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IDENTIFICAÇÃO E CARACTERIZAÇÃO DE MOLÉCULAS ENVOLVIDAS NA INTERAÇÃO DE *PARACOCCIDIOIDES BRASILENSIS* COM O HOSPEDEIRO

Tese de Doutorado

CANDIDATA: SABRINA FONSECA INGÊNITO MOREIRA DANTAS

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Ministério da Educação
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"Quando você tem uma meta, o que era um obstáculo

passa a ser uma das etapas do seu plano"

(Gerhard Erich Boehme)

SUMÁRIO

Lista de Abreviaturas

ATP – adenosina trifosfato

BCIP – 5-bromo-4-cloro-3-indolil fosfato

cDNA – Ácido desoxirribonucléico complementar

DNA - Ácido desoxirribonucléico

DDC – L- aminoacido aromatico - descarboxilase

DHN- melanina - 1,8 – dihidroxinaftaleno-melanina

DOPA-melanina - L-3,4-dihidroxifenilalanina-melanina

EDTA – Ácido etilenodiaminotetracético

EST – Etiqueta de sequência expressa

FAD – Flavina adenina dinucleotídeo (FAD)

FBS – Soro fetal bovino

FCM – Citometria de Fluxo

GAPDH – Gliceraldeído 3-fosfato desidrogenase

FMN – Flavina mononucleotídeo

GP – Glicoproteína

GST – Glutationa S- transferase

GTP – guanosina trifosfato

HPLC – Cromatografia líquida de alta eficiência

HSP – Proteína de choque térmico

H_2O_2 – Peróxido de hidrogênio

hMM – Fração de alta massa molecular

IVIAT – Tecnologia de antígenos induzidos *in vivo*

IPTG - Isopropil-β-D-tiogalactopiranosídeo

kDa – KiloDalton

LS – Lumazina sintase

mRNA – Ácido ribomucléico mensageiro

NBT – Azul de nitrotetrazólio

NMR – Resonância magnética nuclear

NO – Oxido nítrico

PAGE – Eletroforese em gel de poliacrilamida

pb/bp – Pares de bases

Pb – Paracoccidioides brasiliensis

PBS – Tampão fosfato-salino

PCM – Paracoccidioidomicose

PCR – Reação em cadeia da polimerase

PFGE – gel de eletroforese em pulso alternado

PLP – Piridoxal 5´-fosfato

pH – Potencial hidrogeniônico

pI – Ponto isoelétrico

RDA – Análise de diferença representacional

ROS – Espécies reativas de oxigênio

RT-PCRsq – PCR acoplada à transcrição reversa semi-quantitativa

rRNA – Ácido ribonucléico ribossomal

SDS – Dodecil sulfato de sódio

TPI – Triose fosfato isomerase

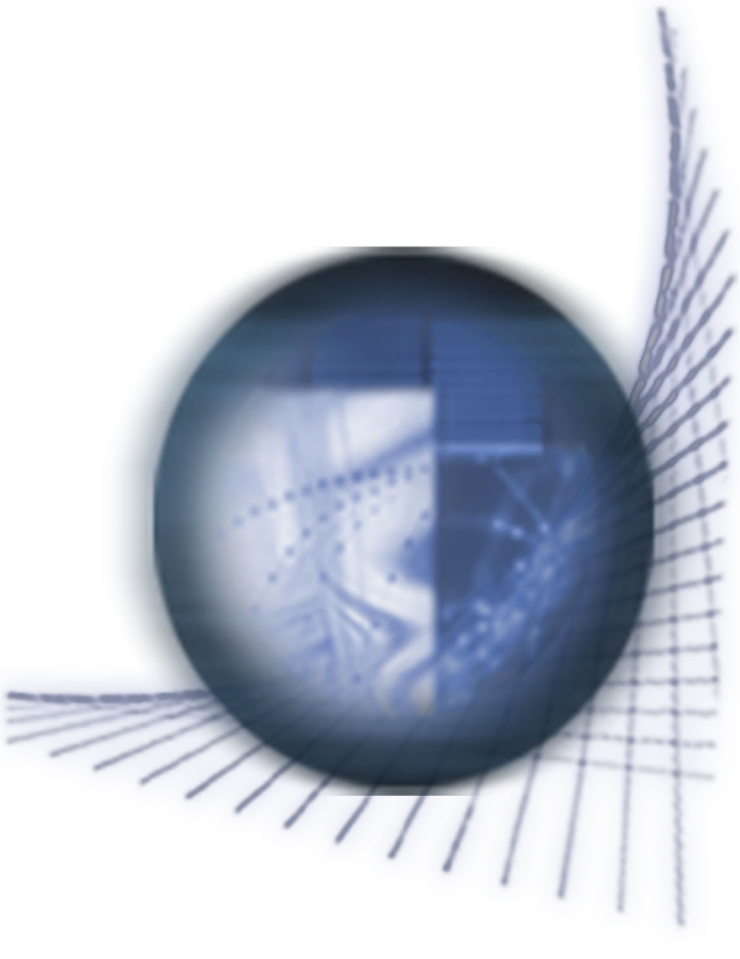
RESUMO

Paracoccidioides brasiliensis é o agente etiológico da paracoccidioidomicose (PCM), uma micose sistêmica, prevalente nos países da América Latina. Uma biblioteca de cDNA de expressão de *Paracoccidioides brasiliensis* foi construída e rastreada com soros de pacientes acometidos por paracoccidioidomicose (PCM). Foram identificados 35 clones de cDNAs que codificam proteínas relacionadas com metabolismo celular, transporte, energia, transcrição, endereçamento de proteínas, transdução de sinal e componentes celulares. Os cDNAs codificantes da L- aminoacido aromatico - descarboxilase (*Pbddc*), da lumazina sintase (*Pbls*) e do transportador de cobre de alta afinidade foram obtidos. As proteínas recombinantes *Pb*DDC e *Pb*LS e o peptídio sintético *Pb*CTR3 foram reconhecidos por soros de pacientes com PCM e não reagiram com soros controle. A técnica de IVIAT (tecnologia de antígenos induzidos *in vivo*) propiciou a identificação de proteínas imunogênicas mais expressas durante o processo infecçioso. RT-PCR em tempo real quantitativa (qRT-PCR) demostrou altos níveis de transcritos de *Pbddc*, *Pbls* e *Pb*ctr3 em células leveduriformes infectando macrófagos. O transcritos de células leveduriformes de *P. brasiliensis* recuperadas de fígado e baço de camundongos foram medidos por qRT-PCR. Estes resultados sugerem o provável papel das proteínas imunogênicas no processo infeccioso de *P. brasiliensis*.

ABSTRACT

Paracoccidioides brasiliensis causes paracoccidioidomycosis (PCM), a systemic mycosis presenting clinical manifestations ranging from mild to severe forms. A *P. brasiliensis* cDNA expression library was produced and screened with pooled sera from PCM patients adsorbed against antigens derived from in vitro-grown *P. brasiliensis* yeast cells. Sequencing DNA inserts from clones reactive with PCM patients sera indicated 35 open reading frames presenting homology to genes involved in metabolic pathways, transport, among other predicted functions. The complete cDNAs encoding aromatic L-amino acid decarboxylase (*Pbddc*), lumazine synthase (*Pbls*) and a homologue of the high affinity copper transporter (*Pbctr*3) were obtained. Recombinant proteins *Pb*DDC and *Pb*LS were obtained; a peptide was synthesized for *Pb*CTR3. The proteins and the synthetic peptide were recognized by sera of patients with confirmed PCM and not by sera of healthy patients. Using the vivo-induced antigen technology (IVIAT) we identified immunogenic proteins expressed at high levels during infection. Quantitative real - time RT-PCR demonstrated high transcript levels of *Pbddc*, *Pbls* and *Pbctr*3 in yeast cells infecting macrophages. Transcripts in yeast cells derived from spleen and liver of infected mice were also measured by qRT-PCR. Our results suggest a putative role for the immunogenic proteins in the infectious process of *P. brasiliensis.*

INTRODUÇÃO



I. INTRODUÇÃO

CAPÍTULO 1

Obtenção e caracterização de antígenos do fungo patogênico humano *Paracoccidioides brasiliensis* por meio da técnica de IVIAT (Tecnologia de antígenos induzidos *in vivo*)

1.1 - *Paracoccidiodes brasiliensis*

Micoses sistêmicas humanas vêm se tornando um sério e crescente problema de saúde pública. O fungo *Paracoccidioides brasiliensis*, agente etiológico da Paracoccidioidomicose (PCM), acomete principalmente trabalhadores rurais do sexo masculino, na faixa etária entre 30 e 60 anos (Lacaz *et al*., 1991). *P. brasiliensis* apresenta a habilidade de crescer tanto na forma leveduriforme a 36ºC *in vitro* e no hospedeiro, quanto na forma miceliana a temperaturas inferiores a 28ºC *in vitro* e no meio ambiente (Restrepo, 1985; San-Blas, 1993).

O exato local e a condição na qual *P. brasiliensis* ocorre na natureza ainda é pouco conhecida em virtude do raro isolamento do fungo do meio ambiente e seu prolongado período de latência no hospedeiro humano (Restrepo, 1985). Terçarioli *et al.* (2007), investigaram o crescimento e a produção de conídeos de *P. brasiliesis* em diferentes tipos de solo. Foi observado que o crescimento de *P. brasiliensis* é mais abundante em solos encharcados e que altos valores de alumínio inibem o seu crescimento. Também foi verificado que alguns isolados produzem grande quantidade de conídeos e a detecção molecular de *P. brasiliensis* foi positiva somente em amostras coletadas em tocas de tatus localizadas em solos arenosos e argilosos. Richini-Pereira *et al.* (2008), por meio de técnicas moleculares, detectaram a presença de *P. brasiliensis* em diversos órgãos de tatus e préa, no pulmão e fígado de porco-espinho e no pulmão de guaxinim. Os resultados sugerem que a infecção por *P. brasiliensis* em animais

selvagens, em áreas endêmicas, pode ser um evento comum. Micélio e conídeos, provavelmente, podem crescer saprofitamente no solo, na água e em plantas, à temperatura ambiente e são consideradas como formas infectivas do fungo (Restrepo *et al*., 2001). *P. brasiliensis* atinge o hospedeiro, usualmente através da via respiratória, por inalação de propágulos de micélio, como conídeos. Nos pulmões esses propágulos se convertem para a fase leveduriforme, de onde podem disseminar-se para diferentes órgãos e tecidos (San-Blas, 1993).

A conversão morfogenética de *P. brasiliensis* está correlacionada com mudanças na composição da parede celular e na estrutura dos polímeros de carboidratos nela presentes (San-Blas, 1982). Estudos citoquímicos e estruturais da parede celular do fungo em suas duas formas foram realizados confirmando a presença de maior teor α-1,3-glucana em formas leveduriformes, β-1,3-glucana em micélio (Paris *et al*., 1986) e quitina em ambas as formas do fungo, apresentando um maior teor em forma leveduriforme quando comparado a micélio (Kurokawa *et al*., 1998).

Os eventos bioquímicos que regulam a transição dimórfica no fungo são ainda pouco definidos, embora informações em nível molecular desse processo estejam parcialmente descritas nos transcriptomas de dois isolados de *P. brasiliensis* (Bastos *et al*., 2007; Felipe *et al*., 2003; Felipe *et al*., 2005; Nunes *et al*., 2005). De acordo com o perfil transcricional de *P. brasiliensis*, Felipe *et al*. (2005), sugeriram que células leveduriformes exibem um metabolismo energético com produção preferencial de etanol, através da fermentação alcoólica, enquanto o metabolismo da fase infectiva é mais aeróbico. Em adição, a análise do transcriptoma de *P. brasiliensis* durante a transição dimórfica, *in vitro*, de micélio para forma leveduriforme Bastos *et al*. (2007) mostraram uma modulação diferencial positiva de transcritos relacionados à síntese de componentes da parede celular/membrana e proteínas de transdução de sinal, sugerindo

que esses processos sejam importantes contribuintes para o dimorfismo. Prováveis fatores de virulência foram expressos durante o processo de transição, sugerindo adaptação ao hospedeiro, pelo fungo, na fase parasítica.

A classificação taxonômica de *P. brasiliensis* é: reino Fungi, filo Ascomycota, subdivisão Euascomycotina, classe Plectomyceto, subclasse Euascomycetidae, ordem Onygenales, família Onygenaceae, subfamília Onygenaceae Anamórficos, gênero Paracoccidioides, espécie única *Paracoccidioides brasiliensis* (San-Blas *et al*. 2002). Matute *et al*. (2006) propuseram a existência de três diferentes espécies filogenéticas de *P. brasiliensis*: S1 (espécie 1), PS2 (espécie filogenética 2) e PS3 (espécie filogenética 3). A espécie filogenética PS3 está geograficamente restrita à Colômbia, enquanto S1 está distribuída no Brasil, Argentina, Paraguai, Peru e Venezuela. Alguns isolados da espécie filogenética PS2 foram encontrados no Brasil nos estados de São Paulo e Minas Gerais e ainda na Venezuela. Carrero *et al*. (2008) demonstraram, por meio de análises filogenéticas que o isolado *Pb*01 separa-se claramente de todos os outros analisados, inclusive daqueles estudados por Matute *et al*. (2006). Estes resultados sugerem que *Pb*01 possa ser uma nova espécie no gênero *Paracoccidioides*, porém, ainda é necessário identificar-se novos isolados com características filogenéticas iguais ao *Pb*01 para validar essa hipótese (Carrero *et al*., 2008).

A organização genômica de *P. brasiliensis* vem sendo objeto de muitos estudos. Feitosa *et al*. (2003), por meio da técnica de eletroforese em gel de campo pulsado (PFGE), sugeriram que o genoma de *P. brasiliensis* varie entre 23 a 31 Mpb, dependendo do isolado e que possivelmente exista isolados haplóides e diplóides. Utilizando a técnica de citometria de fluxo (FCM), Almeida *et al*. (2007) analisaram 10 isolados diferentes de *P. brasiliensis* e observaram em células leveduriformes um genoma variando de 26,3 a 35,5 Mb. O genoma dos conídeos do isolado ATCC 60855

apresentou um tamanho de 30,2 a 30,9 Mb, não mostrando nenhuma diferença significativa com a forma leveduriforme, sugerindo que o fungo seja haplóide (Almeida *et al*., 2007). Montoya *et al*. (1997; 1999) sugeriram, por meio da técnica de PFGE, que alguns isolados clínicos e ambientais de *P. brasiliensis*, possua no mínimo quatro cromossomos. Recentemente, foi desenvolvido um projeto comparativo dos genomas de três diferentes isolados de *P. brasiliensis* (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). Foram sequenciados os genomas completos dos isolados *Pb*01, *Pb*03 e *Pb*18, com o objetivo de se analisar a similaridade entre eles. *Pb*01 apresentou o genoma de 32,94 Mb com um total de 9,132 genes. O isolado *Pb*03 apresentou o genoma de 29,06 Mb com 7,875 genes e *Pb*18 possui um genoma de 29,95 Mb, contendo 8,741 genes. Os resultados confirmaram a presença de cinco cromossomos em cada isolado.

As formas clínicas da PCM são classificadas em: aguda ou subaguda (tipo juvenil) e crônica (Franco *et al.,* 1987; Montenegro, 1986). A forma aguda da micose representa 3 a 5% dos casos descritos e afeta principalmente crianças e adultos jovens. Esta é a forma mais grave e com pior prognóstico (Brummer *et al*., 1993). Seu quadro clínico caracteriza-se por um desenvolvimento rápido e por marcante envolvimento de órgãos como fígado, baço, gânglios linfáticos e medula óssea. O fungo é facilmente isolado do pulmão de pacientes infectados, evidênciando que este órgão seja a via de entrada do patógeno (Restrepo *et al*., 1989). Nesta forma, a PCM conduz à perda gradual de resposta imune celular e induz a produção de altos títulos de anticorpos específicos.

A forma crônica ou adulta representa mais de 90% dos casos, sendo a maioria dos pacientes constituída por homens adultos. Ao contrário da forma aguda, o quadro clínico apresenta um desenvolvimento lento com comprometimento pulmonar evidente

(Brummer *et al*., 1993). Em aproximadamente 25% dos casos, o pulmão é o único órgão afetado - forma unifocal. Muitas vezes, com o desenvolvimento silencioso da doença, o paciente busca auxílio médico somente quando apresenta sintomas de comprometimento extra pulmonar. Nestes casos se constatam o envolvimento de órgãos como pele, mucosas das vias aéreas superiores, tubo digestivo e linfonodos - forma multifocal (Londero, 1986; Brummer *et al*., 1993). A forma crônica apresenta notável tendência à disseminação, sendo pouco frequente os quadros onde há somente comprometimento pulmonar (Restrepo *et al*., 1983).

I.2 – ANTÍGENOS DE *P. brasiliensis*

O fungo *P. brasiliensis*, assim como outros patógenos, expressam diversas moléculas que podem ser reconhecidas por anticorpos produzidos por pacientes humanos ou em animais imunizados em laboratórios. Vários antígenos deste fungo já foram identificados (Puccia *et al*., 1986; Casotto, 1990; Figueroa *et al*., 1994; Fonseca *et al*., 2001), purificados e caracterizados (Diniz *et al*., 2002, Barbosa *et al*., 2006; Pereira *et al*., 2007; Carvalho *et al*., 2008).

Um antígeno de *P. brasiliensis* bem caracterizado é uma glicoproteína secretada que contém uma simples cadeia de oligossacarídeo (Almeida *et al*., 1996), apresenta massa molecular de 43 kDa (gp43) e foi primeiramente descrita por Puccia *et al*. (1986). A gp43 é considerada um importante antígeno do fungo *P. brasiliensis*, pois esta molécula é capaz de induzir a produção de anticorpos em muitos pacientes com PCM (Camargo *et al*., 1994, Travassos *et al*., 1995). A clonagem e a caracterização do gene codificante para gp43 mostrou que sua seqüência de nucleotídeos apresenta 1.981 pares de bases, sendo constituída por dois éxons, que estão separados por um íntron de 78 pares de bases. A seqüência deduzida de aminoácidos apresenta 56-58% de similaridade à exo1-3-β-D-glicanases de *S. cerevisiae* e *C. albicans*, respectivamente (Cisalpino *et*

al., 1996). A gp43 também está relacionada com o processo de ligação de *P. brasiliensis* à laminina na superfície das células do hospedeiro, sugerindo o envolvimento desta glicoproteína no processo de adesão e na patogênese (Vicentini *et al*., 1994). O cDNA codificante da gp43 foi obtido por meio da técnica de RT-PCR, clonado e expresso em *Escherichia coli*. Análises através de *Immunoblotting* mostraram que a gp43 recombinante reage com soros de pacientes com as formas crônica e aguda de PCM e não apresenta nenhum tipo de reação com soros de pacientes com outras micoses (Diniz *et al*., 2002). Carvalho *et al*., (2008) produziram gp43 recombinante de três diferentes isoformas, A, D e E, em levedura *Pichia pastoris* e verificaram que essas proteínas podem ser usadas no diagnóstico de PCM, especialmente as isoformas D e E. Estudos mostraram que a molécula de gp43 inibe a produção de óxido nítrico (NO), de H_2O_2 (peróxido de hidrogênio) e a fagocitose em macrófagos do hospedeiro (Almeida *et al*., 1998; Popi *et al*., 2002).

Konno *et al*. (2009) investigaram que alguns peptídeos derivados da molécula de gp43 podem estar envolvidos com a inibição da produção de NO, H_2O_2 e fagocitose em macrófagos reduzindo sua função e a reação inflamatória no hospedeiro. O peptídeo P10 (QTLIAIHTLAIRYAN) da gp43 foi descrito como ativador de células T específicas, aumentando a proteção contra a PCM em camundongos BALB/c sem produzir resposta humoral (Taborda *et al*., 1998). Marques *et al*. (2006) utilizaram o peptídeo P10, derivado da gp43, em conjunto com a quimioterapia na tentativa de otimizar o tratamento da paracoccidioidomicose e prevenir recaídas. O tratamento combinado mostrou um efeito protetor adicional e seu uso é recomendado para melhorar a quiminoterapia regular e reduzir a duração do tratamento. Braga *et al*. (2009) formularam uma vacina anti-PCM que combina a proteína gp43 ou seu pepítideo P10 com fagelina FliC de *Salmonella enterica*, um agonista da imunidade celular inata.

Camundongos imunizados com gp43 e FliC foram inoculados intraperitonialmente com células leveduriformes de *P. brasiliensis* e após alguns dias apresentaram um aumento da ploriferação do fungo e de danos no tecido pulmonar. Em contraste camundongos imunizados com o peptídeo P10 e FliC foi observado redução do crescimento do fungo e danos no pulmão. Os resultados sugerem que a combinação do peptídeo P10 com FliC seja uma alternativa promissora para geração de novas vacinas anti-PCM.

Um antígeno de *P. brasiliensis* clonado e caracterizado codifica uma proteína de 27 kDa (p27) que não apresenta homologia significativa com outras seqüências já descritas (McEwen *et al*., 1996). A proteína recombinante p27 é reativa com soros de pacientes com PCM e não apresenta reação com soro de indivíduos portadores de outras micoses (Ortiz *et al*., 1997; Díez *et al*., 2003; Correa *et al*., 2007).

Um antígeno de 87 kDa de *P. brasiliensis* também foi sugerido para diagnóstico sorológico de PCM (Gómez *et al*., 1997; 1998). Díez *et al*. (2002) purificaram e caracterizaram este antígeno e o identificaram como pertencente à família das proteínas de choque térmico. Díez *et al.* (2003) descreveram a aplicação da associação deste antígeno com a proteína recombinante p27 de *P. brasiliensis* para utilização no diagnóstico sorológico da PCM.

Panunto-Castelo *et al*. (2003) detectaram onze diferentes antígenos presentes em cinco diferentes isolados de *P. brasiliensis* por meio da técnica de SDS-PAGE e *immunoblotting* usando soros de pacientes com diferentes formas clínicas de PCM. Os antígenos reconhecidos com mais freqüência pelo soro de pacientes com PCM foram as proteínas de 43 kDa (97% dos soros testados), 160 kDa (78% dos soros testados), e 70 kDa (60% dos soros testados).

Diniz *et al*. (2004) fracionaram extratos celulares de formas leveduriformes de *P. brasiliensis* usando sistema de cromatografia líquida de alta eficiência (HPLC). As

frações obtidas (F0, FII e FIII) foram usadas como vacinas em camundongos BALB/c. Após a imunização os animais foram infectados com *P. brasiliensis*. As frações F0 e FII induziram a proteção dos camundongos contra o fungo. Em contraste, camundongos imunizados com a fração FIII desenvolveram doença progressiva, com disseminação para o fígado e baço, embora apresentando níveis significantes de imunidade celular e humoral. Portanto, a resposta imune à fração FIII não ativou eficientemente a proteção contra *P. brasiliensis*.

Pavanelli *et al*. (2007) investigaram a atividade protetora de fração de massa molecular alta (hMM) de *P. brasiliensis* (~380 kDa) em camundongos BALB/c infectados com o fungo. Os resultados avaliados por unidades formadoras de colônia, histopatologia e antigenemia sugeriram que a fração hMM tem um efeito protetor em PCM experimental em camundongos.

Outra glicoproteína expressa em *P. brasiliensis*, gp70, é reconhecida por 96% dos soros de pacientes com PCM. Mattos Grosso *et al*. (2003) produziram anticorpo monoclonal contra a gp70 com o objetivo de isolar esta molécula do extrato total do fungo e investigar seu possível papel na patogênese da PCM. Os autores observaram que a gp70 purificada, assim como a gp43, inibe a liberação de NO, H_2O_2 e a atividade de macrófagos *in vitro*. A imunização passiva de ratos durante a infecção com *P. brasiliensis* usando o anticorpo monoclonal anti-gp70 levou ao decréscimo na formação de granulomas nos pulmões, sugerindo que esta proteína facilite o estabelecimento e a progressão de lesões em infecções primárias causadas por *P. brasiliensis*.

Xander *et al*. (2007) caracterizaram uma proteína antigênica secretada de *P. brasiliensis* presente na superfície de células leveduriformes, de 75 kDa, com atividade de fosfatase. O anticorpo monoclonal produzido contra esta proteína foi utilizado para

imunização passiva em camundongos infectados com *P. brasiliensis* e os resultados sugeriram a drástica redução da resposta inflamatória no hospedeiro.

Estudos do laboratorio de biología molecular, UFG, levaram à identificação e caracterização de moléculas de *P. brasiliensis*, potencialmente associadas à interação fungo hospedeiro. Izacc *et al*. (2001) clonaram, caracterizaram e expressaram em sistema heterólogo, o gene codificante para a proteína mitocondrial HSP60 do fungo *P. brasiliensis*. Análises por meio de *Immunoblotting* mostraram que essa proteína é reativa com soros de pacientes com PCM. Posteriormente, a proteína recombinante HSP60 foi purificada por Cunha *et al*. (2002), e testada com um total de 196 amostras de soros humanos através de *Immunoblotting*. Os resultados obtidos indicaram que a HSP60 apresenta alta sensibilidade e especificidade (97,3 e 92,5% respectivamente) a soros de pacientes com PCM. Portanto, a HSP60 purificada poderia ser usada, tanto isolada, como em associação com outros antígenos, no diagnóstico da PCM. Soares *et al*. (2008) desenvolveram um modelo de PCM pulmonar em camundongos BALB/c com o objetivo de examinar a resposta imune celular contra o antígeno *Pb*HSP60. Foi mostrado que esta proteína tem poder de ativar citocinas e subpopulações de células T, sugerindo sua propriedade protetora no hospedeiro. Este trabalho demonstrou que a vacinação com a proteína recombinante *Pb*HSP60 confere proteção contra PCM pulmonar.

Através das técnicas de eletroforese bidimensional e *immunoblotting* Fonseca *et al*. (2001) identificaram determinantes antigênicos de *P. brasiliensis*, utilizando combinações de soros de pacientes com diferentes manifestações clínicas da PCM. Os antígenos de *P. brasiliensis* caracterizados apresentaram homologia com as proteínas catalase (61 kDa), gliceraldeído 3-fosfato desidrogenase (36 kDa), triose fosfato

isomerase (29 kDa), malato desidrogenase (34 kDa) e frutose bifosfato aldolase (39 kDa).

A proteína de 61 kDa, acima citada, teve cDNA cognato clonado e caracterizado por Moreira *et al*. (2004). A seqüência deduzida de aminoácidos apresentou 78% de identidade e 84% de similaridade com uma catalase peroxisomal de *Histoplasma capsulatum*. Muitos autores vêm identificando catalases de microorganismos patogênicos, plantas e animais como fatores de virulência (Jonhson *et al*., 2002; Shibuya *et al*., 2006), visto que, a produção de catalases no interior do patógeno tem o objetivo de conferir resistência contra os mecanismos oxidativos produzidos por células do sistema imune do hospedeiro. Chagas *et al*. (2008) analisaram a expressão dos três diferentes tipos de catalases de *P. brasiliensis*, *Pb*CatA, *Pb*CatP e *Pb*CatC nas formas leveduriformes e de micelios do fungo em diferentes condições de estresse. Os resultados deste estudo sugerem que *Pb*CatA pode ter um papel protetor principalmente contra o estresse endógeno de *P. brasiliensis* causado por exemplo, pela super utilização da β- oxidação de ácidos graxos. *Pb*CatP preferencialmente protege células do fungo contra o estresse exógeno causado pelas espécies reativas de oxigênio (ROS) e *Pb*CatC apresentou maior atividade enzimática em células expostas a estresse osmótico.

Barbosa *et al*. (2004) caracterizaram a proteína gliceraldeído 3-fosfato desidrogenase (GAPDH) que é reativa com soros de pacientes com PCM. Esta proteína mostrou-se diferencialmente regulada nas formas de *P. brasiliensis*. Altos níveis de proteína foram detectados na forma leveduriforme e durante a transição de micélio para forma leveduriforme, sugerindo o provável papel da proteína na forma parasitária do fungo. Barbosa *et al*. (2006) caracterizaram a GAPDH de *P. brasiliensis* como uma nova adesina do fungo, que pode estar relacionada com o processo de invasão em

células do hospedeiro, passo essencial no processo de infecção e disseminação do patógeno.

Pereira *et al*. (2004) clonaram, caracterizaram e expressaram, em sistema heterólogo, o gene codificante para a proteína triose fosfato isomerase (TPI) do fungo *P. brasiliensis*. A TPI recombinante purificada mostrou forte reatividade com soros de pacientes com PCM e não reagiu com soros de indivíduos controle. Além disso, foi demonstrada a capacidade da TPI em ligar-se a componentes da matriz extracelular do hospedeiro, bem como a sua capacidade em mediar a adesão e invasão do fungo a células cultivadas *in vitro* (Pereira *et al*., 2007).

Formamidase (FMD) é uma molécula potencialmente relacionada com a interação patógeno hospedeiro caracterizada por Borges *et al.* (2005). A FMD de *P. brasiliensis* foi isolada através de sistema de focalização isoelétrica. A proteína purificada mostrou forte reação com soros de pacientes com PCM e não reagiu com soros de indivíduos controle, tornando-se um potencial antígeno no sorodiagnóstico da PCM. Posteriormente, Bailão *et al*. (2007) verificaram que o transcrito codificante da FMD se encontra super-expresso em células leveduriformes durante incubação com plasma humano. Também foi verificada a atividade enzimática da FMD em extrato de células leveduriformes nestas mesmas condições e os resultados foram compatíveis com a acumulação da proteína detectada por análises de *western blot*.

Castro *et al*. (2008) caracterizaram a proteína Dfg5p de *P. brasiliensis* que se encontram na parede celular e tem capacidade de se ligar a laminina, fibronectina e colágeno. A proteína recombinante r*Pb*Dfg5p foi fortemente reativa com soro de pacientes com PCM e não reagiu com soros controle, sugerindo que essa proteína tenha um importante papel na interação patógeno hospedeiro.

I.3 – IVIAT (Tecnologia de antígenos induzidos *in vivo*)

Infecções em geral são processos complexos e dinâmicos que ocorrem no hospedeiro e a expressão de genes de virulência pode ser modulada em resposta a mudança de ambiente encontrada no sítio de infecção (Mekalanos 1992; Mahan *et al*., 1993). Alguns estudos estão sendo realizados em *P. brasiliensis* visando elucidar e compreender a expressão de genes relacionados à interação patógeno hospedeiro.

Bailão *et al*. (2006) identificaram genes diferencialmente expressos por meio de análises subtrativas de cDNAs utilizando a técnica de análise de diferença representacional (RDA). Foram identificados em populações de cDNAs provenientes de fígado de camundongos infectados com *P. brasiliensis* genes super regulados relacionados com a aquisição de ferro, síntese de melanina e defesa celular. Nas populações de cDNAs onde o fungo foi incubado com sangue humano, condição que mimetiza efeitos hematológicos durante a disseminação do fungo, os transcritos super regulados, predominantemente, encontrados são relacionados com o modelamento e síntese de parede celular. Bailão *et al*. (2007) identificaram genes expressos diferencialmente em células leveduriformes de *P. brasiliensis* por análise de população de cDNA em fungos tratados com plasma humano, mimetizando sítios de infecção superficial com inflamação. Os genes super regulados nesta condição são predominantemente relacionados com a degradação de ácidos graxos, síntese de proteínas, detecção de mudanças de osmolaridade, remodelamento da parede e defesa celular. Silva *et al*. (2006) estudaram a interação de células leveduriformes de *P. brasiliensis* melanizadas e não melanizadas com macrófagos de camundongos, modelo de infecção que reflete o contato inicial do fungo com as células do sistema imune no sangue e nos tecidos durante o início da infecção. Os resultados mostraram que a

fagocitose de macrófagos é reduzida na presença de *P. brasiliensis* melanizado. Com o objetivo de melhor compreender o transcriptoma de *P. brasiliensis* durante o processo infeccioso, Costa *et al*. (2007), sequenciaram e anotaram 4.934 etiquetas de seqüências expressas (EST) derivadas de uma biblioteca de cDNA de células leveduriformes obtida de fígado de camundongos infectados por *P. brasiliensis*. As sequencias dos genes de células leveduriformes recuperados de camundongos infectados foram comparadas com as sequencias dos transcritos de *P. brasiliensis* de forma leveduriforme e miceliana. Os resultados deste trabalho sugeriram uma expressão gênica diferencial em resposta ao ambiente do hospedeiro.

No presente trabalho foi utilizada a tecnologia de antígenos induzidos *in vivo* (IVIAT), com algumas modificações, com a finalidade de estudar as interações patógeno hospedeiro. IVIAT é uma técnica de rastreamento imunológico capaz de identificar antígenos que tem expressão induzida durante o processo infeccioso de determinada doença sem o uso de modelos animais (Handfield *et al*., 2000; Rollins *et al*., 2005).

IVIAT vem sendo utilizada no estudo de vários patógenos, visando à descoberta de novos alvos para vacinas, produção de drogas e novas estratégias para diagnóstico. Cheng *et al*. (2003) identificaram 10 genes codificantes de prováveis antígenos de *Candida albicans* mais expressos durante a infecção oral utilizando a estratégia de IVIAT. Os genes identificados possivelmente estão envolvidos em diversas funções, incluindo regulação da morfogênese de formas leveduriformes e hifas, adesão a células do hospedeiro, obtenção de nutrientes, biogênese de fosfolipídios, catabolismo de aminoácidos e genes de virulência. Para confirmar maior expressão durante a infecção foi realizada RT-PCR em tempo real quantitativa a partir de RNA extraído do fungo isolado de pacientes com candidiase oral. Deb *et al*. (2002) identificaram em

Mycobacterium tuberculosis por meio de IVIAT duas enzimas (subunidade ε da DNA polimerase III e dihidrolipoamido desidrogenase) que podem ser potencialmente novos alvos para o desenvolvimento de drogas. Em *Vibrio cholerae* foram identificados genes que codificam proteínas que estão localizadas na superfície celular e que são expressas exclusivamente durante a infecção; alguns desses genes foram clonados em vetor de expressão, purificados e reagiram com soros de pacientes infectados (Hang *et al*., 2003). Kim *et al*. (2003) utilizaram a técnica de IVIAT para identificar genes expressos *in vivo* em *Vibrio vulnificus*. Foram caracterizadas várias proteínas consideradas fatores de virulência em bactérias. A técnica de IVIAT também foi utilizada para a pesquisa de determinantes antigênicos em pacientes com periodontite causada por *Actinobacillus actinomycetemcomitans*. Os resultados deste estudo sugeriram a utilização desses genes no diagnóstico ou no tratamento da doença (Cao *et al*., 2004).

John *et al*. (2005) identificaram proteínas imunogênicas de *E. coli* mais expressas durante a infecção humana, sendo muitas descritas primeiramente neste estudo, como por exemplo: uma glicosil transferase envolvida na síntese de polissacarídeo, uma provável glicosil transferase envolvida na biossíntese de oligossacarídeos e outras proteínas hipotéticas. Salim *et al*. (2005) identificaram 16 quadros de leituras abertos (ORFs) de S*treptococcus* grupo A com homologia a genes que codificam para proteínas relacionadas com o metabolismo e com funções não conhecidas que são super reguladas *in vivo*. Harris *et al.* (2006) utilizaram a estratégia de IVIAT para identificar proteínas imunogênicas de *Salmonella entérica* Sorotipo Typhi. Neste trabalho foram identificados genes que são possivelmente mais expressos durante o processo infeccioso, como: genes codificantes de proteínas relacionadas com a estrutura e biogênese da fimbria, resistência a antibióticos, transporte de metais pesados, adesão, transporte extra-citoplasmático e hidrolases secretadas. Segundo os

autores, todos os antígenos caracterizados podem contribuir para a descoberta de novos tratamentos, testes de diagnóstico, prevenção ou até mesmo elucidar a patogênese da febre tifóide. Yoo *et al*. (2007) identificaram, por meio da técnica de IVIAT, antígenos expressos especificamente em pacientes com periodontite causada por *Tanerella forsythia*, tais como: glucosídeo gluco hidrolase, BspA (um fator de virulência de *T. forsythia*), tRNA sintetase, glicina hidroximentil transferase, dipeptidil pepetidase IV, proteína de reparo de DNA e outras proteínas hipotéticas. As expressões *in vivo* dos genes identificados foram confirmadas por meio de RT-PCR em tempo real, quantitativa. Os genes caracterizados, segundo os autores, poderão contribuir para o entendimento do mecanismo molecular de infecções periodontais por *T. forsythia*. Rollins *et al*. (2008) utilizaram a técnica de IVIAT para identificar em *Bacillus anthracis* genes mais expressos durante o processo infeccioso e foram identificadas proteínas relacionadas com a virulênica, transporte, metabolismo e arquitetura de pepitídeo glicano.

A estratégia de IVIAT utiliza soros de pacientes como sonda para o rastreamento de genes expressos durante o processo infeccioso. A biblioteca de expressão rastreada neste trabalho foi construída a partir de RNAs obtidos de células leveduriformes recuperadas de fígado de camundongos infectados por *P. brasiliensis*, com a finalidade de priorizar a obtenção de genes expressos durante a infecção. Para confirmar o provável papel antigênico das proteínas, dois genes identificados foram selecionados, clonados em vetor de expressão e a proteína recombinante foi purificada para reação com anticorpos presentes nos soros de pacientes com PCM. O esquema do desenho experimental da técnica IVIAT usada para rastrear possíveis antígenos expressos durante a PCM está apresentado na Figura 1. A técnica IVIAT apresenta algumas limitações, como por exemplo, ela só pode ser reproduzida em organismos que

têm a característica de serem cultiváveis, somente detecta genes que apresentam resposta imunológica, nem todos os genes virulentos são identificados e não é uma técnica automatizada.

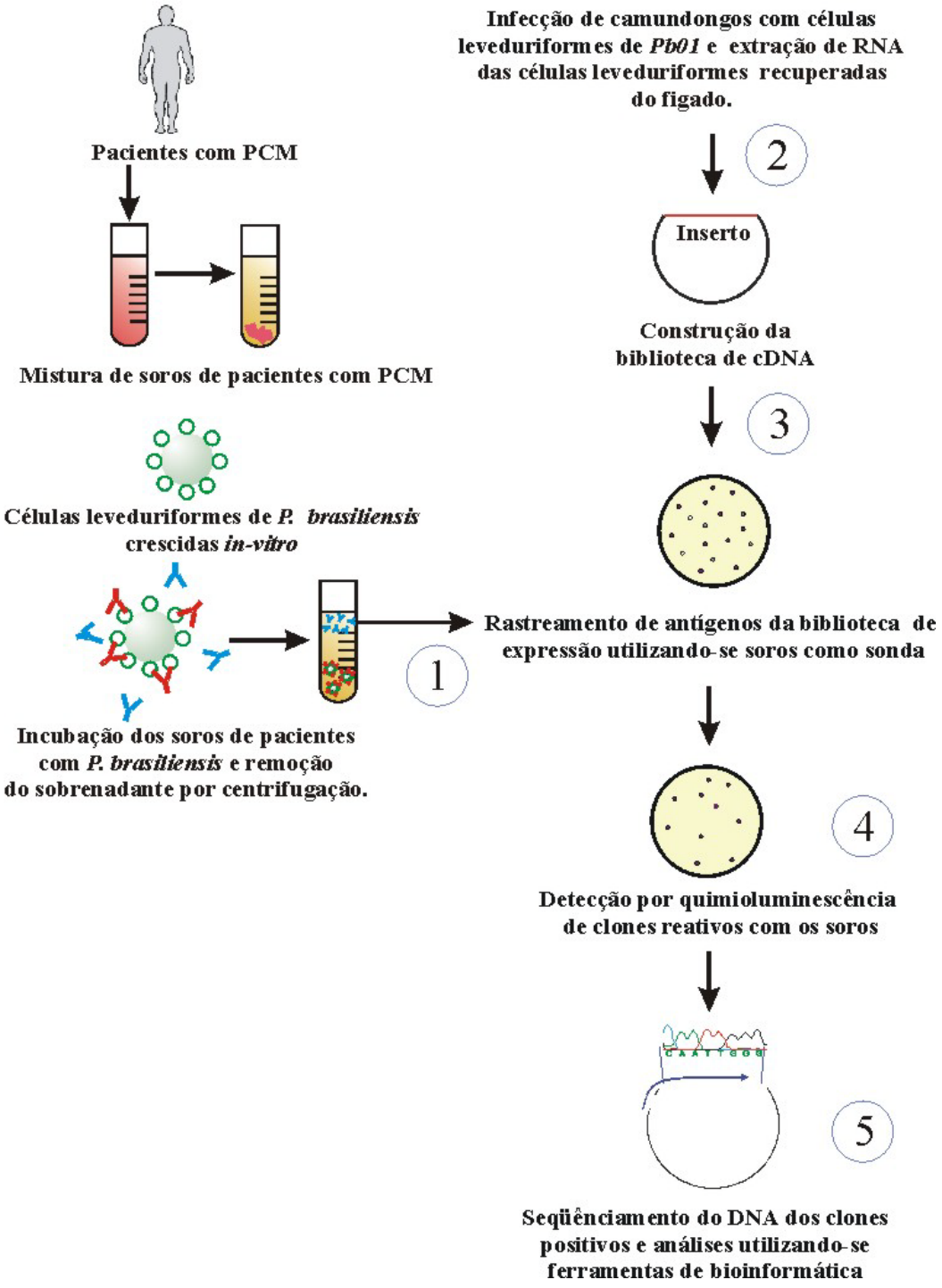


Figura 1 - Estratégia para obtenção de cDNAs codificantes para antígenos expressos durante o processo infeccioso por *P. brasiliensis*. 1 – Combinação de soros de pacientes com PCM seguida de incubação com células leveduriformes do patógeno. 2 – Construção da biblioteca de cDNA utilizando RNAs obtidos de células leveduriformes recuperadas de fígado de camundongos infectados por *P. brasiliensis*. 3 – Rastreamento de antígenos da biblioteca de cDNA utilizando soros (passo1) como sonda. 4 – Detecção de antígenos expressos durante o processo infeccioso, por quimioluminescência. 5 – Sequênciamento de DNA e análises das seqüências obtidas por meio de ferramentas de bioinformática.

Nesse trabalho foram identificados 35 clones de cDNAs que codificam para 29 diferentes proteínas provavelmente envolvidas no metabolismo celular, transporte, energia, transcrição, endereçamento de proteínas, transdução de sinal e biogênese de componentes celulares. Destes genes, alguns já tinham sido identificados anteriormente como expressos durante a infecção por Costa *et al*. (2007); Bailão *et al*. (2006; 2007). Foram selecionados três cDNAs dos 35 rastreados para validar o papel antigênico das proteínas e sua expressão durante a infecção. Os genes escolhidos foram descarboxilase para L-aminoácidos aromáticos (EC 4.1.1.28), lumazina sintase (EC 2.5.1.9), e uma proteína transportadora de cobre de alta afinidade. Os genes codificantes a L-aminoácido aromático e transportador de cobre de alta afinidade foram descritos nos estudos de Costa *et al*. (2007) e Bailão *et al*. (2006) e o gene codificante a lumazina sintase foi descrito como um gene expresso durante a infecção causada por *P. brasiliensis* por Rezende *et al*. (2006).

I.4 – DESCARBOXILASE PARA L- AMINOÁCIDO AROMÁTICOS

A enzima descarboxilase para L-aminoácidos aromáticos (AAAD ou DDC), EC.4.1.1.28 é também conhecida por L-Dopa descarboxilase, triptofano descarboxilase, 5-hidroxitriptofano descarboxilase. DDC participa do metabolismo de aminoácidos com grupos aromáticos, catalisando diferentes reações biológicas de descarboxilação como: a conversão de triptofano em triptamina (um precursor alcalóide), 5-hidroxitriptofano em serotonina, L-Dopa em dopamina e utiliza o grupo piridoxal 5'-fosfato (PLP) como cofator (Toney, 2005). Dentre os possíveis antígenos rastreados neste trabalho, o gene codificante para DDC foi selecionado devido ao seu provável papel na via de biossíntese de melanina, um importante fator de virulência em muitos fungos patogênicos (Langfelder *et al*., 2003).

1.5 – LUMAZINA SINTASE

Como já descrito por Rezende (2006) a 6, 7 – Dimetil – 8 – ribitilumazina sintase (Lumazina sintase; LS), EC 2.5.1.9, catalisa o penúltimo passo na biossíntese da riboflavina em plantas e microorganismos. As enzimas da via de biossíntese de riboflavina não estão presentes em humanos ou hospedeiros animais, sendo essas enzimas, portanto, potenciais alvos para drogas antifúngicas. Devido a estas características, a LS foi selecionada para dar continuidade neste estudo.

A riboflavina (vitamina B2) é o precursor da flavina mononucleotídeo (FMN) e da flavina adenina dinucleotídeo (FAD), considerados os cofatores mais versátis envolvidos em processos de oxidoresução. Ambas as coenzimas são essenciais nos

processos de transferência de elétrons para transmissão de energia em todos os organismos celulares vivos (Fischer e Bacher *et al.*, 2008).

1.6 – TRANSPORTADOR DE COBRE DE ALTA AFINIDADE (CTR3)

O cobre é um metal de transição utilizado como um cofator importante por muitas enzimas que realizam processos biológicos essenciais tais como respiração e crescimento celular, aquisição de ferro, proteção contra o estresse oxidativo, pigmentação (melanização) e outros processos biológicos complexos (Puig & Thiele 2002). O cobre em excesso no meio intracelular torna-se tóxico, pois reage com oxigênio, formando o radical livre hidroxil, altamente destrutivo para a célula, causando danos aos lipídios, às proteínas e ao DNA. Conseqüentemente, os organismos desenvolveram mecanismos para o transporte de cobre na célula, visando manter as concentrações intracelulares em níveis não tóxicos (Marvin e*t al*., 2003). Em *S. cerevisiae* a proteína CTR3 está localizada na membrana plasmática e é responsável pela captura de cobre extracelular (Petris, 2004).

CAPÍTULO 2:

2.1 - DESCARBOXILASE PARA L- AMINOÁCIDO AROMÁTICOS (DDC):

DDC participa do metabolismo de aminoácidos de grupos aromáticos e a degradação desses aminoácidos está associada a outras vias metabólicas. Por exemplo, a fenilalanina e seu produto de oxidação, tirosina, são moléculas precursoras da síntese de melanina. Em fungos patogênicos humanos como *P. brasiliensis* (Gomez *et al*., 2001;

Silva *et al*., 2006), *Fonsecaea pedrosoi* (Alviano *et al*., 2004), *Cryptococcus neoformans* (Casadevall *et al*., 2000; Nosanchuk *et al*., 2000), *Exophiala dermatitidis* (Polak, 1990), *Lacazia loboi* (Taborda *et al*., 1999), *Histoplasma capsulatum* (Nosanchuk *et al*., 2002), *Sporothrix schenckii* (Morris-Jones *et al*., 2003), *Blastomyces dermatitides* (Nosanchuk *et al*., 2004) e *Aspergillus fumigatus* (Youngchim *et al*., 2004) a melanina desempenha um papel de proteção e virulência das células.

O termo melanina não representa uma única substância, mas sim um grande grupo de diversas substâncias, todas com propriedades similares, presentes em diversos organismos (Jacobson, 2000; Butler *et al*., 2001; Langfelder *et al*., 2003). Em geral, melaninas são macromoléculas formadas pela polimerização oxidativa de compostos fenólicos ou indólicos, são moléculas hidrofóbicas e carregadas negativamente. As prováveis funções das melaninas em fungos patogênicos humanos incluem proteção contra UV, radiação gama (Nosanchuk e Casadevall 2003; Mironenko *et al*., 2000), temperaturas extremas (Rosa e Casadevall, 1997), enzimas hidrolíticas (Rosa e Casadevall, 2001), agentes oxidantes, metais pesados e diversos outros componentes tóxicos (Nosanchuk e Casavedall, 2006). Alguns fungos acumulam melanina na parede celular constutivamente (Alviano *et al*., 2004), outros necessitam de L-Dopa para produzir o pigmento (Nosanchuk *et al*., 2002; Nosanchuk *et al*., 2004; Nosanchuk e Casadevall, 1997) e suas particulas podem ser isoladas (Nosanchuk *et al*., 2000; Gomez *et al*., 2001; Silva *et al*, 2006).

A via predominante na síntese de melanina em animais é catalisada por tirosinases (Sanchez-Ferrer *et al*., 1995) enquanto os microorganismos geralmente usam as vias catalisadas pelas lacases e pela poliquetídeo sintase. Em fungos os tipos de melanina predominantemente identificados são dihidroxi fenilalanina (Dopa-melanina

ou eumelanina) e dihidroxinaftaleno (DHN-melanina ou feomelanina) (van de Sande *et al*. 2007).

A via de biossíntese de DHN-melanina foi primeiramente descrita por Wheeler e Bell (1988). Fujii *et al*. (2000) demonstraram que, pelo menos para *Colletotrichum lagenarium*, o primeiro passo na via de biossíntese de DHN-melanina é catalizado pela poliquetídeo sintase, PKS1. Em *P.brasiliensis* sugere-se a existência desta via, visto que esta enzima foi identificada no transcriptoma e no genoma do fungo (Costa *et al*., 2007; www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html).

Fenoloxidases compreende um grupo de enzimas associadas com a produção de Dopa-melanina, que se subdividem em dois grupos: lacases (EC 1.10.3.2) e tirosinases (EC 1.14.18.1). Ambas as enzimas dependem de cobre para sua atividade. Tirosinase e lacase catalisam a hidroxilação de L-tirosina em L-Dopa e a oxidação de L-Dopa em dopaquinona (Langfelder *et al*., 2003). Dopaquinona é um substrato altamente reativo. Na ausência de tiol, dopaquinona forma leucodopacromo que é oxidado em dopacromo. As etapas de hidroxilação e descarboxilação resultam na formação da dihidroxiindol que pode sofer polimerização para formar Dopa-melanina (Ozeki *et al*., 1997; Williamson *et al*., 1998). A melanização em *P. brasiliensis*, assim como em outros fungos patogênicos, requer a presença de componentes dihihroxi phenólicos exógenos como L-Dopa e dopamina (Gomez *et al*, 2001; Silva *et al*., 2006; Williamson *et al*., 1998). DDC catalisa a reação da L-Dopa em L-dopamina e pode participar das reações de descarboxilação para formar o dihidroxiindol, porém pouca descrição tem-se de sua participação na síntese de melanina em fungos (Figura 2). Após a síntese de dopaquinona a reação pode seguir dois caminhos e formar dois tipos de melanina, feomelanina e a eumelanina. Como descrito por Alspaugh *et al*. (1997); Jacobson e Compton (1996); Polacheck *et al*. (1982); Salas *et al*. (1996); Zhu *et al*. (2001); Zhu e

Williamson (2004) esta via sobre regulação por ferro, cobre e baixas concentrações de glicose.

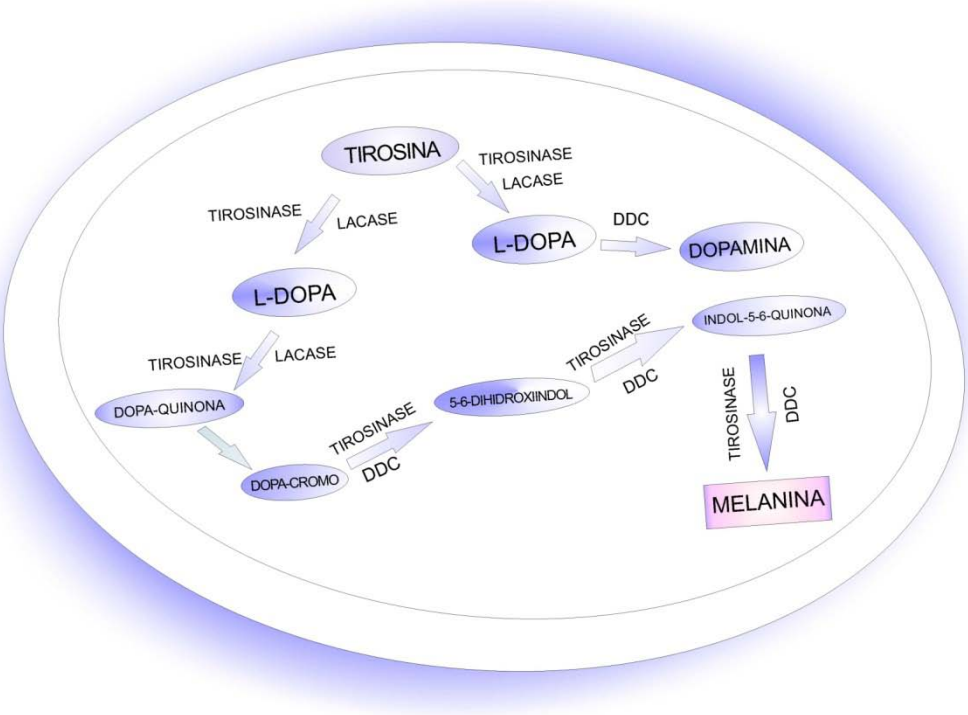


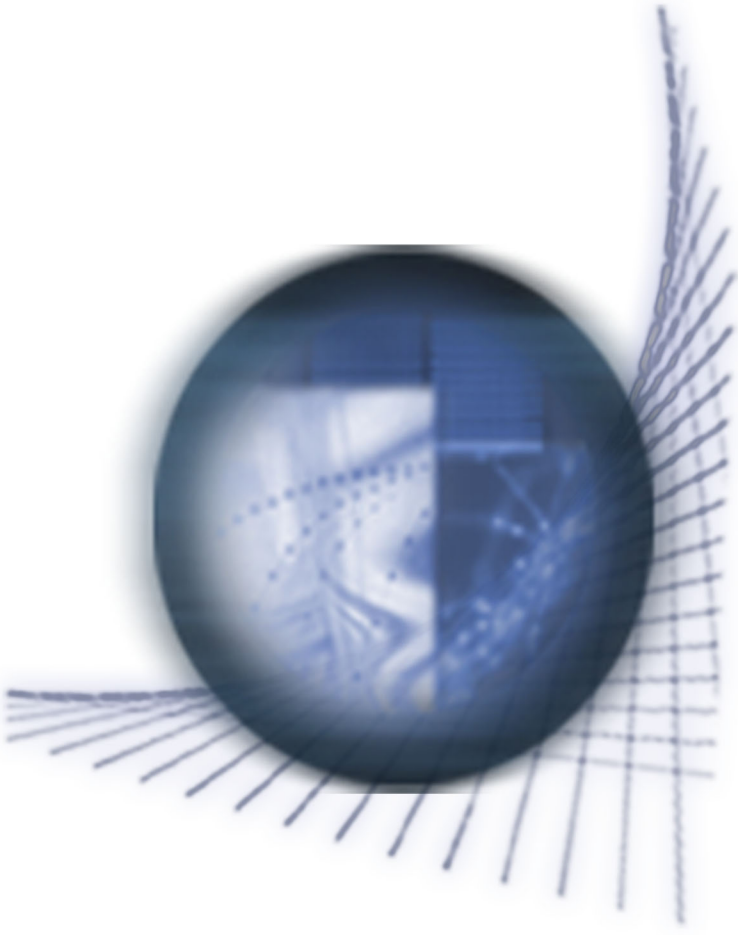
Figura 2 – Via metabólica da formação de Dopa-melanina. As enzimas tirosinase, lacase e DDC participam da via de biossíntese de melanina. Tirosinase e lacase são enzimas que convertem o aminoácido tirosina em L-Dopa, L-Dopa em dopaquinona e que por sua vez, espontaneamente se transforma em dopacromo. Tirosinase e/ou DDC irão converter dopacromo em 5-6-dihidroxiindol, 5-6-dihidroxiindol em indol-5-6 quinona e indol-5-6 quinona em melanina, que por sua vez se deposita no citoplasma ou na parede celular. A enzima tirosina hidroxilase também pode converter o aminoácido tirosina em L-Dopa e DDC converte L-Dopa em dopamina, que também é um substrato para síntese de melanina e neste caso lacase transforma dopamina em dopaquinona.

A melanização em *C. neoformans* envolve a síntese de grânulos de melanina por toda a parede celular (Eisenman *et al*., 2005), porém, este fungo só produz melanina quando entra em contato com substratos exógenos, como a L-Dopa (Tian *et al*., 2003). Schiave *et al*. (2009) investigaram o efeito da exposição de *C. neoformans* a radiação UVB na sobrevivência de células melanizadas e não melanizadas. Após 2, 4, 6 e 8 dias de crescimento celular em meio com ou sem L-Dopa as células foram expostas a radiação UVB e calculou-se a sobrevivência das expostas a radiação em relação as não expostas. Os resultados sugeriram que *C. neoformans* apresentou diferentes tolerâncias

a radiação durante o desenvolvimento de células melanizadas e não melanizadas. Zhong *et al*. (2008), por meio de ressonância magnética nuclear (NMR), monitoraram a formação de L-Dopa, que supostamente é um composto da via de biossíntese de melanina em *C. neoformans*, acrescentando L-Dopa enriquecida com ^{13}C. Os resultados deste trabalho forneceram evidências que L-Dopa seja incorporada na melanina depositada na parede celular deste fungo. Células leveduriformes e conídios de *P. brasiliensis* são capazes de sintetizar melanina tanto *in vitro* como *in vivo*, porém, ainda não se sabe quais os tipos de melanina por elas produzidos (Gómez *et al*., 2001). A atividade da lacase já foi observada em extratos celulares de *P. brasiliensis* e conídios são capazes de produzir pigmentos na ausência de L-Dopa (Gómez *et al*., 2001, Silva *et al*., 2006), sugerindo que o fungo sintetize melanina por outra via, como por exemplo, poliquetídeo sintase, descritas nos isolados *Pb*01, *Pb*18 e *Pb*03 (//www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html).

Bailão *et al*. (2007) verificaram o aumento da expressão de transcritos de DDC durante a incubação de células leveduriformes de *P. brasiliensis* com plasma humano. Provavelmente esse fato possa ocorrer devido à presença de altos níveis de L-Dopa no plasma, que pode ser convertida em melanina. Costa *et al*. (2007) identificaram no transcriptoma de células leveduriformes de *P. brasiliensis* recuperadas pós a infecção de camundongos transcrito em alta redundância que codifica para DDC. Silva *et al*. (2006) descreveram que *P. brasiliensis* melanizado contribui com a virulência por reduzir a fagocitose de macrófagos alveolares e peritoniais, melhorando a resistência das células fúngicas contra o sistema imune do hospedeiro. Células de *P. brasiliensis* melanizadas também diminuem susceptibilidade antifúngica para anfotericina B, cetonazol, fluconazol, itraconazol e sulfametoxazol, drogas comumente usadas no tratamento da doença.

JUSTIFICATIVAS

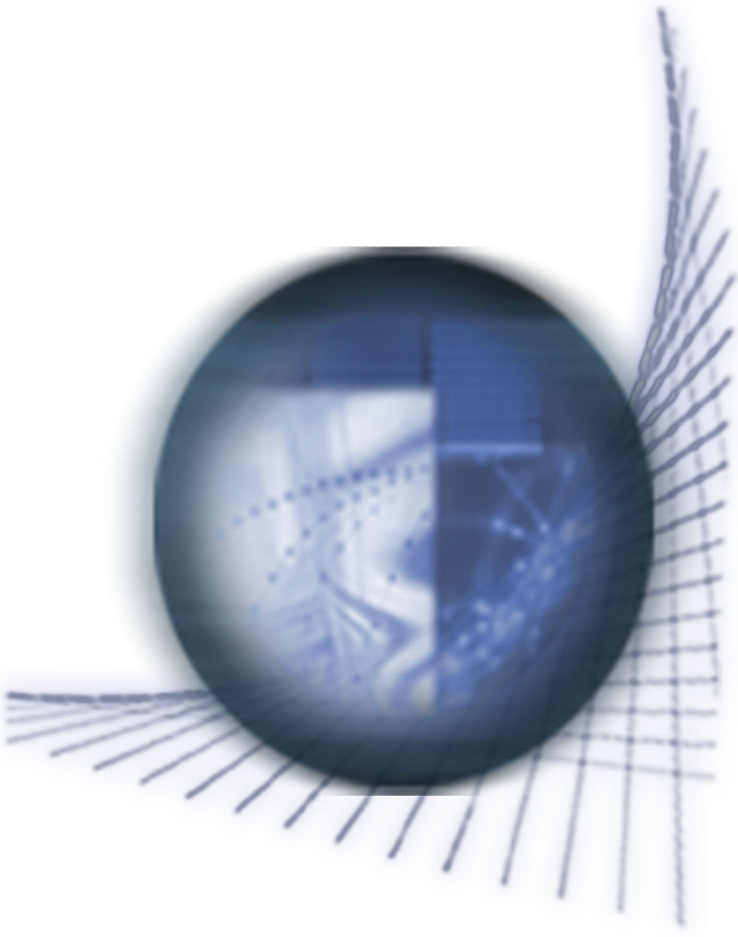


II – JUSTIFICATIVAS

P. brasiliensis vivencia uma mudança ambiental súbita durante o processo infeccioso. Uma alteração de temperatura (22ºC para 36ºC) é um estímulo suficiente para a transição dimórfica. Esse estresse térmico ao qual o fungo é submetido induz a produção de várias moléculas que proporcionam a interação fungo hospedeiro, contribuindo para a patogênese da doença.

A definição de genes expressos diferencialmente por patógenos humanos durante o processo infeccioso pode auxiliar na elucidação das proteínas responsáveis pela patogênese, virulência e interação com o hospedeiro. Por esta razão a utilização de metodologias para a identificação de antígenos de *P. brasiliensis* expressos durante a infecção no hospedeiro humano é importante.

OBJETIVOS



III – OBJETIVOS

III. 1 – OBJETIVO GERAL

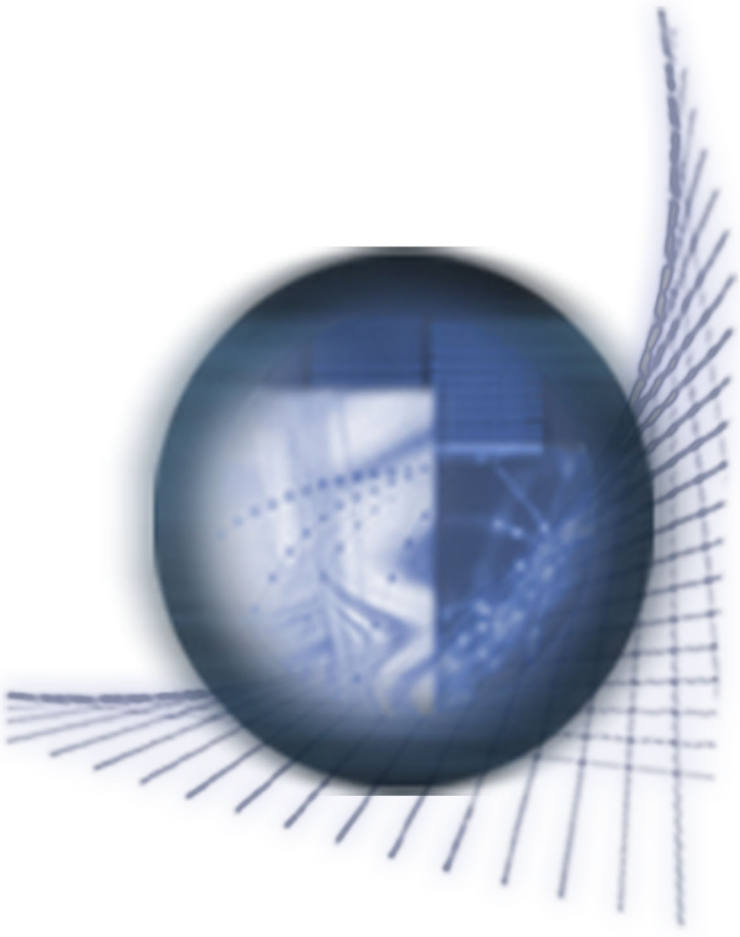
Com a finalidade de identificar e caracterizar moléculas supostamente envolvidas na interação do patógeno com o hospedeiro a técnica de IVIAT foi utilizada para rastrear antígenos de *P. brasiliensis* potencialmente expressos durante o processo infeccioso.

III. 2 – OBJETIVOS ESPECÍFICOS

- ✓ Obtenção de soros de pacientes com PCM apresentando diferentes manifestações clínicas da doença, sem tratamento;
- ✓ Incubação dos soros obtidos com o fungo crescido *in vitro* na temperatura de 36ºC;
- ✓ Construção de uma biblioteca de cDNA de células leveduriformes recuperadas de fígados de camundongos infectados por *P. brasiliensis*;
- ✓ Rastreamento imunológico de possíveis antígenos *P. brasiliensis*;
- ✓ Identificação e classificação das seqüências obtidas quanto a possíveis funções;
- ✓ Obtenção das proteínas recombinantes r*Pb*DDC e r*Pb*LS para a avaliação da reatividade imunológica;
- ✓ Analise da expressão dos transcritos *Pbddc* e *Pbls* durante a infecção de macrófagos de camundongos, por meio da técnica de RT-PCR em tempo real;

- ✓ Analise da expressão dos transcritos *Pbddc* e *Pbls* em células leveduriformes recuperadas de fígado e baço de camundongos, por meio da técnica de RT-PCR em tempo real;
- ✓ Obtenção de anticorpo policlonal anti-DDC;
- ✓ Análise de melanização de células leveduriformes de *P. brasiliensis*, utilizando o anticorpo anti-melanina de *S. schenckii* e anti-DDC de *P. brasiliensis*;
- ✓ Analise da expressão dos transcritos codificantes para DDC, lacase e tirosinase por meio da técnica de RT-PCR em tempo real.

MANUSCRITO



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Original article

Identification and characterization of antigenic proteins potentially expressed during the infectious process of *Paracoccidioides brasiliensis*

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Abstract

Paracoccidioides brasiliensis causes paracoccidioidomycosis (PCM), a systemic mycosis presenting clinical manifestations ranging from mild to severe forms. A *P. brasiliensis* cDNA expression library was produced and screened with pooled sera from PCM patients adsorbed against antigens derived from in vitro-grown *P. brasiliensis* yeast cells. Sequencing DNA inserts from clones reactive with PCM patients sera indicated 35 open reading frames presenting homology to genes involved in metabolic pathways, transport, among other predicted functions. The complete cDNAs encoding aromatic-L-amino-acid decarboxylase (*Pbddc*), lumazine synthase (*Pbls*) and a homologue of the high affinity copper transporter (*Pbctr*3) were obtained. Recombinant proteins *Pb*DDC and *Pb*LS were obtained; a peptide was synthesized for *Pb*CTR3. The proteins and the synthetic peptide were recognized by sera of patients with confirmed PCM and not by sera of healthy patients. Using the in vivo-induced antigen technology (IVIAT), we identified immunogenic proteins expressed at high levels during infection. Quantitative real time RT-PCR demonstrated high transcript levels of *Pbddc*, *Pbls* and *Pbctr*3 in yeast cells infecting macrophages. Transcripts in yeast cells derived from spleen and liver of infected mice were also measured by qRT-PCR. Our results suggest a putative role for the immunogenic proteins in the infectious process of *P. brasiliensis*.

Keywords: *Paracoccidioides brasiliensis*; Immunogenic proteins; Infectious process; DOPA Decarboxylase; Lumazine synthase; High affintiy copper transporter

1. Introduction

Paracoccidioides brasiliensis causes paracoccidioidomycosis (PCM), a human systemic granulomatous disease, prevalent in South America [1]. The fungus is thermo dimorphic and causes by inhalation of airborne propagules of the mycelia phase, which reach the lungs and differentiates into the yeast phase [2].

Although the disease process is well characterized, the fungal expression of genes in vivo is poorly explored. During disease, *P. brasiliensis* must adapt to a range of environments and survival in any one niche should require the differential expression of genes. The in vivo gene expression pattern of *P. brasiliensis* has been examined by our laboratory by transcriptome analysis [3−5]. A wide array of genes involved in nutrient acquisition, melanin synthesis, adhesion, stress response, general metabolism were induced and have been identified.

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CTR3, high affinity copper transporter; DDC, aromatic-L-amino acid decarboxylase IPTG, Isopropyl-β-D-thiogalactopyranoside; IVIAT, in vivo-induced antigen technology; LS, lumazine synthase; NBT, nitroblue tetrazolium; Pb, *Paracoccidioides brasiliensis*; PCM, Paracoccidioidomycosis; TPI, triosephosphate isomerase.

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With the purpose of identifying antigenic proteins potentially expressed during the fungal infectious process, here we applied the in vivo-induced antigen technology (IVIAT). IVIAT has been used to identify genes expressed during human infection by several microorganisms [6–11]. A *P. brasiliensis* cDNA expression library was screened in order to identify clones reactive with sera of PCM patients. Specifically, we hypothesized that by using the IVIAT immunological screening, we could identify proteins that play a role during fungal infection.

Screening of the cDNA library resulted in the identification of 29 genes, putatively playing a role in the fungus-host interaction. The cDNAs, encoding aromatic-L-amino acid decarboxylase (DDC, EC 4. 1. 1. 28), lumazine synthase (LS, EC 2. 5. 1. 9) and high affinity copper tansporter (CTR3) orthologues of *P. brasiliensis* were selected for further analysis. The recombinant proteins (*Pb*DDC and *Pb*LS) and a synthetic peptide (*Pb*CTR3) were obtained and showed strong reactivity with sera of PCM patients. The predictable expression of those transcripts was evaluated by quantitative real time RT-PCR (qRT-PCR) in models of infection. The results suggest a role in the pathogen-host interaction. Due to the relevance of melanin in pathogenesis of microorganisms, we investigated the involvement of *Pb*DDC in this pathway; results demonstrated correlation between the increase of melanin and the enzyme expression in fungal yeast cells.

2. Materials and methods

2.1. P. brasiliensis *isolate growth conditions and differentiation assays*

*P. brasiliensis Pb*01 isolate (ATCC-MYA-826) was cultivated in semi-solid Fava Netto's medium [1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.3% (w/v) proteose peptone, 0.5% (w/v) beef extract, 0.5% (w/v) NaCl, 1% (w/v) agar, pH 7.2] at 36 °C in the yeast form and at 22 °C, for its mycelia phase.

2.2. *Adsorbing PCM patients sera to* P. brasiliensis *grown in vitro*

Human sera were collected from 11 patients with well-documented PCM in chronic disease phase and pooled. The serum samples were selected at the time of diagnosis from patients with mycological confirmed disease. Human control sera obtained from 11 healthy individuals were pooled.

A mixture of equal volumes of sera of PCM patients was diluted with *Escherichia coli* cells lysate and the same volume of PBS 1X. The mixture was incubated at 37 °C for 1 h, and centrifuged at 10,000 × g, 4 °C during 20 min. In the second stage, the supernatant was incubated during 1 h at 37 °C, with the same volume of a mixture containing protein extract of *P. brasiliensis* yeast cells (100 μg/mL) and whole yeast cells. This mixture was centrifuged at 10,000 × g, 4 °C for 20 min; the supernatant was collected. The efficiency of the incubation was monitored by two-dimensional polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

2.3. *Infection of mice with* P. brasiliensis *and RNA extraction*

Female BALB/c mice, 8–12 weeks old, were inoculated with 1×10^7 yeast cells of *P. brasiliensis*. In brief, yeast cells suspension at the 7th day of in vitro growth were washed in PBS 1X and inoculated intraperitoneally in mice. Matched groups of four animals were injected with sterile PBS and used as uninfected controls. The animals were killed on the 15th day after infection. The livers and spleens were removed. Serial dilutions of the lysate were plated in infusion brain and heart (BHI) medium supplemented with 4% (v/v) of fetal bovine serum (FBS) and the plates were incubated at 36 °C for 7 days. The recovered cells were submitted to total RNA extraction by using Trizol reagent (Invitrogen™, Life Technologies), according to the manufacturer's instructions. Total RNA from *P. brasiliensis* yeast cells and mycelium grown in vitro, in the same medium, was also obtained.

2.4. *Construction of a cDNA expression library of* P. brasiliensis

For the construction of a cDNA expression library, the RNA of yeast cells recovered from mice liver on the 15th day after infection was purified by using the Poly (A) Quick® mRNA isolation kit (Stratagene, La Jola, CA). The cDNA library was constructed by using the SUPERSCRIPT™ plasmid system with GATEWAY® technology for cDNA synthesis and cloning.

2.5. *Immunological screening of the cDNA library, identification of inserts and prediction of function of the identified antigens*

The pooled sera were used in the screening of the cDNA library. An aliquot of the cDNA library was diluted and spread onto LB medium plates containing ampicillin (100 μg/ml) to produce 300–600 colonies per plate. The bacterial colonies were grown at 37 °C overnight. The colonies were held up by using nitrocellulose membranes, replica plated onto LB containing ampicillin and 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG), and incubated overnight at 37 °C. The colonies grown onto membrane were exposed to chloroform for 20 min. Following washing and blocking of membranes, they were incubated with the adsorbed sera (1:1000 diluted) in PBS 1X, 0.1% (v/v) Tween 20, at 4 °C for 18 h. The induced proteins reacting with antibodies in the sera were detected by using peroxidase-conjugated goat, anti-human IgG (1:2000 diluted) and revealed with the ECL Advance™ Western blotting detection kit (GE Healthcare, Amersham Biosciences). Reactive cDNAs were identified by their position on the master plate; each positive cDNA was isolated at least by two additional plating and reaction to the pooled sera.

We recovered plasmid DNA from 35 positive clones and sequenced the inserts from their 5′ end by employing the standard fluorescence labeling DYEnamic™ ET dye terminator kit

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(GE Healthcare). An automated DNA sequence analysis was performed in a MegaBACE 1000 DNA sequencer (GE Healthcare). The proteins encoded in the cloned cDNAs were compared against the GenBank non-redundant (nr) database from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) using the BLAST × algorithm and against *P. brasiliensis* genome database (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html).

2.6. Cloning of P. brasiliensis aromatic-L-amino-acid decarboxylase (Pbddc) and lumazine synthase (Pbls) cDNAs, expression and purification of the recombinant proteins

Oligonucleotide primers were designed to amplify the 1.6 kb and 525 bp cDNAs containing the complete coding regions of *Pbddc* and *Pbls*, respectively. The 1.6 kb and 525 bp amplicons were gel-excised and cloned into pGEX-4T-3 (GE Healthcare) to yield the constructs pGEX-4T3-*ddc* and pGEX-4T-3-*ls*. The recombinant plasmids were used to transform *E. coli*, according to standard procedures [12]. The cells were grown to an absorbance of 0.6 at 600 nm and 0.5 mM IPTG was added to the growing cultures. After 16 h incubation, at 15 °C, the bacterial cells were harvested, ressuspended in PBS 1X, lysed by sonication and the recombinant fusion proteins were cleaved by thrombin addition (10 U/mg fusion protein).

2.7. Production of polyclonal antibody anti-PbDDC

The recombinant *Pb*DDC was used to generate specific rabbit polyclonal serum. Rabbit pre immune serum was obtained. The purified protein (300 μg) was injected into rabbit with Freund's adjuvant three times at 2-week intervals.

2.8. Western blotting of the recombinant proteins with sera of PCM patients

The recombinant proteins were fractionated by SDS-PAGE [13]. The gels were either stained with Coomassie blue or blotted onto nitrocellulose membranes that were blocked with 5% non-fat skim milk and reacted with sera from PCM patients or from healthy individuals (1:1000 diluted). The secondary antibody was alkaline phosphatase coupled anti-human IgG. The reactions were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT).

2.9. Preparation and infection of mice macrophages by P. brasiliensis yeast cells

Bone marrow-derived macrophages were obtained by flushing the femurs of 4–12 weeks old female C57BL/6 mice [14]. The prepared cells were cultured at 37 °C under 6% CO_2 in RPMI 1640 medium (Biowhittaker, Walkersville, Md.) supplemented with 10% (v/v) FBS, 1% (w/v) l-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 ng/mL granulocyte macrophage colony stimulating factor (GM-CSF), and 10 μg/mL of gentamicin. After 8 days, the non-adherent cells were discarded and the remaining cells were washed twice with 10 mL of Hank's Balanced Salt Solution (HBSS). The cells were treated with 10 μg/mL of dispase in HBSS at 37 °C for 5 min. Further, macrophages were removed using a cell scraper and washed in HBSS. Cells were centrifuged at 500 × g for 5 min, and resuspended in RPMI 1640 medium (supplemented as described above, minus GM-CSF) at a concentration of 1×10^6 cells/mL. *P. brasiliensis* yeast cells (5×10^6) were added to 2 mL of macrophage suspension plated on 6 well plates. After 24 h of co-cultivation at 37 °C, the non-phagocyted yeast cells were discarded and the bottom cells were washed twice. The RNA of infected mice macrophages and control macrophages were extracted using Trizol.

2.10. Quantitative analysis of RNA transcripts encoding Pbddc, Pbls and Pbctr3 by reverse transcription real-time PCR (qRT-PCR)

The RNA of yeast cells, mycelium, infected macrophages and yeast cells derived from infection of mice liver and spleen after 15 days were used in this analysis. Total RNAs treated with DNAse were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo$(dT)_{15}$ primer. qRT-PCR was performed in triplicate, with samples from three independent experiments in the StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). The PCR thermal cycling was 40 cycles of 95 °C for 15 s; 60 °C for 1 min. The SYBR green PCR master mix (Applied Biosystems) was used as reaction mixture, added of 10 pmol of each primer and 40 ng of template cDNA, in a final volume of 25 μl. A melting curve analysis was performed to confirm a single PCR product. The data were normalized with the ribosomal protein L34 amplified in each set of qRT-PCR experiments. A non-template control was included. A cDNA for a relative standard curve was generated by pooling an aliquot of cDNA from each sample. The standard cDNA was serially diluted 1:5, and a standard curve was generated using four samples from the pooled cDNA. Relative expression levels of genes of interest were calculated using the standard curve method for relative quantification [15].

2.11. Dot blot analysis

To analyze the melanin and DDC accumulation in yeast forms of *P. brasiliensis*, the cells (1.5 g) were sub cultured in Mc Veigh-Morton liquid minimal medium (MMcMi) [16] supplemented or not with 1.0 mM L-Dopa (Sigma) for 15 days at 36 °C. All cultures were incubated in the dark to avoid photo polymerization of L-Dopa into melanin. The viability of fungal suspensions was determined by staining with 0.01% (w/v) Trypan blue in PBS 1X. The cells were collected by centrifugation at 5000 × g for 5 min, frozen in liquid nitrogen and disrupted by maceration. The cellular

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powder was centrifuged at 12,000 × g for 20 min. The cellular extracts were vacuum spotted onto nitrocellulose membranes that were blocked and reacted with the anti-melanin antibody of *Sporothrix schenckii* (1:1000 diluted, kindly provided by Dr Joshua D. Nosanchuck, Albert Einstein College of Medicine, New York) and antibody anti-DDC (1:500 diluted). The anti-melanin secondary antibody was biotin anti-mouse IgM (1:500 diluted) plus streptavidin-HRP (1:1000 diluted) and the reactive bands were developed with hydrogen peroxide and diaminebenzidine. The anti-DDC secondary antibody was alkaline phosphatase coupled anti-rabbit IgG (1:2000 diluted), and the reaction was developed with BCIP/NBT. Dot blot analysis was also performed to assay the reactivity of a peptide synthesized on basis on the deduced sequence of *Pb*CTR3 (Supplementary Fig. S3) to the serum of PCM patients. Reactions were performed as described above.

3. Results

3.1. Identification of P. brasiliensis antigens by IVIAT

We immuno-screened approximately 6000 clones inducible in the *P. brasiliensis* expression library. We identified 35 immuno-reactive clones representing 29 distinct *P. brasiliensis* genes encoding proteins that were persistently reactive after at least three rounds of screening. The predicted proteins encoded by the cDNAs are shown in Table 1. They are implicated in cell metabolism, biogenesis of cellular components, transport, energy, transcription, protein fate and signal transduction.

Table 1
Predicted proteins of *Paracoccidioides brasiliensis* encoded by the cDNAs identified by IVIAT.

Functional category	Gene Product-description, function (reference)	Best Hit/GenBank accession number* or *P. brasiliensis* genome locus†	e-value	EC number	Number of positive cDNAs obtained through IVIAT
Metabolism	Acyl-CoA dehydrogenase	*P. brasiliensis*/CA581965*	5e-101	1.3.1.8	1
	Aromatic-L-amino acid decarboxylase	*P. brasiliensis Pb*01/PAAG_01563.1†	0.0	4.1.1.28	1
	Ubiquinone (COQ9)	*P. brasiliensis Pb*01/PAAG_05083.1†	1e-25	–	1
	Lumazine synthase	*P. brasiliensis*/DQ081183*	4e-78	2.5.1.9	1
	Alfa-1,2-galactosyl transferase	*P. brasiliensis Pb*01/PAAG_02629.1†	1e-8	2.4.1.	1
	Pyridine nucleotide-disulphide oxidoreductase	*P. brasiliensis Pb01*/PAAG_01677.1†	6e-19	1.-	1
Biogenesis of cellular components	Lipopolysaccharide biosynthesis protein	*Trichophyton rubrum*/DW709722*	3e-33	–	1
	Cofilin	*P. brasiliensis Pb*03/PABG_07299.1†	8e-32	–	1
Transport	Coatomer zeta subunit	*Schizosaccharomyces pombe*/AA21186.1*	1e-67	–	1
	High affinity copper transporter	*P. brasiliensis*/ABF93409.1*	2e-59	–	1
	Outer membrane ferric siderophore receptor	*Phakopsora pachyrhizi*/DN739539*	1e-63	–	1
	Carboxylate/amino acid/amine transporter	*Trichophyton rubrum*/DW701041*	1e-29	–	1
	ABC transporter	*P. brasiliensis Pb03*/PABG_07206.1†	1e-7	–	3
Energy	ATP synthase F0 F1 subunit 9	*P. brasiliensis*/YP_537116.1*	7e-15	3.6.3.14	1
	Alcohol dehydrogenase	*P. brasiliensis Pb*01/PAAG_02965.1†	2e-9	1.1.1.1	1
	Mitochondrial cytochrome c oxidase subunit VIIa	*P. brasiliensis*/ABU46290.1*	1e-86	1.9.3.1	1
Transcription	C2H2 finger domain protein	*P. brasiliensis Pb*01/PAAG_04481.1†	0.0	–	1
	Nitrogen regulation protein	*P. brasiliensis*/EH041264.1*	5e-52	–	2
Protein fate	Ubiquitin	*P. brasiliensis Pb*01/PAAG_06536.1†	3e-29	–	1
	Midasin	*P. brasiliensis Pb*01/PAAG_00114.1†	0.0	–	3
Signal Transduction	Protein kinase domain	*P. brasiliensis Pb*01/PAAG_00114.1†	0.0	2.7.1.37	1
	WD repeat protein	*P. brasiliensis Pb*01/PAAG_02429.1†	0.0	–	2
	Diguanylate cyclase	*Trichophyton rubrum*/DW692821*	3e-15	4.6.1.-	1
Unclassified	Conserved hypothetical protein	*P. brasiliensis*/YP_537116*	5e-15	–	1
	Conserved hypothetical protein	*P. brasiliensis*/CN244805*	1e-23	–	1
	Conserved hypothetical protein	*P. brasiliensis Pb*01/PAAG_08269.1†	4e-24	–	1
	Conserved hypothetical protein	*Ajellomyces capsulatus*/XP_001537205.1*	2e-12	–	1
	Hypothetical protein	No hits found	–	–	1
	Hypothetical protein	*Neurospora crassa*/XP_001728522.1*	1e-10	–	1
Total					35

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3.2. Nucleotide and deduced amino acid sequences of aromatic-L-amino-acid decarboxylase (Pb*DDC*), lumazine synthase (Pb*LS*) and high affinity copper transporter (Pb*CTR3*)

We selected the cDNAs encoding *Pbddc*, *Pbls* and *Pbctr*3 for further analysis. The entire cDNA encoding *Pbddc* consisted of 2371 bp and encoded a protein of 545 amino acids, predicted molecular mass of 60 kDa and p*I* of 6.5. The cDNA sequence encoding *Pb*DDC had been deposited on GenBank under accession number ABH03461. The deduced amino acid sequence displayed strong identity to DDCs of fungal origin. The alignment of *Pb*DDC with pathogenic fungi orthologues is presented in Supplementary Fig. S1.

The analysis of *Pbls* showed a single open reading frame (ORF) with 174 amino acids with a molecular mass prediction of 19 kDa and p*I* of 6.6. The cDNA and genomic sequences encoding *Pbls* had been deposited on GenBank under accession numbers DQ081183 and DQ186604, respectively. The sequence of amino acid encoding *Pb*LS was compared with orthologues of fungi (Supplementary Fig. S2).

The analysis of *Pbctr*3 demonstrated a single ORF with 193 amino acids, with a predicted molecular mass of 21 kDa and p*I* of 8.6 (Supplementary Fig. S3). The cDNA encoding *Pbctr*3 had been deposited on GenBank under accession number DQ534496.

3.3. Reactivity of Pb*DDC*, Pb*LS* and Pb*CTR3* to sera of PCM patients

The expression of the recombinant *Pb*DDC and *Pb*LS was obtained. SDS-PAGE was used to verify the composition of

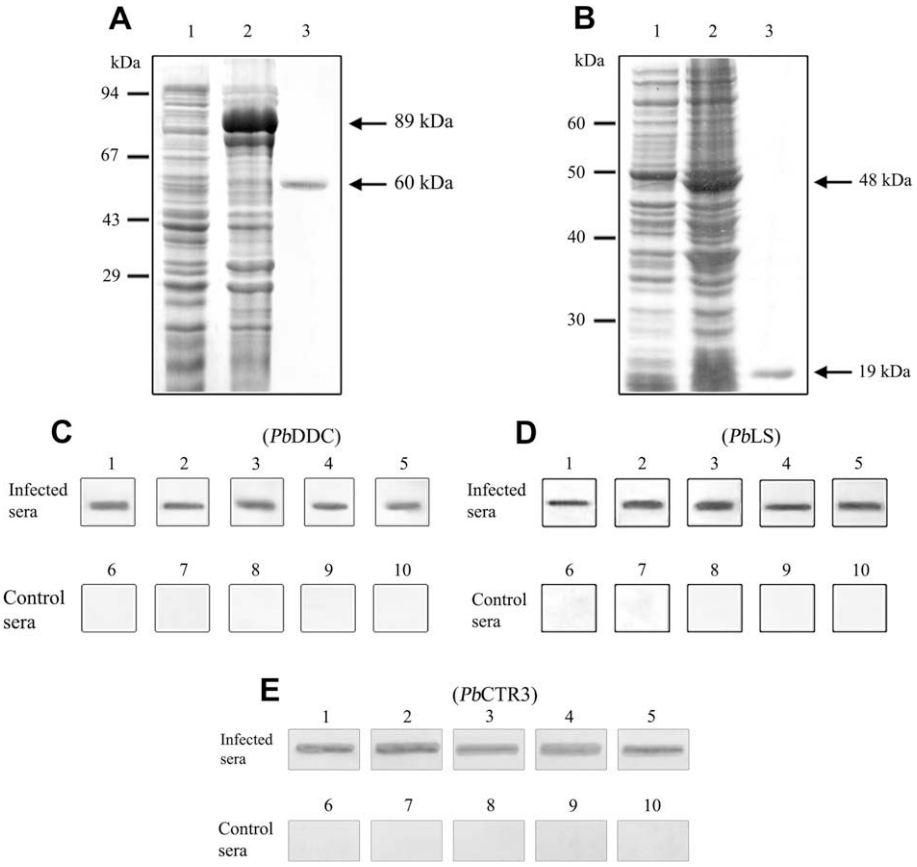

Fig. 1. Reactivity of the recombinant *Pb*DDC and *Pb*LS and of the synthetic peptide of *Pb*CTR3 with sera of PCM patients. The nucleotide sequences of the *Pbddc* oligonucleotide primers were sense 5′-*GGATCC*ATGGACCAGGAAGAATTCAG-3′ and antisense 5′-*CTCGAG*CTAGTTTTTCACAGCCCTGC-3′, which contained engineered *Bam*HI and *Xho*I restriction sites (italicized), respectively. The oligonucleotide primers for *Pbls* were sense 5′-TGGT*GAATTC*CATGGCTACTCTCAAAGG-3′, and antisense 5′-GGTGGT*CTCGAG*CTACGAAAACTTCCCCATTG-3′, which contained engineered *Eco*RI and *Xho*I restriction sites (italicized), respectively. The PCR products were digested with the cited restriction enzymes, electrophoresed on agarose gel cloned into pGEX-4T-3, and used to transform *E. coli* cells. (A) SDS-PAGE analysis of *P. brasiliensis* recombinant DDC. The *E. coli* cells harboring the pGEX-4T-3-*ddc* plasmid were grown to an A_{600} of 0.6 and harvested before (lane1), and after 16 h (lane 2) incubation with 0.5 mM IPTG at 15 °C; the affinity-isolated recombinant *Pb*DDC after thrombin addition and protein purification by affinity chromatography (lane 3). (B) Induced bacterial cells of *E. coli* harboring the pGEX-4T-3-*ls* plasmid were grown at 15 °C to an A_{600} of 0.6 and harvested before (lane 1) and after 16 h incubation with IPTG, at 15 °C (lane 2). The purified recombinant protein was obtained after thrombin digestion and affinity chromatography (lane 3). (C and D) Immunoblot analyses of the recombinant proteins. The recombinant *Pb*DDC and *Pb*LS (1.0 μg) were reacted with sera of five PCM patients (1:1000 diluted), (lanes 1–5) and to control sera (1:1000 diluted), (lanes 6–10). (E) Reactivityof the synthetic peptide from *Pb*CTR3 with the same sera as in C and D. A peptide was synthesized from amino acids 90–130 in the deduced *Pb*CTR3 (Invitrogen, life technologies). One hundred ηg of the synthetic peptide was blotted onto nitrocellulose membrane and reacted with sera from PCM patients and with control sera. After reaction with anti-human IgG alkaline phosphatase coupled antibody (1:2000 diluted), the reaction was developed with BCIP/NBT. Molecular mass of the proteins and standards (kDa) are indicated.

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the cells lysates obtained from *E. coli* cells which had been transformed with the plasmid constructs, as shown in Fig. 1A and B, lane 1. After induction with IPTG, 89-kDa and 48-kDa recombinant proteins were detected in the bacterial lysates (Fig.1A and B, lane 2), respectively, for *Pb*DDC and *Pb*LS, which included the vector-encoded fusion protein at its N-terminus. The fusion proteins were cleaved by the addition of thrombin protease (Fig. 1A and B, lane 3). As observed, highly purified proteins were obtained, that migrated on SDS-PAGE as a single species of 60 kDa and 19 kDa, for *Pb*DDC and *Pb*LS, respectively. A peptide was synthesized toward amino acids 90–130 of the deduced *Pb*CTR3 (Supplementary Fig. S3).

Five sera samples from PCM patients and five control sera samples were reacted with the recombinant proteins *Pb*DDC and *Pb*LS and with the synthetic peptide of *Pb*CTR3 in immunoblot assays (Fig. 1C–E, respectively). Strong reactivity was observed with sera of PCM patients (Fig. 1C–E, lanes 1–5) and no cross-reactivity was observed with control sera (Fig. 1C–E, lanes 6–10).

3.4. Assessment of the expression of Pbddc, Pbls and Pbctr3 by reverse transcription real-time PCR in models of infection

The expression of the genes in a macrophage model of infection is shown in Fig. 2A. In our study, the genes are induced in yeast cells, when compared to mycelia. During macrophage infection, it was detected overexpression of *Pbddc* and *Pbctr*3, when compared to the expression in the mycelium and yeast cells after in vitro growth. Although expressed during macrophage infection, *Pbls* was not upregulated in vivo relative to the highest level of expression in vitro (Fig. 2A).

The expression of the genes was also evaluated by qRT-PCR analysis in yeast cells of *P. brasiliensis* derived from infected mice liver and spleen (Fig. 2B). We have shown that *Pbddc* is upregulated in vivo, with the expression occurring at 15 days post inoculation in spleen, but not in liver (Fig. 2B). We have also shown that *Pbctr*3 is upregulated in liver and spleen (Fig. 2B). Of the genes characterized by qRT-PCR, *Pbls* was not overexpressed in vivo relative to the highest level of expression in vitro (Fig. 2B).

3.5. Melanin accumulation in yeast cells of P. brasiliensis

We directed our experiments toward the analysis of melanin accumulation in *P. brasiliensis*. The fungus was grown on a chemically defined medium supplemented or not with L-Dopa (Fig. 3). The viability was of 62.5% and 75.7%, respectively, for yeast cells grown in media enriched or not with L-Dopa (data not shown). Light microscopy (400× magnification) shows darkly pigmented yeast cells in the presence of L-Dopa (Fig. 3A, panel 2). The accumulation of melanin and DDC were higher when the fungus was grown in medium supplemented with L-Dopa (Fig. 3B and C, respectively). Loading control was performed with the antibody to the recombinant triosephosphate isomerase [17].

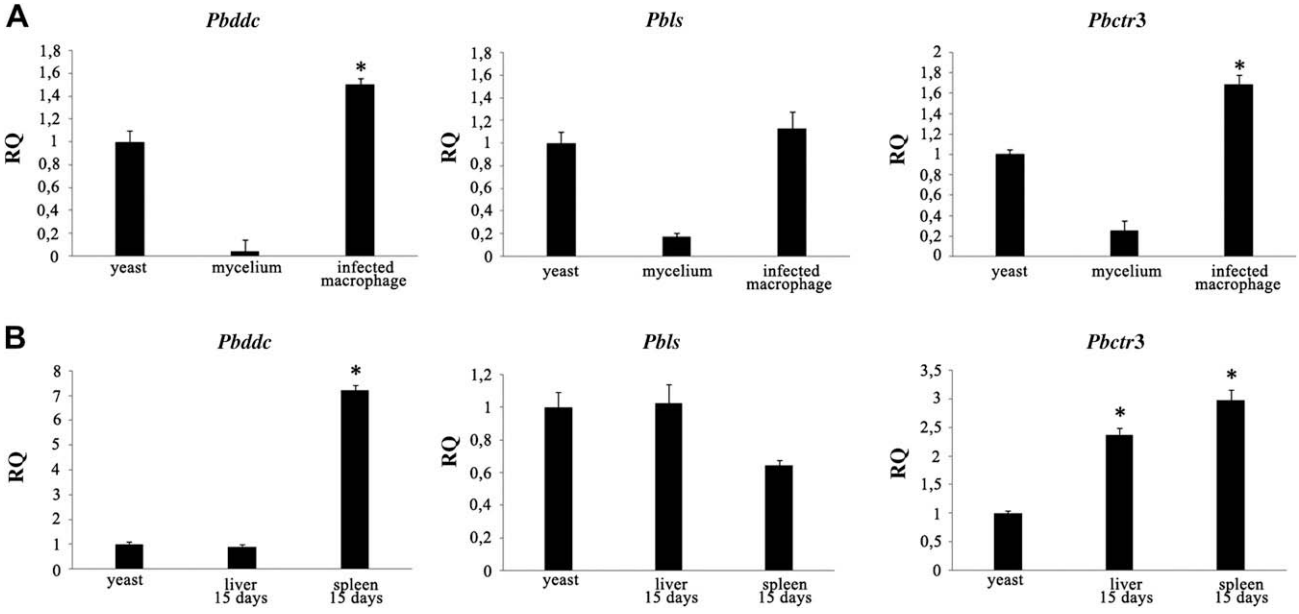

Fig. 2. Average of gene expression of *Pbddc* , *Pbls* and *Pbctr*3 as determined by quantitative real time RT-PCR. (A) qRT-PCR plot of *Pbddc*, *Pbls* and *Pbctr*3 expression levels in mycelium, yeast cells and in a macrophage model of infection. (B) qRT-PCR plot of *Pbddc*, *Pbls* and *Pbctr*3 expression in yeast cells derived from infected tissues of mice. The primers were as following: *Pbddc*, sense 5′-GTACCTTCGTCCTCTTCTTC-3′, antisense 5′-GGGTAAGTCACACAAGAGGG-3′; *Pbls,* sense, 5′-GCCTATTGCTATGGAGAGAATA-3′, antisense, 5′-GTTGACGGTGTTGAATGAGG-3′; *Pbctr*3 sense, 5′-ATGTGAAGC AGCGAGCGG-3′, antisense 5′-CATGGAATGCACGGCGGC-3′ *Pbl34*, sense, 5′-CGGCAACCTCAGATACCTTC-3′, antisense 5′-GGAGACCTGGGAGTATTCAC-3′. The values of expression of the *Pbddc*, *Pbls* and *Pbctr3* were standardized using the values of expression of the constitutive gene encoding to the ribosomal protein L34. The expression level was calculated by relative standard curve method. The standard deviations are presented from three independent experiments. *Significantly increased expression ($P \leq 0.05$).

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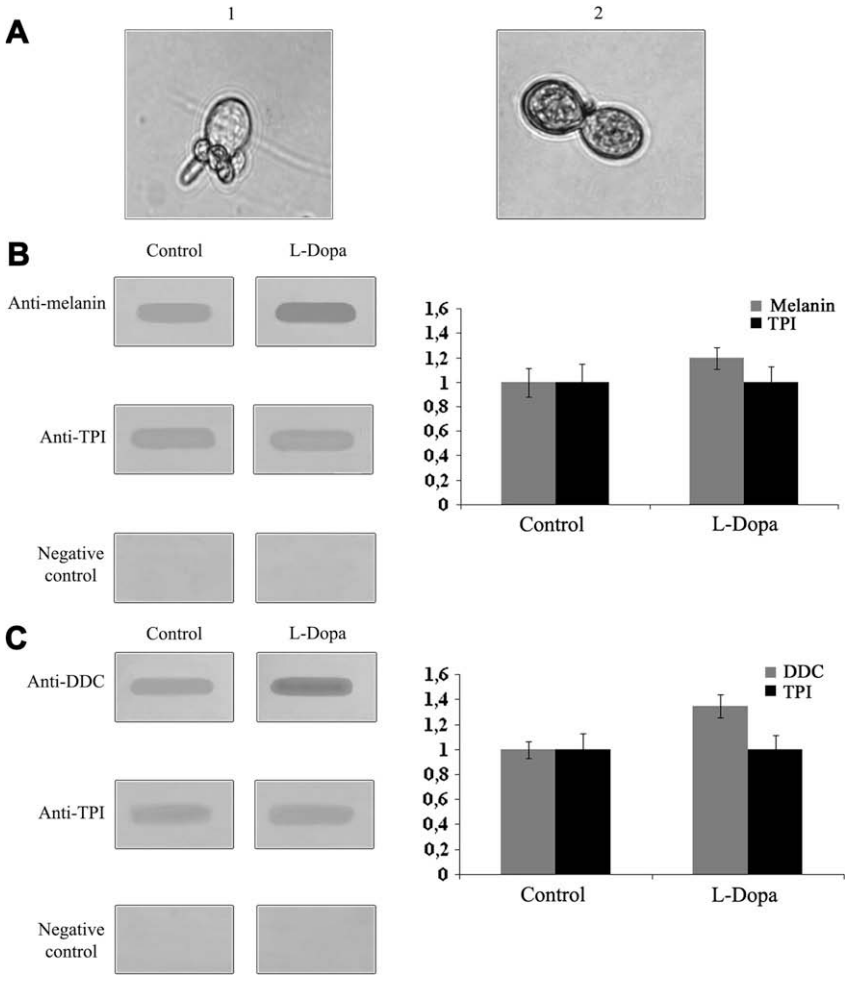

Fig. 3. Analysis of melanization of *P. brasiliensis* yeast cells. A – Light microscopy (400× magnification) of *P. brasiliensis* yeast cells grown in chemically defined liquid medium (1) or the same medium supplemented with L-Dopa (2). B – Dot blot analysis of cellular extracts of *P. brasiliensis* grown in same conditions and reacted with antibody anti-melanin of *S. schenckii* (B) or (C) anti-*Pb*DDC. The antibody to the antigen triosephosphate isomerase (*Pb*TPI), was used as the loading control. The analysis of relative differences were performed by using the Scion Image Beta 4,03 program. In the graphics, the black bars represent the reaction to the antibody anti-*Pb*TPI and the gray bars represent the reaction with the antibodies anti-melanin or anti-*Pb*DDC. The standard deviations are presented from three independent experiments.

4. Discussion

Our objective in the present work was uncovering antigenic proteins that could be expressed during infection with *P. brasiliensis*. Sera from PCM patients were polled and reacted with in vitro-grown *P. brasiliensis*. Antibodies that remain in these sera should be reactive with proteins expressed by the pathogen during the natural infection, as described in other organisms [6–11].

Screening of the *P. brasiliensis* cDNA library with sera from individuals with active PCM resulted in the identification of 35 clones encoding putative immunogenic proteins. Sequence analysis of the reactive clones in the present study identified genes varying from cell metabolism, transport, energy, transcription, protein fate, signal transduction and control of cellular organization, as well as unknown functions. Among the identified transcripts, some encoded molecules presumably present at the fungal cell wall such as high affinity copper transporter, siderophore receptor, carboxylate/amino acid/amine transporter and ABC transporter. Interestingly, some of the identified cDNAs encoded proteins described as immunogenic in organisms, such as DDC [18], acetyl-CoA acetyltransferase [19], LS [20], alcohol dehydrogenase [21]. LS is an enzyme of the family 6,7-dimethyl-8-ribityllumazine synthase, which catalyzes the penultimate step of synthesis of vitamin B_2 (riboflavin). Plants, bacteria and fungi are vulnerable to inhibitors of the synthesis of riboflavin. The lack of such a homologue in humans suggests that *Pb*LS may serve as antifungal drug target, as described [22].

To further confirm the validity of the screening strategy in identifying *P. brasiliensis* antigens potentially relevant to the fungal infection, we selected *Pb*DDC, *Pb*LS and *Pb*CTR3 for further analysis. The recombinant proteins *Pb*DDC and *Pb*LS, as well as the synthetic peptide of *Pb*CTR3 were recognized by sera of PCM patients, validating the IVIAT strategy here employed. LS is an immunogenic molecule and an useful marker in the serological diagnosis of brucellosis in human beings and animals [23].

We selected *Pbddc*, *Pbls* and *Pbctr*3 to follow with experiments concerning to gene expression in models of infection. The

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transcripts encoding *Pbddc* and *Pbctr*3 were overexpressed in fungal yeast cells infecting macrophages and in cells derived from tissues of infected mice. We detected overexpression of the transcript encoding *Pbddc* in yeast cells derived from spleen and not in liver at 15 days postinoculation, which could reflect niche regulation of genes in *P. brasiliensis* microenvironments, as described to fungi [24,25]. Noteworthy, *Pbctr*3 was overexpressed in all analyzed conditions corroborating previous transcriptome analysis from our laboratory [3]. This may not be surprising considering the obvious necessity for upregulating copper acquision during infection [26]. It has long been established that invading pathogens must compete favorably for limited nutrients to both establish and maintain a successful host infection. Studies indicate that copper modulates critical virulence determinants. Genetic analysis in *Cryptococcus neoformans* has demonstrated that a high affinity copper transporter and its corresponding transcriptional regulator are required for infection of the brain [27]. Although expressed in liver and spleen at 15 days postinoculation, *Pbls* was not upregulated in vivo relative to the highest level of expression in vitro. There are possible explanations for this result. The gene expression could be induced earlier or later during the infectious process and we could have missed the time at which the expression increased relative to the in vitro growth. Additionally, the amount of RNA could not reflect the amount of protein if the gene regulation occurs at posttranscriptional level. Further investigation will be required.

The fungus *P. brasiliensis* is known to make Dopa-melanin from L-Dopa [28,29]. The production of melanin-like pigments by *P. brasiliensis* protects the fungus from phagocytosis and increases its resistance to antifungal drugs [29]. We verified in this work a correlation between increase in melanin accumulation and *Pb*DDC in yeast cells incubated in the presence of L-Dopa, which resulted in the presence of dark pigment by yeast cells. The results suggest that *Pb*DDC could be involved in the melanin biosynthesis pathway.

In conclusion, the present study has shown the successful application of IVIAT in the identification of *P. brasiliensis* genes expressed during fungal infection. The identified genes ranged from those involved in metabolic pathways to those with unknown function. The study of the identified genes could improve our understanding of the adaptative mechanisms used by *P. brasiliensis* in the infectious process.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.micinf.2009.05.009.

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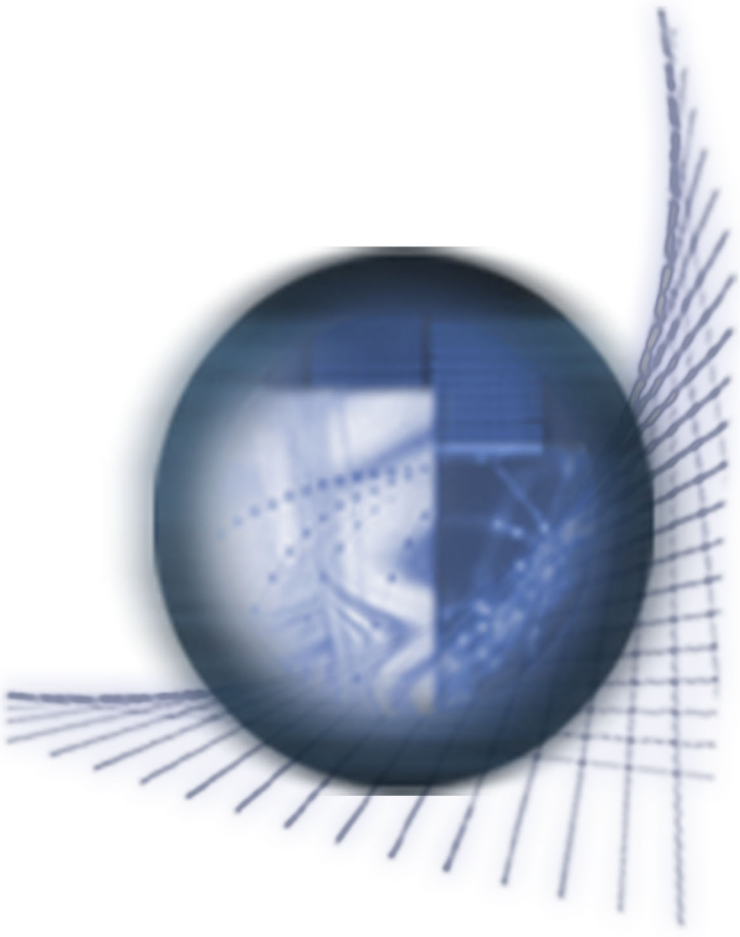
DISCUSSÃO



V - DISCUSSÃO

A interação entre fungos patogênicos e o hospedeiro durante a infecção é muito complexa, envolve múltiplos fatores produzidos pelo patógeno e pelos mecanismos de resistência do hospedeiro. Análises da resposta às mudanças ambientais encontradas no sítio da infecção fornecem explicações para alterações exigidas para o parasita sobreviver no interior do hospedeiro. Qualquer função que contribua para a adaptação do fungo nesse ambiente pode ser percebida como um determinante de virulência (Mahan *et al*., 2000).

Nos últimos anos várias abordagens têm sido desenvolvidas para identificar genes relacionados com a interação fungo-hospedeiro. O perfil transcricional de micélio e de células leveduriformes identificou genes que são potencialmente relacionados com a virulência de *P. brasiliensis* (Marques *et al*., 2004; Felipe *et al*., 2005). Bailão *et al*. (2006) e Costa *et al*. (2007) estudaram genes diferencialmente expressos em células leveduriformes de *P. brasiliensis* recuperadas de camundongos infectados visando analisar as respostas transcricionais que ocorrem no hospedeiro. Tavares *et al*. (2007) usaram tecnologia de microarranjos para avaliar o início da resposta transcricional de *P. brasiliensis* em macrófagos peritoneais de camundongos, com o objetivo de esclerecer os mecanismos utilizados pelo fungo para sobreviver dentro das células fagocitárias. Os dados deste trabalho mostram uma plasticidade transcricional de *P. brasiliensis* no interior de macrófagos que podem conduzir a uma adaptação e conseqüente sobrevivência deste patógeno. Apesar dessas descrições, pouco ainda é conhecido sobre a expressão de genes de virulência de *P. brasiliensis* durante a interação do parasito com o hospedeiro.

Com o objetivo de estudar as características antigênicas de *P. brasiliensis* durante o processo infeccioso, uma biblioteca de cDNA de células leveduriformes, isolado *Pb* 01, recuperadas de fígado de camundongos infectados foi rastreada com soros de pacientes com PCM previamente incubados com o fungo. Os anticorpos que permaneceram no soro após a incubação com células leveduriformes crescidas *in vitro* podem ser reativos com proteínas expressas pelo patógeno durante a infecção (Handfield *et al*., 2000). Esta estratégia identificou 35 clones de cDNAs que codificam para 29 diferentes proteínas provavelmente envolvidas no metabolismo celular, transporte, energia, transcrição, endereçamento de proteínas, transdução de sinal, biogênese de componentes celulares e proteínas com função não identificadas.

Um dos genes identificados codifica uma proteína que participa do metabolismo de aminoácidos com grupos aromáticos, DDC. Essa proteína pode estar envolvida na patogenicidade de muitos organismos, porque ela é uma enzima componente da via de biossíntese de Dopa-melanina, um fator de virulência em muitos fungos patogênicos humanos (Gomez *et al*., 2001; Silva *et al*., 2006; Morris-Jones *et al*., 2003; Nosanchuk *et al*., 2004). Gomez *et al*. (2001) descreveram que *P. brasiliensis* produz melanina *in vitro* e durante a infecção. Silva *et al*. (2006) sugeriram que a melanização de *P. brasiliensis* reduz a fagocitose do fungo por macrófagos alveolares e peritoniais, assim como, a sua susceptibilidade antifúngica, principalmente para anfotericina B. Em conclusão, em *P. brasiliensis* a produção de melanina também contribui com a virulência do fungo.

Outro antígeno identificado foi a enzima LS, já descrito por Rezende (2006), que catalisa o penúltimo passo na biossíntese da riboflavina (vitamina B2) em plantas e microorganismos. A riboflavina é o precursor da flavina mononucleotídeo (FMN) e da flavina adenina dinucleotídeo (FAD), considerados os cofatores mais versáteis

envolvidos em processos de oxidoredução (Fischer e Bacher *et al.*, 2008). As enzimas da via de biossíntese de riboflavina não estão presentes em humanos ou hospedeiros animais, sendo, portanto, potenciais alvos para drogas antifúngicas.

Também foi identificado nesse trabalho o transportador de cobre de alta afinidade (CTR3), que foi anteriormente identificado no transcriptoma de células leveduriformes recuperadas de camundongos infectados (Costa *et al*., 2007; Bailão *et al*., 2006). Foi demonstrado em *C. neoformans* que CTR3 está envolvido no processo de melanização do fungo (Walton *et al*, 2005). Outros prováveis antígenos presentes na membrana de *P. brasiliensis* identificados nesse trabalho foram um transportador ABC, um transportador de carboxilato/ aminoácido/ amina e um receptor de membrana de sideróforo. O transportador ABC pertence ao grupo de proteínas de membrana que catalisam a hidrólise de adenosina trifosfato (ATP) para que a translocação de diversos substratos através da membrana celular ocorra (Locher *et al*., 2002). O transportador de carboxilato/ aminoácidos /amina é uma proteína que contém entre oito e dez regiões transmembrana que provavelmente funciona como transportadores aminoácidos, PecM é o modelo deste transportador mais bem estudado (Rouanet e Nasser, 2001). Em *Pseudomonas putida* o receptor de sideróforo se localiza na membrana externa da célula facilitando o transporte de ferro complexado a sideróforos e sua síntese é induzida na presença de sideróforos específicos sob condições de limitação de ferro (Koster *et al*., 1994).

O cDNA codificante para *Pbddc* apresenta 1.638 pares de bases e uma ORF que codifica para uma proteína de 545 aminoácidos, com massa molecular predita de 60 kDa e pI de 6,5. O alinhamento de *Pb*DDC com outras seqüência de proteínas depositadas em banco de dados revelou altos níveis de identidade e similaridade. O cDNA codificante a *Pbls* apresenta 525 pares de bases e uma ORF codificando para

uma proteína de 174 aminoácidos, com massa molecular predita de 18,6 kDa e pI de 6,6, como já descrito por Rezende (2006). O gene codificante para *Pbctr*3 apresenta 1.655pb, que codifica uma ORF de 193 resíduos de aminoácidos, com massa molecular predita de 21,5 kDa e pI de 8,6.

Com objetivo de iniciar estudos funcionais das proteínas e da reatividade imunológica com soros de pacientes com PCM, *Pbddc* e *Pbls* foram clonadas em vetor de expressão pGEX-4T-3. A análise das proteínas recombinantes *Pb*DDC e *Pb*LS demonstrou espécies monoméricas com massas moleculares de 60 e 19 kDa, que está de acordo com a massa molecular predita para DDC e LS em suas composições de aminoácidos. Também foi sintetizado um peptídio que codifica a *Pb*CTR3.

A detecção de anticorpos em testes sorológicos pode ser útil para um rápido diagnóstico da PCM, mas esses testes podem ser dificultados por variações nas preparações do antígeno. Para se investigar a reatividade imunológica das proteínas recombinantes *Pb*DDC, *Pb*LS e *Pb*CTR3 foram realizadas análises imunológicas por meio de *Western blotting*. Os resultados mostraram que as proteínas recombinantes *Pb*DDC, *Pb*LS e *Pb*CTR3 são reconhecidas por soros de pacientes com PCM e não por soros de indivíduos controle, sugerindo a possível utilização de ambas no diagnóstico sorológico desta micose. Em humanos foi descrito por Bratland *et al*. (2007) que DDC apresenta epítopos imunodominantes em sua molécula. Já LS de *Brucella abortus* é usada como um marcador no diagnóstico sorológico de brucelose em humanos e animais (Baldi *et al*., 1997).

Após a avaliação do papel antigênico de *Pb*DDC, *Pb*LS e *Pb*CTR3 foram feitos estudos por RT-PCR em tempo real para confirmar se os transcritos codificantes a estas proteínas são mais expressos durante o processo infectivo. Os resultados mostraram que

a expressão de *Pbddc*, *Pbls* e *Pbctr*3 em células leveduriformes infectando macrófagos é aumentada. Como *Pbddc* é uma enzima presente na via de biossíntese de melanina, e como já foi descrito por Silva *et al*. (2006) que a fagocitose de macrófagos é reduzida na presença de *P. brasiliensis* melanizado, nossos resultados sugerem que *Pbddc* pode ser capaz contribuir com a proteção das células fúngicas contra o sistema imune do hospedeiro.

Em seguida, foi observado aumento nos níveis de expressão dos transcritos de *Pbddc*, *Pbls* e *Pbctr*3 em células leveduriformes recuperadas de fígado e baço de camundongos quando comparados com células leveduriformes crescidas *in vitro*. *Pbddc* teve uma expressão mais abundante após 15 dias de infecção no baço. *Pbls* apresentou um pequeno aumento de expressão após 15 dias de infecção no fígado. *Pbctr*3 apresentou uma expressão mais abundante após 15 dias de infecção no fígado e no baço. Os resultados sugerem que a expressão dos transcritos analisados seja regulada durante o estabelecimento e a progressão da doença no fígado e no baço. O aumento da expressão de *Pbddc*, *Pbls* e *Pbctr*3 nestes órgãos pode ser um mecanismo de defesa do fungo durante a fase aguda da PCM, visto que nesse período há um acentuado envolvimento do sistema reticuloendotelial (Brummer *et al*., 1993). Além disso, os resultados aqui apresentados corroboram os de Bailão *et al*. (2006; 2007) que mostraram que *Pbddc* é mais expressa em células leveduriformes recuperadas de fígado de camundongos infectados, assim como, em células leveduriformes incubadas com plasma humano.

Devido à importância da melanina durante a patogênese de infecções fúngicas, a proteína antigênica *Pb*DDC foi selecionada para seguir com os experimentos. Após o cultivo do fungo em meio líquido quimicamente definido na presença de L-Dopa observou-se células melanizadas, assim como descrito anteriormente por Gomez *et al*.

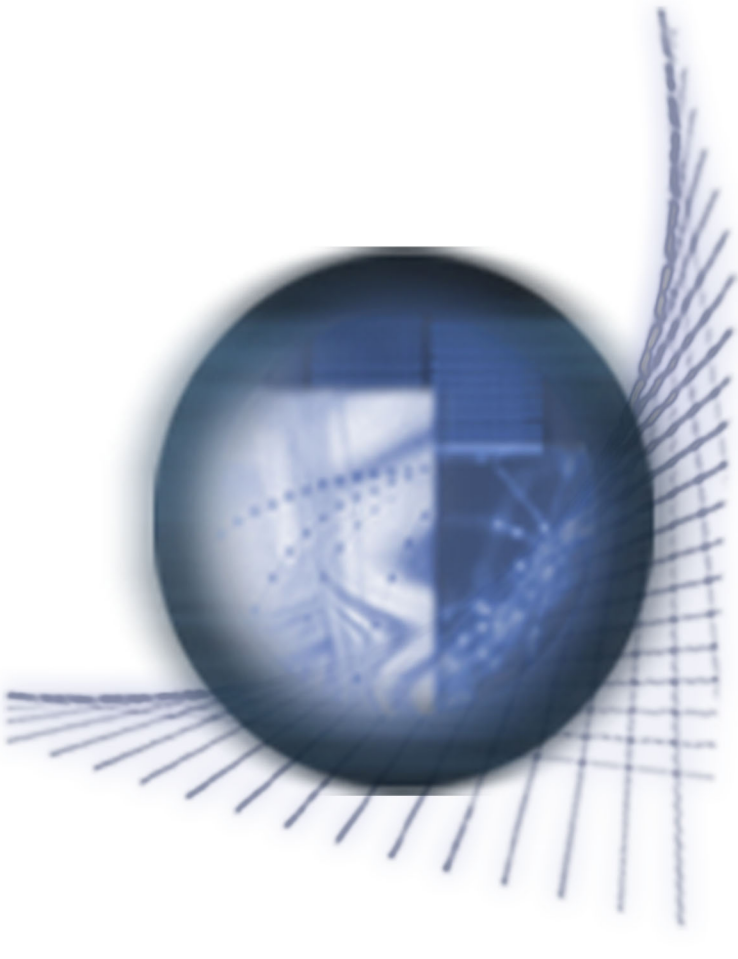
(2001). Extratos protéicos provenientes desta cultura foram obtidos e utilizados em análises por dot blot utilizando-se o anticorpo policlonal anti-melanina de *S. schenckii* (Morris-Jones *et al*., 2003) e anti-*Pb*DDC. Os resultados mostram que na presença de L-Dopa há um aumento na deposição de melanina e da expressão de *Pb*DDC, sugerindo a participação de DDC e sua relevância na via de Dopa-melanina em *P. brasiliensis*.

As enzimas tirosinase, lacase e DDC provavelmente estão envolvidas na via de biossíntese de Dopa-melanina em fungos, sendo as duas primeiras dependentes de cobre para sua atividade. Além disso, já foi descrito que a síntese de melanina é regulada por cobre, ferro, e baixas concentrações de glicose (Alspaugh *et al*., 1997; Walton *et al*., 2005). Por isso, foi analisada a expressão dos transcritos codificantes a *Pbddc*, *Pb*tirosinase e *Pb*lacase durante o cultivo do fungo na ausência de cobre por qRT-PCR em tempo real. Os resultados mostram que a síntese de todos os transcritos analisados é diminuída na ausência de cobre, principalmente o da lacase, sugerindo que em *P. brasiliensis* a síntese de melanina também seja regulada por esse íon. Walton *et al*. (2005) analisaram a mudança de fenótipo de 19 mutantes de *C. neoformans* melanizados após a adição de $CuSO_4$ exógeno ou após a remoção dos íons de cobre do meio com um quelante específico. Seus resultados mostraram que na adição de cobre todos os mutantes foram capazes de produzir melanina, com exceção dos genes codificantes a lacase; e durante a remoção de cobre nenhum mutante foi capaz de sintetizar melanina, sugerindo que, assim como em humanos, a homeostasia do cobre também é requerida para síntese de melanina em fungos.

Em síntese, foram identificados neste trabalho prováveis antígenos que podem ser relevantes no diagnóstico da PCM ou mesmo no desenho de drogas antifúngicas. As proteínas recombinantes *Pbddc* e *Pbls* reagiram com soros de pacientes com PCM,

sugerindo seu uso no diagnóstico da doença. Os experimentos de PCR em tempo real também validaram a hipótese de que essas proteínas sejam mais expressas durante o processo infectivo.

CONCLUSÕES



VI - CONCLUSÕES

✓ 35 clones foram reativos com soros de pacientes com PCM até o terceiro rastreamento imunológico utilizando a tecnologia de IVIAT estes codificam proteínas relacionadas com metabolismo celular, transporte, energia, transcrição, endereçamento de proteínas, transdução de sinal e síntese de componentes celulares.

✓ O cDNA codificante a *Pbddc* apresenta uma ORF codificante para uma proteína de 545 aminoácidos, com massa molecular predita de 60 kDa e pI de 6,5.

✓ Resíduos de aminoácidos conservados provavelmente envolvidos na reação catalítica de um largo grupo de L-aminoácido descarboxilases do grupo II, foram encontrados em *Pbddc*; esses resíduos são: T^{233}, D^{247}, D^{267}, H^{288}, S^{296}, K^{308}, Y^{352}, R^{354}, W^{369}, sendo K^{323} o provável resíduo que se liga à coenzima piridoxal fosfato.

✓ O cDNA codificante a *Pbls* apresenta uma ORF codificante para uma proteína de 174 aminoácidos, com massa molecular predita de 18,6 kDa e pI de 6,6, como já descrito por Rezende (2006).

✓ Possíveis resíduos conservados envolvidos no mecanismo catalítico da LS foram encontrados em *Pbls* (Persson *et al*., 1999). O provável sítio catalítico foi identificado na posição W^{27}, H^{97} e R^{136} e apresenta altamente conservado em todas as LS analisadas.

✓ O gene codificante para *Pbctr*3 apresenta 1.655pb, contendo quatro exons interrompidos por três íntrons e codifica uma proteína predita com 193 resíduos de aminoácidos, massa molecular predita de 21,5 kDa e pI de 8,6.

✓ *Pbddc* e *Pbls* foram clonados no vetor de expressão pGEX-4T-3. As proteínas recombinantes *Pb*DDC e *Pb*LS foram submetidas à análises em SDS-PAGE e exibiram espécies monoméricas com massas moleculares de 60 e 19 kDa, respectivamente.

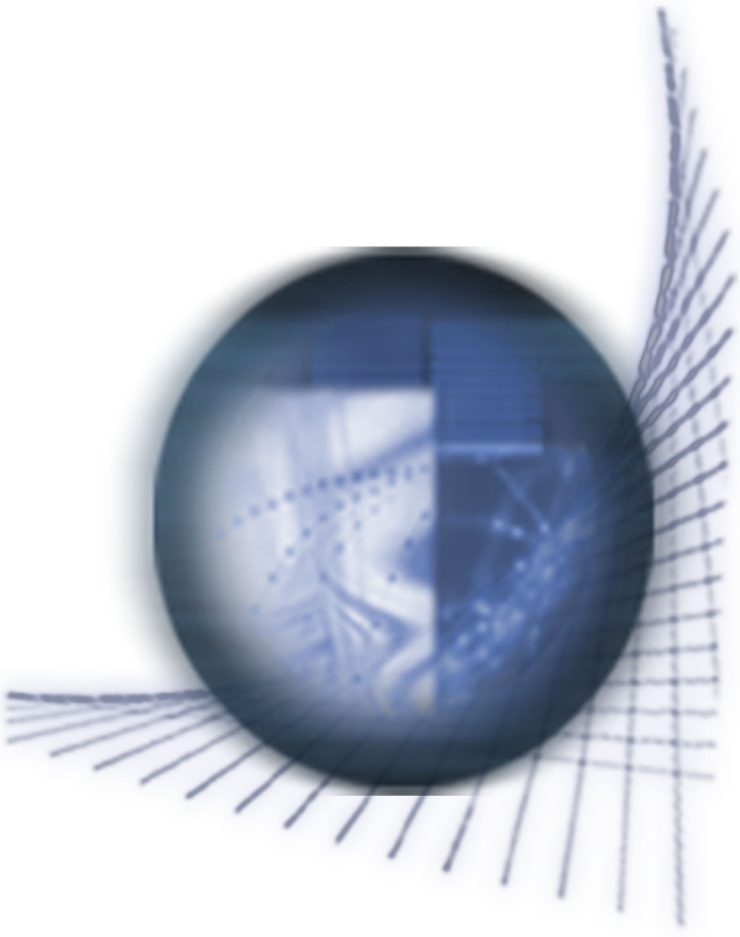
✓ As proteínas recombinantes *Pb*DDC e *Pb*LS e o peptídio sintético codificante a *Pb*CTR3 foram reconhecidos por soros de pacientes com PCM e não por soros de indivíduos controle.

✓ Os resultados de RT-PCR em tempo real mostraram que a expressão de *Pbddc*, *Pbls* e *Pbctr*3 em células leveduriformes infectando macrófagos é aumentada. E células leveduriformes recuperadas de fígado e baço de camundongos tiveram um aumento nos níveis de expressão dos transcritos de *Pbddc*, *Pbls* e *Pbctr*3.

✓ Foram observadas células leveduriformes com múltiplos brotamentos e pigmentação escura após 15 dias de cultivo em um meio líquido quimicamente definido na presença de L-Dopa. Extratos protéicos destas culturas foram obtidos e testados por dot blot utilizando-se anticorpos policlonais anti-melanina de *S. schenckii* (Morris-Jones *et al*., 2003) e anti-*Pb*DDC. Na presença de L-Dopa observou-se um aumento na deposição de melanina e da expressão de DDC.

✓ Os resultados de RT-PCR em tempo real de células leveduriformes cultivadas em meio quimicamente definido desprovido de cobre mostraram que a expressão de *Pbddc*, *Pb*tirosinase e *Pb*lacase apresenta a síntese de todos os transcritos analisados diminuída, sugerindo que em *P. brasiliensis* a síntese de melanina também seja regulada por cobre.

PERSPECTIVAS



VII - PERSPECTIVAS:

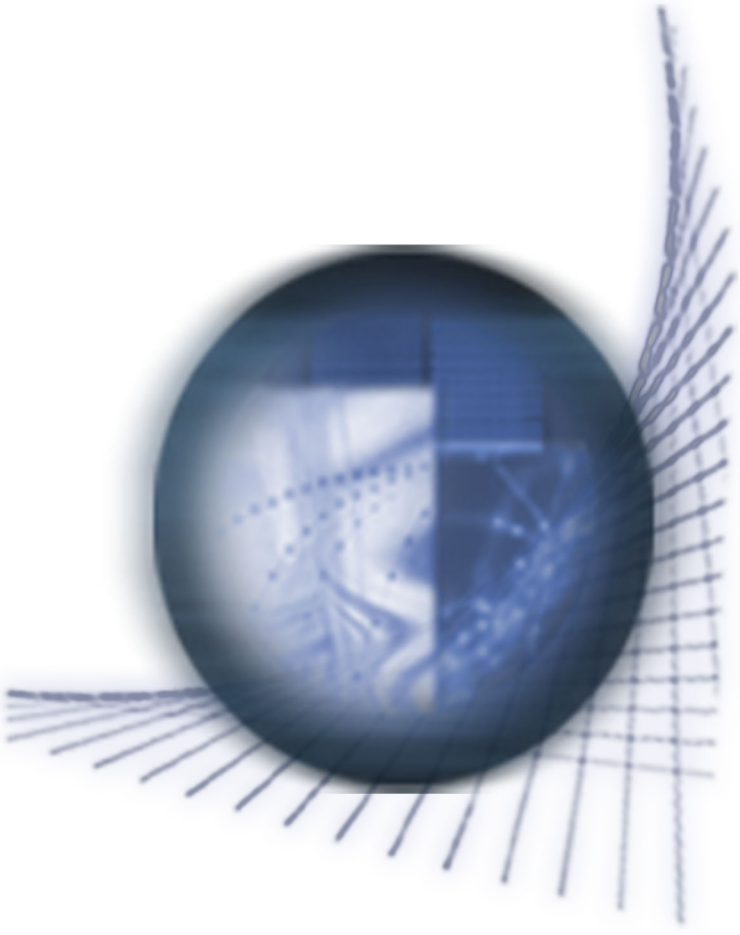
A partir dos resultados obtidos, as perspectivas deste trabalho são:

- ✓ Analisar a localização celular de DDC através de microscopia eletrônica, utilizando seu respectivo anticorpo (em realização);

- ✓ Analisar os transcritos de *Pbddc* de células leveduriformes de *P. brasiliensis* crescidas em meio quimicamente definido na presença do substrato exógeno L-Dopa, por meio de RT-PCR em tempo real (em realização);

- ✓ Estudos de interações intermoleculares da DDC de *P. brasiliensis* através da técnica de duplo-híbrido em *Saccharomyces cerevisiae*.

Produção Científica Durante o Doutorado



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

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

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†These authors contributed equally to this work.

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

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

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

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

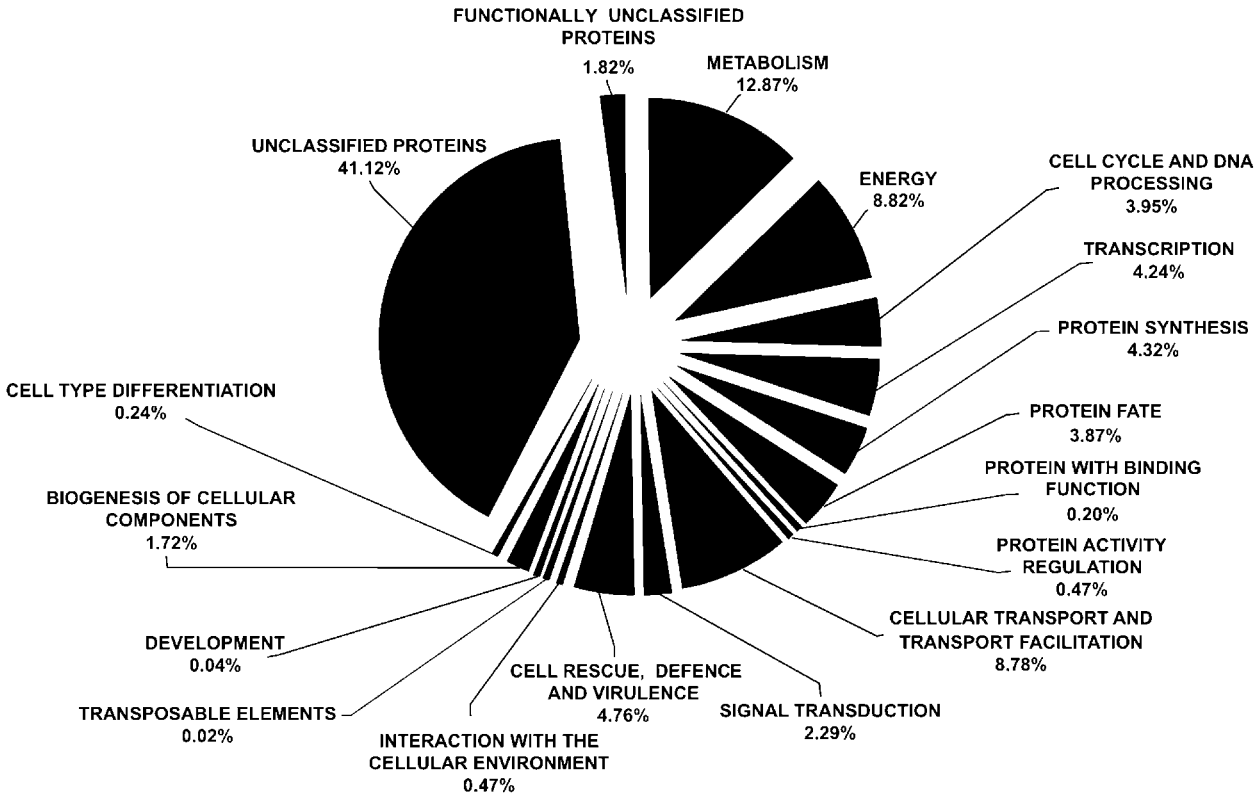

Fig. 1. Overview of ESTs from the *P. brasiliensis* transcriptome. Classification of the ESTs based on *E*-value and according to the functional categories of the MIPS functional annotation scheme.

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

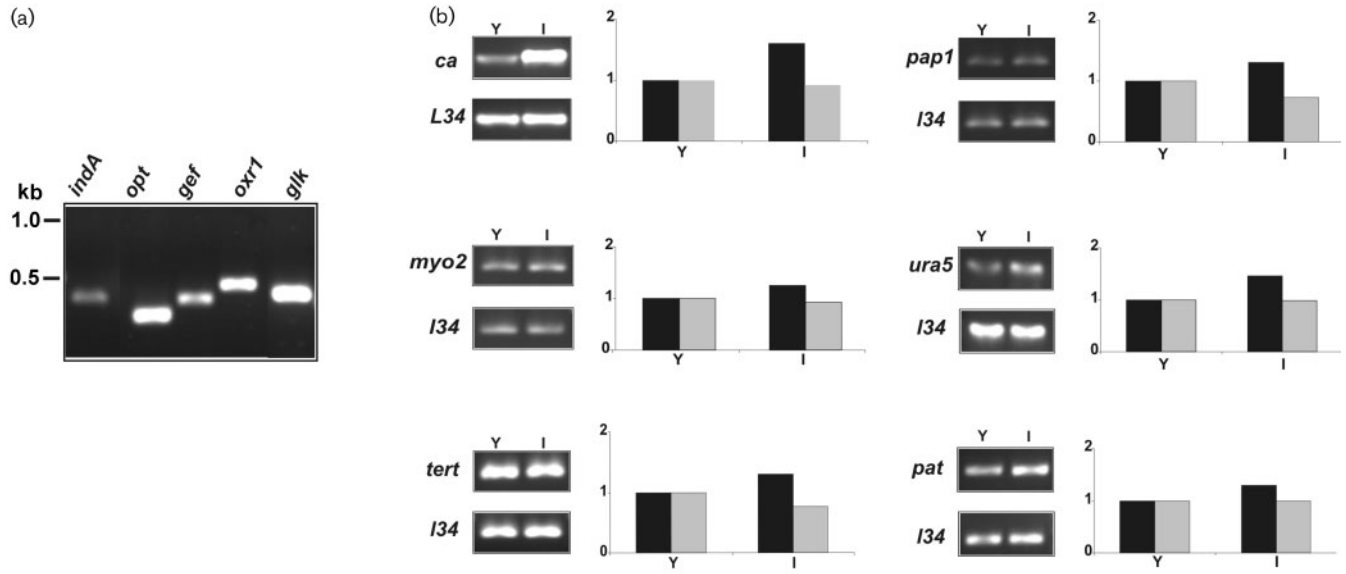

Fig. 2. Validation of the cDNA library for the presence of genes and analysis of redundancy of some transcripts. PCR and sqRT-PCR analysis were carried out with specific sense and antisense oligonucleotide primers, respectively, as described in Table 1. (a) PCR of total genomic DNA of *P. brasiliensis*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

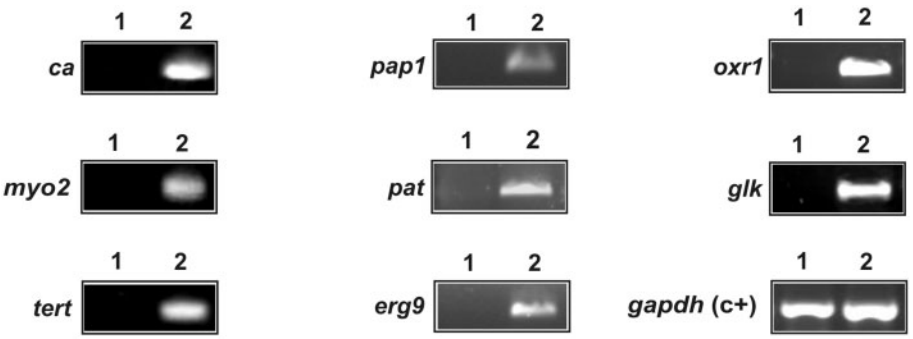

Fig. 3. Transcript analysis of the interaction of *P. brasiliensis* with Vero cells: sqRT-PCR analysis of selected transcripts in yeast-phase cells in the *ex vivo* model of infection. Lane 1, detection of transcripts in control Vero cells; lane 2, detection of transcripts in *P. brasiliensis* yeast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

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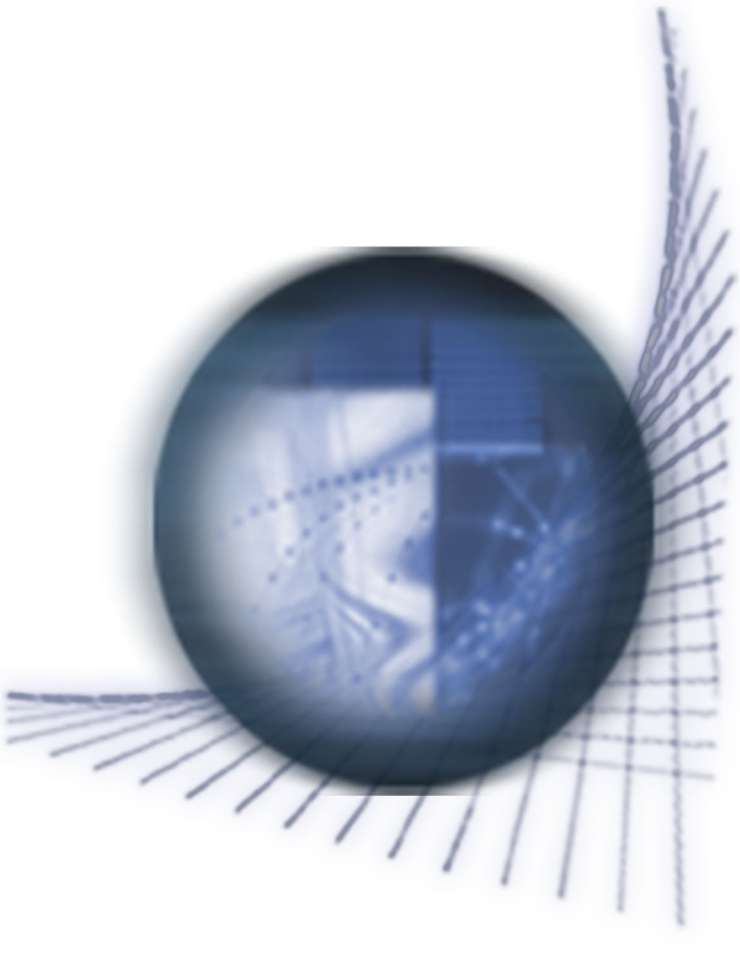
ANEXOS



IX – ANEXO

Anexo I. Etapas do rastreamento imunológico da biblioteca de cDNA utilizando-se a Tecnologia do IVIAT.

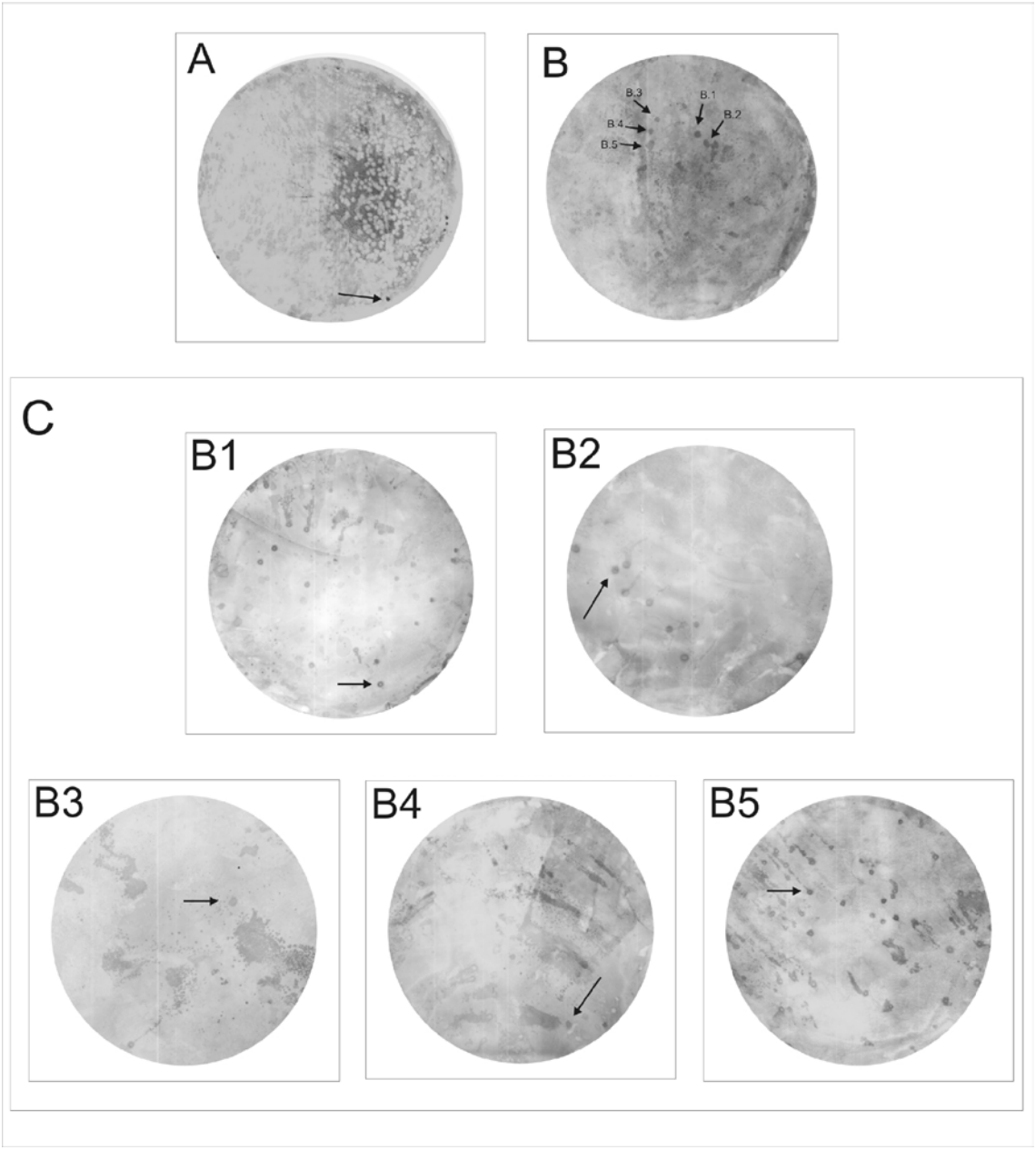


Figura 1 – Esquema das etapas do rastreamento imunológico da biblioteca de cDNA utilizando-se a tecnologia do IVIAT. As membranas foram reagidas com anticorpo primário (soros de pacientes com PCM incubados com extrato celular total de *P.brasiliensis*) diluído à 1:100. O anticorpo secundário (Anti-IgG humano acoplado à peroxidase) foi adicionado à 1:2000 e a reação foi revelada com kit ECL).

Anexo II. Análises de RT-PCR em tempo real dos transcritos codificantes a ddc, lacase e tirosinase de *P. brasiliensis* na presença de um agente quelante pra cobre (BCS). Células leveduriformes (1,5 g) foram cultivadas em meio quimicamente definido na presença de um quelante específico para cobre sulfonato de batocuproina (BCS) a 50 µM, 3 h a 37°C. As células foram coletadas por centrifugação e a viabilidade da suspensão celular foi observada na ausência e na presença de cobre. 68,7% das células foram viáveis na presença de BCS e 77,5% foram viáveis na ausência de BCS. O RNA total foi extraído usando o reagente Trizol (Invitrogen). 1µg de RNA foi usado para síntese de cDNA usando a transcriptase reversa Superscript II (Invitrogen) e oligo$(dT)_{15}$. A temperatura de anelamento para os oligonucleotídeos *ddc*, *tirosinase*, *laccase* e *L34* foi de 60°C. Os níveis de expressão dos genes foram calculados usando o método da quantificação relativa (Bookout *et al*., 2006) e foram normalizados com o gene constitutivo *L34*.

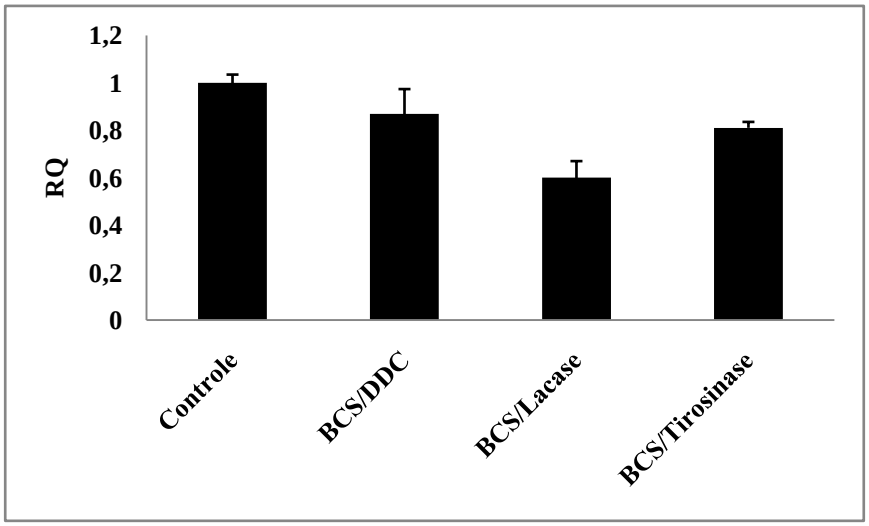


Figura 2 – Análise dos transcritos codificanrtes para *Pb*ddc, *Pb*lacase, *Pb*tirosinase por RT-PCR em tempo real quantitativa. Análises por qRT-PCR foi feita para avaliar os níveis de expressão dos transcritos de células leveduriformes de *P. brasiliensis* na presença de BCS. Os valores da RQ (quantificação relativa) do experimento são médias de determinações em triplicata ± DP. Os valores da expressão de *Pb*ddc e *Pb*ls foram normalizados usando os valores de expressão do gene constitutivo codificante para L34.

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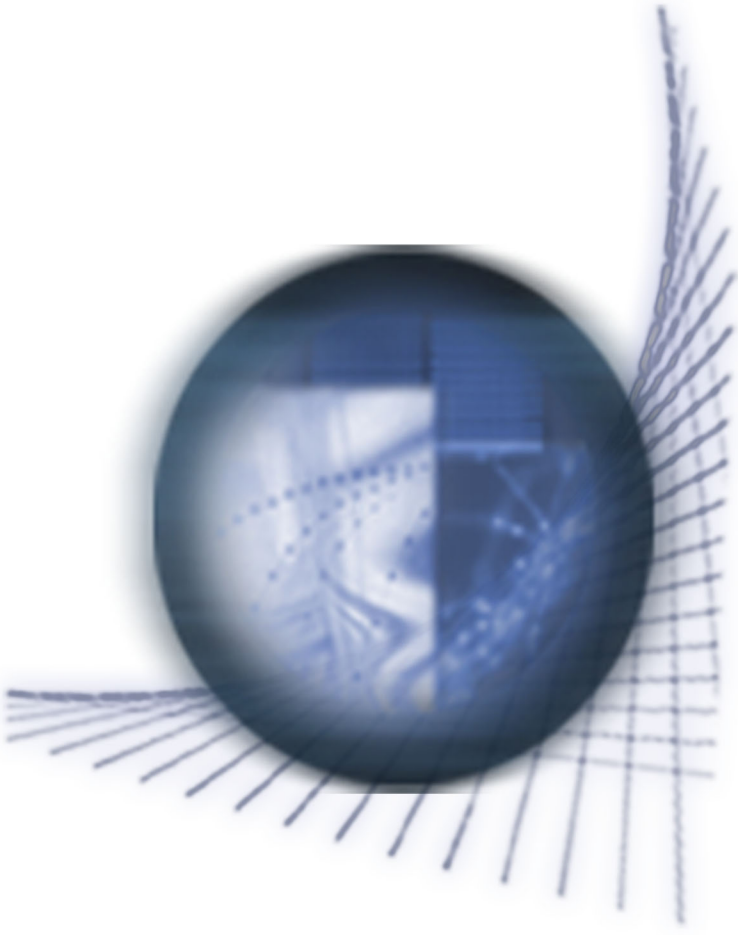

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