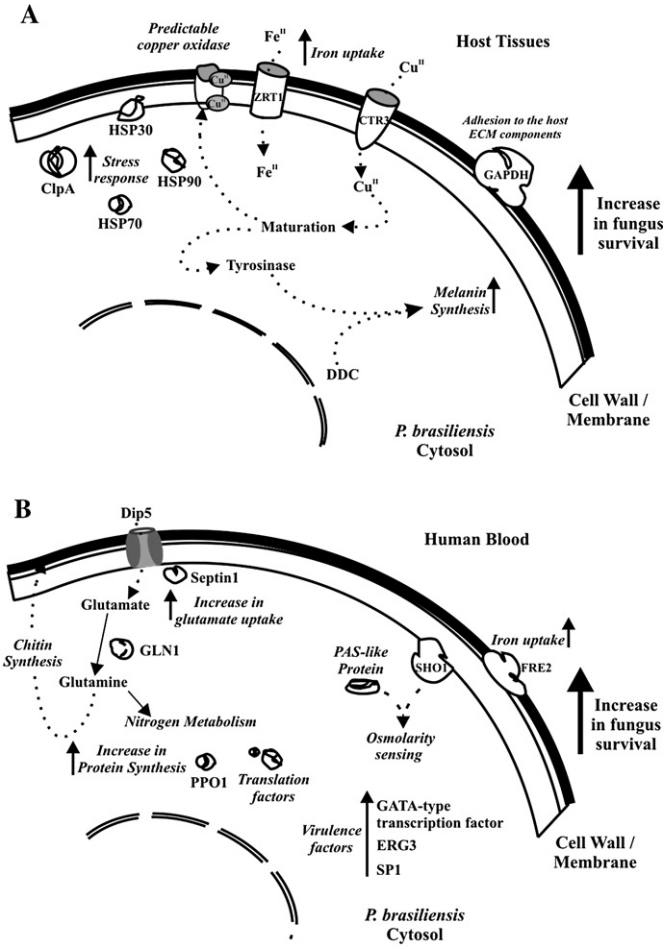

Fig. 4. Model of the adaptive changes of *P. brasiliensis* to the host milieu. (A) Model of how *P. brasiliensis* could enhance its survival in host cells. (B) Model of how *P. brasiliensis* enhance its survival during blood dissemination to organs and tissues. See Section 4 for details.

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*.

Acknowledgments

The work at Universidade Federal de Goiás was funded by grants from CNPq (505658/2004-6). The authors wish to thank Dr. George S. Deepe Jr., Division of Infectious Diseases, University of Cincinnati, for providing invaluable discussion and for critical review of the manuscript.

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RESEARCH ARTICLE

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

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

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

Editor: Alex van Belkum

Keywords
Paracoccidioides brasiliensis; transcription; human plasma.

Abstract

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

Introduction

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

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

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

Materials and methods

Paracoccidioides brasiliensis growth conditions

Paracoccidioides brasiliensis isolate 01 (ATCC MYA-826) has been studied at our laboratory (Bailão *et al.*, 2006; Barbosa *et al.*, 2006). It was grown in the yeast phase at 36 °C, in

Fava-Neto's medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 1% (w/v) agar; pH 7.2] for 7 days.

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

Human blood from 10 healthy donors was collected by venepunctures using heparinized syringes, and centrifuged at 1000 ***g***. *Paracoccidioides brasiliensis* yeast cells were harvested from 7-day-old cultures, and washed twice with phosphate-buffered saline (PBS) (NaCl 137 mM, KCl 2.7 mM, NaH_2PO_4 1.4 mM, Na_2HPO_4 4.3 mM, pH 7.4). The fungal cells (5×10^6 cells mL^{-1}) were inoculated into 7.5 mL of human plasma and incubated for several time intervals at 36 °C with shaking. The fungal cells were collected by centrifugation for 5 min at 1500 ***g***, and washed five times with PBS. As controls, *P. brasiliensis* yeast cells from Fava-Neto's cultures washed five times with PBS and 7.5 mL of the same plasma were taken to prepare control cDNA samples.

RNA extractions, subtractive hybridization and generation of subtracted libraries

Total RNA of the *P. brasiliensis* control yeast cells and of yeast cells incubated with human plasma for 10 and 60 min was extracted by the use of Trizol reagent (GIBCO, Invitrogen, Carlsbard, CA) according to the manufacturer's instructions. The quality of RNA was assessed by use of the $A_{260\,nm}/A_{280\,nm}$ ratio, and by visualization of rRNA on 1.2% agarose gel electrophoresis. The RNAs were used to construct double-stranded cDNAs. For subtractive hybridization, 1.0 μg of total RNAs was used to produce double-stranded cDNA using the SMART PCR cDNA synthesis kit (Clonetech Laboratories, Palo Alto, CA, USA). First-strand synthesis was performed with reverse transcriptase (RT Superscript II, Invitrogen, CA, USA), and the first strand was used as a template to synthesize the second strand of cDNA. The resulting cDNAs were digested with the restriction enzyme Sau3AI. Two subtracted cDNA libraries were made using driver cDNA from 7-day-old-cultures of yeast cells and tester cDNAs synthesized from RNAs extracted from *P. brasiliensis* obtained from yeast cells after incubation with human plasma for 10 and 60 min. The resulting products were purified using the GFX kit (GE Healthcare, Chalfont St Giles, UK). The cDNA representational analysis described by Hubank & Schatz (1994) was used, as modified by Dutra *et al.* (2004). The tester-digested cDNA was bound to adapters (a 24-mer annealed to a 12-mer). For generation of the differential products, 'tester' and 'driver' cDNAs were mixed, hybridized at 67 °C for 18 h, and amplified by PCR with the 24-mer oligonucleotide primer (Dutra *et al.*, 2004; Bailão *et al.*, 2006). Two successive rounds of subtraction and PCR amplification using hybridization tester/driver ratios of 1 : 10 and 1 : 100 were performed to generate second differential products. Adapters were changed between cross-hybridizations, and differential products were purified using the GFX kit. The adapters used for subtractive hybridizations were: NBam12, GATCCTCCCTCG; NBam24, AGGC AACTGTGCTATCCGAGGGAG; RBam12, GATCCTCGGT GA; and RBam24, AGCACTCTCCAGCCTCTCTCACCGAG.

After the second subtractive reaction, the final amplified cDNA pools were submitted to electrophoresis in 2.0% agarose gels, and the purified cDNAs were cloned directly into the pGEM-T Easy vector (Promega, Madison, USA). *Escherichia coli* XL1 Blue competent cells were transformed with the ligation products. Selected colonies were picked and grown in microliter plates. Plasmid DNA was prepared from clones using standard protocols. In order to generate the expressed sequence tags (ESTs), single-pass, 5′-end sequencing of cDNAs by standard fluorescence labeling dye-terminator protocols with T7 flanking vector primer was performed. Samples were loaded onto a MegaBACE 1000 DNA sequencer (GE Healthcare) for automated sequence analysis.

Sequences, processing and EST database construction

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

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

Sequences were placed into three categories: (1) annotated, which corresponds to sequences showing significant

matches with protein sequences with an identified function in databanks; (2) hypothetical protein, which corresponds to sequences for which the e-value was $>10^{-5}$, or for which no match was observed in databanks; or (3) conserved hypothetical protein, which corresponds to protein group sequences for which significant matches ($e < 10^{-5}$) and homology to a protein with no identified function was observed.

ESTs were grouped into 99 clusters, represented by 63 contigs and 36 singlets. With CAP3 assembly information stored in the relational database, SQL queries were performed to determine transcripts unique to a certain EST library and/or present in two or more libraries. Sequences were grouped in functional categories according to the classification of the MIPS functional catalog (Munich Center for Protein Sequences; http://www.mips.gst.de/). The clusters were compared with *P. brasiliensis* ESTs upregulated during incubation of yeast cells with human blood (Bailão *et al.*, 2006) (GenBank accession numbers EB085193–EB086102) and with the *P. brasiliensis* transcriptome database (https://dna.biomol.unb.br/Pb/) using the BLAST program (Altschul *et al.*, 1990). The nucleotide sequences reported here are available in the GenBank database under the accession numbers EH643296–EH643872.

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

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

Dot-blot analysis

Plasmid DNAs of selected clones were obtained. Serial dilutions of DNAs were performed, and the material was applied, under vacuum, to Hybond-N+nylon membranes (GE Healthcare). The DNAs were hybridized to cDNAs, which were obtained under specific conditions, labeled using the Random Prime labeling module (GE Healthcare). Detection was performed using the Gene Image CDP-Star detection module (GE Healthcare). The probes used were as follows: aromatic L-amino acid decarboxylase (*ddc*); translation elongation factor 1, gamma chain (*eEF-1*γ); serine proteinase (*pr1H*); glutamine synthetase (*gln1*); ferric reductase (*fre2*); transmembrane osmosensor (*sho1*); acidic amino acid permease (*dip5*); and eukaryotic translation initiation factor 4A (*eIF-4A*).

Semiquantitative reverse transcriptase (RT)-PCR analysis

Semiquantitative RT-PCR experiments were also performed to confirm the RDA results and the reliability of our approaches. Yeast cells of *P. brasiliensis* treated with human plasma, as well as control yeast cells, were used to obtain total RNAs. These RNAs were obtained from experiments independent of those used in the cDNA subtraction. The single-stranded cDNAs were synthesized by reverse transcription towards total RNAs, using the Superscript II RNAseH reverse transcriptase, and PCR was performed using cDNA as the template in a 30-μL reaction mixture containing specific primers, sense and antisense, respectively, as follows: endoplasmic reticulum to Golgi transport vesicle protein (*erv46*), 5′-CCTTATATGGGGTGAGTGGT-3′ and 5′-CCTCTCGTTCGCACTGCTC-3′; pyridoxamine phosphate oxidase (*ppo1*), 5′-CATCGACGACTGCCTCC TC-3′ and 5′-GGACGGCTTCTGGGTGCT-3′; putative major facilitator protein (*ptm1*), 5′-CGATTCCTCGCAA TTGGTCA-3′ and 5′-CGTTGCGCCCAATGAGTTC-3′; eukaryotic release factor 1 (*eRF-1*), 5′-CAACGTTGACTT TGTCATTGG-3′ and 5′-CCATGGACTTGTCATATACTG-3′; *eEF-1*γ, 5′-GGCTTGGAGAGGGAGTCG-3′ and 5′-CC CTTGTTGGACGAGACCC-3′; *gln1*, 5′-CGTTACCCTCA CCGTAGAC-3′ and 5′-CATACGGCTGGCCCAAGG-3′; *sho1*, 5′-CCACCACCGGCCACTGAC-3′ and 5′-CCCGAAA CAACTGTCTCCG-3′; and ribosomal L34 protein (*l34*), 5′-CAAGACTCCAGGCGGCAAC-3′ and 5′-GCACCGCCATG ACTGACG-3′. The reaction mixture was incubated initially at 95 °C for 1 min, and this was followed by 25–35 cycles of denaturation at 95 °C for 1 min, annealing at 55–65 °C for 1 min, and extension at 72 °C for 1 min. The annealing temperature and the number of PCR cycles were optimized in each case to ensure that the intensity of each product fell within the exponential phase of amplification. The DNA product was separated by electrophoresis in 1.5% agarose gel, stained, and photographed under UV light illumination. The analyses of relative differences were performed with the SCION IMAGE BETA 4.03 program (http://www.scioncorp.com).

Protein extract preparation and Western blot analysis

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

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

Measurement of formamidase activity

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

SDS sensitivity tests

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

Results

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

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

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

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

In addition, a comparison was performed between upregulated transcripts appearing during human plasma incubation and those present during yeast cell incubation in human blood (Bailão *et al.*, 2006). The same batch of blood was used to prepare human plasma and for the incubation of yeast cells in total blood. Table 1 gives the genes

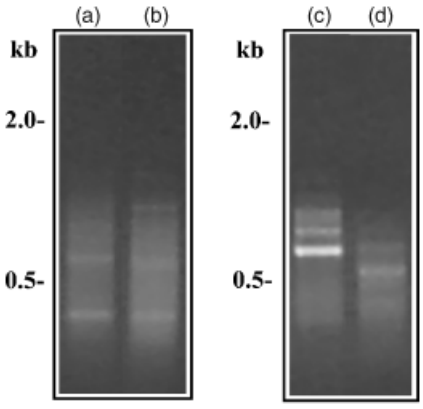

Fig. 1. Agarose gel electrophoresis of subtracted differential cDNA pools derived from *Paracoccidioides brasiliensis* yeast cells incubated with human plasma. Products of the first and second rounds of subtraction performed using as testers the cDNA obtained from RNAs of yeast cells incubated with human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

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

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

Table 1. Continued.

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

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

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

‡Novel genes detected in *P. brasiliensis*.

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

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

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

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

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

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

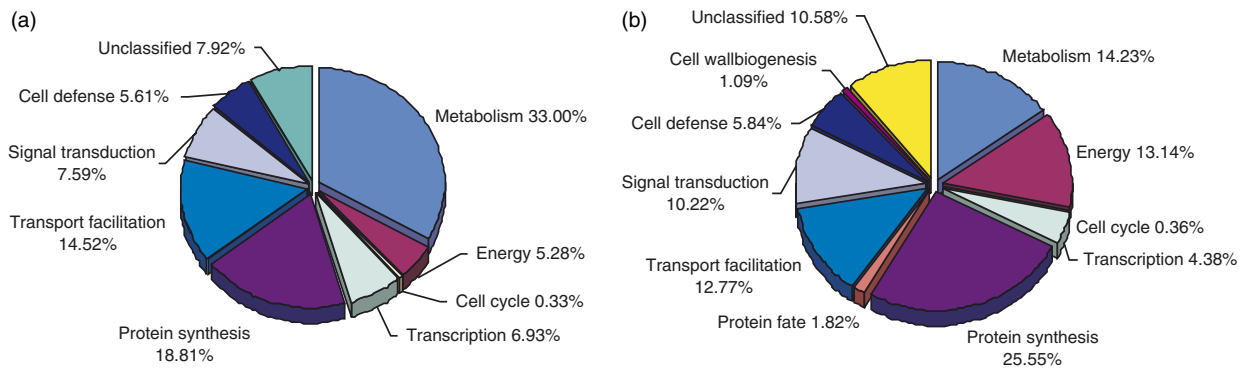

Fig. 2. Functional classification of *Paracoccidioides brasiliensis* cDNAs derived from RDA experiments using as testers the cDNAs obtained from RNA of *Paracoccidioides brasiliensis* yeast cells after incubation with human plasma for 10 min (a) or 60 min (b). The percentage of each functional category is shown (see Tables 1 and 2). The functional classification was based on BLASTX homology of each EST against the GenBank nonredundant database at a significant homology cut-off of $\leq 1e^{-05}$ and the MIPS functional annotation scheme. Each functional class is represented as a color-coded segment and expressed as a percentage of the total number of ESTs in each library.

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

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

*Transcripts not upregulated during yeast cell incubation with human blood (Bailão *et al.*, 2006).
†Transcripts validated by dot blot.
‡Transcripts more abundant in yeast cells during incubation in human plasma than during incubation in human blood (Bailão *et al.*, 2006).
§Transcripts detected in blood of infected mice, as previously demonstrated (Bailão *et al.*, 2006).
¶Transcripts validated by semiquantitative RT-PCR.

Confirmation of the expression of selected genes of *P. brasiliensis*

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

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

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

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

The most prominent adaptations undergone by *P. brasiliensis* during treatment with human plasma are summarized in Fig. 6. As observed, the degradation of fatty acids through β-oxidation, putatively generating acetyl-CoA and propionyl-CoA, could be inferred, as several enzymes are upregulated during the treatment. The flavoprotein dehydrogenase that introduces the double bond passes electrons directly to

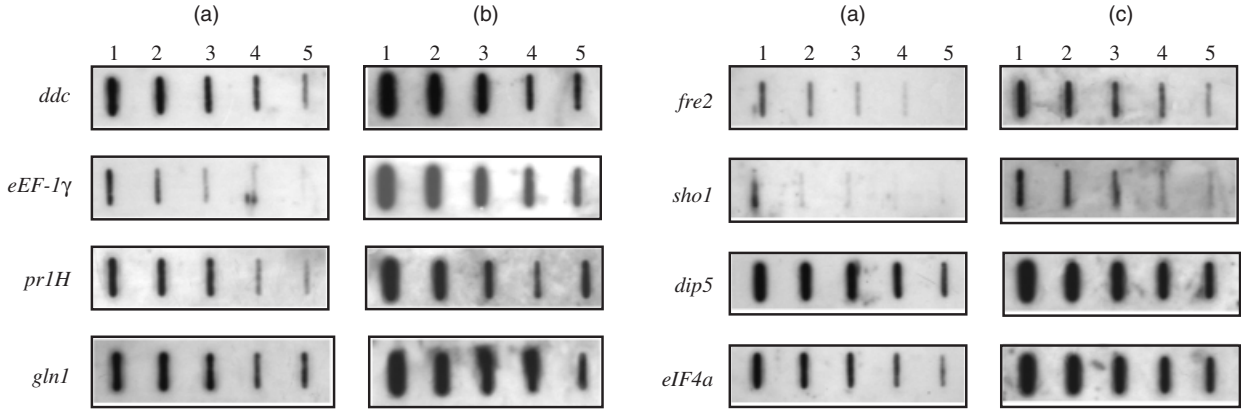

Fig. 3. Dot-blot analysis of *Paracoccidioides brasiliensis* cDNA-RDA clones. DNAs of individual clones were prepared and blotted in several dilutions (1–5). Individual clones were blotted and hybridized to the labeled cDNAs obtained from the control yeast cells (a), and labeled cDNAs obtained from *Paracoccidioides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

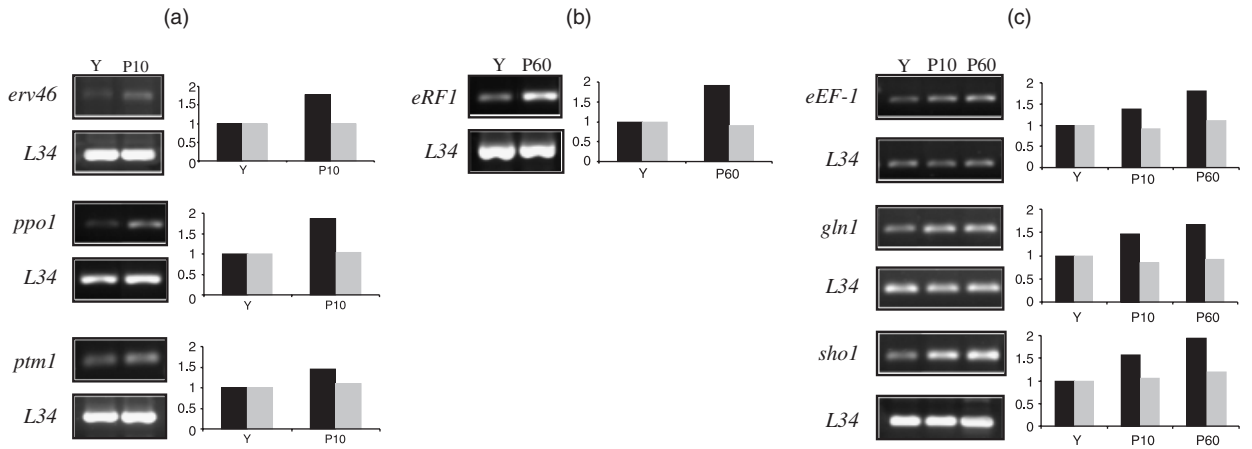

Fig. 4. Validation of RDA results by semiquantitative RT-PCR of RNAs obtained from yeast cells during incubation with human plasma. Semiquantitative RT-PCR analysis was carried out with specific primers, as described. Numbers associated with the bars indicate fold differences relative to the data for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

O_2 during β-oxidation in peroxisomes, producing H_2O_2, a product that could be removed from peroxisomes by catalase A, which is overexpressed in the subtracted cDNA library. Additionally, the methylcitrate cycle could assimilate propionyl-CoA, generating pyruvate. Also, the synthesis of acetyl-CoA from pyruvate and acetate could be performed by the overexpressed enzyme acetyl-CoA synthase. Additionally, soluble fumarate reductase in the cytoplasm could catalyze the conversion of fumarate to succinate during the reoxidation of intracellular NADH, thus providing additional succinate.

Sensitivity of yeast cells to SDS after incubation with human plasma

We tested whether the incubation of yeast cells with human plasma could be reflected in the relative sensitivity of cells to SDS, an anionic detergent that destabilizes the cell wall at

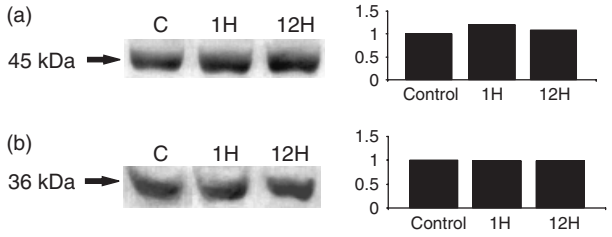

Fig. 5. Validation of the RDA results by Western blot. Total cellular extracts were obtained from yeast cells incubated with human plasma for 1 and 12 h. The proteins (25 μg) were electrophoretically transferred to a nylon membrane and checked by Ponceau S to determine equal loading. The samples were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

Table 3. Formamidase activity of yeast cell protein extracts

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

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

very low concentrations. The yeast cells incubated with human plasma show greater sensitivity to this osmotic destabilizing agent when compared to the control cells (Fig. 7).

Discussion

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

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

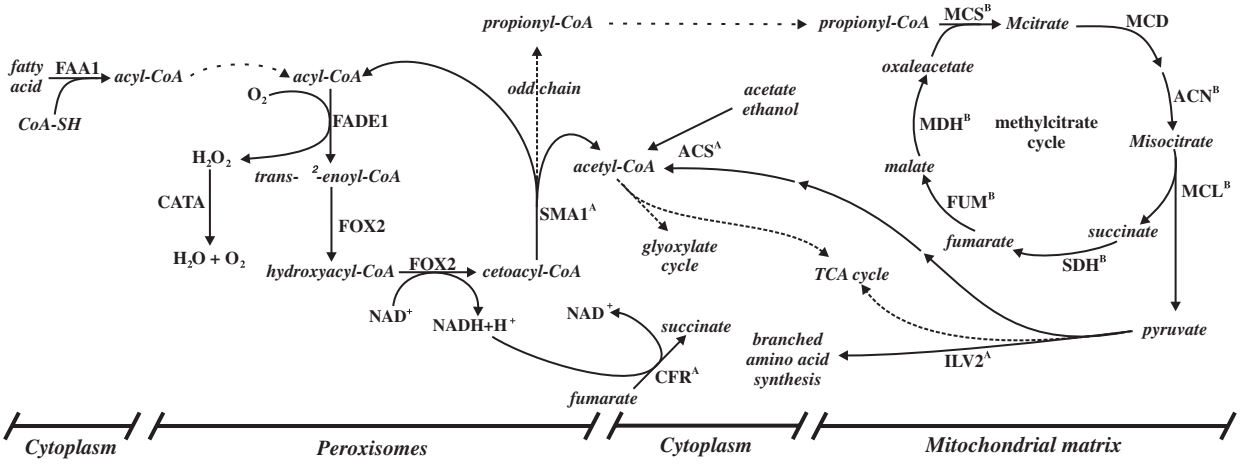

Fig. 6. Some metabolic pathways that are overexpressed during *Paracoccidioides brasiliensis* yeast cell incubation with human plasma. [(A)]Transcripts that are not overexpressed during *Paracoccidioides brasiliensis* treatment with human blood. [(B)]Transcripts present in database. FAA1, long-chain fatty acid-CoA ligase; FADE1, acyl-CoA dehydrogenase; FOX2, multifunctional β-oxidation protein; CATA, catalase A; SMA1, acyltransferase family protein; ACS, acetyl-CoA synthetase; CFR, NADH-fumarate reductase; MCS, methylcitrate synthase; MCD, methylcitrate dehydroghenase; CAN, aconitase; MCL, methylcitrate lyase; SDH, succinate dehydrogenase; FUM, fumarate reductase; MDH, malate dehydrogenase; ILV2, acetolactate synthase; Mcitrate, methylcitrate; Misocitrate, methylisocitrate.

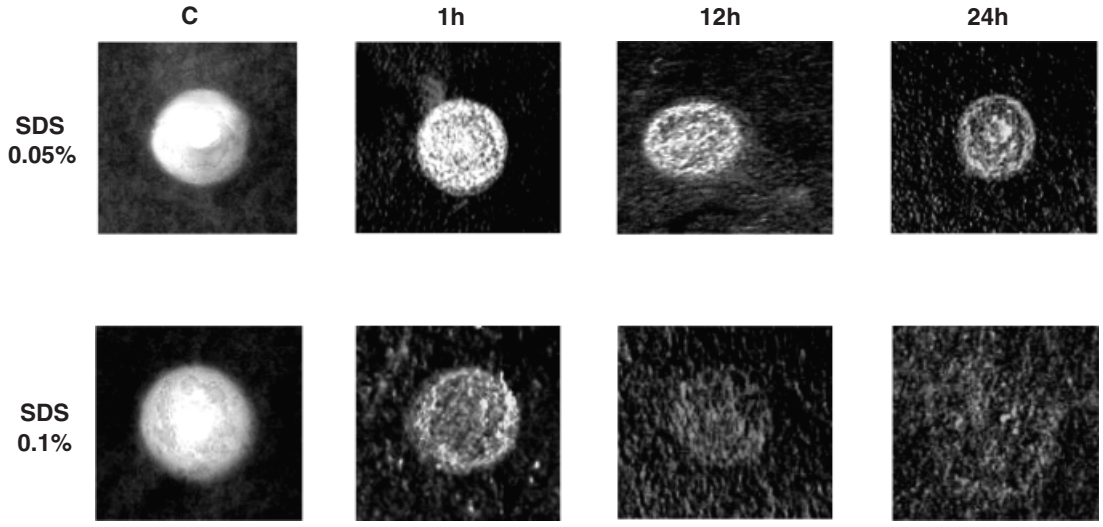

Fig. 7. Phenotypic analysis of *Paracoccidioides brasiliensis* yeast cells after incubation in human plasma for different time periods. Approximately 10^2 cells were spotted onto Fava-Neto's medium plates containing the indicated concentrations of SDS. Plates were incubated at 36 °C for 7 days. Experiments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

dehydrogenase, as described in *Saccharomyces cerevisiae*, *Candida tropicalis* and mammals (Moreno *et al*., 1985; Hiltunen *et al*., 1992; Breitling *et al*., 2001). β-Oxidation of even-chain-length fatty acids yields acetyl-CoA units exclusively, whereas β-oxidation of odd-chain-length fatty acids yields both acetyl-CoA and propionyl-CoA. In several bacteria and fungi, propionyl-CoA is assimilated via the methylcitrate cycle, which oxidizes propionyl-CoA to pyruvate (Brock *et al*., 2000). The growth of fungi on gluconeogenic compounds such as acetate or fatty acids positively regulates enzymes of the glyoxylate cycle, even in the presence of repressing carbon sources such as glucose (Cánovas & Andrianopoulos, 2006). Acetyl-CoA synthetases (EC 6.2.1.1) have been detected as isoforms in microorganisms such as the fungus *Phycomyces blakesleeanus*, in where they can use acetate and propionate as substrates (De Cima *et al*., 2005). Alternatively, conversion of pyruvate to acetyl-coenzyme A can be accomplished by the concerted action of the enzymes of the pyruvate dehydrogenase bypass: pyruvate decarboxylase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase (van den Berg *et al*., 1996).

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

Acetolactate synthase (EC 2.2.1.6) catalyzes the first common step in the biosynthesis of the branched amino acids isoleucine, valine and leucine, starting from pyruvate. Mutants for the homologous gene in *Cryptococcus neoformans* are avirulent and unable to survive in mice (Kingsbury *et al*., 2004). Also, fumarate reductase (EC 1.3.1.6) is upregulated during human plasma incubation of yeast cells of *P. brasiliensis*. In *S. cerevisiae*, two fumarate reductase isoenzymes are required for the reoxidation of intracellular NADH under anaerobic conditions (Enomoto *et al*., 2002). Consistently, the yeast cells of *P. brasiliensis* produce ATP preferentially through alcohol fermentation (Felipe *et al*., 2005). In this sense, aldehyde dehydrogenase (EC 1.2.1.3) can allow the conversion of ethanol into acetate via acetyldehyde, thus providing acetyl-CoA to the glyoxylate cycle. In *P. brasiliensis*, alcohol dehydrogenase is upregulated in the yeast cells, as previously demonstrated (Felipe *et al*., 2005).

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

Plasma treatment also promotes upregulation of transcripts encoding facilitators of transport in *P. brasiliensis* yeast cells. The most upregulated transcripts encode for a

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

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

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

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

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

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

present during blood infection of mice by *P. brasiliensis*, as previously demonstrated (Bailão *et al*., 2006).

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

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

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

Acknowledgements

This work at Universidade Federal de Goiás was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico 505658/2004-6). A.M.B. and C.L.B. are doctoral fellows of CNPq. R.B.F. is a DTI fellow from CNPq. J.A.P. is a doctoral fellow from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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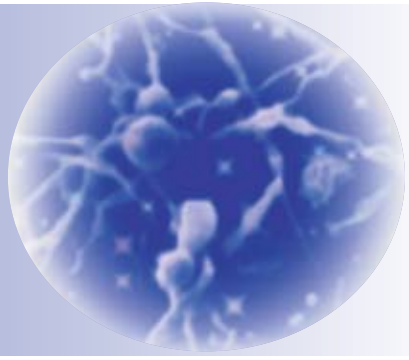
Capítulo III

Artigos Publicados em Colaboração



Yeast
Yeast 2004; **21**: 173–182.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/yea.1077

Research Article

Monofunctional catalase P of *Paracoccidioides brasiliensis*: identification, characterization, molecular cloning and expression analysis

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Received: 4 August 2003
Accepted: 10 November 2003

Abstract

Within the context of studies on genes from *Paracoccidioides brasiliensis* (*Pb*) potentially associated with fungus–host interaction, we isolated a 61 kDa protein, pI 6.2, that was reactive with sera of patients with paracoccidioidomycosis. This protein was identified as a peroxisomal catalase. A complete cDNA encoding this catalase was isolated from a *Pb* cDNA library and was designated *PbcatP*. The cDNA contained a 1509 bp ORF containing 502 amino acids, whose molecular mass was 57 kDa, with a pI of 6.5. The translated protein *Pb*CATP revealed canonical motifs of monofunctional typical small subunit catalases and the peroxisome-PTS-1-targeting signal. The deduced and the native *Pb*CATP demonstrated amino acid sequence homology to known monofunctional catalases and was most closely related to catalases from other fungi. The protein and mRNA were diminished in the mycelial saprobic phase compared to the yeast phase of infection. Protein synthesis and mRNA levels increased during the transition from mycelium to yeast. In addition, the catalase protein was induced when cells were exposed to hydrogen peroxide. The identification and characterization of the *Pb*CATP and cloning and characterization of the cDNA are essential steps for investigating the role of catalase as a defence of *P. brasiliensis* against oxygen-dependent killing mechanisms. These results suggest that this protein exerts an influence in the virulence of *P. brasiliensis*. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: ***Paracoccidioides brasiliensis*; cellular differentiation; gene expression; protein synthesis; oxidative stress**

Introduction

Paracoccidioides brasiliensis is the aetiological agent of paracoccidioidomycosis, which is one of the most prevalent human systemic mycoses in Latin America. It is estimated that among the 90 million people living in the endemic areas, as many 10 million could be infected with *P. brasiliensis*. The fungus causes active disease mainly in immunocompetent individuals. In its most serious form, the infection disseminates to involve multiple organ systems (Brummer *et al*., 1993).

P. brasiliensis is a dimorphic fungus that undergoes a complex differentiation *in vivo*. After entrance of acquired airborne microconidia into a mammalian host, the fungus differentiates into the parasitic yeast form. Within the pulmonary airways *P. brasiliensis* is likely to be subjected to considerable oxidative stress. The first line of defence that *P. brasiliensis* faces during host invasion is the attack of polymorphonuclear leukocytes and alveolar macrophages (Brummer *et al*., 1989). Stimulated phagocytic cells, which migrate to areas of infection, release toxic oxygen radicals such as

H_2O_2 (hydrogen peroxide) as antimicrobial agents (McEwen *et al.*, 1984; Meloni-Bruneri *et al.*, 1996).

Aerobic organisms possess specific enzymes to eliminate H_2O_2. Catalases (EC 1.11.1.6) that are ubiquitous in aerobic metabolism convert H_2O_2 to O_2 and water (H_2O) (Ruis and Koller, 1997). Catalases may be particularly important for human-invasive microbes such as *P. brasiliensis*; they could act as a defensive mechanism against attack by the reactive oxygen species produced by macrophages or neutrophils. There are three separate families of catalases: Mn catalases, bifunctional catalase-peroxidase and monofunctional or true catalases. The last family corresponds to homotetrameric haem-containing enzymes that are composed of two clearly distinct classes, which can be recognized by the size of the subunits. The first class, with subunits of 50–65 kDa, includes a large number of catalases from bacteria, plants, fungi and animals (Ruis and Koller, 1997).

Despite the described importance of the oxidative mechanism in the killing of *P. brasiliensis* by polymorphonuclear cells and the suggested role of reactive oxygen species in cellular differentiation of microorganisms (Hansberg, 1996; Schröter *et al.*, 2000), little has been published on *P. brasiliensis* catalases (McEwen *et al.*, 1984; Meloni-Bruneri *et al.*, 1996). To begin to understand the putative role of catalases of *P. brasiliensis*, our initial focus has been on an immunoreactive protein of 61 kDa, pI 6.2. We previously isolated the protein from the proteome of yeast cells of *P. brasiliensis* (Fonseca *et al.*, 2001). In this work we describe the amino acid sequences of five internal peptides of the native protein. We have obtained a PCR product that was useful for the isolation of a complete cDNA from a yeast cDNA library. The cDNA which contained all the five peptides of the native protein encoded a monofunctional typical peroxisomal catalase and was designated *PbcatP*. *In vivo* cell labelling and Western and Northern blot analysis showed that the protein and the transcript were developmentally regulated in *P. brasiliensis* and induced during the mycelium-to-yeast transition. We have extended our studies of *Pb*CATP on the regulation by the substrate H_2O_2.

Materials and methods

Growth and differentiation of *P. brasiliensis*

P. brasiliensis, isolate *Pb*01 (ATCC-MYA-826), has been studied in our laboratory. It was grown as mycelium or yeast at 22 °C and 36 °C, respectively. The differentiation assays were performed as previously standardized in our laboratory (Silva *et al.*, 1994). Mycelia grown at 22 °C in semi-solid Fava Neto's medium were subcultured every 10 days. The differentiation was performed in liquid medium by changing the culture temperature from 22 °C to 36 °C for the mycelium-to-yeast transition.

Cell labelling

The yeast cells and mycelia were incubated with [^{35}S]-L-methionine (50 μCi) for 12 h. In the differentiation experiments the cells were incubated for 6 h with the radioactive precursor (10 μCi). The cellular extracts were obtained as described below.

Cell treatment with H_2O_2

The yeast cells were incubated in 50 mM phosphate buffer (pH 7.0) containing 15 mM H_2O_2. The cells were washed three times in the phosphate buffer, and the proteins were extracted, as described below.

Obtaining protein extracts and analysis of proteins

The cellular extracts were obtained as described (Fonseca *et al.*, 2001). In brief, the yeast and mycelia were scraped off the medium and washed in Tris–Ca^{2+} 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), 4 mM phenylmethyl-sulphonyl fluoride (PMSF), 5 mM iodoacetamide, 1 mM ethylenediaminetetraacetic (EDTA), 20 μg/ml leupeptine and 1 mM 4-chloromercuribenzoic acid (PCMB). The cells were collected by centrifugation at 5000 × *g* for 5 min, frozen in liquid nitrogen and disrupted by maceration until a fine powder was obtained. The cellular powder was vortexed for 15 min at 4 °C and centrifuged at 12 000 × *g* for 20 min. The supernatant was kept at −80 °C. The proteins were precipitated by addition of 10% (v/v)

trichloroacetic acid (TCA), washed with 100% acetone and processed for one- or two-dimensional gel electrophoresis according to Laemmli (1970) and O'Farrel (1975), respectively.

Isolation and amino acid sequencing of catalase

The spot corresponding to the protein of 61 kDa, pI 6.2, was cut out from the SDS–polyacrylamide gels. The protein (200 pmol) was eluted and digested with the endoproteinase Lys-C. The fragments were separated by reversed-phase high performance liquid chromatography (HPLC) and subjected to Edman's degradation.

Western blot analysis

The cellular extracts were resolved by one-dimensional gel electrophoresis. The proteins were transferred to a nylon membrane and stained by Ponceau S to assess loading of equal amounts of protein. The catalase protein was detected with a mAb raised to the catalase P (peroxisomal catalase) of *Toxoplasma gondii* (kindly provided by Dr Keith A. Joiner). After reaction with alkaline phosphatase anti-rabbit IgG, the reaction was developed with 5-bromo-4-chloro-3-indolylphosphate/nitro-blue-tetrazolium (BCIP/NBT).

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, according to Del Sal *et al*. (1989).

Generation of the catalase DNA probe by PCR

P. brasiliensis genomic DNA was used as a template for PCR amplification of a partial fragment encoding the catalase. Degenerate oligonucleotides primers were designed based on the amino acid sequences of the internal peptides, (Figure 1). The degenerate sense Cat1 5′-GAYAAYCCNGAYTGGCA-3′ and the antisense Cat2 5′-AARACATRCARTANGT-3′ (Figure 1) primers were used in a PCR reaction that was conducted in a total volume of 50 µl containing 50 ng DNA as template. The resulting 690 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*. (1977).

Cloning of the catalase cDNA

A yeast cDNA library was constructed in *Eco*RI and *Xho*I sites of Lambda ZapII (Stratagene, LaJolla, CA). The screening of this library was performed using the 690 bp fragment radiolabelled with [α-^{32}P]-dCTP. Plating 5×10^6 plaque forming units (p.f.u.), DNA transfer to membranes and hybridization were performed as described in standard procedures (Sambrook and Russel, 2001). Twelve positive clones were obtained and phage particles were released from the plaques. The *in vivo* excision of pBluescript phagemids (Stratagene) in *Escherichia coli* XL1-Blue was performed. The nucleotide sequence was determined on both strands.

Sequence analysis

Nucleotide sequence analysis was performed with the Wisconsin Genetics Computer Group (GCG) analysis software package, version 7.0 (Devereux *et al*., 1984). The NCBI BLAST program (**http://www.ncbi.nlm.nih.gov**) was used for search for nucleotide and protein sequences similarity to the *PbcatP*. Protein sequence analysis was performed with the PROSITE (**http://us.expasy.org/prosite**) and Pfam databases (**http://www.sanger.ac.uk/softwer/pfam/index.shtml**).

Protein homology and inferred phylogeny

The phylogenetic relationships of *Pb*CATP and related sequences were generated with 59 catalases available on the Pfam database. The entire amino acid sequences were compared using TreeView software. Robustness of branches was estimated using 100 boot-strapped replicates. The alignment was generated with Clustal X software (Thompson *et al*., 1997).

Northern blot analysis

Total RNA was isolated from *P. brasiliensis* cells with Trizol, according to the manufacturer's instructions (GIBCO™, Invitrogen, Carlsbad, CA). Northern hybridization was performed on a 1.2%

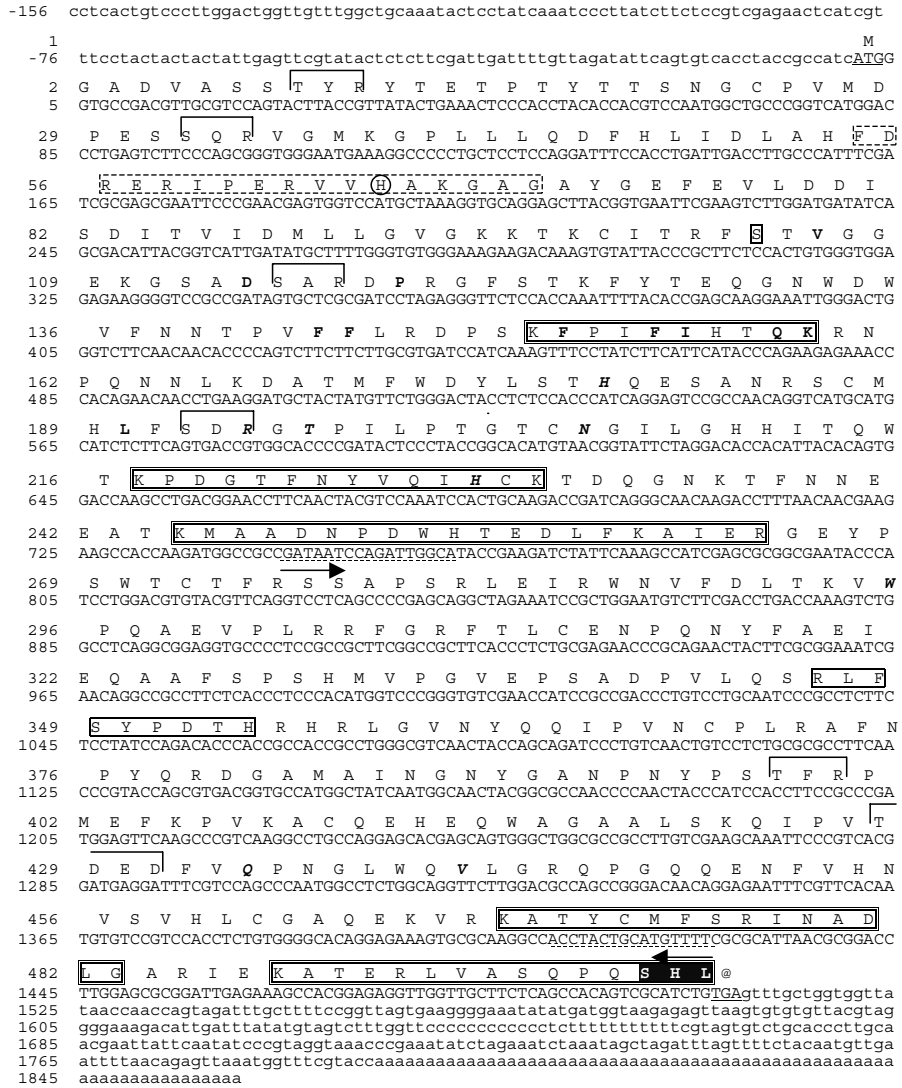

Figure 1. Nucleotide and deduced amino acid sequence of the *P. brasiliensis* catalase cDNA. Amino acids sequences experimentally determined of the five internal peptides of the *P. brasiliensis* catalase are double-boxed. Cat1 (sense) and Cat2 (antisense) oligonuclcotides are underlined with dashed lines and marked by arrows. The non-coding nucleotide sequences are indicated by lower case letters. Superior brackets mark potential phosphorylation sites in the deduced protein. Translational start and termination codons are underlined. The putative catalytic site is boxed with a broken line and the conserved H is marked by a circle. The rectangle identifies the S related to the protein fold. The residues potentially associated to the haem binding are boxed. The amino acids related to the substrate and the NADPH binding are in bold and bold italics, respectively. The PTS-1 signal is marked by a black box

(w/v) agarose-formaldehyde gel; the 690 bp PCR fragment of the catalase gene was used as a probe.

Nucleotide sequence Accession Nos

The sequences of the *PbcatP* and the deduced protein have been filed in the GenBank (Accession No. AF428076).

Results and discussion

Isolation of a catalase of *P. brasiliensis*

A protein of 61 kDa, pI 6.2, was isolated from the proteome of *P. brasiliensis* yeast cells. Edman degradation analysis of the endoproteinase Lys-C digested protein identified 74 amino acids in five

internal peptides, as shown in Figure 1. The amino acids sequence of all the peptides showed identity to catalases P described in the database. Significant identity of those five internal peptides to catalases was observed and suggested the arrangement of the amino acids within the protein. That information was used to synthesize degenerate oligonucleotides (Cat1 and Cat2) that were used to obtain, by PCR, a 690 bp DNA fragment. Homology search analysis suggested that the 690 bp DNA sequence was a homologue of the catalase of *P. brasiliensis*. Also, translation of the gene sequence of this amplicon revealed two of the internal peptides identified by Edmam degradation (Figure 1).

Nucleotide sequence and sequence analysis of the cDNA encoding catalase

The 690 bp PCR fragment was used to screen a cDNA library and the entire coding sequence of this gene was identified (Figure 1). The cloned cDNA was 2016 nucleotides in length and contained a 156 base at the 5′*UTR* (untranslated region). The cDNA had a single ORF. The deduced amino acid sequence indicated a protein of 502 amino acid residues. The ATG codon at base 157 encoded the presumed initiating methionine. This amino acid was in the appropriate position of a consensus translation start codon (Kozak, 1986). The cDNA included 284 bp in the 3′*UTR*, exclusive of the poly-A tail. The stop codon TGA was located at position 1663.

Characteristics of the deduced amino acid sequence

The ORF encoded a calculated 57 kDa polypeptide, pI 6.5, comprising the entire catalase. The deduced amino acid sequence included all five of the endoproteinase Lys-C peptides of catalase shown in Figure 1. A search at PROSITE database defined the canonical motifs of catalases. The amino acids of the conserved catalytic site were present at positions 54–70. The predicted catalase contained the conserved H-65, present in all haem catalases. This residue allows the proper binding and reduction of a peroxide molecule. A S-104 that has been reported to be essential for the correct protein folding was conserved in the deduced catalase. The binding to the substrate H_2O_2 could be related to the amino acid residues V, D, P, F, F, F, F, I, Q, K and L at positions 106, 114, 119, 143, 144, 151, 154, 155, 158, 159 and 190, respectively. The haem binding site was composed of amino acids R, L, F, S, Y, P, D, T and H at positions 346–354. The NADPH–binding site that is present in several catalases were H, R, T, N, H, W, Q and V at positions 179, 194, 196, 205, 228, 295, 434 and 441, respectively, in the deduced protein. Potential phosphorylation sites were found at six positions in the deduced protein, as shown in Figure 1. The C terminus of deduced catalase, S/H/L, matched the consensus PTS-1 signal that includes (S/A/C/K/N)-(K/R/H/Q/N/S)-(L/F/I/Y/M) (Ding *et al*., 2000). The presence of PTS-1 enabled us to designate the deduced sequence and the native protein as a catalase P of *P. brasiliensis, Pb*CATP. In a search of protein databases, appreciable sequence similarities were found between the predicted ORF product and known catalases P, as shown in Figure 2A. The highest identity values of 78%, 78% and 71%, respectively, were with catalases from *H. capsulatum*, *A. nidulans* and *Glomerula cingulata*. High identities of amino acid residues at the catalytic site, PTS-1 signal, haem, NAPDH and substrate binding sites were noted. The results clearly indicated that *P. brasiliensis* contained within its genome a catalase gene that encoded a small subunit, monofunctional catalase, probably localized in the peroxisomes. Based on the lack of a detectable signal sequence, the presence of the targeting signal PTS-1 and the high identity to fungal catalases P, it appears that *Pb*CATP is a peroxisomal catalase.

Phylogenetic analysis

We used the protein families database Pfam to search for all complete protein sequences of 59 catalases P including sequences from plants, fungi, animals and protozoans. To visualize the relationship between *Pb*CATP in terms of amino acid sequence similarity, a phylogenetic tree was constructed. Figure 2B shows the deduced phylogeny of *Pb*CATP, as calculated from the maximum likelihood analysis of amino acid sequences. The catalase P sequences were well resolved into clades A (plant), B (fungi) and C (animals and protozoans). The three groups were clustered separately, suggesting that catalases P segregated early in phylogenetic history. This behaviour has been described for other catalases (Klotz *et al*., 1997). Fungal catalases (clade B) were resolved into two subclades,

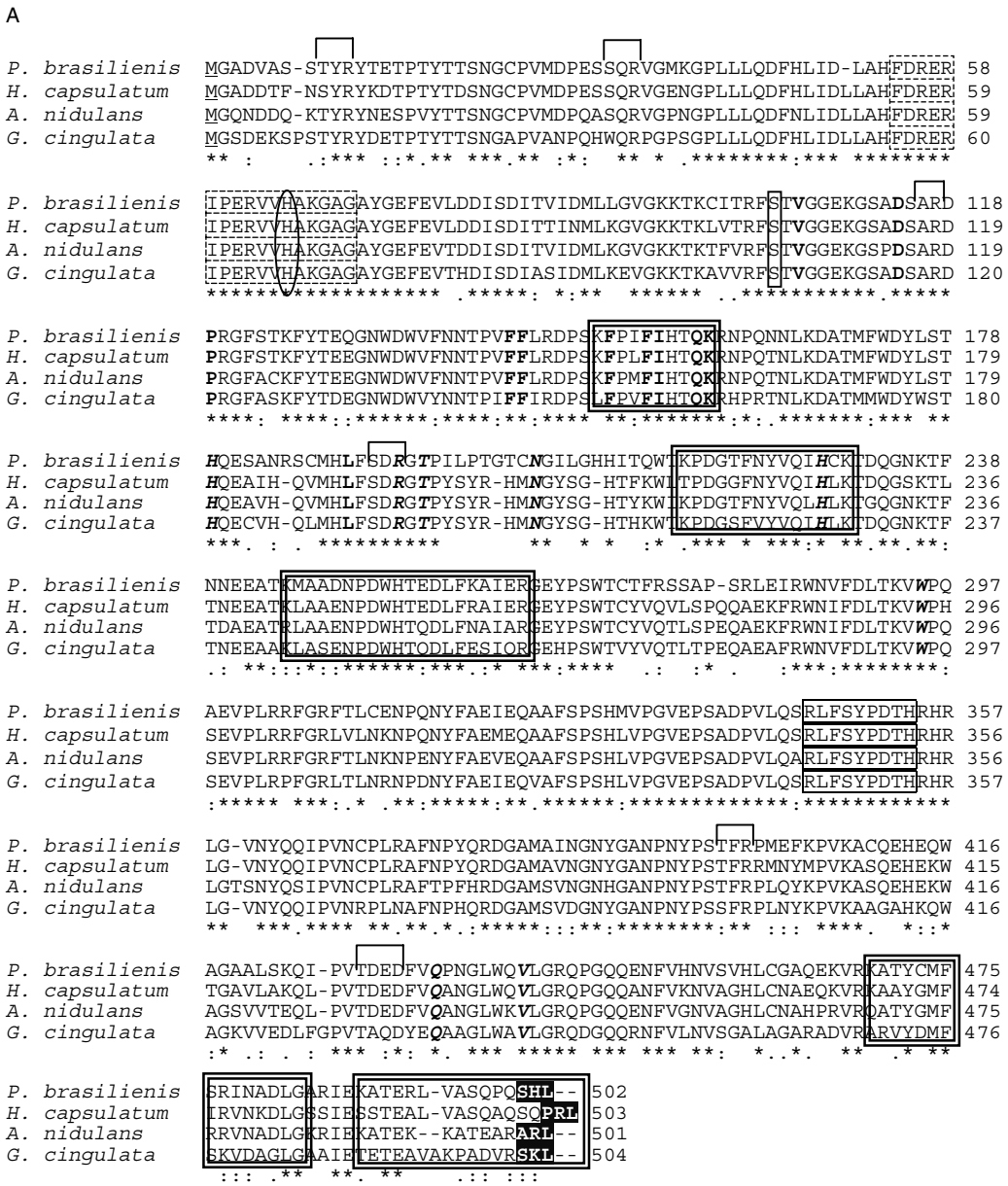

Figure 2. Alignment of sequences of catalases from several species. (A) Comparison of the deduced amino acid sequence of *Pb*CATP with those of catalases from eukaryotes. The amino acids are given in single-letter code. Asterisks indicate conserved amino acid residues. Double and single dots denote a decreasing order of matching similarity between each corresponding amino acid pair. The residues of the five peptides of the native catalase are double-boxed. The residues related to the probable catalytic site are boxed with a broken line and the conserved H is marked by a circle. The residues related to the haem binding are boxed; those concerned to the NADPH are in bold italics and those related to the substrate interaction are in bold. Phosphorylation residues are in brackets. The PTS-1 signal is marked by a black box. The rectangle marks the S described as essential for the folding of catalases. (B) Phylogenetic tree illustrating the relationship of *Pb*CATP to other related sequences. Catalases P from 59 species (named with species binomial name) were aligned and subjected to phylogenetic analysis using maximum parsimony and minimum evolution (neighbour-joining). The numbers on the branches are bootstrap values obtained with 100 replications and indicate the percentage of times all species to the right appear as a monophyletic cluster. GenBank (gb) and Swiss-Prot (sp) Accession Nos are indicated

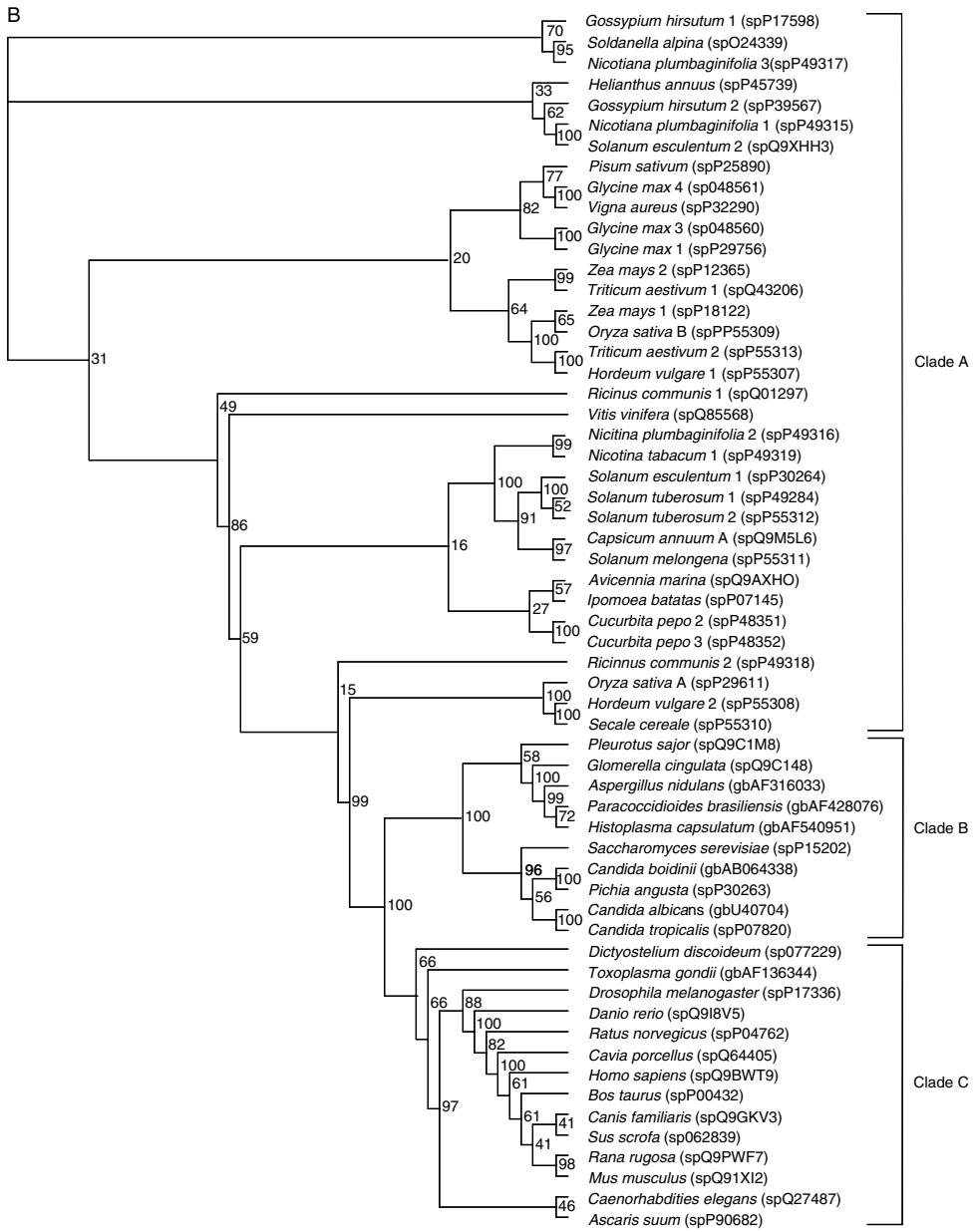

Figure 2. Continued

one containing human fungal sequences, with *Candida* species forming a subgroup of strong evolutionary relationship. The peroxisomal catalase of *Saccharomyces cerevisiae* occupied a derived position in this subgroup. The other subclade comprised catalases P of *P. brasiliensis* and other fungal pathogens of humans and plants. A higher relatedness was found between fungal, animal and protozoan catalases P, that were more closely related to each other than to plants, presenting 100% bootstrap confidence levels of branches.

Catalase P expression in different developmental phases of *P. brasiliensis*

To determine the expression of the catalase P in *P. brasiliensis*, we initially performed two-dimensional gel electrophoresis analysis of newly

synthesized proteins, as shown in Figure 3. We also performed analysis of the transcript. The catalase of 61 kDa, pI 6.2, characterized as a peroxisomal protein, was developmentally regulated in *P. brasiliensis*. *De novo* protein synthesis was strongly detected in the yeast parasitic phase, when compared to the mycelium phase (Figure 3A). Likewise, the transcript of 2.4 Kb was more abundant in the yeast phase (Figure 3B). Catalase mRNA levels were standardized against ribosome protein L35 mRNA levels, that do not vary in the different developmental phases of *P. brasiliensis* (Jesuino *et al*., 2002). We also analysed the levels of catalase P synthesis in the mycelium-to-yeast transition, by pulse-labelling the cells on successive days after the temperature shift from 22 °C to 36 °C. The synthesis of catalase P was increased during the transition, with higher values 10 days after the temperature shift (Figure 4A). At this time 80% of the cells had differentiated (data not shown). The increase in levels of catalase was confirmed by Western blot analysis of the extracts obtained from the cells probed with the mAb to catalase P from *T. gondii* (Figure 4B). The protein level increased during the mycelium-to-yeast transition. The level of catalase transcript was also analysed (Figure 4C). The 2.4 Kb mRNA transcript was increased at 5 and 10 days after the temperature shift, with higher levels at 10 days of differentiation. Adaptation of cellular antioxidant mechanisms during cell differentiation has been described previously. *Neurospora crassa* conidia present 60 times more catalase activity than hyphae growing in a liquid medium (Hansberg, 1996; Díaz *et al*., 2001). Also, the catalase B activity in *A. nidulans* is regulated in a developmental way along the fungus life cycle; the protein is barely detectable in spores and starts to accumulate at mycelium growth (Kawasaki *et al*., 1997; Kawasaki and Aguirre 2001). The generation of reactive oxygen species has been described during the transition of dimorphic fungi. The formation of hyphae, acknowledged as a major factor in *C. albicans* pathogenicity, was shown to be associated with a marked increase of reactive oxygen species (Schröter *et al*., 2000).

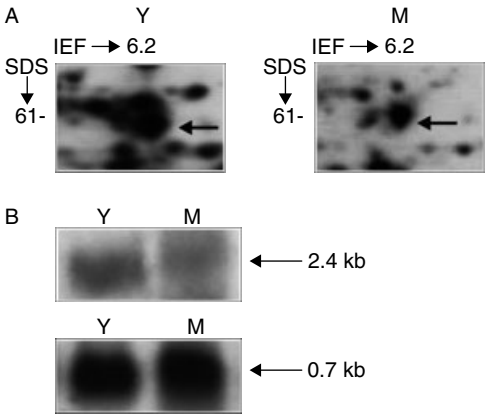

Figure 3. Analysis of the catalase P and its transcript in the developmental stages of *P. brasiliensis*, yeast (Y) and mycelium (M). (A) 2D protein synthesis patterns in yeast and mycelium forms. Yeast cells grown at 36 °C and mycelium at 22 °C were incubated for 12 h with [^{35}S]-L-methionine (50 μCi). The cells were processed and the samples containing 200 000 cpm (counts/min) were submitted to electrophoresis. Arrow indicates the localization of the *Pb*CATP, as determined by microsequencing of the native protein. (B) Northern blot analysis of *PbcatP* transcripts. Total RNA from yeast and mycelium was fractionated on a 1.2% formaldehyde agarose gel and hybridized to the 690 bp fragment of catalase. The membrane was washed and probed to the cDNA encoding a L35 ribosomal protein of *P. brasiliensis* (GenBank Accession No. AY 057112). The RNA sizes were calculated using the 0.24–9.5 marker RNA Ladder (GIBCO™, Invitrogen)

Catalase expression in response to H_2O_2

Western blot analysis was performed to examine the abudance of the *Pb*CATP in cells treated with H_2O_2. The induction of catalase P occurs 20 min after the addition of H_2O_2 (Figure 5B). The protein levels remained unchanged for 6 h after addition of H_2O_2. A rapid response was seen in *Pb*CATP expression when exogenous H_2O_2 was added to cultures. Sensing of H_2O_2 is rapid, suggesting that *Pb*CATP could exert a prompt effect against this toxic species. The enzymatic activity of catalase, in which H_2O_2 is degraded, serves to protect cells from endogenously produced oxygen radicals. One can speculate about protection from exogenous H_2O_2, a mechanism that could potentially facilitate parasite survival during infection. Future work will focus on this subject.

The role of catalases in the virulence of pathogenic fugi is not currently clear. In spite of all the information obtained to date regarding the *Pb*catP, the ability to delete or inactivate this gene or any gene from *P. brasiliensis* has not been accomplished. Once the molecular tools are available, the appropriate studies can be performed

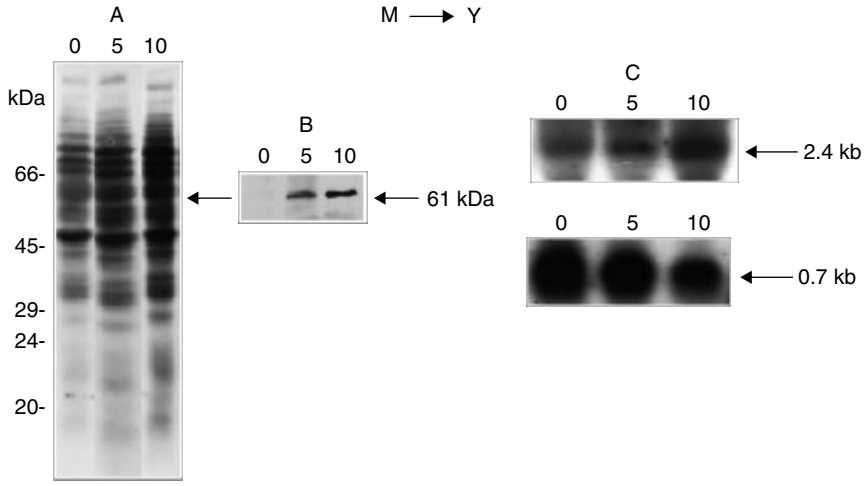

Figure 4. *Pb*CATP expression during the dimorphic transition of *P. brasiliensis*. (A) Phase transition protein patterns synthesis during the transition from mycelium (M) to yeast (Y). M was incubated for 6 h with [^{35}S]-L-methionine (10 μCi), at 0, 5 and 10 days after the temperature shift (22 °C to 36 °C). Samples containing 50 000 cpm were processed and the electrophoresis was performed on a 12% SDS–PAGE. Autoradiography was obtained. (B) Western blot of protein extracts from cells in differentiation at 0, 5 and 10 days after the temperature shift. The protein extracts were probed with the mAb anti-catalase P of *T. gondii*. For each lane 25 μg proteins were electrophoresed on a 12% (SDS–PAGE) Laemmli gel and transferred to nitrocellulose for Western blot experiments. (C) Northern blot analysis of the catalase P transcript during the transition from mycelium to yeast at 0, 5 and 10 days of differentiation. Total RNA (10 μg) from the mycelium in differentiation to yeast was fractionated on a formaldehyde agarose gel (1.2%) and hybridized to the 690 bp fragment encoding catalase. The membrane was washed and probed to a cDNA encoding a L35 ribosomal protein of *P. brasiliensis* (GenBank Accession No. AY057112). The RNA sizes were calculated using the 0.24–9.5 marker RNA Ladder (GIBCO™, Invitrogen)

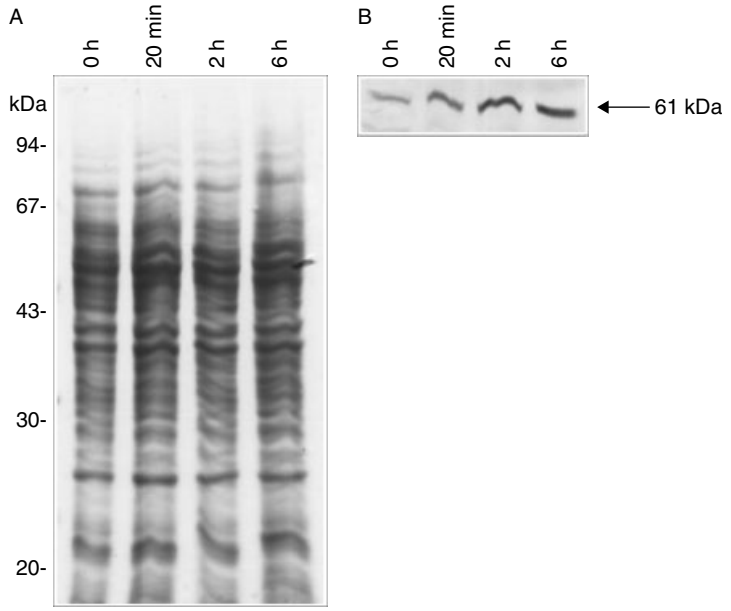

Figure 5. Induction of Catalase P by H_2O_2. (A, B) Yeast cells were treated with 50 mM phosphate buffer (pH 7.0) containing 15 mM H_2O_2, at times denoted on the top of the each lane. The cells were washed and the proteins extracts were obtained. (A) The proteins (25 μg) were fractionated on a 12% SDS–PAGE and stained by Coomassie blue. (B) After transferring to nylon membrane, the proteins were probed to the mAb anti-catalase P of *T. gondii*

to determine the role of this particular gene in the conversion from mycelium to yeast and in thwarting host defences.

Acknowledgements

The authors thank Dr George S. Deepe Jr, Division of Infection Diseases, University of Cincinnati, for providing invaluable discussion and for the critical review of the manuscript. We also wish to thank to Dr Keith A. Joiner, Yale University School of Medicine, New Haven, USA, for kindly providing the anti-catalase antibody of *Toxoplasma gondii*. We also thank Renata de Bastos Ascenço Soares for helpful assistance. This work was supported by grants from CNPq and FUNAPE-UFG.

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Original article

Isolation and partial characterization of a 30 kDa adhesin from *Paracoccidioides brasiliensis*

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Received 27 January 2004; accepted 7 February 2005

Available online 12 April 2005

Abstract

The virulence of *Paracoccidioides brasiliensis* can be attenuated or lost after long periods of repeated subculturing and reestablished after animal inoculation. Only one adhesin (gp43) has been described until now, among the various identified components of *P. brasiliensis*, and gp43 shows adhesion to laminin. Thus, the present study was designed to isolate and characterize factors putatively related to the capacity of this fungus to adhere to the host by comparing *P. brasiliensis* samples, taken before and after animal inoculation. The two samples differed in their pattern of adhesion and invasion. The sample recently isolated from animals (Pb18b) demonstrated a greater capacity to adhere and to invade the Vero cells than the one subcultured in vitro (Pb18a). Extract from Pb18b also showed higher levels of protein expression than that from Pb18a, when two-dimensional electrophoresis gels were compared. A protein species of 30 kDa, p*I* 4.9, was more evident in the Pb18b extract and had properties of adhesin. Laminin, but none of the other extracellular matrix (ECM) components, such as fibronectin, collagen I and IV, bound specifically to the *P. brasiliensis* 30 kDa protein. The roles of 30 kDa and gp43 in cellular interactions were investigated and the adhesion of *P. brasiliensis* yeast cells was intensively inhibited by pre-treatment of epithelial cells with 30 kDa protein and gp43. Thus, this study presents evidence that adhesion capacity could be related to virulence, and that a 30 kDa adhesin accumulated differentially in samples with different levels of pathogenicity. This protein and its adhesion characteristics are being published for the first time and may be related to the virulence of *P. brasiliensis*.

Keywords: *Paracoccidioides brasiliensis*; Adhesin; 30 kDa protein; Laminin

1. Introduction

Paracoccidioides brasiliensis is a dimorphic fungus, known to produce a systemic disease in humans. The disease begins in the lungs and then disseminates to the other organs and systems [1]. The diversity of clinical forms of the disease and the occurrence of asymptomatic or subclinical infection are based on host-related factors, immunological status and characteristics of the infecting agent, mainly its virulence [2,3]. Specific features of the fungus that may play a critical role in infection include cell-wall composition (α-1,3 glucan), ability to grow at 37 °C, dimorphism, production of 43 kDa glycoprotein (gp43), proteinases, and the ability to adhere to host tissues [4–6].

P. brasiliensis synthesizes some substances, with the physico-chemical and biological characteristics of antigens that take part direct or indirectly in the parasite-host relation-

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doi:10.1016/j.micinf.2005.02.005

ship. The antigenic structure of *P. brasiliensis* is relatively complex [7]. Correlation of some of these components with its pathogenicity is of great relevance [8,9] and knowledge of its cell-wall composition and exocellular components has facilitated identification of the cell-surface ligands. Components of the extracellular matrix (ECM) are the host proteins that the fungal cell-wall proteins can bind to [10].

The adhesion of *P. brasiliensis* to epithelial or alveolar cells should provide fertile ground for the study of the mechanisms of fungal adherence and host defense. Previous work in our laboratory has demonstrated that one isolate has the capacity to invade Vero cells [11] and that the adherence phenomenon varied, depending on the strain. Different levels of adhesion were observed between the strains Pb18 and Pb265, the former being the more adherent [9].

The virulence of *P. brasiliensis* can be attenuated or even lost after consecutive cycles of subculturing over long periods [12] and reestablished after passage [13,14] in animals and epithelial cell culture (unpublished data). However, putative factors involved in the virulence of these strains have not yet been identified. Some authors have analyzed the gp43 [15] found in the serum and urine of patients [16,17], which was also involved in the *P. brasiliensis* adhesion mechanisms and laminin binding [8,9]. But it is probable that other fungal components participate in the adhesion process [18].

The successful colonization of the host by this fungus is, therefore, a complex event, generally involving ligands coded by the pathogen (adhesins) and a cell receptor. The identification of these molecules would represent a step towards the discovery of efficient treatments for systemic mycoses. It is known that during hematogenic dissemination, interactions of this nature constitute the first stages in the development of innumerable infections [19]. The aim of the work described here was thus to isolate and characterize factors related to adhesion by comparing *P. brasiliensis* samples before and after animal inoculation.

2. Materials and methods

2.1. Microorganism

Strain 18 of *P. brasiliensis* (Pb18) was isolated from a clinical case of paracoccidioidomycosis (PCM) and maintained in the Faculty of Medicine of the University of Sao Paulo (FM-USP), Brazil. During the current work, Pb18 was grown in PYG medium (peptone, yeast extract and glucose) at 35 °C and subcultured each 3–4 days, 72 times, to yield sample Pb18a.

2.2. Reisolation of P. brasiliensis

Pb18 was inoculated into male hamster intratesticularly, using 0.2 ml of a standard suspension of 2.0×10^6 yeasts per ml per animal. After 30 days, the animals were sacrificed and the testicles macerated and cultured on Sabouraud agar with chloramphenicol at 25 °C until the development of characteristic *P. brasiliensis* mycelial colonies. These were identified and incubated at 35 °C in PYG medium, to obtain the yeast phase [20], and the resulting sample was labeled Pb18b.

2.3. Assay of P. brasiliensis-mammalian cell interaction

Cultures of Vero cells (Africa Green Monkey Kidney) obtained from the American Type Culture Collection were maintained in Medium 199 (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Cult lab, Brazil). Cells were cultured at 36.5 °C in 24-well plates, with the well-bottoms covered with coverslips, and adjusted to 1.0×10^6 cells per well. Next, 1.0×10^6 yeast cells per ml of *P. brasiliensis* (either 18a or b) were added to the epithelial cells and incubated for from 30 min to 5 h at 36.5 °C, to follow the processes of adhesion and invasion. After the period of incubation, the medium was discarded, the cells washed with phosphate-buffered saline (PBS) and the coverslips fixed with 2% paraformaldehyde and stained in May Grünwald–Giemsa. The coverslips were examined by conventional microscopy and the adherent fungal cells were counted and a graph produced, to evaluate the kinetics of interaction of *P. brasiliensis* (samples 18a and b) with Vero cells. Results were expressed as the mean of three determinations ± standard deviations (S.D.).

2.4. Cell-free extract

Cell-free extracts were prepared from the 18a and b isolates in yeast form, as described elsewhere [21]. About 300 mg of *P. brasiliensis* cells were grown for 3–4 days on PYG solid medium, then scraped off and mixed with 1 ml of PBS, pH 7.2. This mixture was vortexed for 30 s and centrifuged at $560 \times g$ for 1 min. The supernatant (cell-free extract) was removed and stored at –20 °C.

2.5. Anti-cell-free sera

Polyclonal sera, against both *P. brasiliensis* cell-free extracts, were prepared in rabbits by intradermal injections of 1.0 ml of extract mixed with 1.0 ml of Freund's complete adjuvant. Subsequent injections of extract mixed with incomplete adjuvant were given weekly for a period of 4 weeks and then monthly for a period of 3 months. The rabbits were bled 7 days after the last dose. The immunoglobulin fractions of the antisera were separated by precipitation with ammonium sulfate and stored at –70 °C.

2.6. Two-dimensional electrophoresis protein separation

The antigenic components of each *P. brasiliensis* sample were submitted to isoelectric focusing, as described by O'Farrell [22]. 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 (0.08 M 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 [23]. Gels were washed and the proteins stained with Coomassie blue [24].

2.7. Affinity chromatography

Affinity chromatography was employed to remove antigens common to the two *P. brasiliensis* samples. The immunoglobulin fraction of Pb18a antiserum was cross-linked with glutaraldehyde to form a solid matrix that would bind the antigens, as described elsewhere [25]. Pb18b cell-free extract was added to the solid matrix and incubated at 25 °C for 18 h. The mixture was centrifuged at 1400 × *g* for 15 min and the supernatant was collected and labeled 18b. The same procedure was followed with Pb18a cell-free extract (using an identical column) and this supernatant was named 18a. Both supernatants were analyzed for protein content by Lowry's method [26], sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [23] and immunoblot [27] were carried out on each sample with the respective anti-cell-free sera.

2.8. Purification of the 18b supernatant

The 18b supernatant was further purified by gel filtration. It was applied to a Sephadex G-50 column (60 by 1.6 cm; Pharmacia) previously equilibrated with PBS. Elution was carried out at room temperature. Fractions of 4 ml were collected at a flow rate of 1 ml/min and monitored for protein content at 280 nm. The protein-containing fractions were pooled and dialyzed against the same buffer for 48 h, then analyzed by SDS-PAGE (using 10% gels) [23] and immunoblotting [27], as previously described [16].

2.9. Protein binding assay by immunoblotting

One of the fractions purified from 18b, containing the 30 kDa protein, was characterized by immunoblotting. SDS-PAGE was performed basically as described by Laemmli [23] with minor modifications. Electrophoretic transfer blotting to a nitrocellulose membrane was carried out as described previously [27]. Blotted protein was assayed for type I and IV collagen, fibronectin and laminin binding as follows. The membranes were incubated with 1% BSA in 10 mM PBS (pH 7.4) containing 0.9% NaCl for 4 h at room temperature and then for 90 min in PBS-T-BSA (PBS, 1% BSA, 0.05% Tween 20) containing fibronectin (30 μg/ml) or laminin (30 μg/ml) or type I and type IV collagen (20 μg/ml). After washing four times (10 min each time) in PBS buffer containing 0.05% Tween 20 (PBS-T buffer), the sheets were incubated for 1 h with either rabbit anti-fibronectin antibody (diluted 1:1000) or rabbit anti-laminin Ab (diluted 1:1000) or rabbit type I and IV anti-collagen Ab (diluted 1:100) in PBS-T-BSA. The blots were washed with PBST and incubated with peroxidase-labeled goat anti-rabbit immunoglobulin (diluted 1:1000 in PBS-T-BSA). Finally, the blots were washed again, and reactive bands were developed with hydrogen peroxide and diaminebenzidine (Sigma) as the chromogenic reagent. As controls, the blots were incubated only with anti-laminin, anti-fibronectin and anti-type I and type IV collagen antibodies, in the absence of ECM proteins (laminin, fibronectin and type I collagen). The positive control was developed with a PCM patient serum (1:40).

2.10. Biotinylation of the protein

A purified fraction of 30 kDa was biotinylated with the ECL protein biotinylation kit (Amersham Pharmacia Biotech) as recommended by the manufacturer. Monolayers of Vero cells were incubated with the biotinylated protein at 37 °C for 5 h and washed with PBS, to remove unbound protein. Next, double-distilled water was added and the cells incubated for 4 h at room temperature, to obtain total lysis. The lysates were centrifuged at 1400 × *g* for 5 min, and the supernatant was submitted to electrophoresis by SDS-PAGE. Proteins in the gel were transferred to a membrane of nitrocellulose and the membrane strips were incubated with blocking buffer (PBS-T with 2% BSA) for 4 h at room temperature. Patterns were revealed with a kit, the Catalyzed Signal Amplification (CSA) System (DAKO). The negative control was developed with the supernatant of Vero cells after lysis them (without incubation with the biotinylated protein).

2.11. Inhibition assays

Vero cells were incubated for 1 h at 37 °C with the following proteins at 25 μg/ml, dissolved in PBS (pH 7.2): 30 kDa, gp43 and 30 kDa + gp43. After this incubation, cells were washed three times in 199 medium and allowed to interact with *P. brasiliensis* yeast cells for 2 and 5 h at 37 °C, as described above. In control experiments, the Vero cells were not pre-incubated with any kind of protein. The percentage of infected cells was determined by randomly counting a minimum of 300 cells on each duplicate coverslip, and experiments were repeated at least three times. The adhesion index was calculated by multiplying the mean number of attached fungi per Vero cell by percentage of infected cells, observed by microscopic examination with an oil-immersion objective. The invasion index was determined in the same way, but using the number of internalized fungi [28]. All experiments were performed in triplicate. The mean and S.D. of at least three distinct experiments were determined. Statistical analysis was calculated by using ANOVA (*F* test followed by Duncan test). *P* values of 0.05 or less were considered statistically significant.

3. Results

3.1. Kinetics of interaction of P. brasiliensis with epithelial cell culture

P. brasiliensis adhesion was observed at the same time, after 30 min of contact, with both Pb18a and 18b, respectively, the subcultured and reisolated samples. Yeast cells of both samples were seen in the interior of the cells (showing invasion) after 3 h of infection. Pb18a and 18b exhibited numerical differences in relation to adhesion and invasion, as demonstrated in Fig. 1. Sample 18b was able to adhere to and invade Vero cells more efficiently than 18a.

3.2. Two-dimensional protein analysis of P. brasiliensis cell-free extracts

The cell-free extracts of the two *P. brasiliensis* samples (Pb18a and b) were analyzed by two-dimensional gel electrophoresis. Sample 18b showed a higher total protein expression than 18a and a 30 kDa protein of p*I* 4.9 was much more strongly expressed by 18b (Fig. 2A and Fig. 2B). Pb18b also expressed other proteins that were not demonstrated in sample 18a, including those at 15 kDa and p*I* 6.7, 18 kDa and p*I* 6.9, 25 kDa and p*I* 5.1, 27 kDa and p*I*s 5.3 and 4.9, 50 kDa and p*I* 8.5, 65 kDa and p*I* 3.5, 66 kDa and p*I* 7.8, 67 kDa and p*I* 7.4, 71 kDa and p*I* 4.1 (labeled 1, 2, 3, 4, 5, 6, 7, 8 and 9, respectively in Fig. 2A′). On the other hand, sample 18a expressed two proteins of 38 kDa, with p*I*s of 6.9 and 7.1, which were not expressed by sample 18b (I and II respectively in Fig. 2B′).

3.3. Isolation and characterization of an adhesin-like protein

Cell-free extracts Pb18a and b were fractionated by affinity chromatography and one protein was isolated from Pb18b. Further purification and SDS-PAGE analysis resulted in the identification of a 30 kDa protein. The purified protein behaved as an adhesin, binding to the Vero cells in the biotinylation experiment (Fig. 3A). It had the capacity to bind to laminin (Fig. 3B), but not to fibronectin, type I or type IV collagen (data not shown).

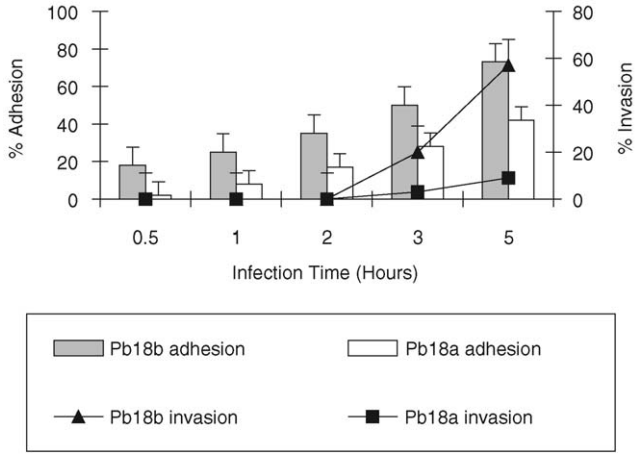

Fig. 1. Percentage of Vero cells showing yeast adhesion (histograms) and invasion (lines), plotted against time of incubation (hours) with two samples of *P. brasiliensis* strain 18, one subcultured in vitro (Pb18a) and the other reisolated from hamster (Pb18b).

3.4. Inhibition of the adhesion to and invasion of the epithelial cells by 30 kDa and gp43 fractions of P. brasiliensis

The adhesion and invasion indices obtained in the interaction of *P. brasiliensis* yeast cells with Vero cells treated with the 30 kDa (p*I* 4.9) and gp43 (p*I* 5.9) fungal proteins were compared with the control (less proteins), to calculate the percentage of adhesion and invasion inhibition by these fractions (Fig. 4). When epithelial cells were pre-treated with the 30 kDa molecule, the inhibition of adhesion and invasion were approximately 73% ($P < 0.05$) and 45% lower than those of non-treated cells after 2 h of contact and 72% ($P < 0.0005$) and 86% ($P < 0.0005$) after 5 h of infection, respectively. The pre-treatment of the Vero cells with gp43 decreased the adhesion and invasion indices of yeast cells by 56% ($P < 0.05$) and 48%, respectively, after 2 h of infection and by 65% ($P < 0.05$) and 70% ($P < 0.0005$), after 5 h.

Moreover, treatment with both the 30 kDa and gp43 proteins exerted significantly stronger inhibition in both the 2 and 5 h assays ($P < 0.0005$). The reduction in the adhesion index was 90% in 2 h and 86% in 5 h, while the invasion index decreased by 93% and 91% in the 2 and 5 h assays, respectively.

4. Discussion

The *P. brasiliensis* virulence mechanisms are not completely clarified. There is a consensus that successive subculturing of this fungus causes the loss of its pathogenicity, which can be reversed by passage in animals, with recovery of some factors of virulence [12]. Although these facts have already been much discussed in the literature, until now no published work has identified the possible factors involved in loss of virulence in the attenuated sample.

Studies have demonstrated the capacity of *P. brasiliensis* for adhesion and invasion [11] and that these attachment phenomena vary with the strain [9]. *P. brasiliensis* strain 18, considered highly pathogenic for animals [6] and more adherent to Vero cells [9], lost the capacity to adhere after consecutive cycles of subculturing over long periods (unpublished data), but it was reestablished after inoculation in animals or recovery from mammalian cell cultures. However, no factor involved in the adhesion and probably in the virulence of the samples has yet been identified. Thus, in the present work we compared protein extracts from *P. brasiliensis* 18 samples isolated before and after animal inoculation, as well as the capacity of each one to adhere to the Vero cells and ECM proteins. Our results showed distinct adhesion and invasion

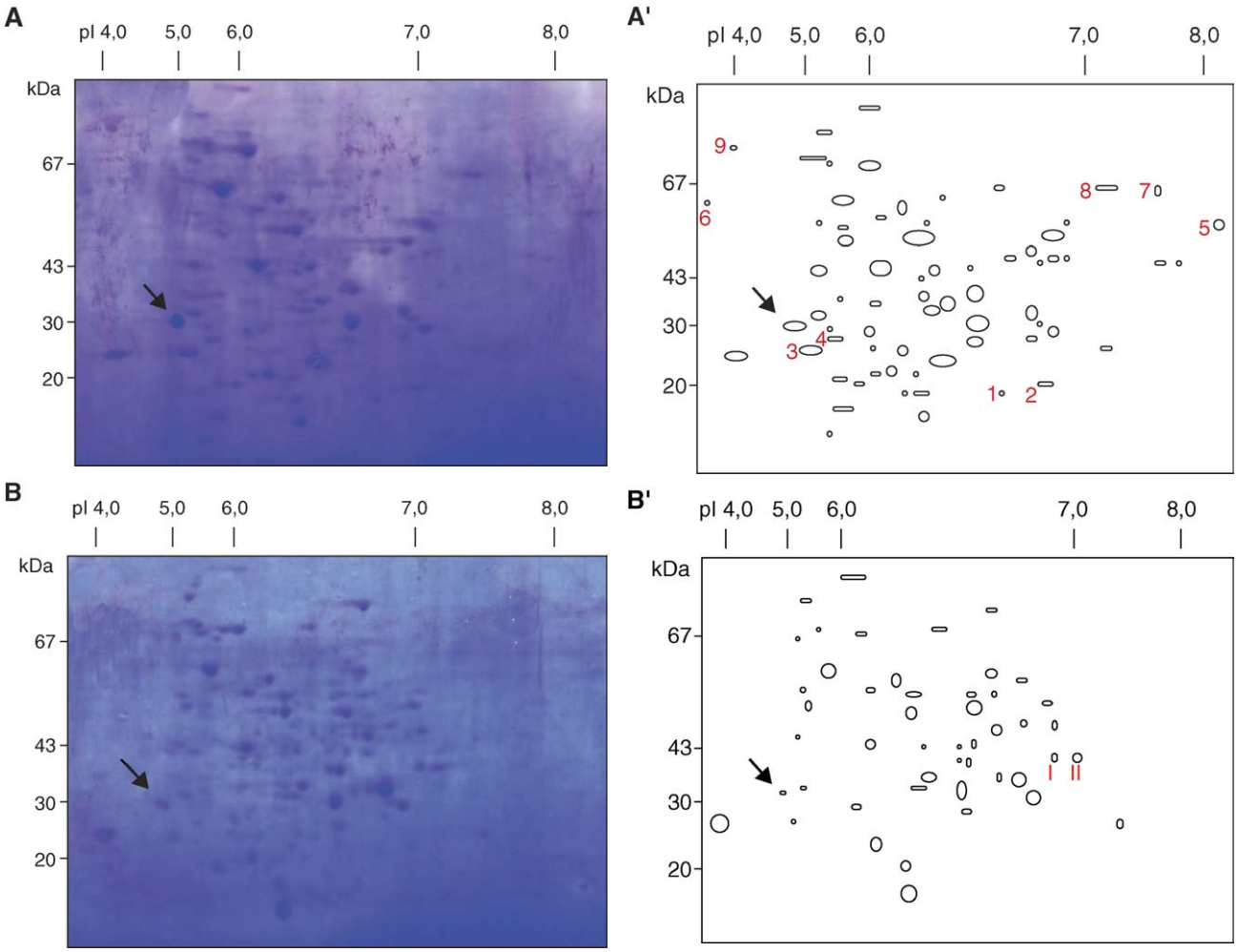

Fig. 2. A) Two-dimensional electrophoresis of *P. brasiliensis* yeast cells extract after passage in animal (Pb18b). Gel stained with Coomassie brilliant blue G250. The arrow points to the protein of mass 30 kDa and p*I* 4.9. A′) Synthetic image of staining pattern of gel in 2A. The arrow indicates the protein at mass 30 kDa, p*I* 4.9. (1) 15 kDa, p*I* 6.7; (2) 18 kDa, p*I* 6.9; (3) 25 kDa, p*I* 5.1; (4) 27 kDa, p*I* 5.3; (5) 50 kDa, p*I* 8.5; (6) 65 kDa, p*I* 3.5; (7) 66 kDa, p*I* 7.8; (8) 67 kDa, p*I* 7.4; (9) 71 kDa, p*I* 4.1. B) Two-dimensional electrophoresis of *P. brasiliensis* extract of yeast cells subcultured 72 times in vitro (Pb18a). Gel stained with Coomassie brilliant blue G250. The arrow points to the protein of mass 30 kDa and p*I* 4.9. B′) Synthetic image of staining pattern of gel in 2A. The arrow indicates the protein at mass 30 kDa, p*I* 4.9. (I) 38 kDa, p*I* 6.9; (II) 38 kDa, p*I* 7.1.

patterns for the two samples. The newly-isolated sample (18b) demonstrated a higher capacity to adhere to and invade the Vero cells than the subcultured sample (18a).

P. brasiliensis produces various substances with or without antigenic characteristics during fungal growth. Such antigens interact with the host in some way, probably in the adhesive processes and/or with the cellular and humoral immune system. Some proteins such as gp43 have been indicated as antigenic molecules of high specificity and great importance in serodiagnosis [15,17], as well as, more recently, being implicated in the adhesion process [8,9]. Hanna et al. [9] also speculated that other components of this fungus might participate, since the inhibition of adhesion by gp43 was only partial. Cell-free extract [21] corresponds to the most superficial components of the fungal cell and probably enters in contact with the host cells most directly. Protein extracts of various microorganisms have been analyzed by two-dimensional electrophoresis. In *P. brasiliensis*, this methodology was used to characterize new antigens of the yeast phase and to compare their expression with that in the filamentous phase [29]. In our study, two-dimensional electrophoresis was also used to characterize and differentiate the protein profiles of the two samples. Sample 18b showed higher protein expression than sample 18a. Nine proteins were expressed differently in the samples and the three most prominent examples had molecular masses of 67, 50 and 30 kDa.

The ability of some fungi to adhere to host surfaces and components of the ECM is an important factor in pathogenesis. Some types of adhesins that interact with receptors seem to exist in a number of different fungi, and host ECM components are of great importance in the modulation of the migration, invasion, differentiation and fungal proliferation. Thus, ECM proteins like laminin, collagen, fibronectin, fibrinogen and the C3 complement are being studied as initial targets for attachment of microorganisms [10].

In recent years, several molecules with receptor-like characteristics have been described in pathogenic fungi such as *C. albicans* [30], *A. fumigatus* [31] and *S. schenckii* [32].

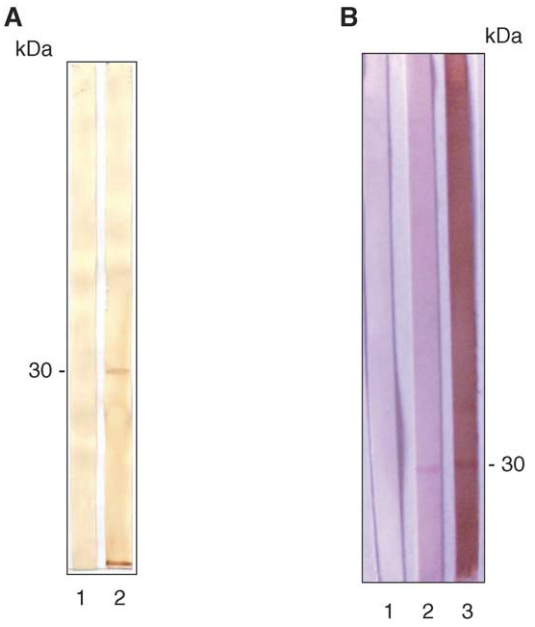

Fig. 3A. A) Characterization of 30 kDa protein as an adhesin. (1) Negative control: lysed Vero cells not treated with biotinylated protein; (2) lysed Vero cell treated with biotinylated 30 kDa protein. B) Immunoblotting of 30 kDa fraction treated with laminin and anti-laminin serum and peroxidase-conjugate anti-rabbit immunoglobulin G. (1) Incubated with anti-laminin serum alone (negative control); (2) treated with laminin, then incubated with anti-laminin serum; (3) serum from patient with PCM at 1:40 (positive control).

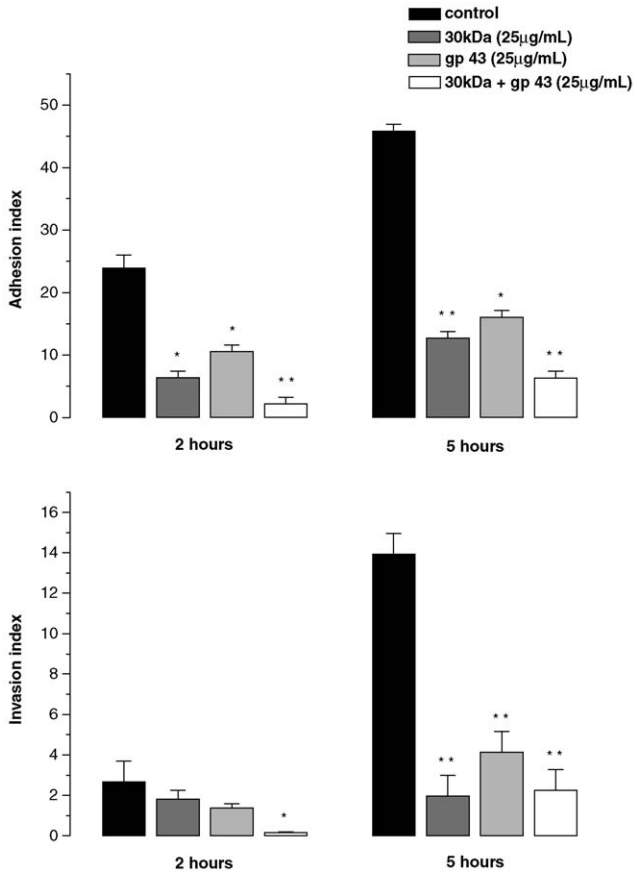

Fig. 4. Assay of inhibition of the interaction between *P. brasiliensis* yeast cells and Vero cells by 30 kDa and gp43 proteins. The Vero cells were pre-treated or not (control) for 1 h with 25 ug/ml of 30 kDa, gp43 and 30 kDa + gp43, before the interaction with yeast cells for 2 and 5 h The adhesion and invasion index values represent the mean ± S.D. of three independent experiments performed in triplicate. One asterisk denotes values statistically different from control ($P < 0.05$) and two asterisks denote $P < 0.0005$.

Most of these microbial molecules are glycoproteins present in the cell-wall and are known as adhesins, displaying properties similar to integrins or lectins [33]. In *P. brasiliensis*, until now only gp43 has been shown to adhere to laminin [8]. Other components of the ECM, such as fibronectin, type I and IV collagens, may also participate in the process of adhesion of this fungus [18].

In the present work, we isolated and characterized a protein of 30 kDa and p*I* 4.9, expressed more strongly in the *P. brasiliensis* sample 18b, with adhesin-like properties according to the binding assay. This protein was capable of binding to laminin, but not to the other ECM components, fibronectin, type I or type IV collagen. Laminin, the main component of the basal membrane [34], is a large glycoprotein that plays an important role not only in cell adhesion, but also during tissue invasion and metastasis in neoplastic cells and with in cell-pathogen interaction [35]. Laminin-linker proteins can contribute to pathogenesis of the infection by mediating the adhesion to the host cells and maintaining the fungus in the tissues.

The presence of laminin binding adhesins on the surface of yeast cells of *P. brasiliensis* and their role in cellular interactions were investigated. Most of our knowledge concerning the virulence determinants of pathogenic fungi comes from the infected host, mainly from animal models and more recently from in vitro studies with cell cultures. The fungi usually exhibit intra- and/or extracellular host-parasite interfaces, where the phenomenon of parasitism depends on complementary surface molecules [18]. Many critical aspects of the interaction between fungal and host cells, such as adhesion, immunosurveillance and immunomodulation, are mediated by host recognition and interaction with the glycoprotein layer [33,36,37]. In support of this observation, the adhesion of *P. brasiliensis* yeast cells was intensely inhibited by pre-treatment of epithelial cells with both 30 kDa protein and gp43. Vero cells, pre-treated with the 30 kDa molecule, showed stronger inhibition of adhesion and invasion than those pre-treated with gp43. On the other hand, a combination of the 30 kDa and gp43 proteins significantly further decreased the adhesion and invasion indices. To conclude, in this study it has been demonstrated that in *P. brasiliensis* the adhesion capacity is related to virulence and some components are differently expressed in the more and less pathogenic samples. Moreover, an adhesin of 30 kDa, that may play a role in the virulence of this fungus, is described here for the first time. *P. brasiliensis* surface molecules that bind to host cell receptors during adhesion and invasion may be of interest for developing vaccines and receptor-blocking therapies.

Acknowledgements

This investigation was financially supported by the Brazilian Organizations, FAPESP, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), CAPES and FCF-UNESP. We thank Tirene Pavanelli, Eliana Regina Cainelli, Rosângela Aparecida Moraes da Silva and Elaine Toscano Miranda for their technical assistance.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 26, Issue of July 1, pp. 24706–24714, 2005

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

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

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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.

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

[S] The on-line version of this article (available at http://www.jbc.org) contains nine additional tables.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) CA580326-CA584263, CN238087-CN253933, and CN373644-CN373755.

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

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

P. brasiliensis genome size was estimated to be ~30 Mb (7). A study of *P. brasiliensis* gene density suggests that this fungus contains between 7,500 and 9,000 genes,[2] which is in agreement with the estimated gene number for ascomycete fungi genomes.

Here are presented the results of an effort to achieve a comprehensive metabolic view of the *P. brasiliensis* dimorphic life cycle based on analysis of 6,022 groups generated from both mycelium and yeast phases. This view arises from both a general metabolism perspective and the identification of the precise metabolic points that distinguish both morphological phases. Overexpressed genes and those that are up- or down-regulated in both stages were identified. Expression levels were assessed by cDNA microarrays and some were confirmed by Northern blot. Drug targets and genes related to virulence were also detected in several metabolic pathways. Finally, the majority of genes involved in signal transduction pathways (cAMP/protein kinase A, Ca^{2+}/calmodulin, and MAPKs) possibly participating in cell differentiation and infection were annotated, and now we are able to describe the corresponding signaling systems in *P. brasiliensis*.

MATERIALS AND METHODS

Fungus—*P. brasiliensis* isolate Pb01 (ATCC MYA-826) was grown at either 22 °C in the mycelium form (14 days) or 36 °C as yeast (7 days) in semisolid Fava Neto's medium. Following incubation, cells were collected for immediate RNA extraction with Trizol reagent (Invitrogen).

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

EST Processing Pipeline and Annotation—PHRED quality assessment and computational analysis were carried out as previously described (8). EST assembly was performed using the software package CAP3 (9) plus a homemade scaffolding program. Sequences of at least 100 nucleotides, with PHRED ≥20, were considered for clustering. A total of 20,271 ESTs were selected by these exclusion criteria. Contaminant and rRNA sequences were then removed to generate a set of 19,718 ESTs, which was submitted to CAP3 clustering, generating 2,655 contigs and leaving 3,367 ESTs as singlets. Contigs plus singlets comprise the base set of 6,022 *P. brasiliensis* assembled EST sequences (PbAESTs) that underwent further analysis. Annotation was carried out using a system that essentially compared these assemblies with sequences available in public databases. The BLASTX program (10) was used for annotation along with GenBank™ nonredundant (nr), cluster of orthologous groups (COG), and gene ontology (GO) data bases. The GO data base was also used to assign EC numbers to assemblies. Additionally, we used the FASTA program (11) to compare assemblies with *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* predicted polypeptides. The INTERPROSCAN program (12) was used to obtain domain and family classification of the assemblies. Metabolic pathways were analyzed using maps obtained in the KEGG Web site (13) with annotated EC numbers, and this information was used to help in assigning function to PbAESTs.

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

cDNA Microarrays and Data Analysis—A set of two microarrays containing a total of 1,152 clones in the form of PCR products was spotted in duplicate on 2.5 × 7.5-cm Hybond N$^+$ nylon membranes (Amersham Biosciences). Arrays were prepared using a Generation III Array Spotter (Amersham Biosciences). Complementary DNA inserts of both *P. brasiliensis* libraries were amplified in 96-well plates using vector-PCR amplification with T3 forward and T7 reverse universal primers. Membranes were first hybridized against the T3 [α-^{33}P]dCTP-labeled oligonucleotide. The amount of DNA deposited in each spot was estimated by the quantification of the obtained signals. After stripping, membranes were used for hybridization against α-^{33}P-labeled cDNA complex probes. The latter were prepared by reverse transcription of 10 μg of filamentous or yeast *P. brasiliensis* total RNA using oligo(dT)$_{12-18}$ primer. One hundred microliters of [α-^{33}P]cDNA complex probe (30–50 million cpm) was hybridized against nylon microarrays. Imaging plates were scanned by a phosphor imager (Cyclone; Packard Instruments) to capture the hybridization signals. BZScan software was employed to quantify the signals with background subtraction. Spots were matched with a template grid. The ratio between vector and cDNA complex probe hybridization values for each spot was used as the reference normalization value. Total intensity normalization using the median expression value was adopted as previously described (15). Gene expression data analyzed here were obtained from three independent determinations for each phase (filamentous or yeast). We used the significance analysis of microarrays method (16) to assess the significant variations in gene expression between both mycelium and yeast. Briefly, this method is based on *t* test statistics, specially modified to high throughput analysis. A global error chance, the false discovery rate, and a gene error chance (*q* value) are calculated by the software.

Northern Blot Analysis—Total RNA (15 μg) was separated in a 1.5% denaturing formaldehyde agarose gel and transferred to a Hybond-N nylon membrane (GE Healthcare). Probes were radiolabeled with the random primers DNA labeling system (Invitrogen) using [α-^{32}P]dATP. Membranes were incubated with the probes in hybridization buffer (50% formamide, 4× SSPE, 5× Denhardt's solution, 0,1% SDS, 100 μg/ml herring sperm DNA) at 42 °C overnight and then washed twice (2× SSC, 1% SDS) at 65 °C for 1 h. Signal bands were visualized using a Typhoon 9210 phosphor imager (GE Healthcare).

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

RESULTS

Transcriptome Features—In sequencing the *P. brasiliensis* transcriptome, EST data were generated from nonnormalized cDNA libraries of mycelium and yeast cells. The size range of the cDNA inserts ranged from 0.5 to 2.5 kb. Single pass 5′ sequencing was performed on 25,598 cDNA clones, randomly selected from both libraries. Upon removal of bacterial and rRNA contaminant sequences, a total of 19,718 high quality ESTs underwent CAP3 assembly, yielding 2,655 contigs and 3,367 singlets, which constitute the so-called 6,022 *P. brasiliensis* Assembled EST (PbAEST) data base. Contigs presented an average size of 901 bp, and the number of ESTs assembled into contigs varied from 2 to 657 in the largest one (PbAEST 1068), which corresponds to M51, a previously reported *P. brasiliensis* mycelium-specific transcript (17). Of the 6,022 PbAESTs, 4,198 (69.4%) showed a probable homologue in GenBank™, and 4,130 (68.3%) showed a fungus homologue (Fig. 1*A* and Supplemental Table I). We had used MIPS functional categories to classify 2,931 PbAESTs into 12 major groups. *P. brasiliensis* showed a slightly higher percentage of PbAESTs (4%) related to cellular communication and signal transduction (Fig. 1*B*) compared with *S. cerevisiae* functional categorization (3.4%).

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

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

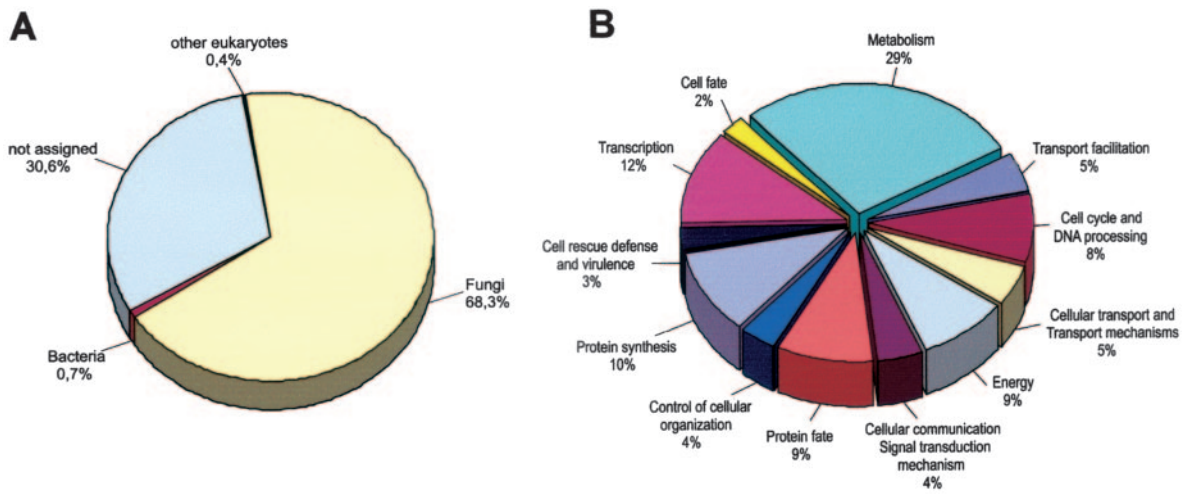

FIG. 1. ***P. brasiliensis* transcriptome characterization.** *A*, distribution of blast best hit among organisms. Each PbAEST was tested against the GenBank™ nr data base, and the best hit organism was computed. A PbAEST was considered as not assigned when the best hit exceed an *E* value of 10^{-10}.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

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

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

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

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

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

In contrast, *P. brasiliensis* yeast cells overexpress the genes encoding alcohol dehydrogenase I and pyruvate dehydrogenase E1 subunit (Table I and Fig. 3); the latter can be detected in high levels in cultures of *S. cerevisiae* grown both anaerobically

TABLE I

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

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

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

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

and aerobically in the presence of ethanol (23). The carbohydrate metabolism is probably shifted toward ethanol production, reflecting the anaerobic behavior of the yeast form as previously reported (24). Several pathways that provide substrates for the glyoxylate cycle are up-regulated in the yeast cells (Table I and Fig. 3). First, isocitrate lyase redirects the metabolic flow using ethanol and acetate as two-carbon sources and generating oxaloacetate, which can be reconverted to glu-

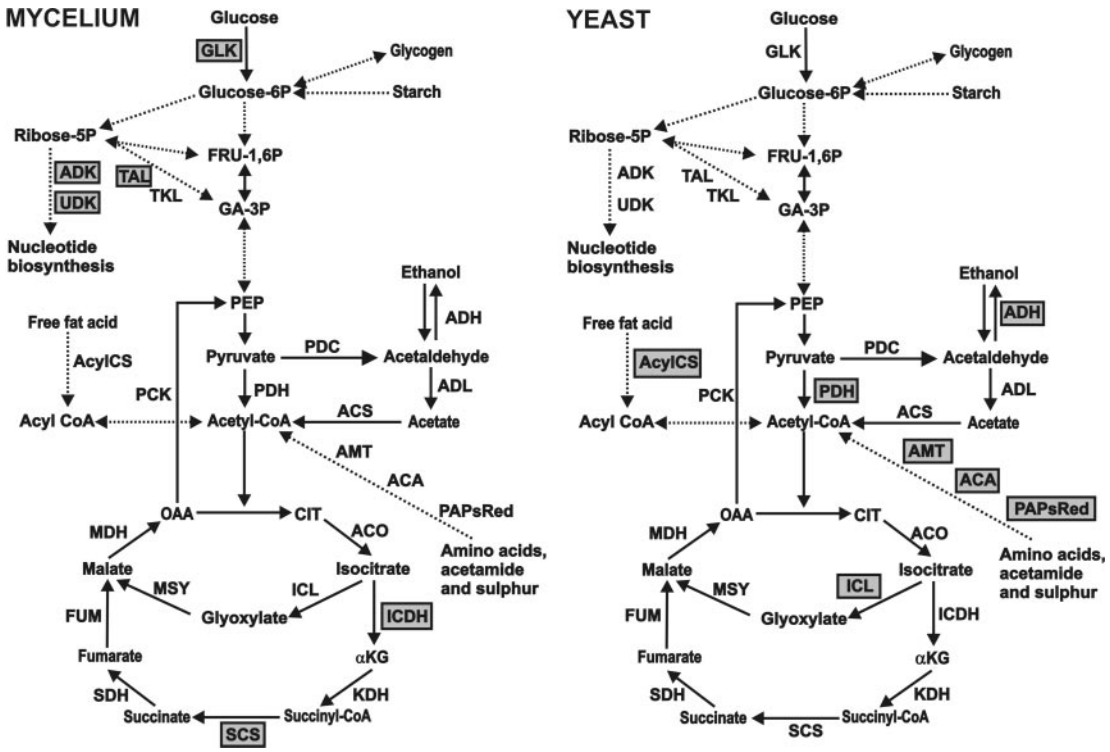

FIG. 2. **Comparison of the expression pattern of genes encoding for enzymes in mycelium-to-yeast cell differentiation of *P. brasiliensis*.** For the detailed metabolic comparison between mycelium and yeast metabolism, see Supplemental Table IV, since we have presented in this figure only the central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

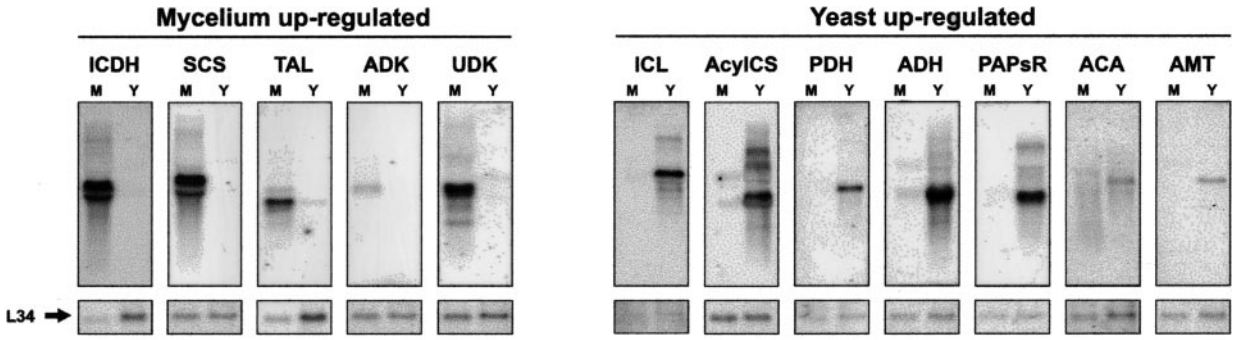

FIG. 3. **Northern blot analysis of mycelium and yeast up-regulated genes of *P. brasiliensis*.** Total RNA samples from both mycelium (*M*) and yeast (*Y*) were blotted onto nylon membranes and hybridized against gene-specific radiolabeled probes. *ICDH*, isocitrate dehydrogenase; *SCS*, β-succinyl-CoA-synthetase,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

cose. In addition, the branched-chain aminotransferase gene is also overexpressed (as are other aminotransferase genes, such as those of aspartate and histidinol-P) and converts valine, leucine, and isoleucine to acetyl-CoA, which is then fed to the cycle. The yeast differential acetamidase also contributes to this pathway by deriving acetate from acetamide. Furthermore, the up-regulated acyl-CoA synthetase generates acetyl-CoA in the first step of β-oxidation, which may also be taken up by the cycle. Finally, the generation of sulfite by phosphoadenylyl sulfate reductase provides acetate for the glyoxylate cycle as mentioned above. The overall analysis suggests that ATP production through alcohol fermentation and the respiratory chain occurs in a biased pattern, the former being preferential in the yeast form and the latter in mycelium.

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

Cell Cycle and Genetic Information—The main genes in-

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

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

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

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

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

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

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

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

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

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

MAPK-related sequences, whose orthologues in *C. albicans* were experimentally correlated to hyphal formation and virulence, were also detected. The extrapolation to the *P. brasiliensis* model is not direct, since yeast, not hyphae, is the pathogenic cell type, but several MAPK homologues are found in species exhibiting diverse morphology and infection habits (35). A *cavps34* orthologue, identified in *P. brasiliensis* transcriptome (*vps34*), is implicated in the protein/lipid transport from the Golgi apparatus/endosome to the vacuole and has been proved to be important to *C. albicans* virulence (36).

Noteworthy is the finding of glyoxylate cycle genes in

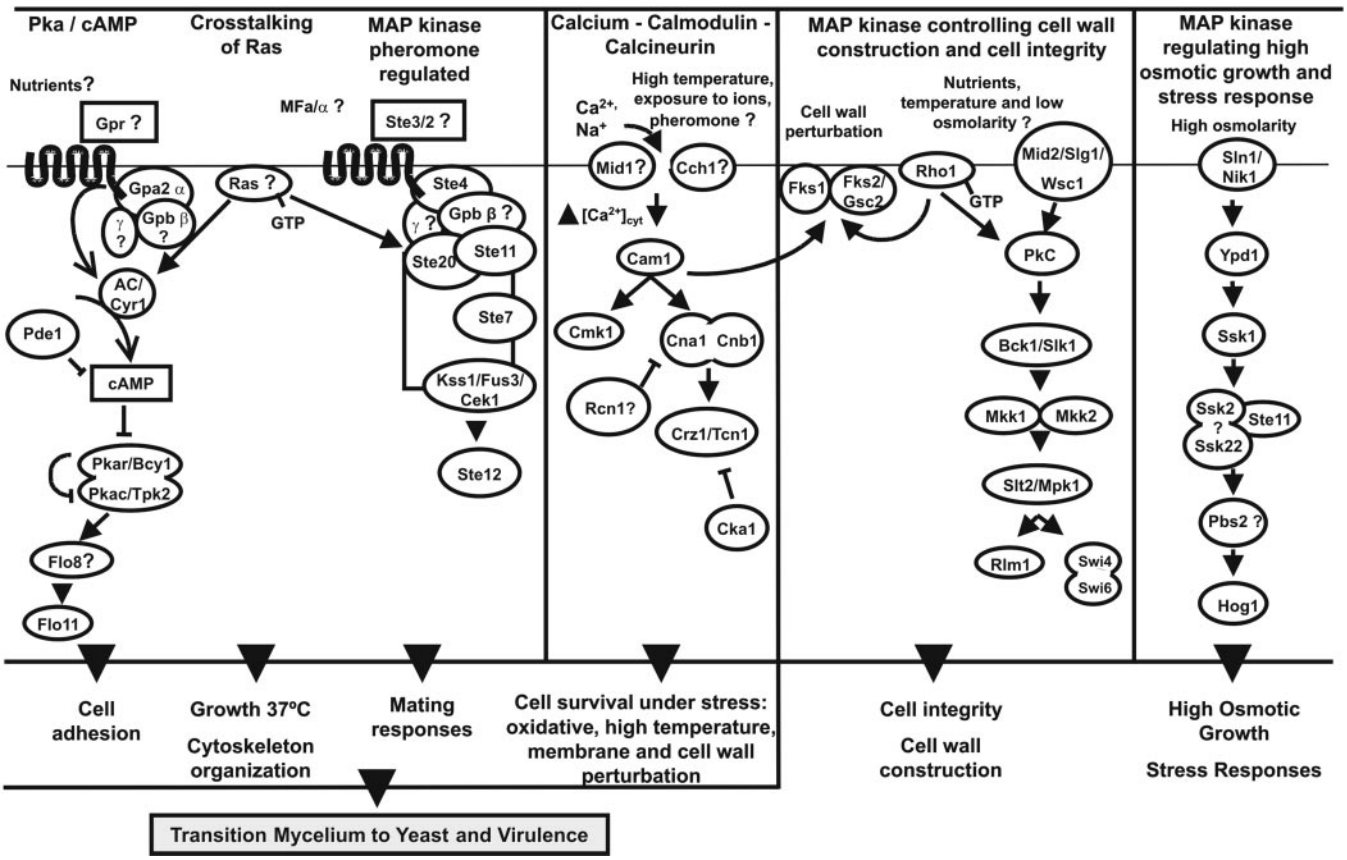

FIG. 4. **Signaling pathways in *P. brasiliensis*.** Shown are cAMP/protein kinase A regulating fungal development and virulence; MAPK signaling for cell integrity, cell wall construction, pheromone/mating, and osmoregulation; calcium-calmodulin-calcineurin controlling cell survival under stress conditions;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

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

The cell wall, as the most obvious difference between human and fungal cells, represents a prime target for antifungals. Genes involved in its biogenesis and assembly can act as virulence factors and therefore are putative drug targets. We have identified orthologues to chitin synthase 3 (*chs3*), glucosamine-6-phosphate acetyltransferase (*gna1*), mannosyltransferase (*pmt1*), and α1,2-mannosyltransferase (*mnt1*) genes and glycosidases Phr1p and Phr2p. The expression of the two last genes in *C. albicans* is responsive to the product of *prr2* (39), a pH-related transcription factor also present in the *P. brasiliensis* transcriptome. The detection of chitin deacetylase, as an overexpressed yeast gene confirmed by cDNA microarray and Northern blot (data not shown), points out to a novel target for drug research in *P. brasiliensis*.

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

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

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

Twenty PbAESTs annotated as related to multiple drug resistance genes were identified (Supplemental Table IX). They include 12 *S. cerevisiae* orthologues, 10 of which are related to the ABC transporter and two to major facilitator superfamilies (46). One of them corresponds to Pfr1, a gene recently described in *P. brasiliensis* (47), and another is related to the *CDR1* gene from *C. albicans*, which is up-regulated in the presence of human steroid hormones (48). It has been speculated that

TABLE II

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

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

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

TABLE III

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

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

steroid hormones are involved in morphological changes as well as in pathogenicity in *P. brasiliensis* and also in drug resistance in *C. albicans*. Interestingly, the process of infection of *P. brasiliensis* is strongly biased toward males, albeit the role of steroid hormones in the expression of ABC transporters in this organism remains to be investigated.

DISCUSSION

The *P. brasiliensis* transcriptome described here is represented by 6,022 EST clusters that may cover about 80% of the fungal total genome, whose gene number has been estimated to be ~8,000 genes.[3] This number greatly exceeds the previous EST studies in this fungus (8, 49). The analysis compares the two fungal cell types as well as their metabolic behavior. The results obtained probably reflect the adaptations associated with the mycelium (soil) and yeast (human host) environments. Most importantly, they provide new insights with respect to

[3] G. San-Blas, personal communication.

signal transduction pathways, virulence genes, and drug targets for this pathogen.

The transcription profile of the mycelium infective phase suggests the shunting of pyruvate into aerobic metabolism, since the expression of the ESTs encoding enzymes of the trichloroacetic acid cycle are up-regulated in this fungal phase. In contrast, the yeast transcription profile evidenced the deviation of pyruvate from the glycolytic pathway into anaerobic metabolism; this observation is consistent with a lower oxygen level in infected tissues. Its putative ability to produce ethanol suggests a potential anaerobic pathway for *P. brasiliensis*, which is dependent on the metabolic state of the cell. It seems that the main regulatory effector on the shunting of the end product of glycolysis into aerobic or anaerobic metabolism is temperature; therefore, it can be hypothesized that this physical factor is the central trigger of all of these molecular events, since it was the only parameter changed in the *in vitro* cultivation of yeast and mycelium of *P. brasiliensis*. Experiments are currently being carried out in order to confirm the *in vivo* expression profile of the differentially expressed genes in macrophages and human pulmonary epithelial cells infected by *P. brasiliensis*.

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

Acknowledgments—We are grateful to Hugo Costa Paes and Robert Miller for English text revision.

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Yeast
Yeast 2007; **24**: 79–87.
Published online 18 December 2006 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1438

Research Article

Cloning, characterization and expression of a calnexin homologue from the pathogenic fungus *Paracoccidioides brasiliensis*

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Received: 10 July 2006
Accepted: 31 October 2006

Abstract

We report the cloning of a *Paracoccidioides brasiliensis* cDNA, here named *Pb*Cnx, encoding the homologue of the endoplasmic reticulum molecular chaperone calnexin. Calnexin specifically recognizes monoglucosylated glycoproteins in the endoplasmic reticulum, thus being an essential component of the complex that interacts with the folded state of nascent secreted glycoproteins. The *Pb*Cnx open reading frame was found in a 1701 base pair (bp) fragment that encodes a 567 amino acid protein with an estimated mass of 62 680 Da. Northern and Southern blot hybridizations showed that *Pb*Cnx is encoded by a single, or a low number of, gene copies. *Pb*Cnx contains the hallmark KPEDWD motifs that are found in all members of the calnexin/calreticulin family proteins. A cDNA-encoding *Pb*Cnx was overexpressed as recombinant protein in *Escherichia coli*. The purified recombinant *Pb*Cnx was recognized by 6 out of 10 sera from PCM patients, a result that rules out its possible consideration for further use in diagnosis. Using confocal microscopy with anti-*Pb*Cnx mouse serum against yeast forms, a cytoplasmic staining pattern was observed. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: chaperone; calnexin homologue; recombinant protein; paracoccidioidomycosis; *Paracoccidioidides brasiliensis*

Introduction

Paracoccidioidomycosis (PCM), a human systemic mycosis prevalent in Latin America, is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. The fungus grows as a mycelial phase at 25 °C, which turns into yeasts at 37 °C. The mycelia generate conidia that differentiate into yeast forms which, when inhaled by the host, establish the infection (McEwen *et al*., 1987).

The yeast forms of *P. brasiliensis* produce a complex of antigenic molecules whose proteins and glycoproteins present important biological functions (Travassos, 1994). Our laboratory has shown that a glycoprotein of 43 kDa may contribute to virulence, as it specifically binds the extracellular matrix protein laminin, enhancing fungal pathogenicity (Vicentini *et al*., 1994). Despite the role of gp43 in the pathogenesis of PCM, it was also shown in our laboratory that another glycoprotein, named gp70, downregulated mouse peritoneal macrophage functions *in vitro*. Furthermore, passive immunization of mice with anti-gp70 mAbs before infection with *P. brasiliensis* practically abolished lung infection (Grosso *et al*., 2003).

These and other proteins, either secreted or endogenous, enter the secretory pathway at the level of the endoplasmic reticulum (ER). In the ER, a number of newly synthesized proteins undergo covalent modifications, such as N-glycosylation

and disulphide-bond formation, by which they acquire their final structure. Due to the high concentration of newly synthesized and resident proteins in the ER, folding in this compartment is a major mechanistic challenge for the cell. Nevertheless, the ER constitutes a cellular compartment optimized for efficiently performing this task. To this effect, the ER contains molecular chaperones and foldases that cooperate to increase the successful acquisition of the tertiary and quaternary structures of proteins. The interactions between proteins to be N-glycosylated and molecular chaperones occur in the ER lumen (Fewell *et al*., 2001).

Calnexin is a molecular chaperone that selectively recognizes monoglucosylated glycoproteins in the ER, being an essential component by interacting with the folded state of nascent secreted glycoproteins (Helenius *et al*., 1997). It has been described as being involved in oligosaccharide binding, and this lectin function was suggested by the identification of a glucose residue at a location similar to that found for sugar binding in a structurally related class of lectins (Schrag *et al*., 2003). Monoglucosylated glycoproteins arise from the consecutive action of the glucosidases I and II, or by reglucosylation of $Glc_0Man_9GlcNAc_2$-containing proteins by UDP-Glc : glycoprotein glucosyltransferase (GT). A distinctive feature of GT is that, *in vitro*, it can only reglucosylate unfolded proteins (Sousa and Parodi, 1995). These observations have led to the current models on quality control of glycoproteins folding in the ER, in which calnexin and GT constitute key elements (Trombeta and Helenius, 1998). The luminal portion of the calnexin molecule shares with calreticulin a highly conserved central domain (hcd) that contains two tandem KPEDWD repeats. However, in *Saccharomyces cerevisiae* this motif appears as a single copy (Virgilio *et al*., 1993).

Considering its central role, it was shown that calnexin is essential for viability in *Schizosaccharomyces pombe* (Jannatipour and Rokeach, 1995). On the other hand, calnexin (*clxA*) is not as essential a gene in *Aspergillus niger* (Wang *et al*., 2003).

In the present study we describe the entire nucleotide sequence of the cDNA corresponding to the calnexin homologue from *Paracoccidioides brasiliensis*. We also report the heterologous overexpression and purification of the recombinant protein *Pb*Cnx. Additionally, we show the recognition of the recombinant purified *Pb*Cnx by sera from paracoccidioidomycosis patients.

Materials and methods

Fungal strain and growth conditions

P. brasiliensis, strain *Pb*01 (ATCC, MYA-826) yeast phase was grown at 37 °C in modified solid YPD medium (0.5% Bacto yeast extract, 0.5% casein peptone and 1.5% dextrose, pH 6.5). The fungus was subcultured every 7 days in fresh medium.

cDNA cloning and sequence analysis

The *P. brasiliensis* cDNA library was constructed with isolated *Pb*01 in λ ZapII, as described by Felipe *et al*. (2003). The library was screened by using the 384 bp fragment that was amplified from the genomic DNA from *P. brasiliensis*. Degenerated oligonucleotides corresponding to peptides obtained by microsequencing from a glycoprotein of 70 kDa from *P. brasiliensis* were used. The cDNA clones were sequenced in MegaBACE 1000 (Amersham Biosciences). The sequences were analysed with Sequence Analyser software using the Base Caller Cimarron 3.12. The program employed to search for nucleotide and protein sequences similarity to *Pb*Cnx was NCBI BLAST. Protein sequence analysis was performed with PROSITE and Clustal X (Bairoche *et al*., 1997; Thompson *et al*., 1997). The nucleotide complete sequences of the cDNA that encoded *Pb*Cnx and the deduced protein have been deposited in the GenBank database under Accession No. DQ 272 490.

Southern and Northern blotting hybridization

Total DNA from *P. brasiliensis* was isolated (Cisalpino *et al*., 1994). Total RNA was isolated with Trizol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Genomic DNA was cut with selected restriction endonucleases. Standard conditions for electrophoresis, Southern and Northern blotting were used (Sambrook *et al*., 1989). The labelled probe of 1984 bp was hybridized to the blot under high-stringency conditions. Hybridizations were carried out at 42 °C for 16 h, in 50% formamide, 5× (1× SSC, 0.15 M

DOI: 10.1002/yea

NaCl, 0.015 M sodium citrate), 5× Denhardt's solution, herring sperm DNA 100 μg/ml and 0.1% SDS, followed by washes in 2× SSC/SDS 0.1% at 42 °C and 1× SSC/SDS 0.1% at 56 °C (twice, for 30 min).

Expression and purification of recombinant *Pb*Cnx

The cDNA of *Pb*Cnx was subcloned into the pHis plasmid. *Sal*I and *Spe* restriction sites were introduced into the oligonucleotides prior to the DNA synthesis. The expression of this pHis–Cnx construct was used to transform *Escherichia coli* Bl21 pLysS. The transformant cells were grown, induced with 0.5 mM IPTG and the extract was obtained by sonication. The bacterial extract was pelleted and resuspended in buffer (NaH_2PO_4 100 mM, Tris–HCl 10 mM, pH 8.0, containing 8 M urea). The recombinant protein was purified by affinity chromatography using a Ni-NTA column, according to the manufacturer's instructions (Quiagen). Proteins were separated by SDS–PAGE as described by Laemmli (1970), followed by Coomassie blue staining (Sigma Chemical Co.).

Preparation of cellular extracts

Yeast cells were scraped from the medium, washed in Tris–Ca^{2+} buffer (20 mM Tris–HCl, pH 8.8, 2 mM $CaCl_2$) containing protease inhibitors N-α-*p*-tosyl-L-lysine chloromethylketone (50 μg/ml), 4 mM phenylmethyl sulphonylfluoride, 5 mM iodoacetamide, 1 mM EDTA and leupeptin (20 μg/ml). Cells were collected by centrifugation at 5000 × *g* for 5 min, frozen in liquid nitrogen and disrupted by maceration. The cellular extract was agitated in a vortex for 15 min at 4 °C, centrifuged at 12 000 × *g* for 20 min and the supernatant was kept at −70 °C.

Preparation and reactivity of polyclonal serum anti-Cnx against the recombinant *Pb*Cnx and native protein

BALB/c mice were immunized by intraperitoneal injections at 1 week intervals for 1 month with macerated polyacrylamide gel containing 25 μg *Pb*Cnx recombinant protein. Before each immunization, the mice were bled through the ocular plexus, serum was separated by centrifugation and stored −20 °C. The reactivity of the polyclonal serum anti-Cnx with the recombinant molecule or native protein was detected by Western blots using peroxidase-conjugated anti-mouse Ig (Sigma) and developed with diaminobenzidine (Sigma).

Immunoblot assay and reactivity of patients' sera against the recombinant *Pb*Cnx

The previously purified *Pb*Cnx protein was electrophoretically separated and transferred to NC membranes, as described by Towbin *et al*. (1979). The membranes were blocked with 5% PBS-milk for 1 h and used to test the reactivity of 10 serum samples of paracoccidioidomycosis patients.

Cellular localization by confocal microscopy

The cellular localization was performed as described by Batista *et al*. (2006). Images of DAPI-stained cells were observed in a Bio-Rad 1024 UV confocal system attached to a Zeiss Axiovert 100 microscope, using a 40× numerical aperture, 1.2 plan-apochromatic differential interference contrast water immersion objective. All images were collected by Kalman averaging at least every eight frames (512 × 512 pixels), using an aperture (pinhole) of 2 mm.

Results and discussion

Cloning an characterization of cDNA encoding *Pb*Cnx

The 384 pb genomic fragment corresponding to gene gp70 from *P. brasiliensis* showed 45% (data not shown) protein sequence identity with a chaperone calnexin homologue of *P. brasiliensis*, and was used to clone the corresponding gene. Figure 1 shows the nucleotide and deduced amino acid sequences from the 1984 bp cDNA clone corresponding to calnexin, obtained by screening the cDNA library. The TGA codon at base 1 encoded the deduced initiating methionine and stop codon TAA was located at position 1702. The sequences contain 3′-untranslated (UTR), as evidenced by the presence of poly(A) tails. The putative protein encoded by the *Pb*Cnx ORF is a 567 amino acid polypeptide with a molecular mass of 62 680 Da and pI 4.92. A search at PROSITE database defined the putative motifs of calnexin. Three internal signature sequences for calreticulins were found. The

DOI: 10.1002/yea

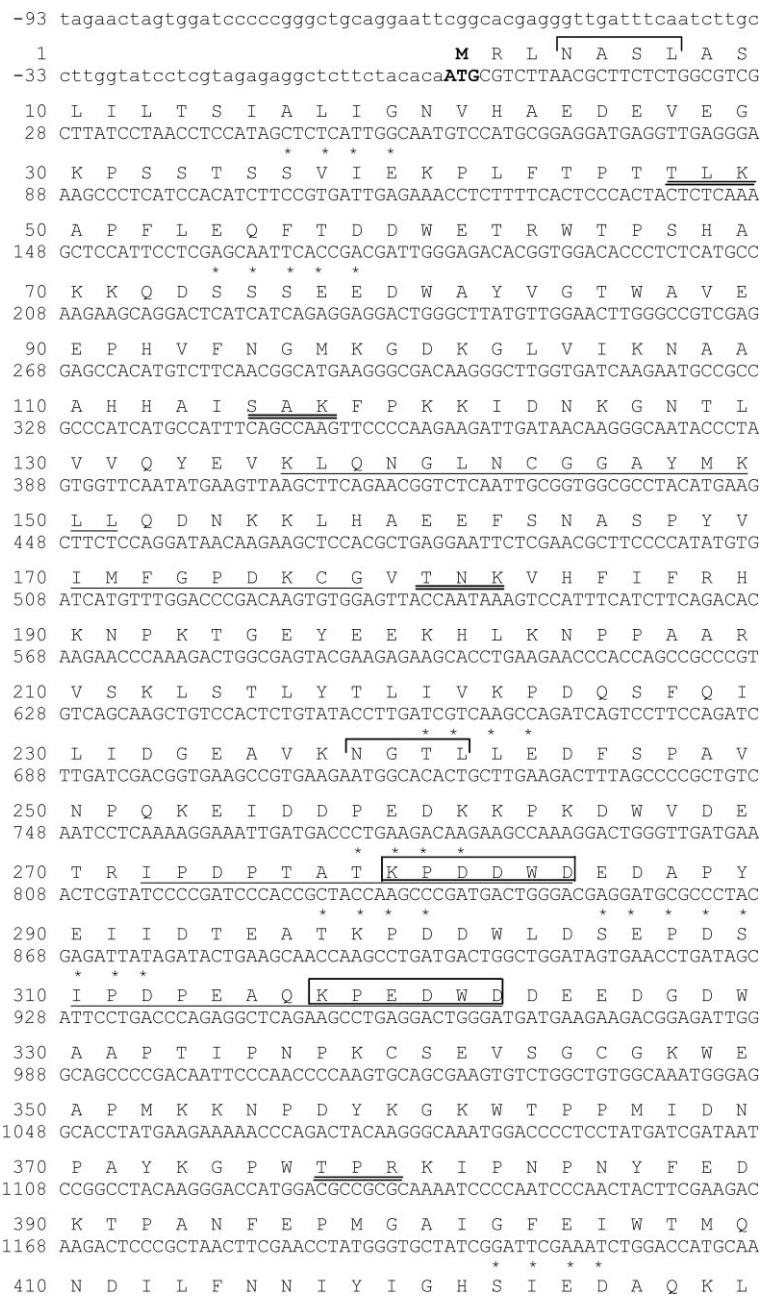

Figure 1. Nucleotide sequence and deduced amino acid sequence of *Pb*Cnx cDNA. Nucleotides and amino acids are numbered at the left. The start, stop codons and poly-A tail are in bold. The non-coding nucleotide sequences are indicated by lower case letters. Boxes represent KPDDWD and KPEDWD motifs. Calreticulin signatures are underlined. Superior brackets mark potential N-glucosylation sites. The casein kinase II phosphorylation sites are indicated by asterisks, and protein kinase C phosphorylation sites are doubly underlined

DOI: 10.1002/yea

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1228 AACGACATTCTCTTCAACAACATCTACATCGGCCACTCAATCGAAGATGCGCAGAAACTC
 430  K  S  E  T  W  D  I  K  H  P  I  E  V  A  E  E  E  A  T  R
1288 AAGTCAGAGACCTGGGACATCAAACACCCAATCGAGGTTGCGGAAGAGGAAGCCACACGC
 450  P  K  D  D  E  K  D  S  S  F  V  S  F  K  E  A  P  V  Q  F
1348 CCCAAGGATGATGAGAAGGACAGCTCCTTTGTCAGCTTCAAGGAGGCTCCCGTACAATTC
 470  V  R  E  K  I  N  L  F  I  S  I  A  R  K  D  P  V  Q  A  A
1408 GTACGTGAGAAGATCAATCTCTTCATCAGCATTGCGCGTAAGGATCCCGTGCAGGCAGCC
        *  *  *  *
 490  K  S  V  P  E  V  A  G  G  L  G  A  L  V  I  T  L  A  L  I
1468 AAAAGCGTTCCGGAGGTCGCTGGAGGTCTGGGTGCTCTTGTCATCACTCTTGCCCTCATC
 510  I  V  G  A  I  G  L  S  S  P  A  P  A  P  A  V  A  K  K  V
1528 ATTGTCGGTGCGATTGGCCTCAGCAGTCCTGCCCCAGCACCCGCTGTTGCGAAAAAGGTG
                                                        *  *
 530  D  G  K  E  K  D  G  A  S  K  E  K  A  A  E  A  V  S  T  T
1588 GATGGAAAGGAGAAGGATGGTGCTTCCAAGGAGAAGGCTGCGGAGGCAGTTAGCACTACG
      *  *
 550  A  D  N  V  K  G  A  A  T  R  R  S  G  K  A  N  N  E  #
1648 GCGGATAATGTGAAGGGGGCAGCTACGCGAAGATCGGGCAAGGCTAATAATGAGTAAgtt
1708 gttttatttttgagatacccgcctggcgaggaagagtgagagtgagggtgagggtgagag
1768 gtgaggtgtgggaggaaggactgggcaacaatgttgttgtaagcctatccaggatgatgt
1828 ctcccgtccacctaaaagagataccttgatgccaaccaaggaaatcatataaaaacccag
1888 aacaagagagcaaagaaatgaaccaaaaaaaaaaaaaaaaaa
```

Figure 1. Continued

cytosolic domain of mammalin calnexin has been shown to be phosphorylated by casein kinase II, and this phosphorylation seems to be involved with the regulation of calnexin function (Wada *et al*., 1991; Cala *et al*., 1993). In this respect, the *Pb*Cnx shows six potential casein kinase II phosphorylation sites in the cytosolic region. This is potentially significant, as the casein kinase II is believed to participate in cell differentiation at the level of signal transduction (Hupp *et al*., 1992). Motifs KLQNGLNCGGAYMKLL (16 residues), IMFGPDKCG (9 residues) and IPDPTATKPDDWD (13 residues) at positions 136–151, 170–178 and 272–284, respectively, with the last motif repeated at positions 310–322, with 13 potential casein kinase II phosphorylation sites found at positions S^{36}, S^{74}, S^{75}, S^{76}, T^{240}, $T^{278/297}$, S^{305}, S^{309}, S^{422}, S^{461}, S^{491}, T^{548} and seven potential protein kinase C phosphorylation sites (T^{47}, S^{115}, T^{180}, T^{377}, S^{461}, T^{558}, S^{561}). Two potential *N*-glycosylation sites were found at positions N^4 and N^{238}. The predicted polypeptide shares 74% identity with calnexin from *Aspergillus fumigatus* (Accession No. XM746454.1) and 73% from *Aspergillus niger* (Accession No. AJ299945). Furthermore, *Pb*Cnx shares additional features with its higher eukaryotic homologues. The encoded polypeptides showed a remarkable degree of evolutionary conservation, especially in the central portion, named highly conserved central domain (hcd), which contains as a hallmark KPEDWD repeats. It is considered to represent the high-affinity calcium-binding site in both calnexin and calreticulins (Tjoelker *et al*., 1994; Baksh and Michalak, 1991).

The lectin-binding site in calnexin is located at the proline-rich P central domain. Importantly, in calnexin, Ca^{2+} is essential for its lectin-like behaviour. In this context, *Pb*Cnx contains two KPEDWD motifs at positions 317–322 and 279–284. Both motifs are also found in *A. fumigatus* and *A. niger*. However, in *Sz. pombe*, cells that have deleted the hcd exhibited increased sensivitity to cell wall lytic enzymes, suggesting that these cells are defective in the biosynthesis of some cell wall component(s), and that calnexin (Cnx1p) could be involved in this process (Elagoz *et al*., 1999). Interestingly, in *S. cerevisiae* the ER glucosidase I, glucosidase II and Kar2p/Bip chaperone were shown to be involved in β-1,6-glucan synthesis (Simons *et al*., 1998), one of the cell wall components. Moreover, genes with specific roles in the cell wall of *S. cerevisiae* present homologies in the *P. brasiliensis* transcriptome, such as those encoding mannosyltransferases, glucan synthases and chitin synthases. This finding suggests similar mechanisms in the construction of the cell walls of *P. brasiliensis* and *S. cerevisiae*. The cell wall plays an essential role in the pathobiology of the former. The morphogenetic changes are directly associated with the life cycle of the fungus and its dimorphism has been cited as a crucial factor in the establishment of infection (Borba and Scaffer, 2002). These features led us to speculate that, as occurs in *S. pombe*, the deletion of the hcd in

DOI: 10.1002/yea

*Pb*Cnx may lead to defective biosynthesis of some cell wall component(s) of *P. brasiliensis*.

Southern blot hybridization detected a single DNA fragment (Figure 2A), indicating that the *Pb*Cnx might occur as a single copy in the genome. The transcript of *Pb*Cnx, by Northern blot hybridization with total RNA from *P. brasiliensis,* was probed with *Pb*Cnx and a single band of approximately 2.2 kb was detected (Figure 2B), reinforcing the presence of only one gene copy in the fungus genome.

To obtain a fusion protein, the cDNA was subcloned into vector pHis and induced with IPTG. The recombinant protein from bacterial lysates was purified by nickel-column affinity and was eluted, collected and analysed by SDS–PAGE. A protein band of approximately 70 kDa was detected (Figure 3A).This fusion protein was purified, blotted and assayed with the polyclonal serum anti-Cnx and, as demonstrated in Figure 3B, a single band approximately of 70 kDa was detected. Moreover, antibodies generated in mice against the recombinant calnexin recognized the native calnexin in immunoblots of *P. brasiliensis* whole cellular extracts (Figure 3C). However, the calculated 70 kDa molecular mass of this protein was unexpectedly smaller than the 78 kDa, as determined by Western blot analysis of the fungal cellular extract. This result is probably due to the fact that modifications such as glycosylation do not occur in this expression system. The purified recombinant *Pb*Cnx was only recognized by six of 10 sera from PCM patients (data not shown), a result that rules out its possible consideration for further use in diagnosis. Furthermore, polyclonal antibody generated against the recombinant *Pb*Cnx fusion protein specifically recognized native calnexin by immunoblotting of the *P. brasiliensis* cellular extract, suggesting that the recombinant

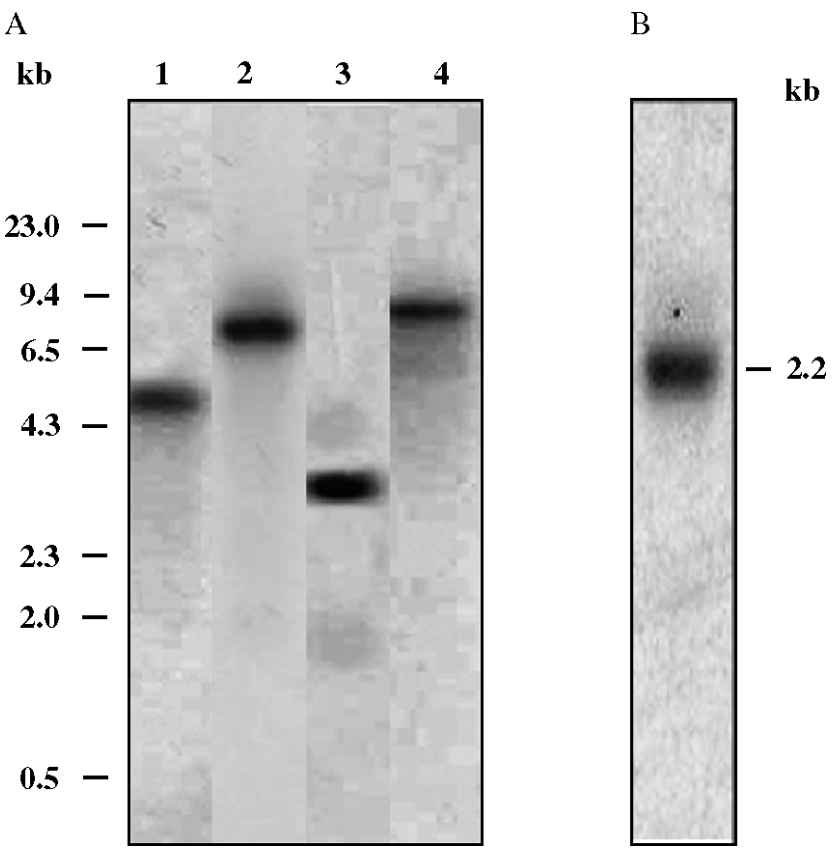

Figure 2. Southern and Northern blotting analyses. (A) Total DNA (10 μg) was digested with restriction enzymes: lane 1, *Pst*I; lane 2, *Not*I; lane 3, *Sal*I; lane 4, *Sma*I. The blot was probed with the 1.9 kb complete nucleotide sequence of *Pb*Cnx, labelled with [α-^{32}P]dCTP). (B) Northern blot analysis of the *Pb*Cnx transcript

DOI: 10.1002/yea

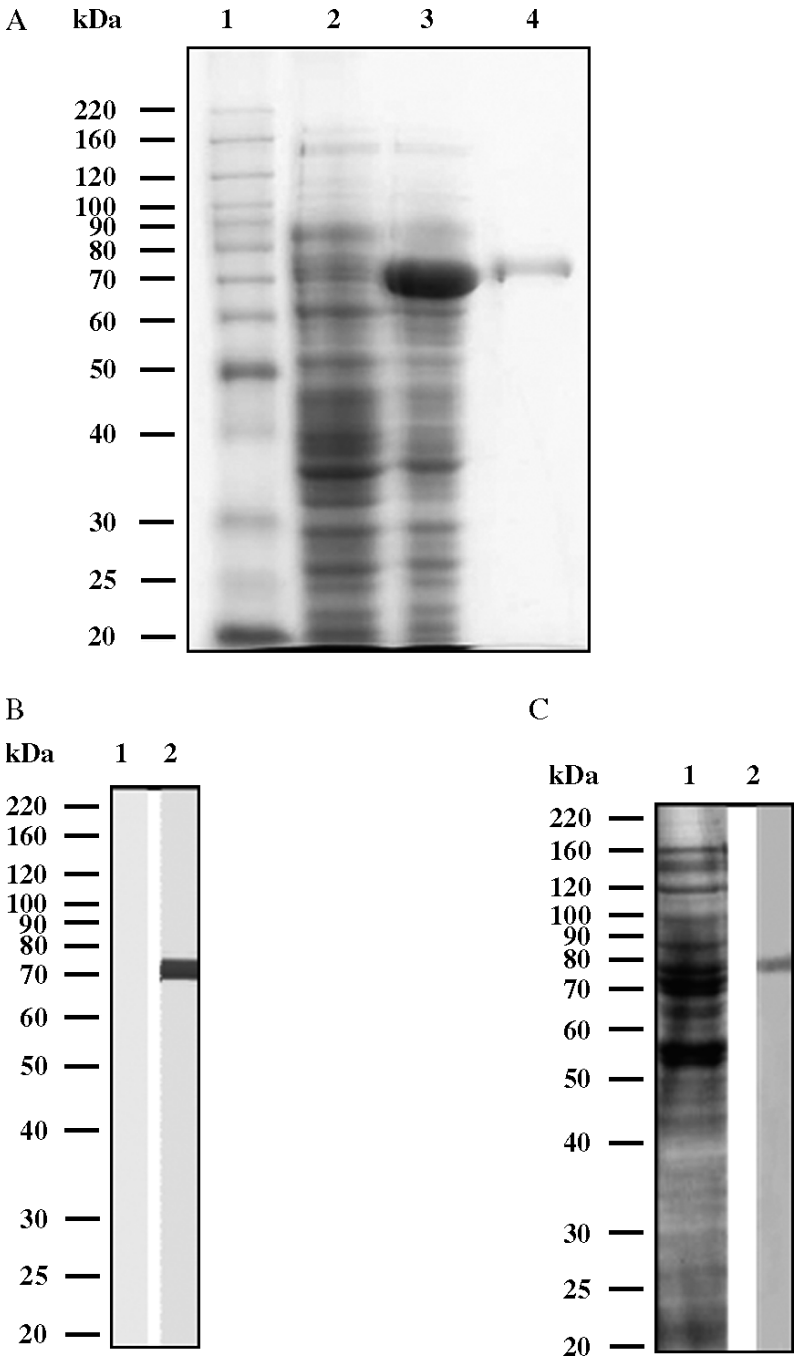

Figure 3. Expression of *Pb*Cnx in *E. coli* and immunological reactivity of recombinant protein and anti-recombinant protein antiserum.(A) The cell lysate from *E. coli*, stained with Coomassie brilliant blue. Lane 1, molecular markers; lane 2, lysate of control cells transformed by pHis-*Pb*Cnx, not induced; lane 3, cells transformed by pHis-*Pb*Cnx induced with IPTG; lane 4, purified recombinant fusion protein. (B) Antibodies generated in mice against the recombinant pHis-*Pb*Cnx recognized the purified recombinant protein. Lane 1, reaction with mouse preimmune serum; lane 2, reaction with the monospecific mouse antiserum. (C) Identification of native calnexin in the crude cellular extract of *Pb*01. Crude cellular extract (lane 1) and the immunoblotted sample (lane 2) was probed with polyclonal antibodies anti-recombinant calnexin. At the left, molecular markers

DOI: 10.1002/yea

*Pb*Cnx shares epitopes with native calnexin, and confirm that the sequenced clone *Pb*Cnx encodes calnexin. Epitopes recognized by murine polyclonal serum anti-Cnx were distributed in the cytoplasm of yeasts, where a very intensive staining was observed by confocal microscopy (Figure 4D). Negative control using cells with secondary antibody only detected no reactions (Figure 4B). The staining pattern is consistent with the fact that calnexin has been described as an ER protein.

The cDNA sequence of *Pb*Cnx will allow the attainment of the genomic clone and promoter region for this gene. Sequences in the promoter region of calnexin (*cnx*1) from *S. cerevisiae* resembled regulatory ones, such as the heat-shock regulatory element and the unfolded protein response element, that were previously identified in the promoters of other stress-induced genes (Pindoux and Armstrong, 1992; Normington *et al*., 1989). As other chaperones of the ER, the expression of *cnx*1 by *S. cerevisiae* was induced when cells were subjected to a variety of stress types causing the accumulation of unfolded and aggregated proteins in the ER, such as heat shock protein (Partaledis and Berlin, 1993). In this context, it is proposed that, during heat stress conditions, the accumulation of denatured or aggregated proteins is responsible for the loss of cell viability, reinforcing the importance of molecular chaperones in these events (Jesuíno *et al*., 2002). Thus, as for *S. cerevisiae*, these data open perspectives for further study of reassembly of regulatory sequences of the *Pb*Cnx gene as well as analyses of expression of this gene by heat-shock treatment. Finally, to better understand the role of calnexin in the biology of *P. brasiliensis*, the knowledge of the calnexin gene may lead to investigation into the consequences of its disruption, thus determining whether it is essential for the viability of the fungus.

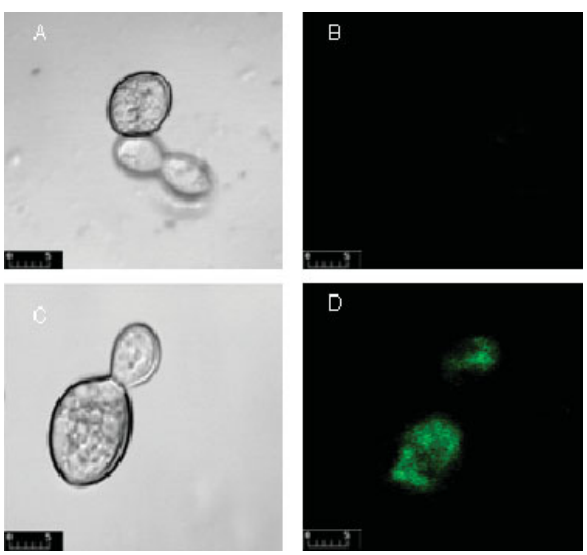

Figure 4. Distribution of calnexin protein in *P. brasiliensis* yeast cells. (A, C) Yeast cells using differential interferential contrast microscopy. (B) Control systems, in anti-calnexin without polyclonal antibodies prior to incubation with FITC-labelled mouse anti-IgG. (D) Confocal microscopy with antibodies generated against the recombinant pHis-*Pb*Cnx. Labelling of *P. brasiliensis* was obtained by anti-calnexin polyclonal antibodies (green). Bar = 5 μm

Acknowledgements

The authors are indebted to Wagner L. Batista, Creuza Rosa Oliveira and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). LSF was recipient of a grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Microbes and Infection 9 (2007) 1415–1421

www.elsevier.com/locate/micinf

Original article

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

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Received 10 November 2006; accepted 12 July 2007
Available online 17 July 2007

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.

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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doi:10.1016/j.micinf.2007.07.005

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

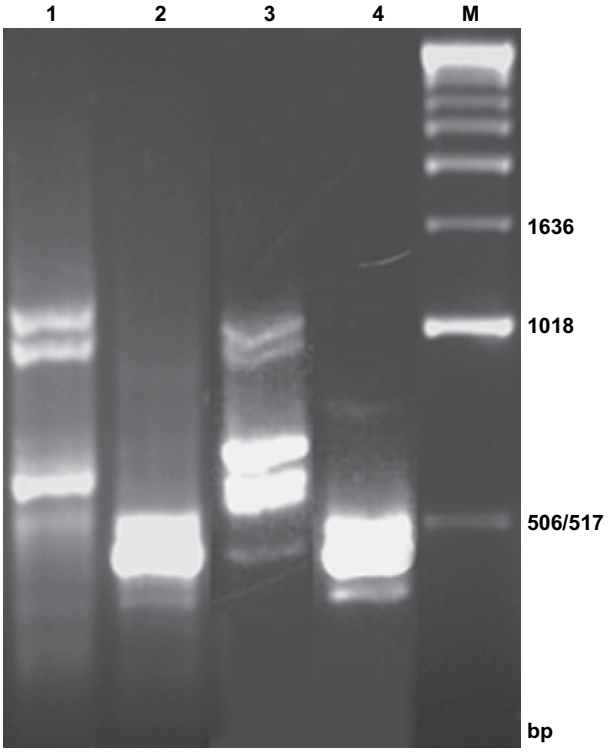

Fig. 1. RDA products analyzed by gel electrophoresis. Lanes 1 and 3: products of the first and second rounds of subtraction, respectively, performed by using tester the cDNA obtained from RNA of *T. rubrum* cultured in the presence of keratin. Lanes 2 and 4: products of the first and second rounds of 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

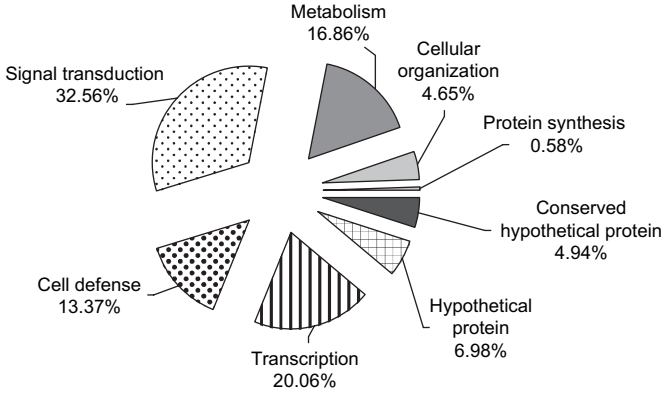

Fig. 2. Functional classification of *T. rubrum* ESTs derived from RDA experiment. This classification was based on BLASTX homology of each EST against the GenBank nr database at a significant homology cut-off of ≤1E−05 and MIPS functional annotation scheme. Each functional class is represented as a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

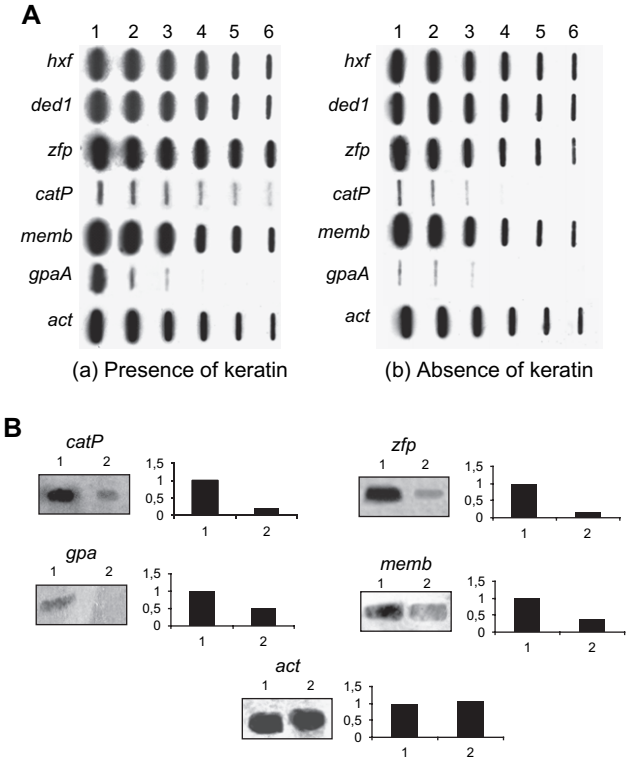

Fig. 3. Validation of the cDNA–RDA results. (A) Dot blot analysis of *T. rubrum*. DNAs of individual clones were prepared and dilutions (1:2000–1:64,000) were blotted (1–6). Panel a: individual clones hybridized to the labeled cDNA obtained from *T. rubrum* cultured in the presence of keratin. Panel 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.

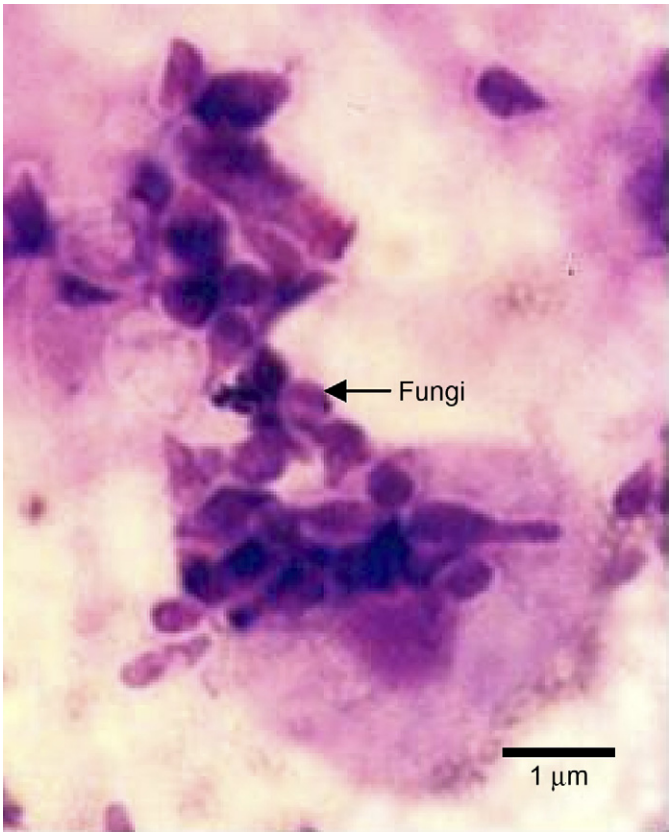

Fig. 4. Interaction between *T. rubrum* and keratinocytes. Cells were seeded into six-well plates and incubated with conidia for 24 h. The wells were washed and stained with the May–Grünwald (Giemsa) for micrographs.

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

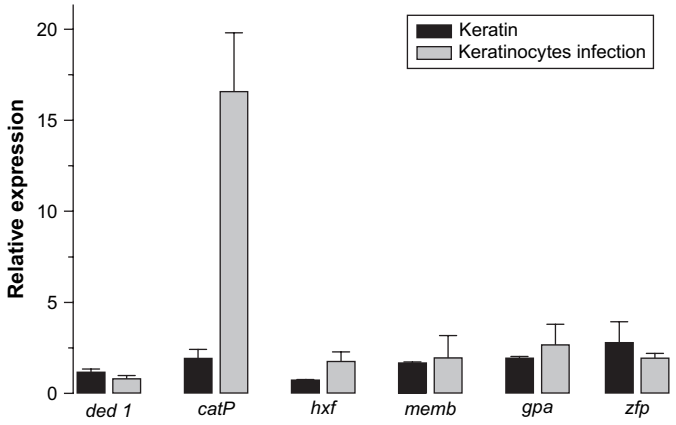

Fig. 5. Quantification of the transcript levels of differentially expressed genes from *T. rubrum* grown in the presence of keratin and after infection in culture of keratinocytes, relative to the fungus cultured in the absence of keratin (control). The results are averages of three repetitions. The 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.

Acknowledgments

We are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), CNPq, and PADC-FCF-UNESP, who provided the financial support for this project.

We thank Prof. Dr. Marisa Roma Herson and Silvana Altran, who processed and kindly supplied the culture of keratinocytes, and Prof. Dr. Mário Hirata who helped with real-time PCR.

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Microbiology (2007), **153**, 4194–4207 DOI 10.1099/mic.0.2007/009332-0

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.

Received 27 April 2007
Revised 31 July 2007
Accepted 13 August 2007

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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.

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

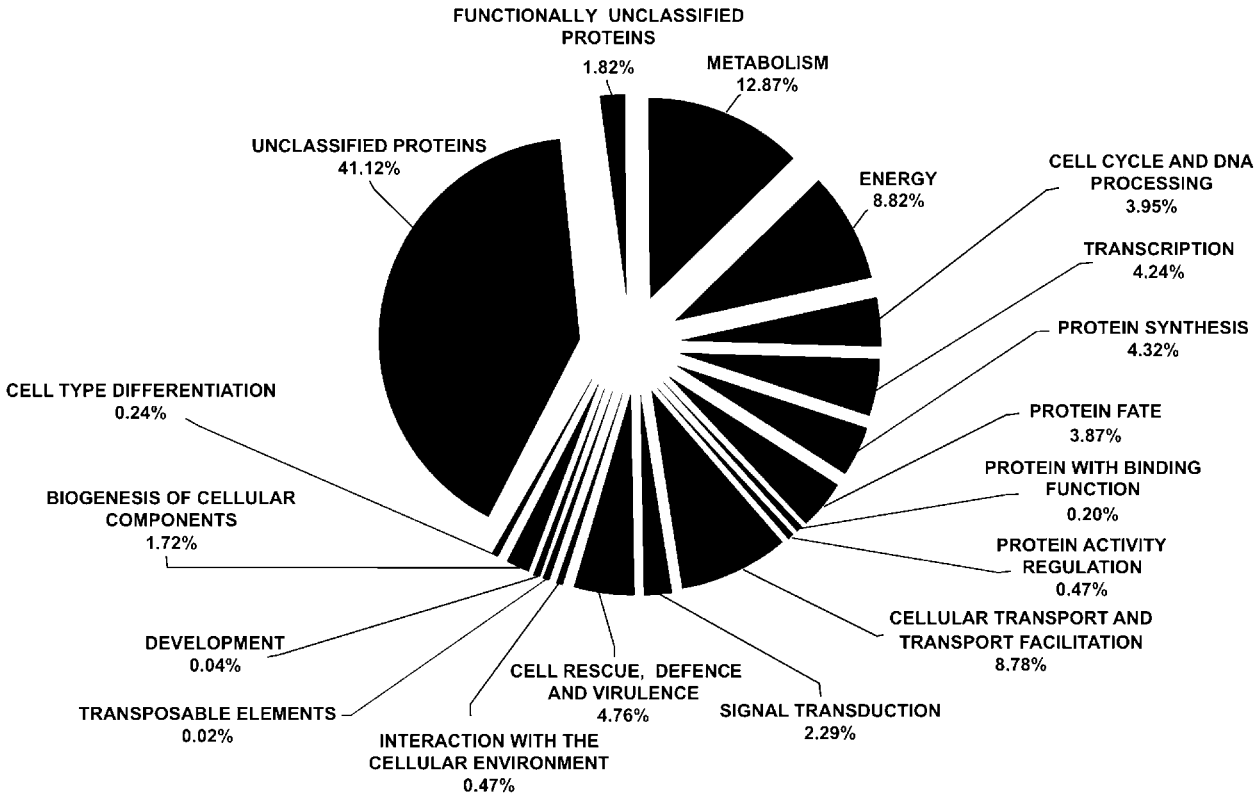

Fig. 1. Overview of ESTs from the *P. brasiliensis* transcriptome. Classification of the ESTs based on *E*-value and according to the functional categories of the MIPS functional annotation scheme.

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 over-expressed 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 over-expressed 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

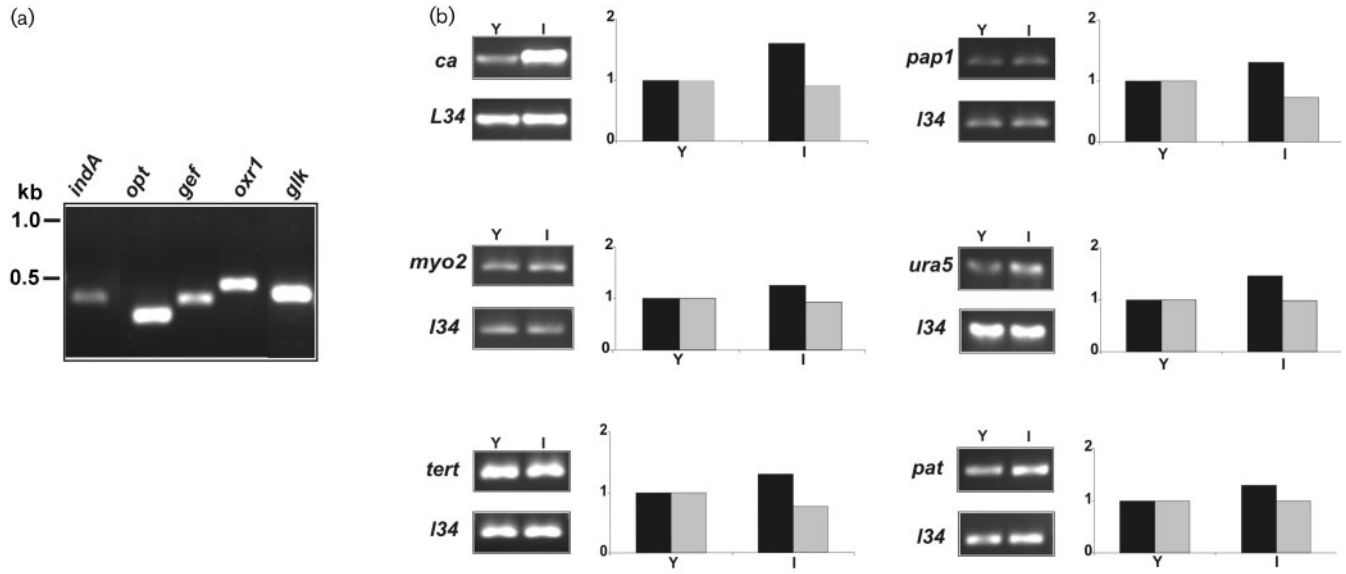

Fig. 2. Validation of the cDNA library for the presence of genes and analysis of redundancy of some transcripts. PCR and sqRT-PCR analysis were carried out with specific sense and antisense oligonucleotide primers, respectively, as described in Table 1. (a) PCR of total genomic DNA of *P. brasiliensis* 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.

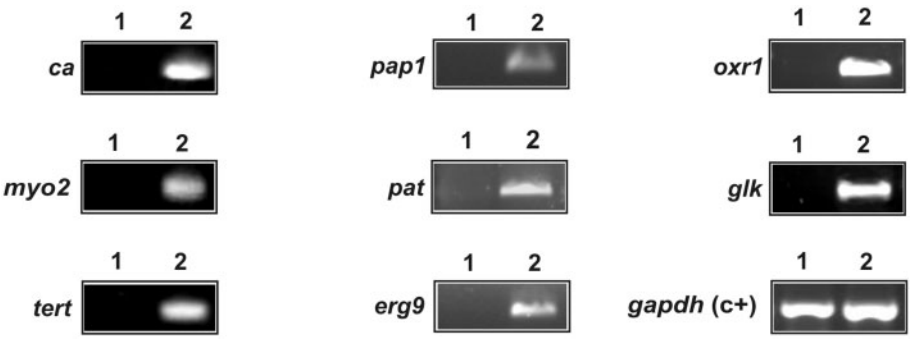

Fig. 3. Transcript analysis of the interaction of *P. brasiliensis* with Vero cells: sqRT-PCR analysis of selected transcripts in yeast-phase cells in the *ex vivo* model of infection. Lane 1, detection of transcripts in control Vero cells; lane 2, detection of transcripts in *P. brasiliensis* yeast 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.

ACKNOWLEDGEMENTS

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors

thank Laboratório Nacional de Computação Científica (LNCC) for bioinformatics support.

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Edited by: D. Sanglard

INSTITUT PASTEUR

Microbes and Infection 9 (2007) 1484–1492

www.elsevier.com/locate/micinf

Original article

A surface 75-kDa protein with acid phosphatase activity recognized by monoclonal antibodies that inhibit *Paracoccidioides brasiliensis* growth

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Received 15 January 2007; accepted 5 August 2007
Available online 10 August 2007

Abstract

Paracoccidioides brasiliensis is a thermo-dimorphic fungus responsible for paracoccidioidomycosis (PCM), a systemic granulomatous mycosis prevalent in Latin America. The fungus releases many antigens which may be transiently bound to its cell surface. Some of them may show enzymatic functions essential for maintaining many cell processes and survival of the microorganism at different conditions. In this study, we report the characterization of a secreted 75 kDa protein from *P. brasiliensis* with phosphatase activity. Biologic function of the molecule was demonstrated using two specific mAbs produced and characterized as IgM and IgG isotypes. Confocal microscopy and flow cytometry analysis demonstrated that both mAbs recognized the protein on the fungus surface, mainly in its budding sites. *In vitro* experiments showed that fungal growth was inhibited by blocking the protein with mAbs. In addition, opsonized yeast cells with both mAbs facilitated phagocytosis by murine peritoneal macrophages. Passive immunization using mAbs before *P. brasiliensis* mice infection reduced colony-forming units (CFU) in the lungs as compared with controls. Histopathology showed smaller inflammation, absence of yeast cells and no granuloma formation.

Keywords: *P. brasiliensis*; Monoclonal antibodies; Acid phosphatase

1. Introduction

Paracoccidioides brasiliensis causes paracoccidioidomycosis, a prevalent systemic mycosis in Latin America, which starts by inhalation of fungal propagules that differentiate into infective yeast forms in the lungs. It induces formation of granulomatous lesions in the lungs and lymph nodes rich in viable fungi that can disseminate to virtually all organs and tissues (reviewed in Ref. [1]).

P. brasiliensis presents complex antigenic structure, some of which has been related with pathogenicity. The surface glycoprotein gp43 is considered as virulence factor since it binds to extracellular matrix (ECM) components such as laminin [2], and fibronectin [3]. A 15-amino-acid peptide (P10) from gp43 is responsible for T-cell activation and induces protection against PCM in BALB/c mice [4].

Other antigens present important biological functions. A 70-kDa secreted glycoprotein down-regulated mouse peritoneal macrophage functions *in vitro* and passive immunization of mice with specific mAbs practically abolished lung infection [5]. Recently, a glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detected in *P. brasiliensis* cell wall bound to ECM and mediated the process of fungal internalization *in vitro* [6]. However, knowledge on fungal cell wall composition and exocellular components is still scarce.

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doi:10.1016/j.micinf.2007.08.001

The vast majority of proteins secreted are transiently found in the cell surface [7] and some have enzymatic functions required for growth in different natural environments [8]. Characterization of surface components might disclose new targets for antifungal agents and a better comprehension of *P. brasiliensis* pathogenesis.

Herein is reported the characterization of a secreted 75 kDa protein from *P. brasiliensis* with phosphatase activity. Treatment with specific mAbs affects *in vitro* fungal growth. The antigen is expressed on the cell surface, mainly in fungal budding sites. Passive immunization with mAbs along *P. brasiliensis* mice infection drastically reduced fungal burden and inflammation as compared with controls.

2. Materials and methods

2.1. Animals

Six-week-old male BALB/c (H-2^d) mice, from the animal facilities of the Federal University of São Paulo, Brazil, were used throughout. Animals handling and housing were performed according to NIH guide for care and use of laboratory animals.

2.2. Fungal strains

Pb18, 339, 9673, Ap and Pb01 (ATCC, MYA-826) were maintained by frequent subculturing on modified solid YPD (0.5% bacto yeast extract, 0.5% casein peptone, 1.5% dextrose, 1.5% agar [pH 6.3]). Yeast forms grown at 36 °C were subcultured every 5th day.

2.3. Preparation of fungal antigens

P. brasiliensis yeast cells were grown in YPD agar at 36 °C for 5 days and transferred to 50 ml of liquid YPD. Preinoculum was transferred to 450 ml YPD-containing Fernbach flasks after 3 days. One week later, cells were separated by paper filtration; filtered material representing the crude exoantigen. Protein content was always determined [9].

2.4. Hybridoma production

Comparison of Western blot assays performed with PCM patients' and infected mice sera showed coincident reactions closer to bands of approximately 75 kDa. Molecular species, described originally as 70–75 kDa, recognized by patients' sera [10], probably represent a family of analogous glycoproteins showing microheterogeneity of carbohydrate chains [11]. These results were previously confirmed in our lab by Western blot for the purpose of this investigation. Pb18 exoantigens of *P. brasiliensis*, fractionated by SDS-PAGE under reducing conditions [12], had proteins with molecular mass ranging from 72 to 90 kDa were macerated and injected intraperitoneally in a group of five mice at 2-week intervals for 4 months. Before each immunization mice were bled through the ocular plexus, serum was separated by centrifugation and stored at −20 °C.

Cells of the murine myeloma line SP_2 were fused with spleen cells from immunized mice according to Lopes and Alves [13]. Supernatants from cell colonies were screened by enzyme immunoassay (EIA) as described in Ref. [5] by using crude exoantigen as target antigen (50 μg/ml). Hybridomas producing mAbs that bound to exoantigens by ELISA assay were tested by immunoblotting [5].

Positive supernatants were checked for immunoglobulin isotype with Mouse Antibody Isotyping kit (BD, Biosciences, San Diego), following the manufacturer's instructions. Higher amounts of antibodies were obtained from ascites fluids [13]. IgG mAbs were purified by affinity chromatography in a protein G column and IgM mAbs were purified by gel filtration chromatography with Sephadex G-200 (Pharmacia, Uppsala, Sweden) [14].

2.5. Identification of the mAb-binding antigen

Purification of antigen was performed by affinity chromatography of Pb18 exoantigen in a CNBr-Sepharose column (Pharmacia) coupled with mAbs 5E7C or 1G6, both yielding a single protein band of approximately 75 kDa.

One microgram of purified antigen was analyzed by isoelectric focusing (IEF) using PhastGel (pH range of 3–9, Pharmacia), according to the manufacturer's instructions. Standard markers for isoelectric point (IP) determination of proteins in the same pH range (Pharmacia) were used. The gel was silver stained (PhastGel Silver Kit, Pharmacia).

2.6. Competition ELISA

MAbs were biotinylated using succinimide ester of biotin (Sigma) as previously described in Ref. [15]. Polyvinyl microplates (Costar) were coated with 0.1 μg/ml of 75 kDa purified protein in PBS (50 μl/well) during 1 h at 37 °C. After blocking free sites with PBS–5% fat-free milk, wells were treated with a constant concentration of one of the biotinylated IgG mAb 5E7C (25 μg/ml) and incubated with varying amounts of another non-biotinylated IgM mAb 1G6 (125 and 250 μg/ml) for 1 h at 37 °C. After washing, peroxidase-conjugated streptavidin (Zymed) was added for 30 min at 37 °C. Binding of the biotinylated mAb was detected as described in Ref. [5]. Results were expressed as optical density values.

2.7. Confocal microscopy and flow cytometry analysis (FACS)

Confocal microscopy labeling was performed according to Ref. [16]. MAbs 1G6 or 5E7C were added at a concentration of 50 μg/ml diluted in PBS–0.5% skim milk and incubated for 4 h at room temperature (RT). Fluorescein-conjugated secondary antibody was added with 50 μM DAPI (4′,6′-diamidino-2-phenylindole) for nuclei staining (Sigma) and 0.01% saponin (Sigma) diluted in PBS–0.5% skim milk for 1 h at RT. All steps were followed by constant washing with PBS. As controls, no mAbs anti-75-kDa protein were added. Samples were observed

on Bio-Rad 1024 UV confocal system attached to a Zeiss Axiovert 100 microscope.

Labeling for FACS was performed as described by Soares et al. [17]. Pb18 yeast cells were incubated with 50 μg/ml of each mAb overnight at 4 °C, washed with PBS, and sequentially labeled with FITC-polyclonal anti-mouse IgG (Bio-Rad, Hercules, CA) or FITC-polyclonal anti-mouse IgM (Sigma) at 1:100 dilutions for 1 h at RT in the dark. As controls, cells were incubated with irrelevant mAbs. Analyzes were performed in a FACSCalibur (Becton Dickinson, Mountain View, CA).

2.8. Assays on direct effect of mAbs on P. brasiliensis

The growth rate of Pb18 in the presence of specific mAbs was compared with growth irrelevant antibodies or medium alone. Yeast cells were grown in liquid brain-heart infusion (BHI) and 50 μg/ml from each mAb were added at 96-h intervals. Each culture was taken at 48-h intervals, and cell numbers were counted with a hemocytometer.

2.9. Phagocytosis assays

Macrophages were collected from the abdominal cavities of BALB/c mice as described in Ref. [18]. Pb18 yeast forms (10^6) were incubated with optimal concentration (50 μg/ml of each mAb) for 1 h at 37 °C. After washing with PBS to remove unbound mAbs, macrophages were challenged with 10^6 opsonized yeasts for 1, 2 and 8 h. Preparations were analyzed by contrast optical microscopy. Macrophages viability was checked by trypan blue exclusion showing at least 90% viability. An average of 200 phagocytic cells were counted for each slide. Phagocytic indexes (PI) were calculated as the percent of phagocytic cells multiplied by the mean number of internalized particles.

The number of viable fungi after phagocytosis was made by CFU counts. Colonies per plate were counted after 8–10 days of incubation at 37 °C. Control groups were performed with fungi alone or incubated with an irrelevant IgM mAb or IgG mAbs.

2.10. Evaluation of production of nitric oxide (NO)

Concentration of NO_2 was evaluated by Griess reaction [19] in the culture supernatant of macrophages challenged with opsonized yeasts. All determinations were performed in triplicates.

2.11. In vivo studies

Six groups of five mice each were used. All groups were intratracheally infected with 10^6 Pb18 yeast cells/animal and received different treatments by the intravenous (i.v.) route. The first group received 100 μl of PBS, the second group 100 μg of irrelevant IgM 5B3 mAb, the third group 100 μg of irrelevant IgG 5D11 mAb, the fourth group 100 μg of mAb 1G6, the fifth group 100 μg of mAb 5E7C and the sixth group 100 μg of both mAbs, 1G6 and 5E7C. MAbs were administered 3 days before, following the same protocol used for IgG. They were also given together with the fungus (*P. brasiliensis* was resuspended in mAb solution containing 100 μg of each mAb/10^6 yeast cells) and every 3 days after infection to ensure their availability from time-to-time along infection for the next 45 days.

Mice of each group were sacrificed after 45 days of infection and the numbers of viable microorganisms in the lungs, liver and spleen were determined by CFU [20]. Fragments of mice organs of different groups were fixed in 10% formalin for 24 h and embedded in paraffin. At least three non-successive tissue sections (5 μm thick) from each mouse were obtained, stained with hematoxylin and eosin and observed by optical microscopy.

The ability of mAbs to reduce lung CFU burden if administered after infection was studied in animals infected with Pb18, as above. At 7 or 15 days after infection each mouse received i.v. PBS, 100 μg of 5E7C mAb plus 100 μg of 1G6 mAb or 100 μg of 5D11 mAb plus 100 μg of 5B3 mAb every 3 days. The therapeutic efficacy was determined by CFU after 45 days of infection.

2.12. Isolation and amino acid sequencing of target antigen

All fractionation and isolation steps were performed at the Molecular Biology Laboratory, Federal University of Goiás, Brazil. Pb01 yeast cellular extract was fractionated by two-dimensional gel electrophoresis according to O'Farrell [21]. After immunoblotting, the single reactive spot was cut out from the *P. brasiliensis* extract gel. Peptides were obtained by cleavage with endoproteinase Lys-C, purified on high-performance liquid chromatography (HPLC) and subjected to Edman's degradation at the Structure Core facility, Department of Biochemistry and Molecular Biology, Nebraska Medical Center, Omaha, USA. Homology searches were conducted by using the BLAST program.

2.13. Enzyme assay

Phosphatase activity was determined as described by Kneipp et al. [22] with modifications. Approximately 0.1–1 μg of protein was incubated at RT for 60 min in a reaction mixture (0.5 ml) containing 50 mM sodium acetate buffer at pH 5.5 and 5 mM *p*-nitrophenyl phosphate (*p*-NPP) (Sigma). The reaction was stopped by adding 0.5 ml and 1 M NaOH to each sample. The amount of *p*-nitrophenol (*p*-NP) released by the monoesterase activity was determined by measuring the absorbance value at 425 nm and using *p*-NP as standard.

2.14. Statistics

Results are expressed as the mean ± S.D. Data were analyzed by Student's *t*-test or by analysis of variance (ANOVA) followed by the Tukey–Kramer test (INSTAT software, GraphPad, San Diego, CA). $P < 0.05$ indicated statistical significance.

3. Results

3.1. Generation of mAbs against P. brasiliensis proteins

After fusion of immunized mice spleen cells, cloning and selection, two stable mAbs specifically recognizing a 75-kDa

protein from Pb18 exoantigen were obtained (Fig. 1A), one IgM (1G6) and one IgG2a isotypes (5E7C), both κ light chains. MAbs recognized a 75-kDa protein from exoantigen produced by several *P. brasiliensis* isolates (Fig. 1C).

The recognized antigen was purified by affinity chromatography with mAbs. The purified protein was submitted to SDS-PAGE and a single band with 75 kDa was detected (Fig. 1A). IEF assay showed the protein as a single isoform with an IP of 3.75 (Fig. 1B). However, the yield of protein was always very scarce, thus difficulting further biological assays.

To verify whether both mAbs recognized the same epitope, competition ELISA was performed. Binding of biotinylated mAb 5E7C was inhibited by mAb 1G6 at concentrations greater than 125 μg/ml in assays with constant concentration of 5E7C (Fig. 1D). MAbs 1G6 and 5E7C showed to compete but interference with each other's through steric hindrance was not ruled out. They may recognize the same or related carbohydrate epitope since their binding was inhibited after antigen treatment with sodium metaperiodate/borohydride (data not shown).

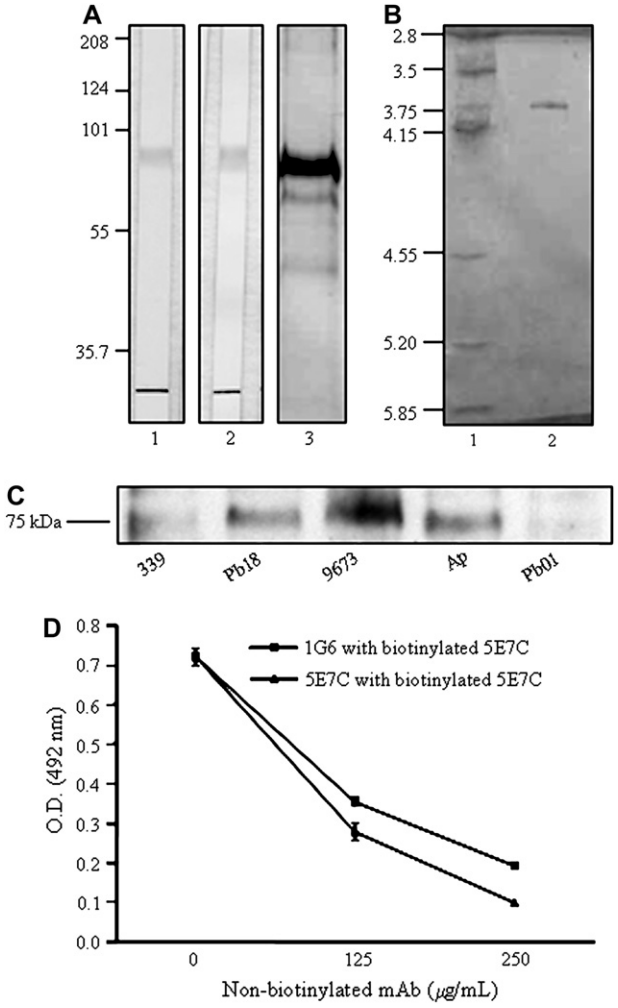

Fig. 1. MAbs and antigen identification. (A) Recognition by mAb 1G6 (lane 1) and 5E7C (lane 2) of immobilized proteins from crude exoantigen (isolate Pb18). Silver stained gel from SDS-PAGE showing purified 75-kDa protein (lane 3). Molecular mass standards are shown at the left. (B) IEF of the purified protein (lane 2); the pI of 75-kDa protein is approximately 3.75. Lane 1, pI standards. (C) Recognition by mAb 5E7C of 75-kDa protein in exoantigens produced by different *P. brasiliensis* isolates. (D) Competition ELISA using biotinylated mAb 5E7C and non-biotinylated mAb 1G6. The concentration of 5E7C was maintained constant (25 μg/ml) whereas the amount of 1G6 was five or 10 times greater than 5E7C concentration. Each point denotes the average of three measurements and error bars denote SD. This experiment was performed twice with similar results.

3.2. Localization of 75-kDa protein in P. brasiliensis yeast cells

Localization of the 75-kDa protein in Pb18 yeast cells with the mAbs was made by confocal microscopy. Yeasts showed heterogeneous labeling but both mAbs bound as small aggregates on the cell surface (Fig. 2). Fig. 2B and D suggest budding labeling with mAb 5E7C. Pattern of reaction with mAb 1G6 is shown in Fig. 2F. Reactions were sometimes detected in the intracellular milieu (data not shown). Controls were performed by labeling the cells with only secondary antibody (data not shown).

Flow cytometry of yeast forms labeled with each mAb confirmed antigen localization on the cell surface, with lesser frequency with IgM (11.65%) (Fig. 2H) than with IgG, which stained 42.44% of cells (Fig. 2G). These results might reflect different affinities. Control assays, as described in Section 2, were always negative (Fig. 2J and I).

3.3. Evaluation direct effects of mAbs on fungal growth

Given that mAbs anti-75-kDa protein mainly bound to the surface and preferentially to budding sites of *P. brasiliensis* cells, we evaluated their influence on fungal growth. Irrelevant mAbs were also used. The addition of antibodies to 75-kDa protein to the cultures clearly inhibited fungal growth (Fig. 3). Cell numbers were always smaller when yeasts were incubated with each mAb, with the difference to controls after 10 days being statistically significant ($P < 0.01$).

3.4. In vitro phagocytosis

Both mAbs showed opsonic activity with peritoneal macrophages. Phagocytic index was higher than control to all mAb 1G6 concentrations (from 10 to 100 μg/ml) and to mAb 5E7C it occurred in a dose-dependent manner (Fig. 4A). This finding was confirmed by measuring the phagocytic index for 1, 2 and 8 h (Fig. 4B). No differences between IgM and IgG were seen after 8 h. The mAb-mediated increase in phagocytic index was statistically significant ($P < 0.05$) relative to PBS at 8 h.

Although with phagocytic index increased in opsonized yeast cells, CFU showed that macrophages did not change the pattern of fungal burden after 1, 2 and 8 h of phagocytosis (data not shown). However, small increase in NO production was observed after 2 and 8 h of phagocytosis of opsonized cells (Fig. 4C).

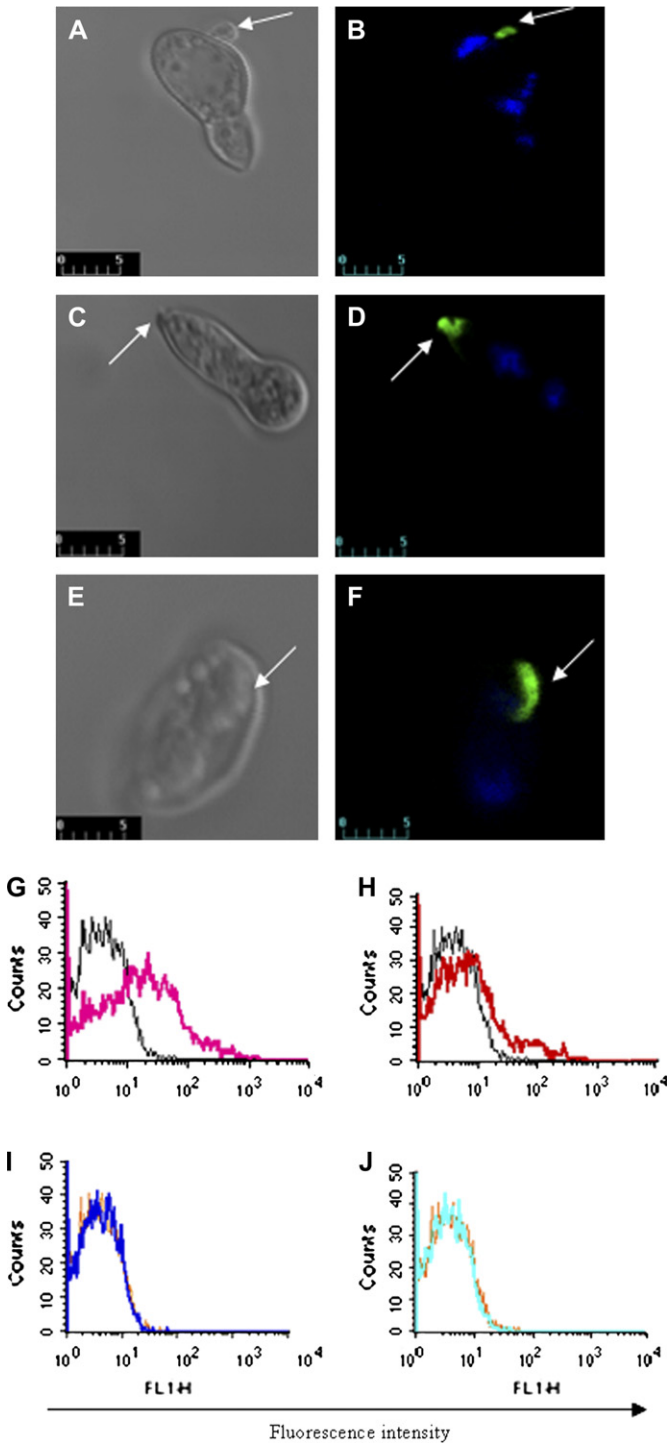

Fig. 2. Distribution of 75-kDa protein in *P. brasiliensis* yeast cells. Confocal microscopy and FACS with mAbs anti-75-kDa protein revealed with anti-mouse IgM or IgG coupled with fluorescein isothiocyanate (FITC). In control systems, in which no mAbs anti-75-kDa protein were added prior the incubation with FITC-labeled anti-IgM mouse or anti-IgG, and no detectable fluorescence was observed (data not shown). Left panels (A, C and E) show the same yeast cells labeled using differential interferential contrast microscopy. (B and D) Labeling of *P. brasiliensis* by mAb 5E7C (green) and nuclei labeled by DAPI (blue). Arrows show the budding region. (F) Labeling of yeast cells by mAb 1G6 (green) while nuclei labeled with DAPI (blue). Magnification bars = 5 μm. (G) FACS analysis of yeasts labeled with 5E7C, (H) with 1G6, (I) irrelevant mAb IgG and (J) irrelevant mAb IgM.

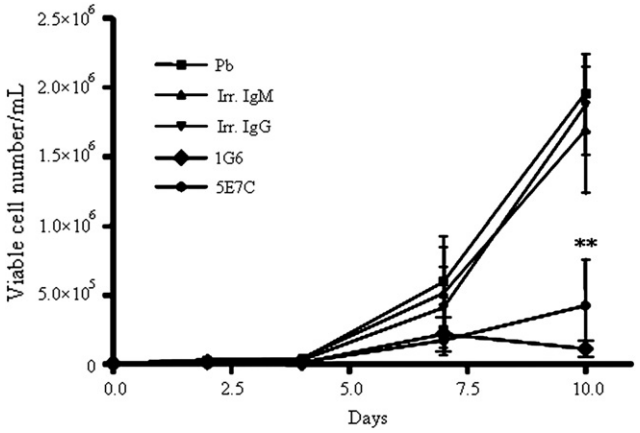

Fig. 3. Influence of antibodies 1G6 and 5E7C on fungal cell growth. Antibodies were added at the first day of culture and at 96-h intervals. Experiments were done in triplicates. ANOVA, followed by the Tukey–Kramer test, showed statistical difference ($^{**}P < 0.01$). This experiment was done three times with similar results. Points denote the average of three counting and error bars denote SD.

3.5. Passive immunization

Passive immunization using mAbs 1G6 and 5E7C was assayed to verify *in vivo* effects. BALB/c mice were intratracheally infected with *P. brasiliensis* opsonized with each mAb and intravenously treated with mAbs anti-75-kDa protein to evaluate whether their administration, either IgG, IgM, or both could modify the course of PCM. Treated mice showed significantly reduced CFU average in the lungs as compared with controls (Fig. 5A). Also, histological examination revealed that mice treated with mAbs had almost no inflammatory response to infection (Fig. 5). Therefore, histopathology of lungs from infected and treated animals confirmed the CFU results. The lungs of control animals presented lung parenchyma with large cellular infiltrates, mainly of the mononuclear type, and well-organized granulomas containing great numbers of viable fungi (Fig. 5B). The lungs of animals that received mAb 1G6 (Fig. 5C) or 5E7C (Fig. 5D) or both (Fig. 5E) showed well-preserved parenchymas with peribronchial cellular infiltrates and did not present yeast cells or granuloma formation.

Therapeutic efficacy of mAbs was studied by treating BALB/c mice for 7 or 15 days after *P. brasiliensis* infection. Fig. 5F shows that mAbs administration after 7 days reduced CFU burden in the lungs despite not statistically different when compared with controls. No differences were observed with mAbs given after 15 days of infection (Fig. 5G).

3.6. Isolation and amino acid sequencing of recognized antigen

Amino acid sequences of the 75-kDa protein led to the identification of a 15-amino-acid peptide sequence (LYVELLAIYQEDYD) (Table 1). A BLAST search revealed homology of this sequence with hypothetical proteins of *Magnaporthe grisea* (XP_359460), *Neurospora crassa* (XP_956396), *Chaetomium globosum* (EAQ88996, EAQ90805), *Gibberella zeae*

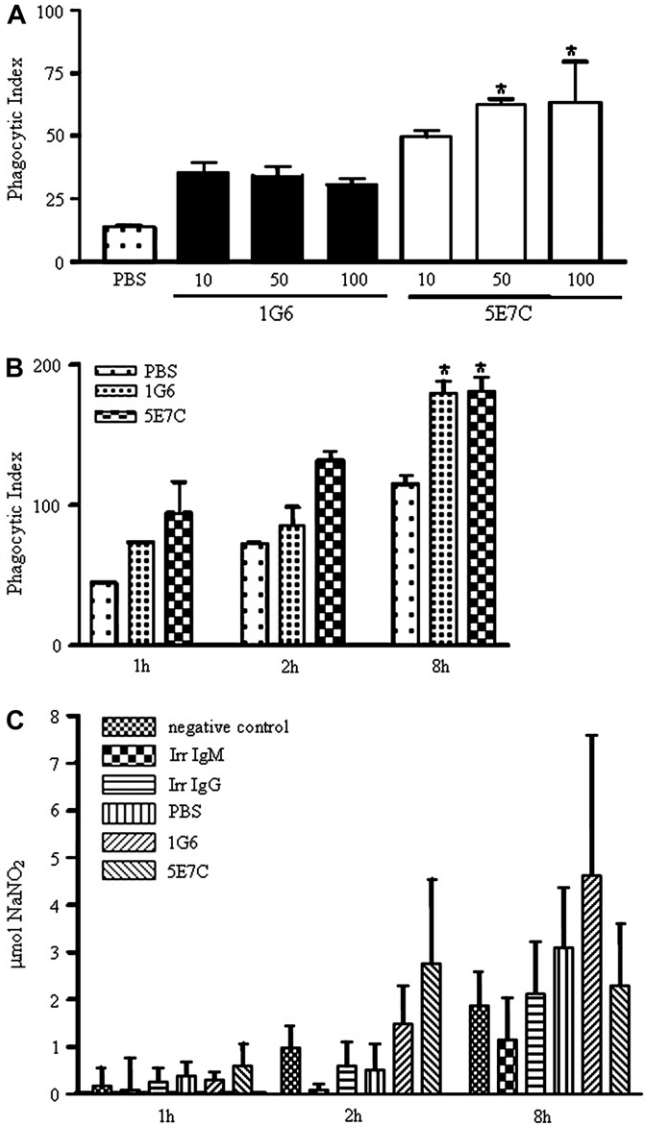

Fig. 4. Phagocytosis of *P. brasiliensis in vitro* by peritoneal macrophages in the presence of mAb 1G6 or mAb 5E7C or the addition of a similar volume of PBS. (A) MAbs 5E7C or 1G6 were incubated with yeast cells at concentrations 10, 50 or 100 μg/ml. In control assays no mAb was added. Phagocytic index was always higher for mAb 5E7C than for mAb 1G6 at the same concentrations. Bars denote the average of three measurements and error bars denote SD. Experiments were done in triplicates and different fields were counted. These results are representative of three experiments. Statistically significant when compared with control (ANOVA, followed by the Tukey–Kramer test, $*P < 0.05$). (B) Phagocytic index for yeast cells opsonized or non-opsonized with 50 μg/ml of each mAb, 1G6 or 5E7C, after 1, 2 and 8 h of incubation. Phagocytic indexes (PI) were calculated as described in Section 2. Bars denote the average of three measurements and error bars denote SD. Experiments were done in triplicates and different fields were counted. Statistically significant when compared with control (ANOVA, followed by the Tukey–Kramer test, $*P < 0.05$). (C) NO released by peritoneal macrophages challenged with opsonized *P. brasiliensis* with 1G6, 5E7C, irrelevant IgM, irrelevant IgG or fungal alone. Bars denote the average of three measurements and error bars denote SD. This experiment was done at least twice with similar results.

(XP_391115) and *Coccidioides immitis* (EAS33689) with identities of 80%, 69%, 69%, 69% and 87%, respectively. There was 87% identity with a putative esterase of *Aspergillus fumigatus* (XP_753654).

3.7. Enzyme assay

Two substrates were employed to verify possible esterase activity of the antigen. Preliminary results showed that the protein recognized by mAbs probably contained a phosphomonoesterase activity (data not shown). To confirm that result, the artificial substrate *p*-NPP was used to check whether it was hydrolyzed by the purified protein. As seen in Fig. 6, the antigen was active against monophosphate ester in a dose-dependent fashion.

4. Discussion

P. brasiliensis expresses several antigenic molecules recognized by antibodies produced by human patients or raised in laboratory animals [10,11]. Da Fonseca [23] identified and partially sequenced antigens reactive with pools of sera from patients with PCM. Evaluation of these antigens allowed exploring the biological functions of several proteins. However, studies on the immunochemical characterization of these proteins as well as their relationship to *P. brasiliensis* virulence is poorly understood.

We report characterization of a secreted 75-kDa protein from *P. brasiliensis* with phosphatase activity related with fungal growth and establishment of experimental PCM. Two specific mAbs against the molecule were produced. Recognition of the same 75 kDa protein by both mAbs was probably due to the higher immunogenicity of this specific antigen. A similar specific band was detected for all isolates tested. The antigen was located preferentially at cell budding sites and flow cytometry demonstrated its presence on the cell surface. Inhibition assays *in vitro* using those mAbs altered fungal growth. The mAb-dependent-inhibition effect was statistically significant after 7 days of culture. Some growth was always maintained, as seen after 48 h, increasing notably after that period.

Some antibodies against cell wall components were reported to direct antimicrobial properties. Such findings were seen for *Candida albicans* [24] and *Cryptococcus neoformans* [25]. Moragues [24] characterized a mAb against a mannoprotein of >200 kDa on the cell wall of *C. albicans* with direct candidacidal activity. Human antibodies against glucosylceramide from *C. neoformans* inhibited cell budding and fungal growth probably by interfering with the biosynthesis and organization of the cell wall polymers [25].

Extracellular acid phosphatases (EC 3.1.3.2) have been described in several fungi [22,8]. An acid phosphatase was recovered from *A. fumigatus* culture filtrate and cell wall fraction after cleavage of glycosylphosphatidylinositol (GPI) anchor. Biochemical and sequential studies showed that the molecule is an 80 kDa glycoprotein [8].

The presence of such enzymes in fungal surfaces is not understood, but a role in cell wall biosynthesis, in protection from acidic conditions, in the adhesion to mammalian cells, and as in fungal nutrition by hydrolyzing organic phosphates cannot be discarded. They seem to be also involved with development, morphogenesis and cell cycle (reviewed in Ref. [26]). In contrast, acid phosphohydrolases were associated with virulence

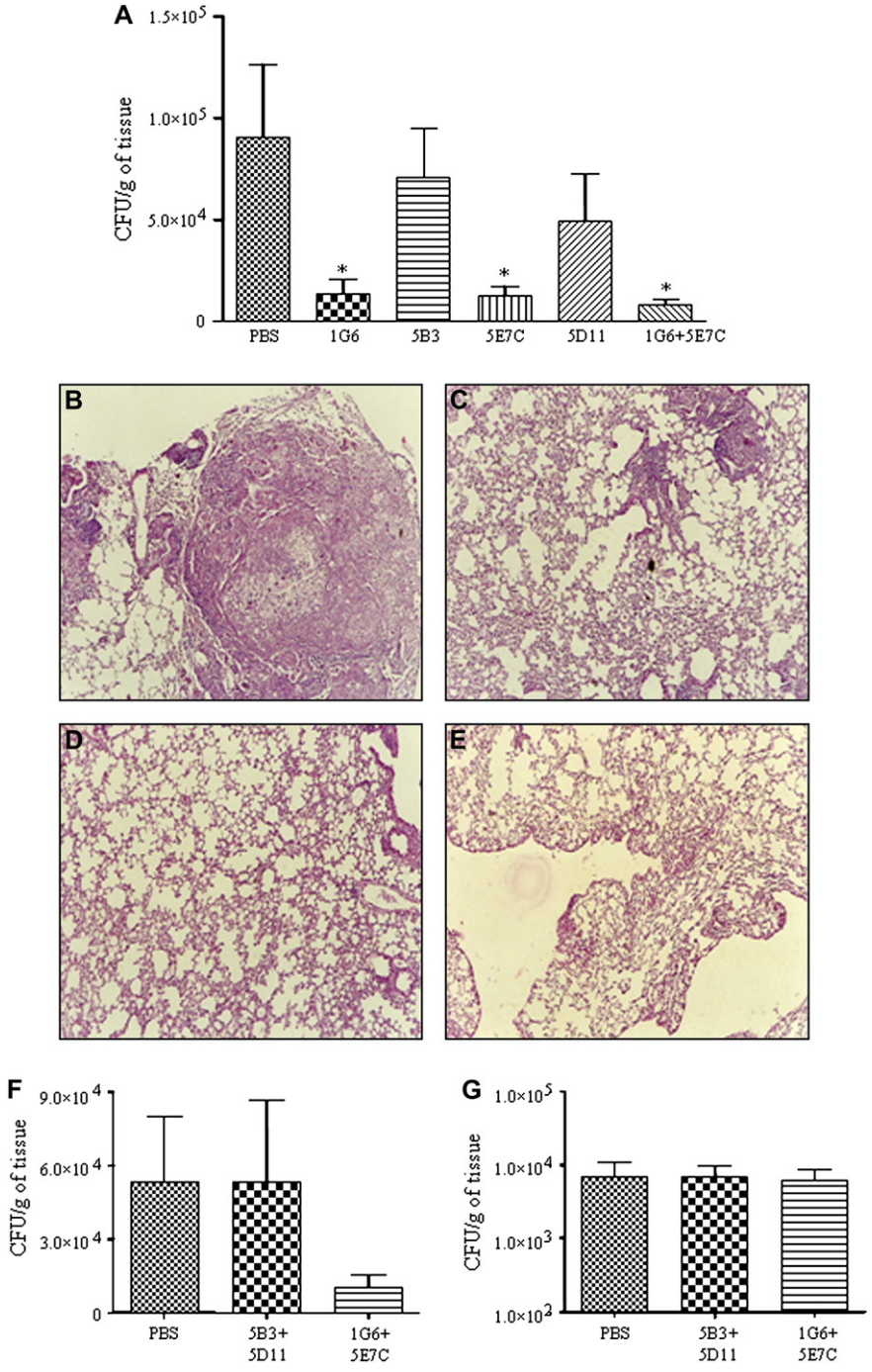

Fig. 5. Analysis of effects of passive immunizations with mAbs in infected mice. (A) CFU from lungs of BALB/c mice pretreated with 100 μg of mAbs against 75-kDa protein, nonspecific mAbs (5D11 or 5B3), or PBS and infected with 10^6 *P. brasiliensis* (Pb18) opsonized with each mAb and intravenously treated or not with mAbs anti-75-kDa protein or irrelevant mAbs, as described in Section 2. Both mAbs protected mice in this model. Each bar denotes the average of five measurements and error bars denote SD. Statistically significant difference when compared with control (ANOVA, followed by the Tukey–Kramer test, $^*P < 0.05$). (B) Histopathology of lungs of control mice showed well-organized granulomas and high number of viable yeast cells. (C) Lungs of mice treated with mAb 1G6 (100 μg) showed smaller granulomas; (D) mAb 5E7C and (E) both mAbs showed absence of granulomas and yeast cells. Magnification, ×100. (F) CFUs from lungs of BALB/c mice after 45 days of infection with 10^6 *P. brasiliensis* (Pb18) intravenously treated with mAbs anti-75-kDa protein or irrelevant mAbs after 7 days of infection. (G) CFU from lungs of BALB/c mice after 45 days of infection with 10^6 *P. brasiliensis* (Pb18) intravenously treated with mAbs anti-75-kDa protein or irrelevant mAbs after 15 days of infection.

of some microorganisms such as *Francisella tularensis* that inhibits respiratory burst of neutrophils [27].

Our findings led us to investigate by passive immunization in experimental *in vivo* PCM whether those anti-75-kDa protein mAbs could modify the course of infection. With this purpose, mAbs were injected before, together with and during infection and led to a significant reduction in lung fungal burden when 1G6 and 5E7C were administered alone or concomitantly.

Table 1
Amino acid sequence from 75 kDa protein compared with higher homology fungal proteins from BLAST

Protein	Sequence	% Identity
P. brasiliensis 75-kDa protein	LYVELLAIRYQEDYD 1 8 15	
Magnaporthe grisea hypothetical protein	LTLRYQEDYD 6 15	80
Neurospora crassa hypothetical protein	VEGVTVRYQEDYD 3 15	69
Chaetomium globosum hypothetical protein	VEGLTVRYQEGYD 3 15	69
Chaetomium globosum hypothetical protein	VILLTVRYPEDY 3 14	66
Gibberella zeae hypothetical protein	YVELLGAVRYEMD 2 15	69
Aspergillus fumigatus esterase, putative	IRYQPDYD 8 15	87
Coccidioides immitis hypothetic protein	IRYQPDYD 8 15	87

Histopathology showed reduction in the number of fungi particles and granulomas within the lungs, with a clear improvement in inflammation in treated mice. These mAbs, when given after the infection, showed some protection only until the first week. Such results could be the in vivo counterpart of the effect observed *in vitro* where fungal growth was inhibited. There are possibilities of immunomodulation or interactions with effector cells (Fc) through the constant region of the immunoglobulin. Despite the assumption that IgM penetrates poorly in the lungs, our results showed that either this supposition is not completely true or that the amount given allowed some penetration in that tissue. Also, infection with fungus pre-incubated with MAbs improved the outcome of the disease, as already shown in Ref. [28].

Antibodies are natural products of the immune system and interact with other immune components. Protective mAbs may act through complement-mediated lysis, enhancement or inhibition of phagocytosis, Fc-mediated cytokine release and direct antimicrobial effects. Specific immunoglobulin G (IgG)–Fc receptor interactions can inhibit the inflammatory response; thus antibody therapy could be effective by reducing the resulting damage (reviewed in Ref. [29]). Our results demonstrated that yeast opsonized with anti-75-kDa protein mAbs had increased phagocytic index. This finding was not unexpected. Opsonic IgM in a serum-free system was observed with antibodies against histone-like protein from *Histoplasma capsulatum* [28] and glucuronoxylomannan from *C. neoformans* [30]. In both cases, CR3 (CD11b/CD18) was involved in complement-independent antibody-mediated phagocytosis. Macrophages ingested more opsonized than non-opsonized *P. brasiliensis*, but this finding was not correlated with increased CFU in groups treated with mAbs, suggesting that macrophages could only partially control fungal burden. This statement is supported by the small increase of NO production by macrophages incubated with mAbs opsonized *P. brasiliensis*.

Contrariwise, our results *in vitro* led to formulate two hypotheses to explain protection of lung infection *in vivo*. First, mAbs could be reacting by inhibiting fungal growth through direct fungistatic effect. Second, yeast opsonization activated macrophages that could produce reactive oxygen intermediates which could partially contribute to the cytotoxic effect. Besides that mAbs against the 75-kDa protein clearly demonstrated a protective role in experimental PCM, they can also become a tool to understand the significance of the target protein in this mycosis.

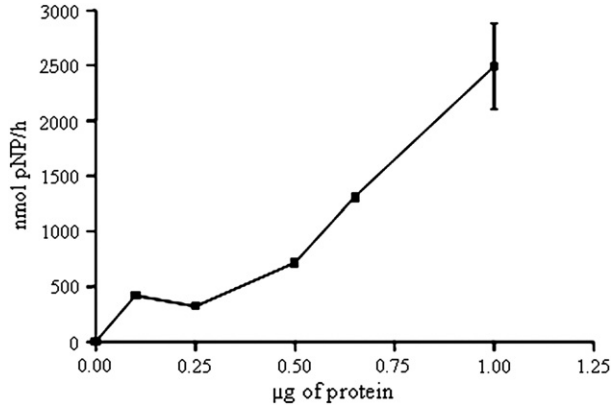

Fig. 6. Phosphatase activity of 75-kDa purified protein. Experiments were done in duplicates. These results are representative of two experiments. Error bars denote SD.

Acknowledgements

We are indebt to Creuza Oliveira and Ronni Brito for technical assistance, Dr. Rosana Puccia and Dr. Vera Lúcia Garcia Calich for *P. brasiliensis* isolates and Dr. Zoilo Pires de Camargo for equipments. This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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Genetics and Molecular Biology, 30, 1, 219-224 (2007)

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Research Article

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

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Abstract

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

Key words: Mycoplasma, kinases, genomes.

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

Introduction

Edmond Nocard and Emile Roux successfully cultivated the agent of the contagious bovine pleuropneumonia, *Mycoplasma mycoides*, over a century ago (Nocard and Roux, 1898). Since that time, approximately 111 species of the genus *Mycoplasma* have been identified in animals. These and other 102 species comprise the class of Mollicutes (Minion *et al*., 2004). These prokaryotes are known as the smallest self replicating organisms (Glass *et al*., 2000; Westberg *et al*., 2004). Most members of this class are pathogenic and colonize a wide variety of hosts, such as animals, plants and insects. Mollicutes represent a group of Low-G+C-content eubacteria that are phylogenetically related to the *Clostridium-Streptococcus-Lactobacillus* branch of the phylum (Woese *et al*., 1980; Rogers *et al*., 1985; Maniloff, 1992). As a consequence of the reduced biosynthetic machinery, Mollicutes live in nature as obligate parasites and depend on the uptake of many essential molecules from their hosts (Papazisi *et al*., 2003). Thus, they have been considered model systems for defining the minimal set of genes required for a living cell (Morowitz, 1984).

Although, Mollicutes have a simple genome, mycoplasma diseases are complex and relatively unknown (Minion *et al*., 2004). One hallmark of these diseases is the chronicity (Ross, 1992), but equally important is the ability to alter or circumvent the immune response and to potentiate diseases caused by other pathogens (Ciprian *et al*., 1988; Thacker *et al*,. 1999; Muhlradt, 2002). A key factor in the ability of mycoplasmas to establish a chronic infection is their genome flexibility, which allows them to produce a highly variable mosaic of surface antigens (Citti and Rosengarten, 1997; Chambaud, *et al*., 1999; Shen *et al*., 2000 Assunção *et al*., 2005).

In the last years, the genomes of ten mycoplasma species have been completely sequenced (Himmelreich *et al*., 1996; Glass *et al*., 2000; Chamabaud *et al*., 2001; Sasaki *et al*., 2002; Berent and Messik, 2003; Papazisi *et al*., 2003; Westberg *et al*., 2004; Jaffe *et al*., 2004; Minion *et al*., 2004). Recently, the complete genomes of a pathogenic (7448) and nonpathogenic (J [ATCC 25934]) strains of *Mycoplasma hyopneumoniae,* as well as the complete genome of a strain (53) of *Mycoplasma synoviae* (Vasconcelos *et al*., 2005) were obtained. Both species cause great adverse impact on animal production. *M. hyopneumoniae* is the causative agent of porcine enzootic pneumonia, a mild, chronic pneumonia of swine, commonly complicated by opportunistic infections with other bacteria (Ross, 1992). Like most other members of the order *Mycoplasmatales*, *M. hyopneumoniae* is infective for a single species, but the mechanisms of host specificity are unknown. *M. synoviae* is the major poultry pathogen

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

Kinases play indispensable roles in numerous cellular metabolic and signaling pathways, and they are among the best-studied enzymes at the structural, biochemical, and cellular levels. Despite the fact that all kinases use the same phosphate donor (in most cases, ATP) and catalyze apparently the same phosphoryl transfer reaction, they display remarkable diversity in their structural folds and substrate recognition mechanisms, probably due largely to the extraordinarily diverse nature of the structures and properties of their substrates (Cheek *et al*., 2005).

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

Methods

By using previous results from the complete genomes of *M. synoviae* and *M. hyopneumoniae,* J and 7448 strains as input to BLAST search tools we obtained 17 ORFs encoding kinase homologues in *M. synoviae* and 15 in both strains of *M. hyopneumoniae*. Putative biological functions of the kinases were deduced by using Pfam interface and InterPro information. The classification of enzymes into fold groups and families was performed by following the scheme described by Cheek *et al*. (2005). In brief, all kinase sequences from the NCBI non-redundant database were assigned to a set of 57 profiles describing catalytic kinase domains by using the hmmsearch program of the HMMR2 package (Eddy, 1998). Sequences from each Pfam/COG profile presenting significant PSI-BLAST (Altschul *et al*., 1997) hits to each other were clustered into the same family. Families in the same fold group share structurally similar nucleotide-binding domains that have the same architecture and topology (or are related by circular permutation) for at least the core of the domain. Multiple sequence alignments were generated using the ClustalX 1.81 software (Thompson *et al*., 1997). The amino acid sequence relationships were generated with the predicted protein sequences obtained from 47 kinase-encoding ORFs identified in the complete genome sequences of *M. synoviae* and *M. hyopneumoniae*. A phylogenetic tree was constructed by multiple sequence alignments (pairwise alignments) using the Clustal X 1.81 program (Thompson *et al*., 1997) and visualized by using the TreeView software. The tree was constructed by using the minimum evolution (neighbor-joining) method (Saitou and Nei, 1987). Robustness of branches was estimated using 100 bootstrap replicates.

Results and Discussion

Mycoplasma kinases

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

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

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

of *M. hyopneumoniae* (7448) were also identified in the nonpathogenic strain (J). This finding could be explained by the fact that such enzymatic activities may be essential to Mollicutes which have a reduced metabolism.

Kinase classification

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

Nucleotide metabolism and kinases

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

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

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

*The classification was based on Cheek *et al*. (2005).

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

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

Kinases involved in the metabolism of carbohydrates

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

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

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

Riboflavin metabolism and kinases

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

Amino acid sequence relationships

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

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

Concluding Remarks

In the complete genomes of *M. synoviae* strain 53, *M. hyopneumoniae* strains J and 7448 we identified kinases in-

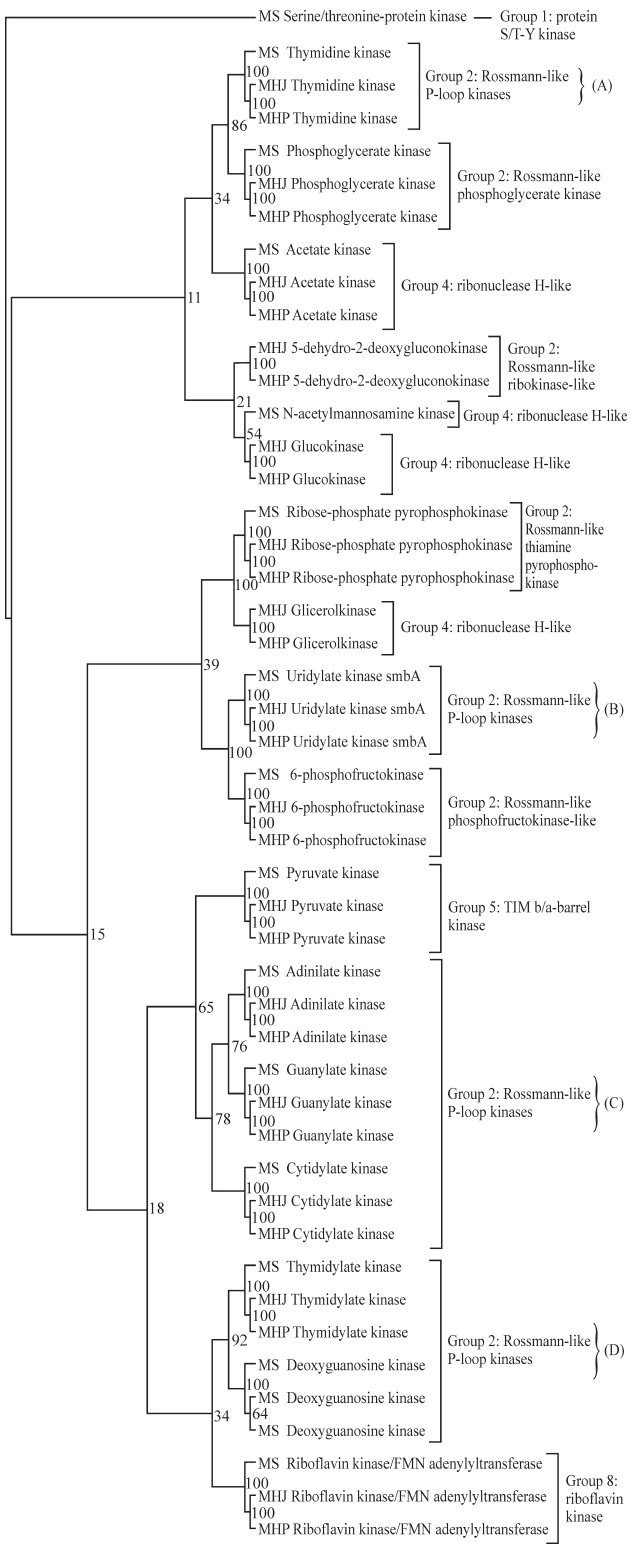

Figure 1 - Phylogenetic tree obtained from kinase amino acid sequence relationships. The kinase fold groups and families are shown in brackets on the right side. The Group 2: Rossmann-like P-loop kinases were clustered into four sub-groups (A, B, C and D). The numbers on the branches are bootstrap values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

volved in many essential metabolic pathways such as carbohydrates, purine, pyrimidine and cofactors metabolism. The presence of those enzymes evidenced the metabolic machinery utilized by these organisms which are considered minimalist models.

Acknowledgments

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

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Internet Resources

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

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

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

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

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

Associate Editor: Arnaldo Zaha

Mycopathologia (2008) 165:259–273
DOI 10.1007/s11046-007-9078-8

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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Received: 12 July 2007 / Accepted: 15 October 2007

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

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.

Electronic supplementary material The online version of this article (doi:10.1007/s11046-007-9078-8) contains supplementary material, which is available to authorized users.

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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^{TM}, 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.

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%).

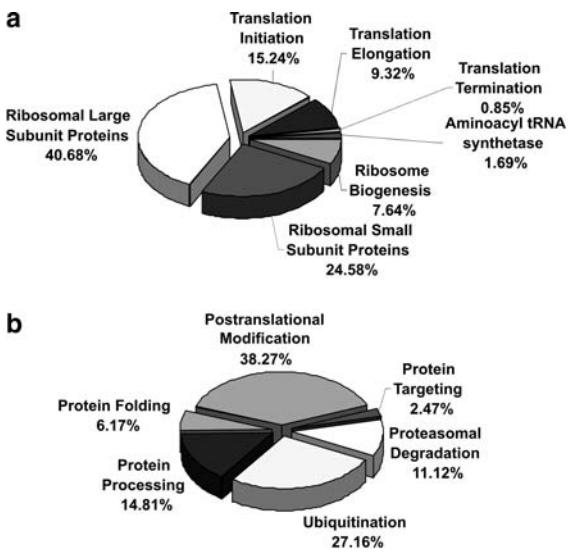

Fig. 1 Distribution of ESTs from *P.brasiliensis* mycelia ongoing transition to yeast according to their cDNA products. (**a**) Protein synthesis; (**b**) protein fate. The classification was based on *E*-value and performed according to the functional categories developed on the MIPS functional annotation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

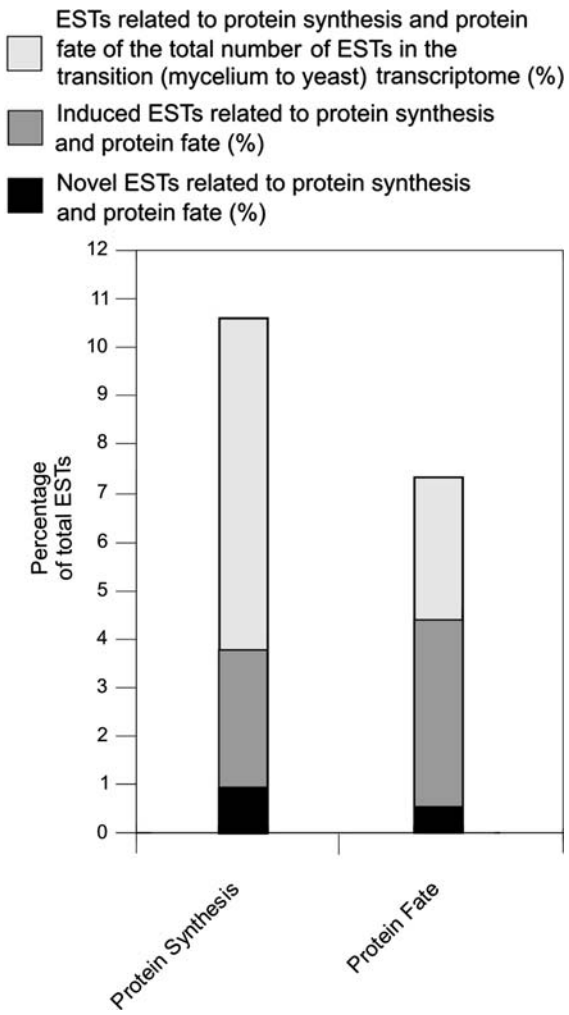

Fig. 2 Prevalence distribution of ESTs from *P. brasiliensis* mycelia ongoing transition to yeast. The percentage of ESTs related to protein synthesis and fate in the total ESTs are represented along with the percentage of over expressed and novel genes of *P. brasiliensis* in the transition library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

Acknowledgments This work at Universidade Federal de Goiás was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico- 505658/2004-6). Juliana A. Parente and Clayton L. Borges are PhD fellows from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq, respectively.

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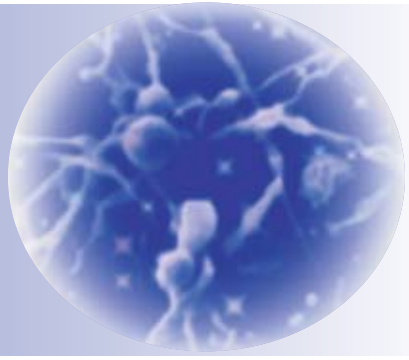
Capítulo IV

Trabalho com autoria compartilhada



BMC Microbiology

Research article

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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Published: 10 April 2007

BMC Microbiology 2007, **7**:29 doi:10.1186/1471-2180-7-29

Received: 4 December 2006
Accepted: 10 April 2007

This article is available from: http://www.biomedcentral.com/1471-2180/7/29

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-

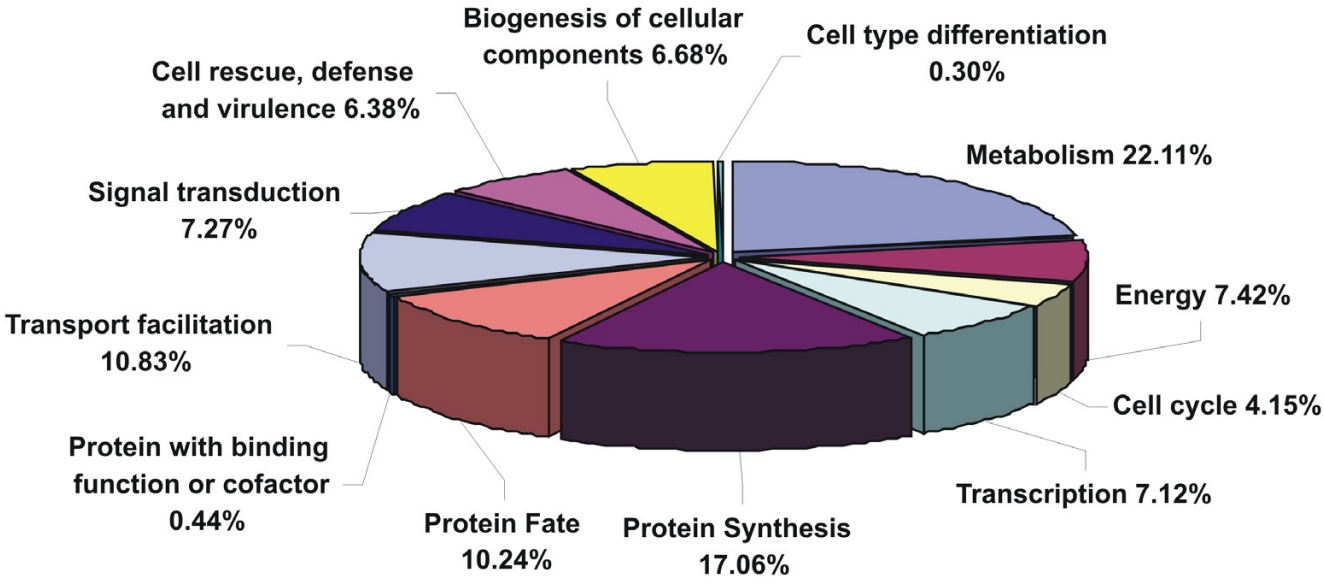

Figure 1
Classification of ESTs from the transition cDNA library of *P. brasiliensis*. The classification was based on *E* value and performed according to the functional categories developed on the MIPS functional annotation scheme.

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

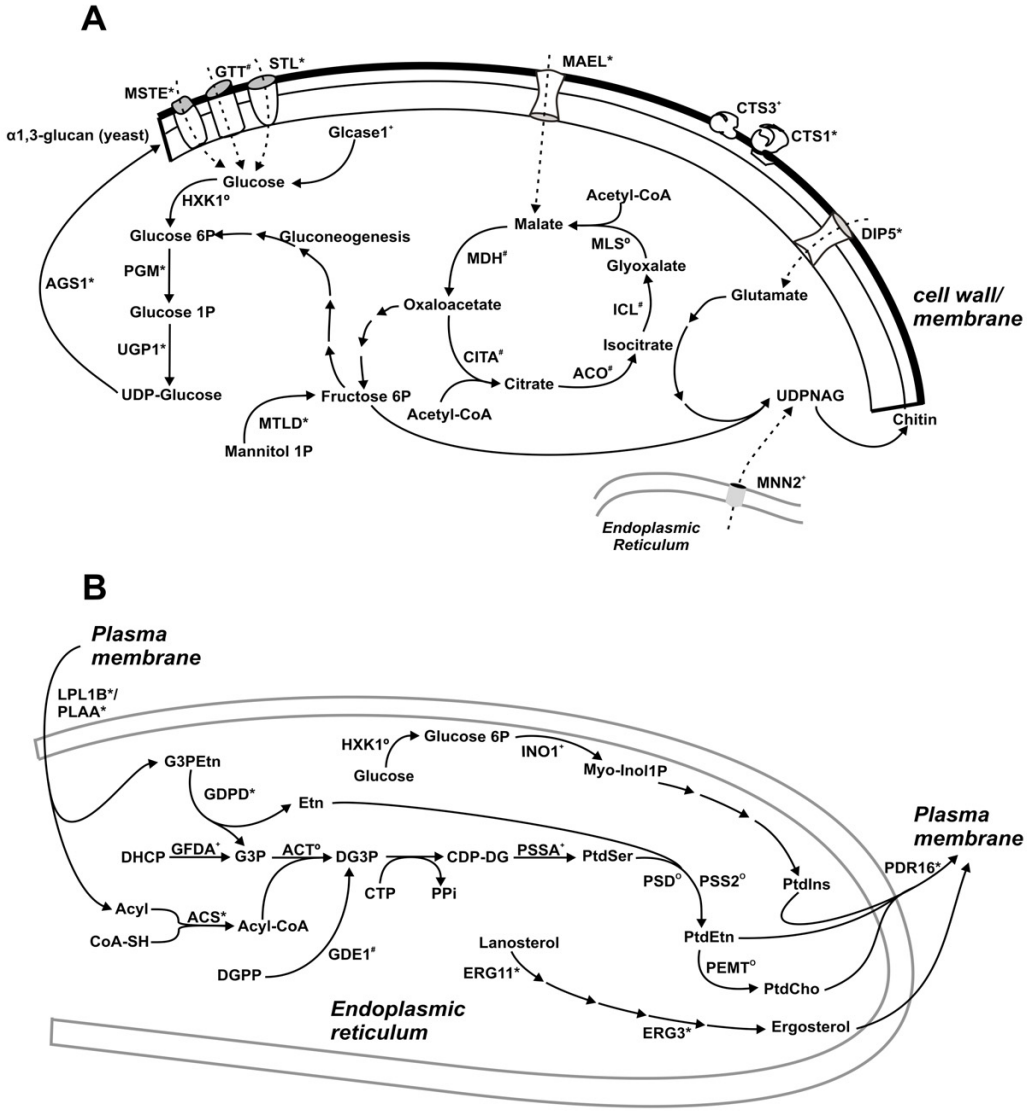

Figure 2

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,

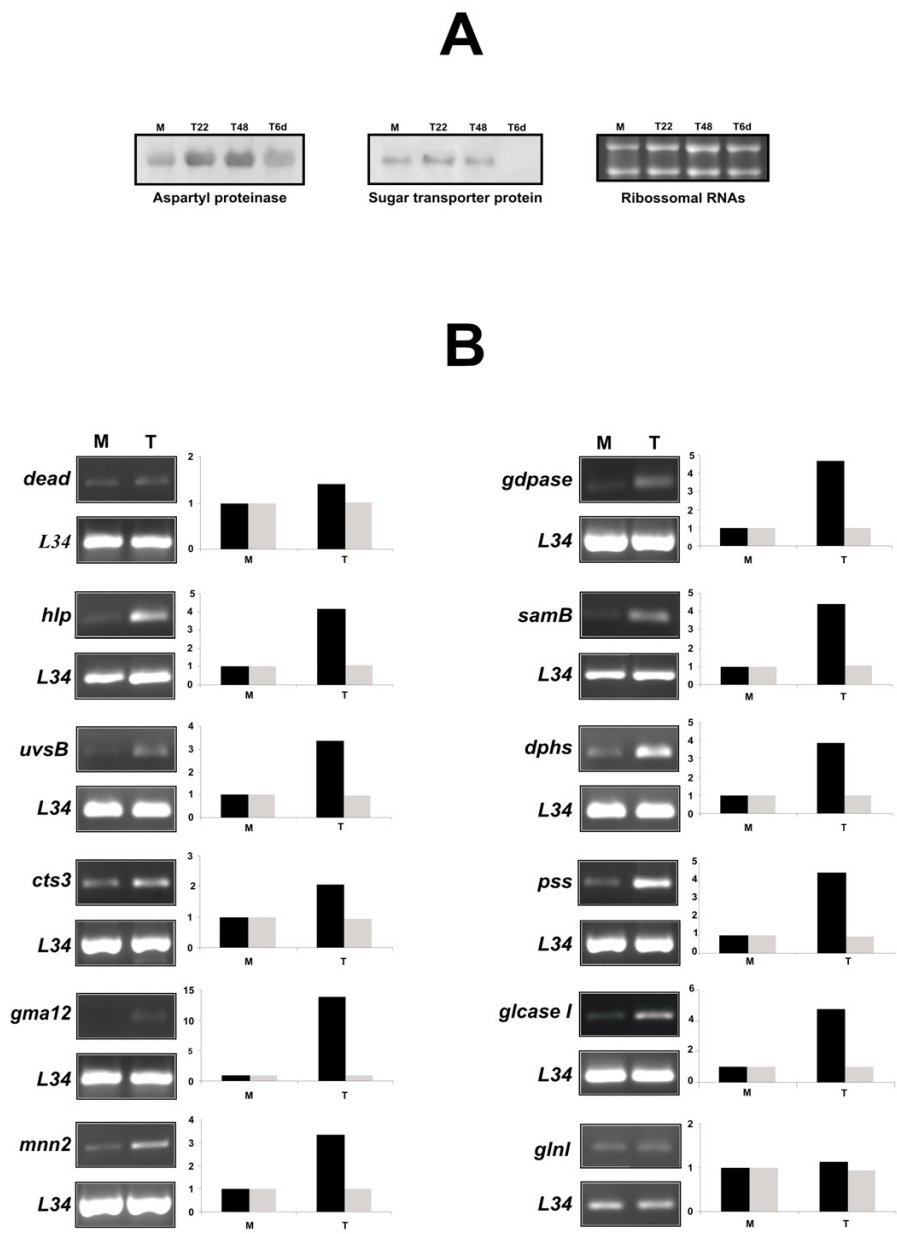

Figure 3

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 (glcasel)	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.*

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[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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[http://www.biomedcentral.com/content/supplementary/1471-2180-7-29-S2.doc]

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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[http://www.biomedcentral.com/content/supplementary/1471-2180-7-29-S3.doc]

Acknowledgements

This work at Universidade Federal de Goiás was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico-505658/2004-6). AMB, CLB, MGS and RBF have fellowship from CNPq. The authors wish to thank Dr. George S. Deepe Jr, Division of Infectious Diseases, University of Cincinnati, Ohio, USA, for providing invaluable discussion and for the critical review of this manuscript.

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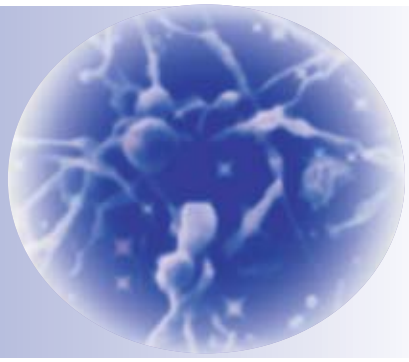
Capítulo V

Discussão



6 – DISCUSSÃO

Microrganismos desenvolvem uma miríade de estratégias para sobrevivência quando no ambiente hostil do hospedeiro. A complexidade mecanística do sistema adotado por *P. brasiliensis* para detectar e responder ao ambiente do hospedeiro está começando a ser elucidada. Com o objetivo de se determinar as bases moleculares dos mecanismos de sobrevivência do fungo em diferentes nichos do hospedeiro o presente trabalho analisou o perfil transcricional do patógeno em condições que mimetizam diferentes etapas do processo infeccioso. O padrão de expressão de genes diferencialmente expressos foi avaliado de células de *P. brasiliensis* recuperado de fígados de camundongos infectados. Como este patógeno utiliza a rota hematogênica de disseminação seu transcriptoma foi estudado após incubação com sangue humano durante 10 minutos e 60 minutos. Além disso, este patógeno pode ser exposto ao plasma humano em conseqüência de resposta inflamatória aguda no sítio inicial de infecção. Desta maneira, para elucidar a influência do plasma no perfil transcricional, foram isolados e caracterizados os transcritos induzidos nesta condição.

6.1 – Estratégias adaptativas de *P. brasiliensis* na rota hematogênica de disseminação e na infecção de fígado em animais experimentais

A anotação e comparação dos genes induzidos durante a infecção de fígado de camundongos bem como, durante a incubação com sangue humano permitiu a identificação dos vários processos biológicos envolvidos na sobrevivência do *P. brasiliensis* nestes ambientes.

Para que ocorra uma colonização bem sucedida do hospedeiro os microrganismos devem inicialmente aderir a tecidos alvos e concomitantemente obter nutrientes essenciais para o seu crescimento. Ferro (Fe) é geralmente um desses nutrientes essenciais e a habilidade em obter esse componente é considerado um fator de virulência (Weinberg, 1999), uma vez que a quantidade de ferro livre nos tecidos é extremamente

baixa (Ratledge and Dover, 2000). Devido e importância metabólica do ferro na sobrevivência de patógenos, a limitação da disponibilidade deste metal é uma estratégia de defesa do hospedeiro (Finkelstein *et al.*, 1983; Jurado, 1997). *In vivo*, todo o Fe extracelular está ligado a proteínas séricas e de muco como a transferrina. Tal ligação diminui substancialmente o acesso do micróbio ao Fe (Finkelstein *et al.,* 1983). Uma das estratégias utilizadas por macrófagos na morte de patógenos através da deprivação de Fe é a redução do nível de receptores para transferrina, mecanismo que diminui o nível de ferro intracelularmente e conseqüentemente diminui o nível do nutriente para os microrganismos residentes em fagossomos (Theurl et al., 2005).

Durante a infecção o nível de ferro disponível é significativamente reduzido e sistemas de aquisição deste composto tornam-se mecanismos essenciais para virulência, como demonstrado em vários organismos. Foi demonstrado que excesso de ferro promove a exacerbação de meningite em um modelo animal de infecção cerebral por *C. neoformans* (Barluzzi *et al.,* 2002). Mutantes do gene codificante para ferro oxidase (contendo cobre) relacionada à captação de ferro em *C. albicans* , são menos letais do que o tipo selvagem em camundongos (Ramanan and Wang, 2000). Em *C. albicans* a expressão aumentada de transcritos por privação de ferro inclui uma variedade de genes, desde aqueles relacionados a propriedades da parede celular aos relacionados com atributos de virulência, como os genes codificantes para hidrolases secretadas (Lan *et al.,* 2004). A colonização de estômago por *Helicobacter pylori* requer expressão de um regulador de captação de ferro (*Fur*), o qual promove a expressão de vários genes em condições de privação de ferro. Mutantes para o gene *Fur* são menos hábeis em colonizar o hospedeiro em comparação ao fenótipo selvagem (Gancz *et al.,* 2006). O fator de transcrição ferro-dependente (*Cir* 1) de *C. neoformans* controla a regulação de vários genes para aquisição de ferro, bem como a expressão de fatores de virulência como a produção de cápsula e de melanina (Jung *et al.,* 2006).

A captação de íons de ferro envolve transportadores de alta e baixa afinidade; o sistema de alta afinidade é assistido por uma proteína da família de oxidases de cobre, que oxida o ferro para sua forma férrica (Stearman *et al.,* 1996). Este sistema é encontrado em fungos e é induzido em condições de baixa concentração de ferro. Por meio de análises de SAGE foi demonstrado que a limitação de Fe resulta em um alto

nível de transcritos codificantes para componentes de transporte de ferro em *C. neoformans* (Lian *et al.,* 2005).

Um dos cDNAs mais abundantes na condição de infecção de fígado codifica uma permease de ferro/zinco de alta afinidade predita, a qual é ortóloga ao gene *zrt1* (transportador de zinco/ferro de alta afinidade) de *S. cerevisiae* (Zhao and Eide, 1996), possivelmente relacionada com a captação de ferro por *P. brasiliensis*. A redundância observada para este transcrito foi de 140 ESTs (etiquetas de seqüências expressas) no fungo recuperado de tecido infectado. O transcriptoma do *P. brasiliensis*, isolado *Pb*01, cultivado *in vitro* revelou uma redundância de 8 ESTs para o *zrt1* (Felipe *et al.,* 2005). A superexpressão do *zrt1* foi confirmada por meio de dot blot e northern blot, durante a infecção de fígado de camundongos, fato este que reflete o potencial papel desta molécula na infecção do *P. brasiliensis*. Estes dados podem refletir a limitação de ferro encontrada nos tecidos do animal.

A captação de ferro de alta afinidade requer uma eficiente captação de cobre, pois a maturação de uma proteína que contêm cobre, a qual participa do processo de captação de Fe, necessita de cobre e a ausência do gene responsável por esta atividade resulta na diminuição do transporte de Fe de alta afinidade (Dancis *et al.,* 1994). O transporte de cobre em células leveduriformes recuperadas de tecidos infectados parece estar exacerbado neste momento celular. A alta redundância de transcritos de um possível transportador de alta afinidade (56 ESTs) sugere que *P. brasiliensis* apresenta um transporte de Fe dependente de cobre. Além disso, a indução da expressão do *ctr3* (transportador de cobre de alta afinidade) durante a infecção de camundongos, foi confirmada pelo uso de dot blot, northern blot e RT-PCR semiquantitativo (RT-PCRsq) suportando seu potencial papel na patogênese do fungo. Ressalte-se que dados recentes de nosso grupo revelam que o transcrito *ctr3* é superexpresso em *P. brasiliensis* durante a infecção de macrófagos murinos (dados não mostrados).

Embora a concentração de ferro livre no sangue seja baixa, os genes que participam do transporte de alta afinidade não foram induzidos durante a incubação de células leveduriformes de *P. brasiliensis* em sangue humano. Este fato pode refletir uma condição de ferro não limitante para o fungo nesta etapa de disseminação. A captação de ferro poderia ser dependente da FRE2 (ferro redutase detectada no tratamento com

sangue) que estaria removendo ferro de moléculas do hospedeiro que possuem grupos ferro, tal como heme (Timmerman and Woods, 2001). Corroborando nossos resultados, atividade de ferro redutase extracelular foi recentemente descrita em *P. brasiliensis* (Zarnowski and Woods, 2005).

A hipótese que a disponibilidade de ferro seja restrita no citoplasma de células do hospedeiro foi fortalecida pela observação da indução de genes que participam da captação de ferro. Os dados sugerem que o sistema de captação de alta afinidade é o principal sistema de transporte de ferro durante a infecção intracelular. Além disso, os resultados sugerem a existência de um sistema de obtenção de ferro regulado pela disponibilidade do nutriente e que estes genes são induzidos em resposta à estarvação de ferro na infecção do hospedeiro. A confirmação de transcritos destes genes em *P. brasiliensis* recuperado de fígado de camundongo infectado suporta esta hipótese, o que permite ainda inferir que a captação de ferro pela permease de alta afinidade representa, não somente nutrição, mas também a participação deste sistema na patogênese do fungo. Em *C. albicans* existem dois genes codificantes para permeases de ferro e um destes codifica um transportador de alta afinidade o qual é requerido para virulência no modelo de infecção de camundongos (Ramanan and Wang, 2000).

Vários produtos diferenciais identificados codificam para proteínas de choque térmico. A produção de HSPs contribui tanto para proteção celular quanto para o reparo de danos causados por estresse, danos estes que podem ocorrer durante a infecção. Além disso, as HSPs realizam importantes papeis em diferentes processos celulares tais como divisão celular, síntese de DNA, transcrição, tradução, enovelamento e trasnporte protéico, e translocação transmembrana (Mager and Ferreira, 1993; Liberek *et al.*, 2008). Notavelmente, o transcrito codificante para a HSP30 foi altamente expresso em leveduras de *P. brasiliensis* recuperadas de fígado de camundongo infectado. Este fato é particularmente interessante, pois esta HSP é fortemente induzida por tratamentos que aumentam a fluidez da membrana, como no choque térmico (Seymour and Piper, 1999; Coucheney *et al.*, 2005). Novas funções para HSPs de baixa massa molecular foram descobertas. Sugere-se que um conjunto destas moléculas desempenha um papel na proteção celular, modulando o estado físico de lipídeos em condições de choque térmico (Coucheney *et al.,* 2005). Esta função incomum atribuída às HSPs é reforçada pela

localização destas moléculas na membrana, bem como pela observação que a indução da expressão destas proteínas em algumas condições de estresse parece não ser regulada por fatores de transcrição regulados por estresse (Seymour and Piper, 1999). Anteriormente, observou-se que a HSP30 foi preferencialmente expressa na fase leveduriforme de *P. brasiliensis*, onde 51 ESTs codificantes para a proteína foram identificadas no transcriptoma de células leveduriformes cultivadas *in vitro* (Felipe *et al.,* 2005). Apesar deste achado, a alta expressão da HSP30 em tecidos infectados (33,67% dos transcritos totais) reforça o envolvimento desta proteína no processo infeccioso. Adicionalmente, a expressão diferencial da HSP30 nesta condição foi confirmada utilizando-se dot blot e northern blot. A manutenção da homeostase das células de *P. brasiliensis*, bem como a fluidez da membrana podem ser possíveis funções da proteína.

O tratamento de leveduras com sangue humano induz uma alta expressão de genes possivelmente associados com a remodelação da parede celular. O gene com maior expressão na condição que mimetiza a rota hematogênica de disseminação foi aquele codificante para glutamina sintase (GLN1). Esta proteína é uma enzima reguladora da assimilação de nitrogênio em bactérias e fungos fornecendo glutamina para as células. A indução deste gene em *P. brasiliensis* é uma forte evidência que a remodelação da parede celular/membrana pode ser uma das maneiras pelas quais *P. brasiliensis* detecta e responde a mudanças na osmolaridade externa. Corroborando, a síntese de quitina foi demonstrada como sendo essencial na resposta compensatória ao estresse na parede celular de fungos, prevenindo a morte celular (Popolo *et al.,* 1997). O composto doador de açúcar na síntese de quitina é a UDP-N-acetilglicosamina. A via metabólica que leva à formação de UDP-N-acetilglicosamina a partir de frutose 6-fosfato consiste em 5 passos, dos quais o primeiro passo desta via é a formação de glicosamina-6-fosfato a partir de glutamina e frutose, uma etapa limitante neste processo. A resposta ao estresse da parede celular em *Aspergillus niger* envolve a indução da expressão do gene *gfa*A codificante para glutamina:frutose-6-fosfato amidotransferase e uma acentuada deposição de quitina na parede celular (Ram *et al.,* 2004). De forma similar especulamos que o aumento da expressão de transcritos para glutamina sintase pode estar relacionado com deposição de quitina em resposta à mudança na osmolaridade externa que o fungo encontra na rota de disseminação hematogênica. Ressalte-se que a expressão diferencial do gene da *gln1* foi

confirmada por dot blot, northern blot e RT-PCRsq durante incubação de células leveduriformes com sangue. O transcrito também foi detectado em leveduras durante infecção sanguínea de camundongos BALB/c.

A hipótese de que o aumento na síntese de glutamina, durante contato com sangue humano, possa fornecer um mecanismo de reassimiliação/desintoxicação para o fungo *P. brasiliensis*, não pode ser desconsiderada. Entretanto, foi demonstrado que somente uma pequena parcela de amônia é incorporada no grupo amina da glutamina de *S. cerevisiae* (Magasanik, 1992). O entendimento completo do papel desempenhado pela glutamina sintase na infecção seria possível por meio de ruptura/silenciamento gênico para avaliar-se se o hospedeiro poderia suprir toda demanda de glutamina requerida pelo patógeno durante a patogênese. O desenvolvimento de ferramentas genéticas para avaliação funcional de genes de *P. brasiliensis* estão ainda em fase de desenvolvimento (Almeida *et al.*, 2007).

A permease de aminoácido ácido (DIP5) pode mediar a captação de glutamato e aspartato (Regenberg *et al.,* 1998). O glutamato poderia ser utilizado como substrato da glutamina sintase e, conseqüentemente, estaria relacionado com a deposição de quitina do fungo *P. brasiliensis* como descrito anteriormente. Neste sentido, a alta expressão desta permease estaria relacionada com a captação deste aminoácido no ambiente sanguíneo, resultando em um alto teor de glutamina disponível para síntese de quitina. Além disso, o glutamato é requerido para manter o interior celular em balanço osmótico com o meio externo. A biossíntese de pirodoxal-5-fosfato (PLP) depende da catálise de duas enzimas: piridoxamina quinase e piridoxamina fosfato oxidase (PPO1). A alta expressão do transcrito codificante para PPO1 em *P. brasiliensis* após 60 minutos de incubação com sangue total pode também estar relacionado com a produção de glutamato por amidotransferases que requerem como coenzima o PLP. A indução na captação deste aminoácido foi associada com a expressão de uma septina (*spn1*) em astrócitos humanos, que modula a atividade de um transportados de glutamato (Kinoshita *et al.,* 2004). Durante a incubação com sangue humano um transcrito codificante para um ortólogo de septina foi identificado como produto diferencial. Interessantemente, estudos em *Blastomyces dermatitidis* demonstram que a *spn1* provavelmente é um gene essencial e está relacionado com morfogênese e esporulação deste fungo (Krajaejun *et al.,* 2007).

Os eucariotos respondem a diferentes condições de estresse, incluindo estresses osmóticos e de temperatura, pela ativação da via das MAPK. A via HOG (alta osmolaridade do glicerol) está relacionada com controle e detecção de estresse osmótico através de dois reguladores acoplados a membrana, *sln1* (quinase sensor) e *sho1* (osmosensor transmembrana) (Winkler *et al.,* 2002; Roman *et al.,* 2005). A função do osmosensor *sho1* foi caracterizada em *C. albicans*. A molécula desempenha um papel discreto na transmissão do sinal de fosforilação para MAPK Hog1 em resposta ao estresse oxidativo, o qual ocorre principalmente por meio do braço *sln1* da via HOG. Por outro lado, *sho1* está relacionado com morfogênese do fungo interconectando duas vias envolvidas com biogênese da parede celular e estresse oxidativo (Roman *et al.,* 2005). O transcrito de *sho1* de *P. brasiliensis* apresentou-se induzido tanto no tratamento com sangue humano quanto durante a infecção sanguínea de camundongos, o que sugere sua importância no processo de detecção de mudanças da osmolaridade pelo fungo durante a etapa de disseminação hematogênica, possivelmente controlando o remodelamento da parede celular.

Proteínas fotoativas em amarelo (PYPs) são fotorreceptores que mediam a resposta fototática negativa em bactérias. Proteínas PYP são membros da superfamília PAS (Per-Arnt-Sim) que desempenham papéis em processos de sinalização, detecção de estímulos e podem também mediar interações proteína-proteína (Taylor and Zhulin, 1999). Um homólogo a esta família foi superexpresso durante tratamento com sangue. Tal fato suporta o papel predito destas proteínas sensores na percepção das diferentes condições encontradas por *P. brasiliensis* no sangue humano durante a disseminação para órgãos e tecidos.

Dentre os produtos diferencias, foi identificado um transcrito homólogo ao fator de transcrição *ap-1*, durante contato do *P. brasiliensis* com sangue total humano. Seqüências homólogas a este fator de transcrição *ap-1* de mamíferos foram descritas em fungos, tais como *C. albicans*, *Schizosaccharomces pombe, Kluyveromyces lactis*, *S. cerevisiae* e *Cochilobolus heterostrophus*. Análises genéticas revelaram que estas proteínas podem desempenhar funções na reposta ao estresse oxidativo, resistência a cádmio e controle do tráfego de complexos ubiquitinizados (Lev *et al.*, 2005; Phelan *et al.*, 2006).

A síntese protéica foi um dos processos mais prevalentes na incubação do *P. brasiliensis* em sangue humano durante 60 minutos. As ESTs mais freqüentes corresponderam a seqüências que codificam para proteínas que compõem a maquinaria de síntese protéica (28% do total de ESTs). Os transcritos mais abundantes foram o fator de elongação da tradução, subunidade 1-gama (eEF-1γ) (20 ESTs), e fator de iniciação da tradução, subunidade 4A (eIF-4A) (43 ESTs). O grupo amino da maioria dos aminoácidos deriva do glutamato via reações de transaminação. O nitrogênio do grupo amina do glutamato é uma fonte de grupo amina em uma ampla variedade de processos biossintéticos. Conseqüentemente, a alta captação de glutamato no tratamento de *P. brasiliensis* com sangue poderia prover as células do fungo com precursores na alta taxa de síntese protéica. Corroborando, enzimas envolvidas na síntese dos cofatores tetrahidrofolato (dihidropteroato sintase) e S-adenosilmetionina (S-adenosil metionina sintase) foram superexpressos no sangue, o que insinua um metabolismo ativo de compostos nitrogenados. A superexpressão de genes que compõem a maquinaria de síntese protéica pode refletir tanto a adaptação molecular a uma nova condição celular, bem como a transferência do *P. brasiliensis* de um ambiente pobre em nutrientes para um ambiente relativamente rico, nutricionalmente, como o sangue. Resultados similares foram descritos para o fungo patogênico *C. albicans* (Fradin *et al.,* 2003).

Vários genes identificados por meio de RDA, neste trabalho, foram previamente implicados na patogênese de organismos. A entrada do patógeno nas células do hospedeiro é iniciada pela aderência do fungo à superfície celular (Mendes-Giannini *et al.,* 2000). Um fator de virulência potencialmente significante inclui a eficiência de *P. brasiliensis* na adesão e consequente invasão aos tecidos do hospedeiro. A gliceraldeido-3-fosfato-desidrogenase (GAPDH) é altamente transcrita principalmente em células obtidas de fígado de animais infectados. Esta enzima apresenta-se como uma molécula multifuncional encontrada extracelularmente em eucariotos, apresentando funções não relacionadas à glicólise. Foi elucidado que a GAPDH de *P. brasiliensis*, reconhecida por soros de pacientes com PCM (Fonseca *et al.,* 2001; Barbosa *et al.,* 2004), está localizada na parede celular do fungo. A proteína funciona como uma adesina de *P. brasiliensis* que liga-se a componentes da matriz extracelular e é capaz de mediar a aderência e internalização do *P. brasiliensis* a culturas de células, o que sugere seu envolvimento na

patogênese deste fungo e de outros microrganismos (Barbosa *et al.*, 2006; Kinoshita *et al.*, 2008).

Várias evidências sugerem que as serinas proteases são solicitadas para uma invasão eficaz de células do hospedeiro por patógenos. Uma serina protease tiol-dependente foi caracterizada em células leveduriformes de *P. brasiliensis*. Esta protease cliva os principais componentes da membrana basal *in vitro*, sendo, portanto potencialmente relevante para disseminação do fungo (Puccia *et al.,* 1999). Estas enzimas podem desempenhar um papel importante na degradação de proteínas do hospedeiro tanto durante a invasão de células hospedeiras quanto durante a disseminação através dos órgãos. Em particular, a serina protease com predominante expressão nas condições analisadas apresenta um motivo de ligação ao zinco, o qual é comum dentre as proteases de patógenos (Parente *et al.,* 2005). Dados recentes evidenciaram que a serina protease é uma proteína secretada pelo fungo sob deprivação de nitrogênio (Parente, comunicação pessoal)

Transcritos codificantes para tirosinase e para descarboxilase de aminoácidos aromáticos (DDC) foram superexpressos em células recuperadas de fígado de camundongos B10A infectados. A tirosinase é responsável pelo primeiro passo da biossíntese de melanina e é uma proteína que contem cobre (Arredondo and Nunez, 2005). A melanina está implicada na patogênese de varias doenças humanas, incluindo algumas infecções microbianas. Vários tipos diferentes de melanina de fungos foram identificados. Os dois tipos mais importantes são DHN-melanina (nomeada com base em um dos intermediários da via, 1,8-dihidroxinaftaleno) e DOPA-melanina (nomeada com base no precursor L-3,4-dihidroxifenialanina). Ambos os tipos de melanina estão implicados na patogênese de organismos. O processo de melanização de *C. neoformans* é dependente de vários genes. Os genes *ccc2* e *atx1*, codificantes para homólogos de transportador de cobre e chaperona de cobre, respectivamente, coordenam e mantêm a homeostase de cobre e estão relacionados com a melanização de *C. neoformans* (Walton *et al.,* 2005). Pigmentos similares à melanina foram detectados em *P. brasiliensis* cultivado *in vitro* e durante infecção (Gomez *et al.,* 2001). Adicionalmente, estudos demonstraram que leveduras melanizadas de *P. brasiliensis* são mais resistentes à atividade fungicida de macrófagos murinos (Silva *et al.,* 2006). A alta expressão da DDC

em *P. brasiliensis* tratado com plasma pode refletir os altos níveis de L-DOPA, neste ambiente (Machida *et al.,* 2006), que pode ser convertida em melanina pelo fungo. Os dados sugerem o envolvimento da melanina na virulência de *P. brasiliensis*.

Uma família conservada de reguladores transcricionais que apresentam dedo de zinco, conhecidos como fatores-GATA asseguram a utilização eficiente de fontes de nitrogênio por fungos. Em *C. albicans* o gene codificante para o ativador transcricional do tipo GATA foi identificado com um mediador da regulação de nitrogênio, função importante para virulência (Limjindaporn *et al.*, 2003; Liao *et al.*, 2008). Adicionalmente, o gene *gln3* codificante para um fator de transcrição do tipo GATA de *Pichia pastoris*, é fator responsável pelo controle do sistema de captação de ferro deste fungo (Miele *et al.*, 2007). A identificação de um ortólogo de fatores GATA em *P. brasiliensis*, tratado com sangue, sugere o envolvimento desta molécula no metabolismo do nitrogênio deste fungo, o que parece ser ativo nesta condição. Um possível fator de virulência que apresenta similaridade a RAS GTPses foi isolado em células do fungo recuperadas de fígado de camundongos. Estas proteínas estão relacionadas com direcionamento de hifas, crescimento invasivo e possivelmente participam como mediador de sinais extracelulares durante a invasão do hospedeiro por *C. albicans* (Hausauer *et al*., 2005). Um transcrito codificante para delta-5,6-desaturase (ERG3) foi superexpresso por *P. brasilienses* tratado com sangue total. A ruptura gênica do ortólogo de *C. albicans* atenua a virulência, a habilidade de este organismo realizar a transição morfológica de levedura para micélio (Chau *et al.,* 2005), bem como aumenta a sensibilidade do fungo ao fluconazol (Miyazaki *et al.*, 2006).

Um homólogo do gene da histona desacetilase de *A. nidulans* também foi descrito neste trabalho. Este gene não fora descrito nos transcriptomas anteriores de *P. brasiliensis.* No fungo filamentoso *Cochliobolus carbonum* este gene afeta a virulência como resultado de uma reduzida eficiência de penetração em tecidos de plantas (Baidyaroy *et al.*, 2001). Trabalhos futuros serão focados nestes possíveis fatores de virulência de *P. brasiliensis*.

A metodologia de RDA permitiu a identificação de novos transcritos de *P. brasiliensis* não descritos no transcriptoma do fungo, formas leveduriforme e miceliana, cultivadas *in vitro* (https://dna.biomol.unb.br/*Pb*/). Isto sugere a utilidade da estratégia de

cDNA-RDA na identificação de genes diferencialmente expressos em condições especificas e de novos genes de um organismo. Dois novos genes descritos para *P. brasiliensis* estão possivelmente relacionados com síntese e compartimentalização protéica. Para eucariotos, estabelece-se que a especificidade de reconhecimento do códon de parada está associada com o fator de liberação da tradução 1 (eRF1), mais que com o ribossomo, assegurando a alta fidelidade de terminação da síntese protéica (Kervestin *et al.,* 2001). A presença de um tetrapeptideo NIKS na seqüência da proteína é essencial nesta molécula (Frolova *et al.,* 2002). Este fator foi presente durante a condição que mimetiza a disseminação hematogênica e sua expressão foi confirmada utilizando-se o modelo de infecção sanguínea em camundongos. De forma similar, uma proteína envolvida com transporte retículo endoplasmático-Golgi, predita em transitar entre os dois compartimentos, foi descrita. Os resultados estão em conformidade com a alta expressão de genes que compõem a maquinaria de síntese protéica durante incubação com sangue humano.

Dentre os novos genes identificou-se um novo transcrito que apresenta homologia com serina proteases. Este transcrito foi detectado por RT-PCR em células de *P. brasiliensis* presentes em sangue de camundongos BALB/c infectados, indicando o possível envolvimento desta nova proteína no evento de disseminação para órgãos e tecidos. O gene *dbr1* de *S. cerevisiae*, que apresenta como produto gênico uma 2'-5'-fosfodiesterase e que participa do processo de remoção de íntrons de RNA e da biossíntese de snoRNAs (Samarsky *et al.,* 1998), foi identificado em células leveduriformes recuperadas de fígado de camundongos infectados. Muitos dos novos transcritos identificados codificam para proteínas hipotéticas fato que insinua a especificidade do perfil de estratégias utilizadas por *P. brasiliensis* no processo infeccioso.

6.2 – Mecanismos de adaptação do *P. brasiliensis* durante exposição ao plasma humano

O perfil transcricional do *P. brasiliensis* incubado com plasma humano ilustrou uma grande diversidade funcional dos fatores que compõem a natureza das estratégias

adaptativas, utilizadas pelo fungo, nesta condição que mimetiza um nicho encontrado no hospedeiro.Vários transcritos codificantes para enzimas relacionadas com β-oxidação apresentaram expressão exacerbada durante tratamento com plasma humano. Ressalta-se que dentre as enzimas que compõem a via de oxidação de ácidos graxos, identificou-se uma enzima peroxissomal multifuncional que é possivelmente uma 2-enoil-CoA-hidratase/3-hidroacil-CoA-desidrogenase (FOX2), assim como observado em *S. cerevisiae*, *Candida tropicalis* e mamíferos (Moreno de la Garza *et al.,* 1985; Hiltunen *et al.,* 1992; Breitling *et al.,* 2001).

A oxidação de ácidos graxos de cadeia ímpar produz acetil-CoA e propionil-CoA. Em bactérias e fungos, o propionil-CoA é assimilado via ciclo do metilcitrato, que oxida o composto a piruvato (Brock *et al.,* 2000). Acetil-CoA sintases foram detectadas em fungos e podem utilizar acetato e propionato como substratos (De Cima *et al.,* 2005). Alternativamente, a conversão de piruvato à acetil-CoA pode ser realizada pela ação seqüencial das enzimas: piruvato descarboxilase, acetaldeído desidrogenase e acetil-CoA sintase (Van Den Berg *et al.,* 1996).

Genes de *M. tuberculosis* que participam do metabolismo de ácidos graxos apresentam alta expressão na infecção de camundongos e de macrófagos. Adicionalmente, o ciclo do metilcitrato é requerido para o crescimento da bactéria em fagossomos de macrófagos murinos derivados de medula óssea (Munoz-Elias *et al.,* 2006). O efeito do acúmulo do propionil-CoA, oriundo da oxidação de ácidos graxos de cadeia ímpar, sobre o metabolismo celular foi investigado em *A. nidulans* e *A. fumigatus* (Brock and Buckel, 2004; Maerker *et al.*, 2005). Caracterizações bioquímicas detalhadas mostraram que o complexo da piruvato desidrogenase e a succinil-CoA sintase são competitivamente inibidas por propionil-CoA (Brock and Buckel, 2004). Mutantes de *Aspergillus sp*., que possuem genes do ciclo do metilcitrato rompidos, acumulam quantidades significantes de propionil-CoA e, conseqüentemente o crescimento é retardado (Brock *et al.,* 2000; Maerker *et al.,* 2005). Além disso, a inabilidade de *A. fumigatus* remover o propionil-CoA tóxico, que provavelmente resulta da degradação protéica e de ácidos graxos durante o crescimento invasivo, reduz consideravelmente a capacidade do fungo estabelecer aspergilose invasiva (Ibrahim-Granet *et al.,* 2008). Adicionalmente, o metabolismo secundário também é afetado pelo acúmulo de propionil-

CoA, sendo que a formação de precursores de melanina é inibido em altas concentrações de propionil-CoA (Maerker *et al.,* 2005). Ensaios de ruptura gênica de *A. nidulans* revelaram a importância do ciclo do metilcitrato na detoxificação do propionil-CoA (Fleck and Brock, 2008). O teor de RNAm da metilcitrato desidratase foi aumentado durante incubação de *P. brasiliensis* com plasma humano e, portanto, este complexo metabólico poderia funcionar como fornecedor de piruvato para processos biossintéticos, bem como para a manutenção dos metabolismos primário e secundário, que participariam do repertório de estratégias de adaptação do fungo a esta etapa da infecção.

A acetolactato sintase catalisa o primeiro passo em comum na síntese dos aminoácidos ramificados leucina, isoleucina e valina, a partir do piruvato. Mutantes para o ortólogo de *C. neofor*mans perdem a virulência e conseqüentemente são incapazes de sobreviver em animais (Kingsbury *et al.,* 2004). Assim como a acetolactato sintase, a produção de RNAms codificantes para fumarato redutase foi induzida em leveduras de *P. brasiliensis* incubadas com plasma humano. Duas isoenzimas fumarato redutase são necessárias para reoxidação do NADH intracelular em condições de anaerobiose. Corroborando, a fase leveduriforme do *P. brasiliensis* produz ATP preferencialmente por meio da fermentação alcoólica (Felipe *et al.,* 2005). Ainda neste contexto, a aldeído desidrogenase pode converter o etanol em acetato via acetaldeído, fornecendo, portanto acetil-CoA para o ciclo do glioxalato.

Fatores participantes do mecanismo de defesa celular também foram induzidos durante incubação de *P. brasiliensis* com plasma humano. Transglutaminases (TGAse) são enzimas que inserem ligação irreversível tanto intramolecular quanto intermolecular, utilizando resíduos específicos de glutamina de uma proteína e o grupo amina da outra molécula. As moléculas modificadas são resistentes à ação de proteases e desnaturantes (Greenberg *et al.,* 1991). Enzimas com atividade de TGAse estão associadas com o ancoramento de proteínas Pir (proteína com repetições internas presentes na parede celular) ao polímero de β-1,3-glicana em *S. cerevisiae* (Ecker *et al.,* 2006). Esta enzima foi localizada na parede celular de fungos. TGAse de *C. albicans* é um importante fator na manutenção da organização estrutural do fungo, pois a molécula estabelece pontes entre proteínas estruturais e a inibição desta função resulta na alta sensibilidade de protoplastos a choque osmótico (Ruiz-Herrera *et al.,* 1995)

A NADPH-quinona redutase induzida no plasma humano, pode desempenhar função no controle do estresse oxidativo. Esta enzima catalisa a transferência de elétrons do NADPH para quinona, cuja forma reduzida é importante para o gerenciamento do estresse oxidativo. Resistência ao estresse oxidativo é uma propriedade essencial que capacita microrganismos patogênicos a sobreviver aos efeitos das espécies reativas de oxigênio produzidas pelo hospedeiro. A capacidade de *H. pylori* colonizar estômago de camundongos é dependente da atividade antioxidante da NADPH-quinona redutase (Wang and Maier, 2004). O transcrito codificante para catalase A, uma enzima de resposta ao estresse oxidativo, também foi identificado. Catalases são descritas como fatores que conferem resistência ao estresse oxidativo em fungos (Giles *et al.,* 2006). A β-oxidação, induzida nesta condição, é responsável pela produção de peróxido de hidrogênio, o que sugere que a catalase A de *P. brasiliensis* estaria protegendo a célula do peróxido de hidrogênio endógeno.

O contato com o plasma também promoveu o aumento da transcrição de genes que podem estar associados com facilitadores de transporte que participam na obtenção de nutrientes. Os transcritos com maior abundância codificam as proteínas DIP5 e FRE2. Um alto nível de transcritos da DIP5 pode estar relacionado com a obtenção de glutamato para o remodelamento da parede. O alto teor de FRE2 no plasma sugere uma ativa redução do Fe^{+3} em Fe^{+2} que seria internalizado por uma permease de ferro. O papel de serinas proteases no processo de invasão de tecidos do hospedeiro por patógenos é estabelecido. Esta proteína apresenta um papel essencial na clivagem de proteínas do hospedeiro durante a invasão tecidual e disseminação. Serina protease de *Bacillus subtilis* facilita a captação de ferro da molécula transferrina através da clivagem da proteína (Park *et al.,* 2006). Complementando, a incubação de *A. fumigatus* em meio contendo soro humano estimula a secreção de proteases, sendo a atividade de serinas proteases a classe catalítica com a maior atividade (Gifford *et al.*, 2002). Desta maneira a produção de proteases no tratamento com plasma poderia se uma das estratégias utilizadas por *P. brasiliensis* para a captação do micronutriente ferro.

Assim como observado no tratamento de *P. brasiliensis* com sangue total humano, a incubação com plasma induziu a expressão de transcritos associados com biossíntese de proteínas, como, por exemplo, fatores eucarióticos da tradução. Esta alta

taxa de síntese protéica reflete uma mudança no estoque protéico em busca da adaptação à nova condição. Assim como especulado no tratamento com sangue, este fato também pode refletir a adaptação do fungo ao um meio nutricionalmente rico (Fradin *et al.*, 2003). Além disso, outros transcritos também foram identificados como produtos diferenciais tanto no tratamento com plasma quanto no tratamento com sangue humano. Dentre eles estão aqueles codificantes para osmosensor transmembrana, glutamina sintase e a descarboxilase de aminoácidos aromáticos. Tal fato sugere que os mecanismos de resposta a estresse osmótico, remodelamento da parede celular e síntese de melanina são processos importantes para sobrevivência do *P. brasiliensis* nestas condições. Além disso, a diferença da redundância de ESTs para estes genes nas duas condições sugere a influência das células do sangue no perfil transcricional do fungo, assim como observado para *C. albicans* (Fradin *et al.,* 2003). Adicionalmente, a comparação entre os perfis transcricionais do *P. brasiliensis* incubado com sangue humano e o fungo incubado com plasma humano demonstrou que 16,63% dos transcritos induzidos pelo plasma não foram encontrados no tratamento com sangue humano, reforçando a influencia das células sanguíneas no padrão de expressão deste patógeno.

Este trabalho foi o primeiro a utilizar a tecnologia de cDNA-RDA na caracterização do perfil transcricional de um fungo patogênico humano em condições que mimetizam o processo infeccioso. A análise dos dados mostra que o fungo *P. brasiliensis* apresenta diferentes respostas frente às diferentes condições analisadas, apresentando um favorecimento da captação de micronutrientes e manutenção da fluidez da membrana nos tecidos infectados. De forma confirmatória vários genes superexpressos em células leveduriformes recuperadas de fígado infectado, também foram identificados como altamente expressos no transcriptoma do *P. brasiliensis* nesta mesma condição (Costa *et al.*, 2007). Durante a rota de disseminação hematogênica o remodelamento da parede celular e a resposta ao estresse osmótico seriam as estratégias adaptativas preferencialmente utilizadas. Os dados sugerem que o direcionamento do metabolismo para a β-oxidação associado com a resposta ao estresse oxidativo é um mecanismo importante para a sobrevivência do fungo no contato com plasma, em sitio de infecção com extravasamento de plasma. As análises de RDA e a comparação entre o repertório de genes induzidos em cada uma das três condições experimentais sugere que *P. brasiliensis*

apresenta uma resposta nicho-específica de adaptação e sobrevivência nos diferentes ambientes encontrados no hospedeiro que compõem as diferentes etapas do processo infeccioso. A Tabela 1 sumariza as alterações metabólicas que predominam nas condições analisadas evidenciando os mecanismos que compõem as estratégias específicas que *P. brasiliensis* utiliza na sobrevivência em diferentes microambientes encontrados no hospedeiro.

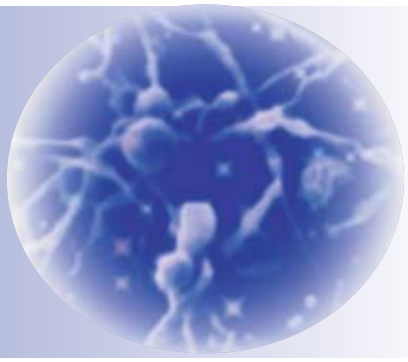
Tabela 1 – Mecanismos adaptativos ativados em diferentes nichos do hospedeiro

	Sangue	Plasma	Infecção de fígado
Captação de ferro	+	+	+
Síntese de melanina	-	+	+
Remodelamento da parede celular	+	+	+
Fermentação alcoólica	-	-	+
Ciclo do glioxalato	-	-	+
Metabolismo de nitrogênio	+	+	+
Ciclo do metilcitrato	+	+	-
β-oxidação	+	+	-

7 – CONCLUSÕES

- O *P. brasiliensis* apresentou um perfil de expressão diferencial nas condições estudadas
- A técnica de RDA foi utilizada com sucesso na análise diferencial do perfil de expressão de *P. brasiliensis* em condições que mimetizam diferentes nichos do hospedeiro, permitindo a identificação de:
    - 240 genes superexpressos
    - 18 novos genes
- A mudança do nível de expressão também foi observada a nível protéico
- O *P. brasiliensis* apresenta resposta específica frente a diferentes nichos encontrados no hospedeiro

Perspectivas



8 – PERSPECTIVAS/TRABALHOS EM DESENVOLVIMENTO

1. Análise do padrão de expressão gênica em vários nichos do hospedeiro, como pulmão e baço.
2. Análise temporal da expressão gênica do fungo em diferentes nichos do hospedeiro.
3. Confirmação, por meio de RT-PCR quantitativo, de vias metabólicas induzidas temporalmente e espacialmente em *P. brasiliensis* durante o processo infeccioso.
4. Análises proteômicas em condições que mimetizam o processo infeccioso.
5. Caracterização dos sistemas de captação de micro-nutrientes em *P. brasiliensis.*
6. Caracterização das vias de produção de melanina e do papel biológico do composto.
7. Desenvolvimento de ferramentas genéticas para análise do papel dos genes diferencialmente expressos.
8. Construção de biblioteca de mutantes de *P. brasiliensis*, rastreamento e caracterização dos transformantes.

Capítulo VI

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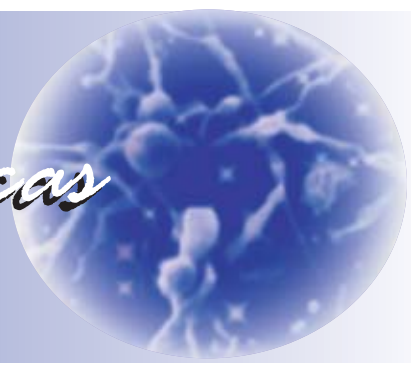

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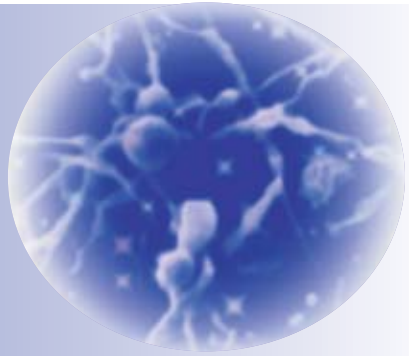
Anexos



10 – ANEXOS

10.1 – Artigos publicados em revistas internacionais

Moreira, S. F., Bailao, A. M., Barbosa, M. S., Jesuino, R. S., Felipe, M. S., Pereira, M. and de Almeida Soares, C. M. (2004). "Monofunctional catalase P of *Paracoccidioides brasiliensis*: identification, characterization, molecular cloning and expression analysis." Yeast 21(2): 173-82.

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10.2 - Totais de Produção

Produção bibliográfica:

Artigos completos publicado em periódico 12

Comunicações em anais de congressos e periódicos 22

Demais produções bibliográficas 2

Eventos:

Participações em eventos (congresso) 3

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