UNIVERSIDADE DE BRASÍLIA
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS

Rastreamento de ESTs, Expressão Heteróloga de cDNAs e Análise de Proteases no Estudo da Interação Patógeno-Hospedeiro por *Paracoccidioides brasiliensis*

Tese apresentada ao Programa de Pós-Graduação em Ciências Médicas como requisito para obtenção do título de Doutor em Ciências Médicas.

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SUMÁRIO

LISTA DE ABREVIATURAS

ATP – adenosina trifosfato

cAMP – adenosina monofosfato cíclico

cDNA – DNA complementar

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

bp – pares de bases

BSA – soro albumina bovina

CTS – quitina sintase

DNA – ácido desoxiribonucléico

EDTA - ácido etilenodiaminotetracético

EGTA - ácido etilenoglicoltetracético

EST – etiqueta de sequência expressa

E-64 - L-*trans*-Epoxisuccinil-leucilamido (4-guanidino) butano

FBS – soro fetal bovino

GP – glicoproteína

GM-CSF - Fator Estimulador de Colônia Granulócito Macrófago

GST – glutationa S- transferase

GTP – guanosina trifosfato

HBSS – solução salina tamponada Hanks

HSP – proteína de choque térmico

IPTG - isopropil-β-D-tiogalactopiranosídeo

kDa - kiloDalton

L-DOPA - L - Di-hidroxi fenil alanina

MAPK – proteína quinase ativada por mitose

NBT - nitro blue tatrazólio

ORF – região de leitura aberta

PAGE – eletroforese em gel de poliacrilamida

Pb – Paracoccidioides brasiliensis

PBS – solução de tampão fosfato

PCM – paracoccidioidomicose

PCR – reação em cadeia da polimerase

PFGE - gel em eletroforese de pulso alternado

pH - potencial hidrogeniônico

p-HMB - p-hidroximercurio benzoato

pI - ponto isoelétrico

PMSF – fluoreto de fenilmetilsulfonil

RDA - análise de diferença representacional

RNA – ácido ribonucléico

RT-PCR – PCR acoplada à transcrição reversa

SAP – aspartil protease secretada

SDS – dodecil sulfato de sódio

SP – serine protease

RESUMO

Paracoccidioides brasiliensis é um fungo patogênico humano, agente etiológico da paracoccidioidomicose (PCM). *P. brasiliensis* apresenta dimorfismo térmico, apresentando-se sob a forma miceliana a temperaturas inferiores a 28 ºC e sob a forma leveduriforme no hospedeiro humano e em temperaturas superiores à 28 ºC. Proteases são enzimas que clivam proteínas e desempenham funções como o processamento intracelular e a clivagem de proteínas do meio extracelular para obtenção de nitrogênio. Em microrganismos patogênicos proteases podem atuar como fatores de virulência, clivando proteínas do hospedeiro para facilitar a penetração pelos tecidos. No presente trabalho, foi realizado o rastreamento e identificação de proteases em banco de dados de ESTs oriundas de células leveduriformes de *P. brasiliensis.* Foram detectadas 53 ORFs codificantes para proteases. As seqüências preditas de aminoácidos foram obtidas e classificadas por homologia em banco de dados de proteases, sendo agrupadas em 3 aspartil, 8 cisteíno proteases, 10 metalo-, 10 serino proteases e 22 proteínas relacionadas às subunidades proteassomais. O presente trabalho inclui também o rastreamento e a identificação de ESTs codificantes de proteínas relacionadas à síntese e ao processamento de proteínas em banco de dados de ESTs oriundas de células de *P. brasiliensis* durante a transição dimórfica de micélio para levedura por 22 horas. Foram identificadas 200 ORFs apresentando homologia com seqüências codificantes a proteínas relacionadas aos processos de síntese e processamento de proteínas. Destas, 16 ORFs codificam para genes não descritos anteriormente em *P. brasiliensis*. Foi possível identificar ESTs que compõem subunidades ribossomais, assim como fatores de iniciação transcricional induzidos durante a transição dimórfica, sugerindo um aumento no nível de proteínas sintetizadas durante este processo. Também foram identificadas ESTs codificantes para chaperonas, para glicosiltransferases e para proteínas relacionadas com a aceleração do enovelamento de proteínas, sugerindo aumento da produção e do controle de qualidade durante a transição dimórfica de micélio para células leveduriformes. Alguns genes codificantes para proteases são induzidos após a indução da transição termodimórfica: uma aspartil protease A01, uma lon protease S16 e uma metaloprotease M28, o que sugere aumento dos processos de controle de qualidade das proteínas produzidas e da aquisição de aminoácidos do meio extracelular. Uma serino protease S08 foi caracterizada. As seqüências de nucleotídeos (*Pb*sp) e aminoácidos (*Pb*SP) foram obtidas e analisadas. O cDNA codificante para *Pb*sp foi clonado em vetor de expressão para sistema bacteriano e a proteína recombinante foi utilizada para obtenção de anticorpo policlonal em camundongos. O anticorpo policlonal reconheceu especificamente uma espécie protéica de 66 kDa em extrato protéico e em sobrenadante de cultura de células leveduriformes de *P. brasiliensis*, sugerindo que *Pb*SP seja uma molécula secretada. Ensaios de deglicosilação *in vitro* com endoglicosidase H demonstraram que *Pb*SP é uma molécula N-glicosilada. *Pb*SP tem seu nível de expressão induzido durante a privação de nitrogênio tanto em extrato protéico quanto em sobrenadante de cultura de células leveduriformes de *P. brasiliensis*, sugerindo que *Pb*SP tenha importância na captação de nitrogênio. A expressão de *Pb*sp é aumentada durante a internalização de células leveduriformes por macrófagos murinos, sugerindo a importância do produto deste gene na resposta adaptativa de *P. brasiliensis* à internalização por macrófagos. A interação de *Pb*SP com moléculas de *P. brasiliensis* foi avaliada utilizando o sistema de duplo-híbrido em *Saccharomyces cerevisiae*. Proteínas relacionadas com enovelamento protéico, controle de qualidade e destinação de proteínas, além de uma proteína de parede celular de *P. brasiliensis* foram identificadas.

ABSTRACT

Paracoccidioides brasiliensis is a human pathogenic fungus, the causative agent of paracoccidioidomycose (PCM). *P. brasiliensis* is a thermodimorphic fungus presents as mycelium form in temperatures below 28º C and as yeast form in the human host and in temperatures above 28ºC. Proteases are enzymes that cleave proteins and are related to the intracellular protein processing and cleavage of extracellular proteins to nitrogen acquisition. In pathogenic microorganisms proteases could act as virulence factors by cleaving host proteins facilitating the invasion process in the host tissues. The present work identified proteases in ESTs database constructed with cDNAs sequences of *P. brasiliensis* yeast form. It was detected 53 ORFs encoding proteases. The predicted amino acids sequences were obtained and classified by homology in protease database: 3 sequences were classified as aspartil, 8 as cysteine, 10 as metallo-, 10 as serino and 22 proteins related to proteasomal subunits. The present work also includes the identification of ESTs encoding proteins related to protein synthesis and processing in ESTs database of *P. brasiliensis* during transition from mycelium to yeast cells. It was identified 200 ORFs presenting homology to sequences encoding proteins related to protein synthesis and processing. Sixteen ORFs were described as novel genes of *P. brasiliensis.* It was identified ESTs related to ribosomal subunits and initiation transcription factors, suggesting intense synthesis of new ribosome particles, affecting the rate of protein synthesis. It was identified ESTs encoding chaperones, glycosiltransferases and proteins related to acceleration of protein folding, suggesting high protein production and high quality control in the protein production during transition from mycelium to yeast cells in *P. brasiliensis.* Transcripts encoding proteases were induced in *P. brasiliensis* during transition from mycelium to yeast: an aspartil protease A01, a lon protease S16 and a metaloprotease M28. The higher expression of these genes during transition in *P. brasiliensis* suggests high control quality of proteins and nitrogen acquisition. A serine protease S08 was characterized. The cDNA (*Pb*sp) and deduced amino acids (*Pb*SP) sequences were obtained and analyzed. *Pb*sp was cloned into expression vector in bacterial system and used to generate polyclonal antibody in mice. The polyclonal antibody recognized specifically a 66 kDa protein species in protein extracts and culture supernatants of *P. brasiliensis* yeast cells, suggesting *Pb*SP is a secreted molecule. *In vitro* deglycosylation assays with endoglycosidase H demonstrated that *Pb*SP is a N-glycosylated molecule. *Pb*SP is increased during nitrogen starvation both in protein extracts and cultures supernatants of *P. brasiliensis* yeast cells, suggesting *Pb*SP is important in nitrogen acquisition. *Pb*sp expression was induced during internalization of *P. brasiliensis* yeast cells by murine macrophages, suggesting the *Pb*sp product is important in the *P. brasiliensis* adaptative response to macrophage internalization. *Pb*SP interaction with *P. brasiliensis* proteins was evaluated by two-hybrid assay in *Saccharomyces cerevisiae.* Proteins related to protein folding, quality control of translation, protein destination and a cell wall protein of *P. brasiliensis* were identified.

Capítulo I

Introdução

I. INTRODUÇÃO

I.1. O fungo *Paracoccidioides brasiliensis*

O fungo termodimórfico e patogênico humano *Paracoccidioides brasiliensis* foi descrito por Adolpho Lutz em 1908 e é o agente etiológico da paracoccidioidomicose (PCM) (Franco, 1987, San-Blas 1993). *P. brasiliensis* apresenta-se sob a forma miceliana, a qual se presume que ocorra na natureza em temperaturas inferiores a 28 °C e, sob a forma leveduriforme em tecidos infectados e *in vitro* a temperaturas acima de 28 ºC (Restrepo 1985, Bagagli *et al* 2006). A forma leveduriforme apresenta brotamentos múltiplos formados pela evaginação da célula-mãe; neste caso, a célula central é circundada por várias células periféricas. A forma miceliana é caracterizada por filamentos septados com conídeos terminais ou intercalares (Queiroz-Telles 1994; Restrepo-Moreno 2003).

O habitat de *P. brasiliensis* ainda é indeterminado em virtude de fatores como o prolongado período de latência no hospedeiro humano e a não identificação de hospedeiros intermediários do fungo (Montenegro *et al.* 1996, Bagagli *et al.* 2003). Sabe-se que *P. brasiliensis* pode ser isolado a partir de solos, como já realizado no Brasil (Montenegro *et al.* 1996) e na Venezuela (Albornoz 1971), sugerindo que o solo pode ser um importante elemento na biologia do fungo. As diferentes condições do solo alteram a capacidade de crescimento da forma miceliana e a produção de conídeos por *P. brasiliensis* (Franco *et al.*, 2000). Terçarioli e colaboradores (2007) realizaram o cultivo de vários isolados de *P. brasiliensis* em diferentes tipos de solo e observaram que este fungo apresenta a capacidade de crescimento em solos arenosos e argilosos, com alta umidade. Neste caso, a produção de conídeos foi observada em alguns isolados. Foi possível observar também a inibição de crescimento de *P. brasiliensis* em solos com altos níveis de alumínio.

O isolamento de *P. brasiliensis* de animais também tem sido relatado, como o morcego frugívoro *Artibeus lituratus* (Greer & Bolanos 1977), o pingüim da Antártida

Uruguaiana *Pygoscelis adeliae* (Garcia *et al.* 1993) e duas espécies de tatus, *Dasypus novemcinctus* e *Cabassous centralis* (Bagagli *et al.* 2003, Corredor *et al.* 2005). A infecção natural em alguns animais selvagens e domésticos tem sido observada e manifestações clínicas da doença foram relatadas em cachorros (Ricci *et al.* 2004). Richini-Pereira e colaboradores (2008) realizaram estudos objetivando a detecção molecular de *P. brasiliensis* em tecidos de 19 animais mortos em rodovias, pela técnica de PCR-Nested. As reações apresentaram amplificações específicas para *P. brasiliensis* em vários tecidos de tatus e de porco da índia, pulmão e fígado de porco espinho e pulmão de furão. Estes dados sugerem que a presença de *P. brasiliensis* na natureza, em animais de áreas endêmicas, é maior do que inicialmente postulado.

A classificação taxonômica de *P. brasiliensis* é difícil dada o não conhecimento da forma sexuada do fungo. Entretanto, análises da subunidade maior 25 do RNA ribossomal foram realizadas para fungos dermatófitos e dimórficos por Leclerc e colaboradores (1994). Os resultados demonstraram que estes dois grupos apresentam-se filogeneticamente separados. Neste estudo, foi possível classificar *P. brasiliensis* juntamente com outros fungos dimórficos, tais como *Blastomyces dermatitidis* e *Histoplasma capsulatum* como pertencente ao filo Ascomycota, à ordem Onygenales e à família Onygenaceae. Atualmente, com base em análises filogenéticas moleculares, *P. brasiliensis* é classificado com pertencente ao 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).

Foram realizados estudos filogenéticos utilizando-se oito regiões de cinco genes nucleares com sessenta e cinco isolados de *P. brasiliensis*. O fungo foi classificado em três espécies filogenéticas distintas: S1 (contendo 38 isolados), PS2 (contendo seis isolados), e

PS3 (com 21 isolados). S1 compreende isolados do Brasil, Argentina, Paraguai, Peru e Venezuela. PS2 compreende cinco isolados brasileiros e um isolado da Venezuela. PS3 compreende isolados somente da Colômbia e é considerada uma linhagem filogeneticamente independente (Matute *et al.*, 2006).

Em estudo recente pela técnica de concordância genealógica para reconhecimento de espécies filogenéticas, Carrero e colaboradores (2008) analisaram 14 genes de 21 isolados de *P. brasiliensis*. Com exceção das análises filogenéticas realizadas para os genes codificantes para quitina sintase 1 e catalase A, o isolado *Pb*01 apresentou-se filogeneticamente distinto dos outros isolados analisados. O isolado *Pb*01 agrupou-se com o isolado IFM 54648, obtido a partir de um paciente do estado do Paraná. Os outros isolados apresentaram-se agrupados segundo a região geográfica das quais foram obtidos. Estes resultados sugerem a possibilidade de ocorrência de mais de três espécies filogenéticas em *P. brasiliensis*. Os resultados sugerem que os isolados *Pb*01 e IFM 54648 apresentam-se geneticamente separados dos outros isolados por um longo período de tempo. Especula-se também que o isolado *Pb*01 possa constituir uma nova espécie do gênero *Paracoccidioides*; entretanto, esta hipótese requer que sejam encontrados outros isolados geneticamente similares ao *Pb*01 para ser validada (Carrero *et al.*, 2008).

A organização genômica tem sido investigada em *P. brasiliensis*. Feitosa e colaboradores (2003) em estudos utilizando-se técnica de gel em eletroforese de pulso alternado (PFGE) analisaram 12 isolados clínicos de *P. brasiliensis* de diferentes regiões geográficas. Foi estimado um genoma compreendendo em torno de 23 a 31 Mb, com a identificação de 4-5 cromossomos com tamanhos variáveis de 2 a 10 Mb. Análises utilizando-se o fluorocromo DNA - específico DAPI (4', 6-diamidino-2-fenilindol) foram realizadas em células leveduriformes de dois isolados de *P. brasiliensis* (isolados B339 e 113). Através desta técnica, foi estimado um genoma entre 45,7 e 60,9 Mb, sugerindo que as leveduras

destes isolados sejam diplóides (Cano *et al.*, 1998). Em estudos utilizando-se citometria de fluxo na análise de conídeos e leveduras de 10 isolados de *P. brasiliensis* foram descritos genomas de tamanhos entre 26,3 a 35,5 Mb nos conídeos e 30,2 a 30,9 Mb nas leveduras, não havendo, portanto, diferenças significativas entre as duas formas (Almeida *et al.*, 2007).

Recentemente, o seqüenciamento dos genomas estruturais dos isolados *Pb*01, *Pb*03 e *Pb*18 de *P. brasiliensis* foi realizado. As análises dos resultados confirmaram a presença de 5 cromossomos em cada isolado. O genoma do isolado *Pb*01 é composto de 32,94 Mb, com um total de 9.132 genes. Este isolado apresenta o genoma maior tanto em número de bases quanto em quantidade de genes comparado aos outros dois isolados analisados, que apresentaram genomas do tamanho de 29,06 e 29,95 Mb, com número de genes de 7.875 e 8.741 (dados dos isolados *Pb*03 e *Pb*18, respectivamente). Essas informações, além de auxiliar a elucidar as diferenças existentes entre os isolados, serão importantes na caracterização de genes e regiões promotoras e, conseqüentemente, na melhor caracterização da biologia de *P. brasiliensis* (http://www.broad.mit.edu/science/projects/msc/data-release-summary).

I.2. A Paracoccidioidomicose

A PCM é uma micose sistêmica que se apresenta sob duas formas principais: aguda e crônica. A primeira, também denominada forma juvenil é a forma mais severa da doença, acomete principalmente jovens e crianças e representa 3 a 5% dos casos. A segunda, também denominada adulta, tem progressão mais lenta e representa mais de 90% dos casos (Franco *et al.,* 1987; Montenegro, 1986). A PCM apresenta distribuição geográfica restrita à América Latina, com maior incidência no Brasil, Colômbia e Venezuela (Wanke e Londero, 1994; Rivitti e Aoki, 1999). Estima-se que 80% dos casos descritos estejam no Brasil (Brummer *et al.,* 1993; Coutinho *et al.,* 2002), principalmente nas regiões Sul, Sudeste e Centro-Oeste (Blotta *et al.,* 1999; Paniago *et al.,* 2003) com alta prevalência em áreas rurais. Nas áreas

endêmicas da doença, a incidência estimada é de, aproximadamente, 1 a 3 casos clínicos por ano para cada 100.000 habitantes (Restrepo 1985). A doença acomete principalmente indivíduos do sexo masculino entre 30 e 60 anos de idade, que trabalham em atividades agrícolas. Acredita-se que o manejo da terra, realizado por homens na maioria das propriedades rurais, leva-os ao contato com propágulos do fungo aumentando a incidência da doença nesta população (Brummer *et al.,* 1993). Análises da transição de micélio para levedura em 3 isolados de *P. brasiliensis* foram realizadas na presença do hormônio feminino 17-β-estradiol. Os resultados demonstraram que, na presença do hormônio, o número de formas micelianas que transitaram para as células leveduriformes era menor do que na ausência do hormônio. Foi possível observar também que o número de células que realizavam a transição dimórfica era inversamente proporcional à concentração do hormônio utilizada. Estes resultados sugerem que o hormônio 17-β-estradiol, presente em mulheres em idade reprodutiva confere resistência à infecção por *P. brasiliensis*, sendo um importante fator que justifica a menor incidência da doença em mulheres (Restrepo *et al.,* 1984). A transição da forma miceliana para forma leveduriforme de *P; brasiliensis in vivo* foi realizada em camundongos. Após a infecção intranasal, a transição dos conídios para formas intermediárias e leveduriforme foi avaliada em lavado bronquioalveolar nos tempos de 24 a 96 horas. A transição foi observada nos camundongos machos. Células leveduriformes foram encontradas 24 horas após a inoculação, aumentando em número após 48 e 96 horas. Nos camundongos fêmeas, a transição não ocorreu. Estes resultados corroboram dados epidemiológicos e observações *in vitro*, sugerindo que o hormônio feminino bloqueia a transição da forma miceliana para leveduriforme em *P. brasiliensis* e pode ser responsável pela menor incidência da PCM em mulheres em idade fértil (Aristizabal *et. al.*, 1998).

Acredita-se que a inalação dos propágulos aéreos seja a etapa inicial da infecção e que os mesmos convertam-se em células leveduriformes nos pulmões. (Franco 1987, San-Blas *et*

al, 2002). A partir dos alvéolos pulmonares o fungo pode disseminar-se por vias hematogênica ou linfática para outros órgãos como fígado, baço, ossos e sistema nervoso central (Camargo & Franco 2000; Valera *et al.,* 2008).

I. 3. Perfis de expressão gênica de *P. brasiliensis*

Estudos envolvendo perfis de expressão de genes das formas miceliana e leveduriforme de *P. brasiliensis* têm sido realizados com o objetivo de elucidar respostas adaptativas do fungo ao dimorfismo e ao hospedeiro. Neste sentido, foram construídas bibliotecas de cDNAs obtidos das formas leveduriforme e miceliana do isolado *Pb*01 de *P. brasiliensis*. As bibliotecas de cDNAs obtidas foram clonadas e os produtos seqüenciados. Foram identificados 6.022 genes expressos nas fases miceliana e leveduriforme. Entre os transcritos identificados, incluem aqueles codificantes pra chaperonas diferencialmente expressas e genes que não apresentam homologia no genoma humano. Entre os genes não homólogos a genes humanos estão aqueles codificantes para isocitrato liase e α-1,3-glucana sintase, considerados alvos para drogas antifúngicas, ambos expressas preferencialmente na fase leveduriforme (Felipe *et al.,* 2003; Felipe *et al.,* 2005). Características metabólicas diferenciais das formas miceliana e leveduriforme de *P. brasiliensis* foram descritas. A alta expressão de genes que codificam enzimas que participam da fosforilação oxidativa, como a isocitrato desidrogenase e succinil coenzima-A sintase sugere que o micélio apresente metabolismo aeróbio. Já na fase leveduriforme, genes como álcool desidrogenase I tem seus níveis de expressão aumentados, sugerindo que o metabolismo, nesta fase, seja predominantemente anaeróbio (Felipe *et al.* 2005).

Goldman e colaboradores (2003), analisando 4.692 ESTs (Expressed Sequence Tags) do isolado *Pb*18 de *P. brasiliensis* identificaram vários genes expressos na fase leveduriforme de *P. brasiliensis* homólogos a fatores de virulência descritos em *C. albicans*, que

possivelmente atuam na sobrevida do fungo no ambiente do hospedeiro. Os resultados sugerem que os mecanismos para patogenicidade e virulência são conservados entre as espécies analisadas. A expressão de alguns genes foi analisada por RT-PCR em tempo real. Os resultados identificaram genes com níveis de expressão aumentados na fase miceliana, tais como isocitrato liase, malato desidrogenase e oxidases. Outros genes, tais como ubiquitina e chaperonas de choque térmico (HSP70, HSP82 e HSP104) apresentaram níveis de expressão aumentados na transição dimórfica de micélio para levedura e na fase leveduriforme. O aumento da expressão destes transcritos durante a transição dimórfica sugere a importância do controle da qualidade das proteínas produzidas nesta etapa, tanto no enovelamento, realizado pelas chaperonas, quanto na degradação de proteínas mal-enoveladas, realizada pela ubiquitina.

A transição da fase miceliana para a fase leveduriforme em *P. brasiliensis* tem sido alvo de vários estudos, visto a importância deste processo para a sobrevida do fungo nas condições de temperatura encontrada nos tecidos do hospedeiro. Estudos utilizando-se a técnica de microarranjo de DNA em diferentes etapas da transição dimórfica foram realizados por Nunes e colaboradores (2005). Alguns genes relacionados às vias de transdução de sinais apresentaram níveis de expressão aumentados, sugerindo que as vias de sinalização associadas ao dimorfismo são controladas pelo cAMP e MAPK. Muitos genes apresentaram níveis de transcritos aumentados durante a transição dimórfica, como aqueles relacionados ao metabolismo de aminoácidos, síntese de proteínas, metabolismo da parede celular, estrutura do genoma, resposta ao estresse oxidativo e controle de crescimento e desenvolvimento celulares.

Bastos e colaboradores (2007), em nosso laboratório, avaliaram a expressão de 1.107 transcritos obtidos a partir de biblioteca de cDNA de *P. brasiliensis*, isolado *Pb*01, após 22 horas de indução da transição dimórfica de micélio para levedura. Foram identificados genes

com níveis de expressão aumentados relacionados ao remodelamento da parede celular, bem como transcritos que codificam para enzimas relacionadas com a síntese e degradação de carboidratos de membrana. Também foram induzidos nesta condição genes codificantes para transportadores e de enzimas precursoras da síntese de carboidratos de membrana e genes codificantes para enzimas da síntese de lipídeos de membrana. Os dados sugerem que o remodelamento da parede celular ocorra nos estágios iniciais da transição. Um novo transcrito que codifica para alfa-glicosidase, possivelmente relacionado ao processamento de beta-1,6 glicana também foi identificado durante a transição dimórfica de micélio para levedura. Genes codificantes para quitinase 1 (CTS1) e 3 (CTS3) foram induzidos sugerindo que o processamento de quitina, o maior componente da parede celular, é importante para a transição dimórfica em *P. brasiliensis.* Um transcrito que codifica para amino ácido permease ácida, relacionada à captação de glutamato, precursor para a síntese de quitina também foi induzido durante a diferenciação de micélio para levedura (Bastos et al., 2007).

Bastos e colaboradores (2007) detectaram também aumento nos níveis de expressão de genes relacionados ao metabolismo de enxofre, como sulfito redutase, sugerindo a importância do enxofre durante a transição, corroborando trabalhos anteriores (Marques *et al.*, 2004; Ferreira *et al.*, 2006). Transcritos relacionados ao ciclo do glioxalato, tais como isocitrato liase, malato desidrogenase, citrato sintase e aconitase sugerem que esta via seja funcional durante a transição dimórfica. Além disto, genes relacionados às vias de transdução de sinais (MAPK, serina/treonina quinase e histidina quinase) apresentaram níveis de expressão aumentados, sugerindo o controle destes genes na adaptação e sobrevivência do fungo durante os estágios iniciais da transição (Bastos et al., 2007).

Com o objetivo de elucidar as respostas adaptativas de *P. brasiliensis* à infecção em modelo animal, análises subtrativas de cDNAs utilizando a técnica de RDA (análise de diferença representacional) foram realizadas em nosso laboratório a partir de cDNAs obtidos

de células leveduriformes recuperadas de fígado de camundongos infectados. A regulação positiva da transcrição do gene transportador de alta afinidade de ferro/zinco foi observada, justificada pelas baixas concentrações destes compostos no fígado de camundongos. A super-expressão de transcritos codificantes para proteínas de choque-térmico (HSPs) foi relatada, sugerindo o papel destas no reparo de danos causados por estresse, além de atuarem em processos como divisão celular, síntese de DNA, transcrição, tradução, enovelamento, transporte protéico e translocação transmembrana. Transcritos que codificam para enzimas das vias de biossíntese de melanina, tais como L-amino ácido descarboxilase aromática, tirosinase e policetídeo sintase foram induzidos em células leveduriformes de *P. brasiliensis* recuperadas de fígado de camundongos e demonstram a relevância da síntese de melanina no processo infeccioso em camundongos (Bailão et al., 2006). A importância da síntese de melanina na patogênese de fungos já é descrita (Hamilton & Gomez 2002, Taborda et al., 2008). O cultivo de *P. brasiliensis* na presença L-DOPA, precursor da melanina, resulta na melanização das células fúngicas, demonstrando a capacidade do fungo de produzir melanina (Gomez et al., 2001). Células de *P. brasiliensis* melanizadas são mais resistentes à fagocitose por macrófagos murinos e à ação dos antifúngicos anfotericina B, cetoconazol, fluconazol, itraconazol e sulfametoxazol (Silva et al., 2006).

Com o objetivo de ampliar os conhecimentos sobre as mudanças transcricionais de *P. brasiliensis* durante a infecção em modelo animal, nosso laboratório realizou análises do transcritoma de células leveduriformes recuperadas de fígado de camundongos infectados. Foram obtidas e analisadas 4.932 ESTs. Dos transcritos obtidos, 37,47% foram descritos como novos genes em *P. brasiliensis* e 23,75% dos transcritos apresentaram níveis de expressão aumentados. Os transcritos codificantes para proteínas relacionadas à glicólise, biossíntese de aminoácidos e de lipídeos, biossíntese do ergosterol, transportadores de membrana e proteínas relacionadas ao estresse celular apresentaram-se regulados

positivamente nesta condição. Uma avaliação global dos resultados leva a sugerir que *P. brasiliensis* utilize várias fontes de carbono durante a colonização do fígado, incluindo glicose e substratos do ciclo do glioxalato. Pode-se sugerir também que metabolismo de nitrogênio esteja mais ativo durante o processo infeccioso, quando comparado com dados de leveduras cultivadas *in vitro*, sugerindo que alguns compostos nitrogenados podem ser adquiridos dos tecidos do hospedeiro, enquanto outros devem ser sintetizados pelo patógeno.

Análises subtrativas de cDNAs utilizando a técnica de RDA foram realizadas após a incubação de células leveduriformes de *P. brasiliensis* na presença de sangue humano, condição semelhante àquela que o fungo é submetido durante a disseminação no hospedeiro. Neste estudo foi detectado aumento da expressão de genes como aqueles codificantes para glutamina sintase e permease de aminoácidos ácidos. O aumento da transcrição destes genes poderia estar relacionado ao acúmulo de quitina para remodelamento da parede em resposta a mudanças de osmolaridade externa. O transcrito codificante para uma serina protease S08 foi regulado positivamente em *P. brasiliensis* durante a incubação com sangue humano (Bailão et al., 2006). Estudos envolvendo culturas de *A. fumigatus* na presença de soro demonstraram que a secreção de proteases está associada ao aumento da concentração de soro em meio de cultura, provavelmente para promover o acúmulo de aminoácidos no meio extracelular, para captação pelo fungo (Gifford *et al.,* 2002).

Análises de transcritos através da técnica de RDA em células leveduriformes de *P. brasiliensis* após incubação com plasma humano foram realizadas, condição na qual o fungo pode ser submetido em conseqüência de resposta inflamatória aguda em sítios de infecção. Foi possível detectar-se a super-expressão de genes relacionados à síntese de proteínas, respostas celular à mudança de osmolaridade do meio, remodelamento de parede celular e defesa. Os transcritos codificantes de enzimas da β-oxidação foram induzidos durante a incubação de leveduras de *P. brasiliensis* em plasma humano sugerindo que o fungo, nesta

condição, desvie o seu metabolismo para a degradação de lipídeos. Neste estudo os transcritos codificantes de duas proteases apresentaram-se induzidos, sendo codificantes para uma serina protease S08 relacionada à captação de nitrogênio em fungos e uma protease de membrana mitocondrial relacionada ao processamento intracelular de proteínas (Bailão *et al.*, 2007).

A resposta transcricional de *P. brasiliensis* à internalização por macrófagos murinos foi descrita sendo possível identificar genes regulados positivamente nesta condição, principalmente àqueles relacionados à biossíntese de aminoácidos e o gene codificante da proteína de choque térmico HSP60, sugerindo que estes genes são relevantes durante a fagocitose por macrófagos (Tavares et al., 2007). Posteriormente, genes relacionados ao ciclo do glioxalato foram avaliados durante a internalização por macrófagos e os resultados mostraram que os genes necessários para metabolização de compostos com dois carbonos apresentaram-se regulados positivamente nesta condição. Estes dados sugerem que *P. brasiliensis* utilize o ciclo do glioxalato como uma via metabólica para obtenção de carbono durante a internalização por macrófagos (Derengowski et al., 2008).

I.5. Proteases

Proteases são enzimas que clivam proteínas, catalisando a hidrólise de ligações peptídicas (Barrett *et al.*, 1998). De acordo com o Comitê de Nomenlatura da União Internacional de Bioquímica e Biologia Molecular (International Union of Biochemistry and Molecular Biology - IUBMB http://www.chem.qmul.ac.uk/iubmb/enzyme/EC34/), o termo mais correto para denominar esta classe de enzimas é peptidase. Em 2006, análises realizadas em banco de dados SwissProt (http://www.ebi.ac.uk/swissprot/) mostraram que cerca de 18% das seqüências anotadas neste banco estão relacionadas ao processamento proteolítico. Além disto, cerca de 2% de todos os genes já depositados no banco de dados SwissProt codificam para peptidases e seus homólogos (Rawlings et al., 2006). Rawlings e colaboradores (2008)

descrevem que o banco de dados de proteases MEROPS (http://merops.sanger.ac.uk) contém 66524 seqüências de peptidases depositadas, incluindo 2403 espécies de proteínas, organizadas em 185 famílias, que, por sua vez, são classificadas em 51 clãs. As peptidases podem ser classificadas quanto ao seu mecanismo catalítico em: aspartil-, metalo-, cisteíno-, serino- e treonino- proteases. Proteases dos dois primeiros grupos utilizam uma molécula de água para atacar a ligação peptídica do substrato, enquanto as outras classes utilizam um resíduo de aminoácido localizado no sítio ativo (Rawlings & Barrett, 1993; Barrett *et al.,* 1998). As proteases são divididas também em dois grupos maiores de acordo com seus sítios de ação: exoproteases clivam o peptídeo próximo à região amino ou carboxi terminais no substrato, enquanto endoproteases, também denominadas proteinases, clivam peptídeos distantes das regiões terminais do substrato (Watson, 1976).

Em todas as células, procarióticas e eucarióticas, as estruturas estão continuamente sendo renovadas. Assim, a homeostase entre as vias anabólicas e catabólicas deve ser continuamente mantida (Voges *et al.*, 1999). As funções das peptidases vão além da digestão generalizada de proteínas, englobando processos tais como ativação de zimógenos, coagulação do sangue, lise de fibrina e ativação de hormônios e de precursores de proteínas (Neurath *et al.*, 1984). Nas células eucarióticas, a grande maioria das proteases apresenta localização restrita, geralmente associada a lisossomos, endossomos, grânulos secretores, vesículas transportadoras e mitocôndrias. Neste caso, as proteases têm suas funções associadas à ativação de zimógenos, endereçamento e processamento de outras proteínas, apresentando localização restrita e, geralmente associadas à condição ideal de pH (Bond & Butler 1987). Proteínas envolvidas no processo de ubiquitinação e de deubiquitinação regulam vários processos celulares essenciais, como a degradação de proteínas, progressão do ciclo celular, sinalização e reparo de DNA (revisado por Rytkonen & Holden 2006). A morte

programada da célula, processo denominado apoptose, é dependente da ativação de caspases, que são proteases cistenil-aspartato específicas (Nicholson et al., 1995).

Algumas proteases são secretadas e apresentam suas funções fora do ambiente celular. Em microorganismos esta atividade está geralmente associada à degradação de peptídeos para aquisição de nitrogênio, principalmente em condições onde há privação de carbono e/ou nitrogênio. Alterações no perfil transcricional de genes codificantes para proteases foram detectadas para o fungo entomopatogênico *Magnaporthe grisae* em condições de privação das fontes de nitrogênio no meio extracelular. Nesta condição, foi possível detectar-se o aumento de expressão de serino proteases. Acredita-se que este aumento de expressão de proteases faça parte de uma resposta global ao estresse nutricional (Donofrio *et al.*, 2006). No fungo filamentoso *Podospora anserina*, um gene codificante para uma aspartil protease é induzido quando o organismo é submetido à privação de carbono, tanto na presença quanto na ausência de fontes de nitrogênio. Este fato pode ser explicado pela presença do motivo de ligação 5'-SYGGGG-3'na região promotora do gene codificante para aspartil protease. Este domínio é descrito como de ligação à proteína CreA que reprime a expressão dos genes aos quais ela se liga quando há fonte de carbono disponível no meio de cultura (Paoletti *et al.*, 1998).

Em *Schizosaccharomyces pombe,* o gene *isp6* codificante para uma serina protease tem seus níveis de expressão inversamente proporcionais à concentração de nitrogênio em meio de cultura (Sato *et al.*, 1994). O cultivo de *S. pombe* em condição de privação de nitrogênio promove a autofagia, que está associada à degradação de proteínas em larga escala para obtenção de fontes de nitrogênio. Mutantes para o gene *isp6* (Δ*isp6*) apresentam disfunção na degradação de proteínas durante a privação de nitrogênio, sugerindo a importância do produto gênico neste processo. A avaliação do desenvolvimento sexual em células Δ*isp6* mostrou que há uma diminuição significativa na eficiência de esporulação e de promoção de diploidia. Análises utilizando-se a técnica de *Northern blot* mostraram que esta

deficiência está associada à expressão do gene *stel 1*, responsável pelo desenvolvimento sexual e induzido em resposta à privação de nitrogênio. Os resultados demonstram que *stel 1* apresenta níveis de expressão basal nas células Δ*isp6* e são expressos em níveis aumentados em células selvagens, durante a privação de nitrogênio. Estes dados demonstraram que *isp6* afeta a expressão de *stel 1* (Nakashima *et al.*, 2006).

Em fungos, a utilização de fontes de nitrogênio alternativas é controlada pelos fatores de transcrição GATA. Em *C. albicans*, a aspartil protease SAP2 é preferencialmente expressa em meio de cultura quando soro albumina bovina é utilizada como única fonte de nitrogênio (Banerjee *et al.*, 1991). A transcrição de SAP2 é influenciada por fatores de transcrição GATA, visto que mutantes duplos para dois fatores de transcrição GATA, Gln3p e Gat1p, são incapazes de expressar SAP2 e de crescer em meio com soro albumina bovina como única fonte de nitrogênio (Dabas & Morschhauser 2008).

Além de todas as funções em nível celular, as proteases estão associadas a processos mais complexos em eucariotos, incluindo condições patológicas. É descrito que metaloproteases atuam no processo de metástase clivando componentes de matriz extracelular (revisado por Rydlova et al., 2008). Uma aspartil protease humana BACE 1, que é uma peptidase de processamento da proteína precursora β-amilóide, apresenta níveis de expressão maiores em casos de doença de Alzheimer e, tem sido foco como alvo para drogas inibidoras no tratamento desta doença (revisado por Ghosh et al., 2008).

Muitas proteases apresentam utilidade comercial, principalmente aquelas que possuem pH ideal de ação alcalino. Peptidases produzidas por bactérias apresentam pH de ação que variam de 5 a 8 e são relativamente termos-tolerantes. Muitas são utilizadas pelas indústrias de alimentos, couro e de detergentes para hidrólise de resíduos, substituindo muitas vezes tratamentos químicos, mais agressivos ao meio ambiente (Rao et al., 2008).

I.4.1. Aspartil proteases

Aspartil-proteases (EC 3.4.23) são um grupo de peptidases que apresentam sítio catalítico conservado, possuem pH ideal de ação ácido e tem preferência por clivagem de aminoácidos hidrofóbicos. O pH de ação desta classe limita a localização celular e a atuação destas peptidases a condições mais específicas, sendo menos abundantes que serino- e metaloproteases. Nesta família incluem-se as peptidases estomacais, tais como, pepsina e quimosina e peptidases lisossomais, tais como catepsinas D e E. As aspartil proteases são sintetizadas como pré-propeptídeo, com uma região de peptídeo sinal e uma região pro-peptídica. A auto-remoção destas seqüências é determinante para que a ativação ocorra e é dependente das condições de pH (Tang & Wong 1987).

Aspartil-proteases são encontradas desde retrovírus a plantas e mamíferos. Possuem massa molecular em torno de 40 kDa, com seqüência pro-peptídica que varia de 27 a 60 aminoácidos. Todas as peptidases desta classe são inibidas por pepstatina A. A seqüência de aminoácidos das peptidases desta família apresenta duas regiões conservadas, DSG (onde D corresponde ao resíduo de ácido aspártico, S, serina e G, glicina) e DTG (onde D corresponde ao resíduo de ácido aspártico, T, treonina e G, glicina). Os resíduos de ácido aspártico compõem o sítio ativo destas proteínas que utilizam uma molécula de água para mediar o ataque nucleofílico da ligação peptídica (Szecsi 1992; Rawlings & Barrett 1995). A classe das aspartil-proteases é dividida em sete clãs e 14 famílias, sendo a de maior representatividade o clã das pepsinas, AA (http://merops.sanger.ac.uk).

I.4.2. Cisteíno-proteases

Cisteíno-peptidases (EC 3.4.22) são peptidases que apresentam um resíduo do aminoácido cisteína no sítio ativo. Atualmente esta classe se subdivide em 9 clãs e em 58 famílias (http://merops.sanger.ac.uk). O maior clã é o CA, que inclui a cisteíno peptidase mais

caracterizada, a papaína. Neste clã agrupam-se também várias catepsinas. Esta classe de proteases geralmente localiza-se no citosol ou em lisossomos (Barrett & Rawling 2001). Evolutivamente, as cisteíno peptidases apresentam pelo menos sete origens evolutivas diferentes: clãs CA, CD, CE, CF, PA (que provavelmente evoluíram a partir da família das serino peptidases), PB e CH, sendo estas duas últimas auto-ativadas por clivagem. Em comum, todas as cisteíno peptidases dependem dos resíduos de cisteína e de histidina do sítio ativo (Barrett & Rawlings 2001). O clã CA, além destes resíduos, possui também o resíduo de arginina no sítio ativo (Cohen *et al.*, 1986), porém outros sítios ativos são conhecidos para outros clãs, como as caspases envolvidas no processo de apoptose, que posssuem sítio ativo formado pelos resíduos QACXG (onde Q é um resíduo de glutamina, A, alanina, C, cisteína, X pode ser glicina, arginina ou glutamina, e G, glicina) (Nicholson *et al.*, 1995).

O clã da papaína é composto por peptidases de baixa massa molecular, que varia de 20 a 35 kDa, com exceção da catepsina C, que se apresenta sob a forma oligomérica, com cerca de 200 kDa. As catepsinas B, C, H and L, são encontradas em lisossomos de todos os animais. Com exceção da catepsina C, que é uma dipeptidil peptidase, todas as peptidases deste clã são endopeptidases. As cisteíno proteases atuam geralmente em pHs ácidos, que podem variar de 5.5 a 6.8 (Turk *et al.*, 1997). Cisteíno proteases são inibidas irreversivelmente pelo composto E-64 (L-*trans*-Epoxisuccinil-leucilamido (4-guanidino) butano) (Barrett *et al.*, 1982) e por antígenos 2-beta de células T ativadas (Delaria *et al.*, 1994).

I.4.3. Metaloproteases

Metaloproteases (EC 3.4.24) são um grupo de peptidases que possuem em comum a presença de um íon metálico no sítio ativo. Geralmente, o íon é o zinco, que atua no posicionamento da molécula de água que será hidrolisada no processo da clivagem do peptídeo. A grande maioria das metaloproteases, incluindo as termolisinas e

metaloendopeptidases, apresenta uma região HEXXH (onde H corresponde a resíduos de histidina E, corresponde a resíduo de glutamina e X a qualquer aminoácido), responsável pela ligação ao íon Zn^{2+} (Jongeneel *et al.*, 1989). Algumas famílias de metalopeptidases como as insulinases, as carboxipeptidases, e as proteases III bacterianas apresentam o motivo HXXEH, que possuem a mesma função do motivo HEXXH de ligação ao íon de zinco. (Becker & Roth 1992). Atualmente, para as metaloproteases, são descritos 15 clãs que se agrupam em 54 famílias (http://merops.sanger.ac.uk). As metaloproteases são inibidas por quelantes de íons bivalentes tais como ácido etilenodiaminotetracético (EDTA), ácido etilenoglicoltetracético (EGTA) e 1,10 fenantrolina (Bond & Butler, 1987).

As proteases desta classe possuem pH ideal de atividade variando de neutro a alcalino. A peptidase mais bem caracterizada desta família é a termolisina, uma protease neutra, sintetizada na forma monomérica, com 34 kDa. É uma proteína termoestável, apresentando meia vida de uma hora, a 80°C. Outras peptidases importantes nesta classe são as colagenases, que degradam além de colágeno, gelatina. As colagenases foram primeiramente caracterizadas em *Clostridium hystolyticum*, mas já foram identificadas em outros microorganismos, incluindo fungos (Rao *et al.*, 1998).

I.4.4. Serino-proteases

Serino proteases (EC 3.4.21) são peptidases que utilizam um resíduo de serina do sítio ativo para clivar peptídeos. É uma família altamente distribuída em todos os reinos e tem sido agrupada em clãs que compreendem peptidases de acordo com a origem evolutiva, mecanismo catalítico ou mecanismo de ativação, conformação tridimensional e função biológica. (Barrett & Rawlings, 1995, Rawlings *et al.*, 2008). Esta classe inclui 43 famílias agrupadas em 13 clãs, compiladas em banco de dados MEROPS (http://merops.sanger.ac.uk).

As peptidases desta família apresentam região catalítica diferenciada entre alguns clãs, o que sugere diferentes origens evolutivas. Por exemplo, enquanto a família das quimiotripsinas possui uma tríade catalítica formada pelos resíduos de aminoácidos histidina, asparagina e serina (HDS), as subtilisinas apresentam os mesmos resíduos de aminoácidos em ordem diferenciada (DHS) e clivam preferencialmente a seqüência de aminoácidos Ala-Ala-Pro-Phe (Alanina, Alanina, Prolina e Fenilalanina, respectivamente) (Hartley 1970). Mais recentemente, serino proteases com outros sítios ativos tem sido descritas: Ser-His-Glu, Ser-Lys/His, His-Ser-His. Serino peptidases atuam preferencialmente em pH alcalino e tem sido descritas em todas os grupos taxonômicos de fungo. São peptidases inibidas por PMSF (fenilmetilsulfonil fluorido). Algumas também são inibidas por reagentes tiol, como p-cloromercuribenzoato. A inibição pelos compostos tiol, reflete a proximidade de resíduo de cisteína ao sítio ativo, que pode interferir na ligação com o substrato (Dodson & Wlodawer 1998).

Serino peptidases geralmente apresentam massa molecular pequena, que varia de 18,5 a 35 kDa. Porém, fungos como *A. niger* e *A. nidulans* apresentam serino peptidases com massas moleculares maiores. A maior peptidase desta família já descrita apresenta 126 kDa. Muitas das serino peptidases já descritas em fungos apresentam carboidratos associados e apresentam pI variando de 4.4 a 6.2, com algumas exceções que podem chegar a 8.9, ou mais, como descrito para *Neurospora crassa* (revisado por North 1982).

I.5. Proteases de microorganismos como fatores de virulência

Proteases de várias famílias têm sido associadas à virulência em patógenos humanos. Em *Trycophyton rubrum*, o rastreamento de biblioteca genômica permitiu a detecção de sete possíveis genes codificantes para serina proteases da subfamília das subtilisinas, duas delas capazes de clivar queratina, sugerindo atuação destas no processo invasivo da infecção

fúngica (Jousson *et al.,* 2004). Análise de sobrenadantes de cultura do fungo dermatófito *T. rubrum* utilizando-se queratina como única fonte de nitrogênio permitiu identificar várias aminopeptidases e dipeptidil peptidases que possivelmente sejam importantes na virulência deste fungo, visto que *T. rubrum* inicia seu processo de invasão no hospedeiro por tecidos queratinizados (Monod *et al.*, 2005).

C. albicans apresenta, pelo menos, 10 isoformas de aspartil proteases secretadas (SAPs) (Monod *et al.,* 1994). As SAPs são detectadas em sobrenadantes de cultura e são associadas a danos teciduais em epitélio vaginal, facilitando a invasão e disseminação do fungo no hospedeiro (Schaller *et al.,* 2003). SAPs são expressas diferencialmente durante as etapas de infecção. SAP9 é a protease encontrada em grande parte dos pacientes que apresenta candidíase oral e vaginal. Entretanto, a SAP5 apresenta os maiores níveis de expressão durante a infecção em tecido epitelial. Durante a infecção oral, as SAPs que apresentam os maiores níveis de expressão são SAP4-6. SAP1-2 não variam os níveis de expressão nos diferentes modelos de infecção enquanto SAP3, SAP7 e SAP8 apresentam baixos níveis de expressão durante o processo de infecção. Estudos envolvendo mutantes para SAP3 mostraram a expressão de SAP5 é aumentada nestes mutantes numa tentativa compensatória da ausência de SAP3. Da mesma forma, mutantes para SAP4-6 apresentam níveis de expressão de SAP2 aumentados (Naglik et al., 2008). Em *A. fumigatus*, uma aspartil protease extracelular foi identificada com capacidade de clivar proteínas tais como elastina, colágeno e laminina. Neste caso, sugere-se que esta protease possa contribuir para disseminação do fungo nos tecidos do hospedeiro (Lee *et al.,* 1995). Estudos envolvendo culturas de *A. fumigatus* demonstraram que a secreção de proteases está associada ao aumento da concentração de soro em meio de cultura, provavelmente para promover o aumento de aminoácidos no meio extracelular, que é então captado pelo fungo (Gifford *et al.,* 2002). Em *Cryptococcus neoformans*, análises proteômicas utilizando sobrenadantes de cultura e frações de parede

celular identificaram proteases secretadas e associadas à parede relacionadas à virulência do fungo, apresentando a capacidade de clivar citocinas e componentes da matriz extracelular (Eigenheer *et al.* 2007).

O transcrito codificante para uma serina protease vacuolar é aumentado em *M. grisae* durante privação de nitrogênio. Esta serina protease foi associada ao processo de patogenicidade, visto que mutantes para o gene codificante desta proteína causaram níveis significativamente menores de lesões em folhas de arroz, quando comparados às linhagens selvagens (Donofrio *et al.,* 2006). A resposta eficiente à depleção de nitrogênio pode ser importante para sobrevivência de microorganismos patógenos no ambiente do hospedeiro, onde são encontradas baixas concentrações deste elemento químico (Rubin-Bejerano *et al.,* 2003). Em *S. cerevisiae* e *C. albicans,* a internalização de leveduras por neutrófilos induziu uma resposta transcricional similar àquela apresentada pelos fungos durante privação de aminoácidos, sugerindo que o ambiente do fagossomo apresente baixas concentrações de nitrogênio (Rubin-Bejerano *et al.,* 2003). Bactérias como *Natrialba magaddi*, apresentam níveis significativamente aumentados da produção de proteases secretadas e do nível de atividade proteolítica em sobrenadantes de cultura com o decréscimo da concentração de fontes de nitrogênio em meio de cultura quimicamente definido (D'Alessandro *et al.,* 2007).

I.6. Proteases de *P. brasiliensis*

Poucos estudos foram realizados em *P. brasiliensis* no que concerne a proteases. Um gene codificante para uma metaloprotease Lon foi descrito, clonado e caracterizado (Barros & Puccia 2001). O rastreamento de biblioteca genômica de *P. brasiliensis* permitiu a identificação de um gene codificante para uma ClpB protease. Análises pela técnica de *Northern blot* demonstraram que o transcrito codificante desta chaperonina é preferencialmente expresso na fase leveduriforme do fungo (Jesuíno et al., 2002). O cDNA

codificante para o protease ClpA de *P. brasiliensis* foi identificado e caracterizado. Estudos filogenéticos foram realizados utilizando-se seqüências completas de cDNAs codificantes para ClpA de fungos e bactérias. Os resultados demonstraram uma seqüência de peptídeos na região interna das proteínas ClpA de fungos, ausente nas proteínas ClpA de bactérias (Oliveira et al., 2005). Uma atividade de serina-tiol protease extracelular foi detectada em filtrados de cultura de *P. brasiliensis* com capacidade para clivar proteínas associadas à membrana basal como laminina e fibronectina (Carmona *et al.,* 1995), tendo sua atividade modulada por polissacarídeos neutros (Matsuo *et al.,* 2006) e inibida pela presença de S-[3-nitro-2-piridinesulfenil]. A interação de serina-tiol protease com os polissacarídeos de galactomananas endógenos pode ser importante na termoestabilidade e no aumento da afinidade da proteína com seu substrato (Matsuo *et al.,* 2007).

Transcritos codificantes para proteases foram detectados em bibliotecas de cDNAs das formas leveduriforme e miceliana de *P. brasiliensis* (Felipe *et al.,* 2005) e foram classificados, permitindo a identificação de 53 cDNAs codificantes para proteases, assim distribuídas: proteases ATP - independentes (15 seqüências), ATP-depedentes (12 seqüências), subunidades de proteassoma (22 seqüências) e proteínas de deubiquitinação (4 seqüências) (Parente *et al.,* 2005).

Vários genes codificantes para proteases apresentam-se regulados positivamente durante a transição dimórfica de micélio para levedura em *P. brasiliensis*. Transcritos codificantes para uma aspartil-protease, para uma zinco metaloprotease e para uma protease pertencente à classe M28 foram induzidos durante a transição dimórfica (Parente *et. al.*, 2008). Aspartil-proteases são fatores de virulência em *Aspergillus fumigatus,* sendo associadas a danos teciduais em pulmão de camundongos, atuando na facilitação do processo de invasão do fungo no hospedeiro (Lee & Kolattukudy 1995). Zinco-metaloprotease são associadas ao desenvolvimento de esporos em *Schizosacharomyces pombe,* evidenciando a

importância desta protease durante processos de diferenciação na célula (Nakamura *et al*., 2004). Metaloproteases da classe M28 estão associadas à aquisição de nitrogênio em *Trichoderma harzianum*, apresentando a expressão protéica aumentada durante a privação do elemento químico (Suarez *et al*., 2007).

Transcritos codificantes para algumas proteases apresentaram regulação positiva em *P. brasiliensis*, formas leveduriformes recuperadas de infecção em fígado de camundongos. incluindo uma serina protease S08, objeto de estudo deste trabalho e uma aspartil aminopeptidase, ambas descritas como fatores de virulência. Também foram identificadas proteínas relacionadas ao complexo de ubiquitinação para degradação, provavelmente relacionadas ao controle de qualidade de proteínas com enovelamento incorreto (Costa *et al.*, 2007).

O transcrito codificante para uma aspartil protease classificada na família A01, que apresentou regulação positiva durante a transição dimórfica de micélio para levedura (Bastos *et al.*, 2007) teve sua seqüência de cDNA e a seqüência predita da proteína caracterizadas, tendo sido detectada em sobrenadante de cultura e na parede celular e de leveduras de *P. brasiliensis* (Tacco *et al.*, *in press*).

Uma serina protease da família das subtilisinas (S08) que apresenta níveis de transcritos aumentados em leveduras de *P. brasiliensis* isoladas de camundongos (Costa *et al.,* 2007) também é induzida durante incubação de leveduras de *P. brasiliensis* com sangue e plasma humanos (Bailão *et al.,* 2006; Bailão *et al.,* 2007). A seqüência de nucleotídeo e seqüência predita de aminoácidos foram obtidas e analisadas. O cDNA codificante para serino protease foi clonado em vetor de expressão, transformado em sistema bacteriano e a proteína recombinante obtida foi utilizada para obtenção de anticorpo policlonal em camundongos. A expressão de serino protease é aumentada na privação de nitrogênio, sugerindo a importância desta protease na aquisição desse elemento. A expressão do transcrito codificante para a

serino protease é induzida durante a internalização de células leveduriformes de *P. brasiliensis* em macrófagos murinos. A interação de serino protease com outras moléculas protéicas de *P. brasiliensis* foi avaliada através do sistema de duplo-híbrido em leveduras *S. cerevisiae* (Parente et al., em preparação).

Justificativa

II. Justificativa

O laboratório de Biologia Molecular do Instituto de Ciências Biológicas da Universidade Federal de Goiás há vários anos tem estudado moléculas de *P. brasiliensis*, com foco principal naquelas potencialmente associadas à interação do fungo com o hospedeiro humano. Neste contexto, proteases apresentam grande relevância, visto que há evidências que proteases extracelulares de fungos patogênicos são importantes na degradação de proteínas nos tecidos do hospedeiro. A sobrevivência de microorganismos patogênicos no ambiente hostil do hospedeiro também é facilitada por proteases intracelulares, visto que atuam na manutenção da qualidade das proteínas do fungo, degradando proteínas não corretamente traduzidas ou enoveladas.

A identificação e caracterização de proteases com níveis de expressão aumentados durante o processo infeccioso e durante a transição dimórfica de *P. brasiliensis* são necessárias para o melhor conhecimento do processo de invasão no hospedeiro, contribuindo para o aumento de conhecimento da patobiologia do fungo.

Objetivos

III. OBJETIVO

O presente trabalho tem como objetivo o rastreamento e identificação de seqüências codificantes para proteases de *P. brasiliensis* obtidas a partir de bancos de dados de ESTs de *P. brasiliensis* bem como a caracterização de uma serina protease S08 do fungo.

III.2. Objetivos específicos

1. Identificação *in silico* de proteases de *P. brasiliensis* em bancos de dados de ESTs oriundas de células leveduriformes de *P. brasiliensis*.

2. Identificação *in silico* de genes relacionados à síntese e processamento de proteínas de *P. brasiliensis* durante a diferenciação celular da forma miceliana para células leveduriformes.

3. Caracterização de uma serino protease de *P. brasiliensis*

Obtenção da seqüência de nucleotídeos e da seqüência predita de aminoácidos codificantes para a serino protease;

Clonagem do cDNA codificante para a serino protease em vetor de expressão e obtenção da proteína recombinante em sistema bacteriano;

Produção de anticorpo policlonal em camundongos;

Avaliação da expressão da serino protease em extrato protéico de células leveduriformes e sobrenadante de cultura de *P. brasiliensis* durante privação de nitrogênio;

Avaliação da presença de N-glicosilação na molécula de serino protease*;*

Avaliação da expressão de transcritos codificantes para serino protease em micélio, células leveduriformes e durante a infecção de macrófagos por células leveduriformes de *P. brasiliensis;*

Rastreamento de proteínas de *P. brasiliensis* interagindo com serino protease através da construção de biblioteca de duplo híbrido em *S. cerevisiae;*

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

Confirmação das interações protéicas por meio de co-imunoprecipitação.

Capítulo II

Proteases de P. brasiliensis

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Review

Transcriptome overview of *Paracoccidioides brasiliensis* proteases

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ABSTRACT. Proteases perform a wide variety of functions inside and outside cells, regulating many biological processes. Infectious microorganisms use proteases, either secreted or attached to their cell surface to weaken and invade their hosts. Therefore, proteases are targets for drugs against a diverse set of diseases. *Paracoccidioides brasiliensis* is the most prevalent fungal pathogen causing systemic mycosis in Latin America. The development of paracoccidioidomycosis depends on interactions between fungal and host components and proteases have been described as important factors implicated in the mechanism of host colonization by fungi. The primary goal for this study is to present an overview of the transcriptome sequences - identified cDNAs that encode proteases. We obtained a total of 53 cDNAs encoding proteases; 15 were classified as ATP-independent, 12 as ATP-dependent, 22 as proteasome subunits, and 4 as deubiquitinating proteases. The mechanisms and biological activity of these proteases differ in substrate specificity and in catalytic mechanisms.

Key words: *Paracoccidioides brasiliensis*, Host-fungus interaction, Proteases, Proteasome

INTRODUCTION

Proteases are enzymes that cleave proteins catalysing the hydrolysis of peptide bond. Based on their catalytic mechanisms, proteases can be classified into five main classes: i) aspartyl proteases; ii) metalloproteases; iii) cysteine proteases; iv) threonine proteases, and v) serine proteases. Proteases in the first two classes use an activated water molecule as a nucleophile to attack the peptide bond of the substrate, whereas in the last three classes the nucleophile is a catalytic amino-acid residue located in the active site (Rawlings and Barrett, 1993; Barrett et al., 1998). Proteases are also grossly subdivided into two major groups, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxyl termini of the substrate whereas endopeptidases cleave peptide bonds distant from the termini of the substrate (Watson, 1976).

Proteolytic enzymes play many physiological roles and are essential factors for homeostatic control in organisms. Proteases are widely produced amongst fungi and serve a number of different roles within fungal systems including nutrient cycling and post-translational processing (North, 1982). In some instances a correlation between protease production and pathogenicity was reported. There is accumulating evidence of the direct involvement of fungi proteases in different phases of the host-fungi interactions. The physiological role of proteases during colonization of the host is thought to be the degradation of the skin and mucosal barriers, digestion of host proteins to provide nutrients and attack the lymphocytes and macrophages, affecting the immune defenses of the host (Hube, 2000; Yang, 2003).

A fraction of the proteolytic activity of cells is ATP independent. In addition, studies have identified three ATP-dependent systems involved in protein degradation in eukaryotic organisms (Menon and Goldberg, 1987). These systems include: i) proteases that require the binding and hydrolysis of ATP for proteolytic activity (Lon, ClPs and the 26 S protease have been identified in detail; ii) the ubiquitin conjugating system, and iii) chaperone proteins.

Paracoccidioides brasiliensis is a dimorphic fungus that alternates between a mycelium phase in the free environment and a yeast phase in the human host. Primary infection starts in the lungs after inhalation of fungal propagules which then transform into the pathogenic yeast form. Primary infection is usually spontaneously healed; active paracoccidioidomycosis is estimated to develop in approximately 2% of the infected individuals (McEwen et al., 1995). *P. brasiliensis* thus represents a serious public health challenge, with social and economical importance. The observation that only a percentual of infected individuals can develop the disease points to both the pathogenic potential of *P. brasiliensis* and the importance of host defense in controlling fungal infection. *P. brasiliensis* expresses some molecules that account for its ability to evade efficiently the host protective immune system and proteases should be included with these molecules. Protease-like activity in the culture filtrates of *P. brasiliensis* was originally noted and an exocellular serine protease has been characterized as a molecule that cleaves *in vitro* the main components of the basal membrane (Carmona et al., 1995; Puccia et al., 1999). Although potentially associated with the invasion process the role of *P. brasiliensis* proteases in the fungus ability to cause disease remains to be elucidated.

The objective of this review is to summarize the information about the transcriptome-based identification of proteases of *P. brasiliensis*. The availability of primary structural information about a group of the identified expressed sequence tags (ESTs) allows further analysis and a better overall understanding of pathogen interactions with human host. This insight may

shed light on the elucidation of specific functions in which these proteins are involved as well as discover the role of these proteins in the pathogenesis of *P. brasiliensis*.

METHODS

Annotated ESTs encoding energy-dependent and -independent proteases were obtained in *P. brasiliensis* Transcriptome Project database (http://www.biomol.unb.br/Pb). We screened available databases of proteases, including MEROPS (http://merops.sanger.ac.uk/) and Pfam (http://pfam.wustl.edu). The search for similarity was conducted using the BLAST search tools, using the interface web of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Domains and predicted active sites were screened using the ProfileScan (http://hits.isb-sib.ch/cgi-bin/PFSCAN?) and ScanProsite algorithms (http://ca.expasy.org/tools/scanprosite/). Multiple sequence alignments were generated using the program ClustalX 1.81 software (Thompson et al., 1997).

RESULTS AND DISCUSSION

Proteases of *Paracoccidioides brasiliensis*

By using the primary information that was retrieved from the *P. brasiliensis* transcriptome (http://www.biomol.unb.br/Pb), combined with data from the MEROPS database (http://merops.sanger.ac.uk/) we have annotated 53 ORFs encoding energy-independent and -dependent proteases, including proteasome subunits, aspartyl, cysteine, metallo, and serine proteases. These cDNAs that encode protease homologues in the fungus transcriptome were annotated, as shown in Figure 1. The proteases of *P. brasiliensis* are distributed as following: 5.6% aspartyl proteases, 11.3% cysteine proteases, 22.6% metalloproteases, 18.8% serine proteases, and 41.5% proteasome subunits.

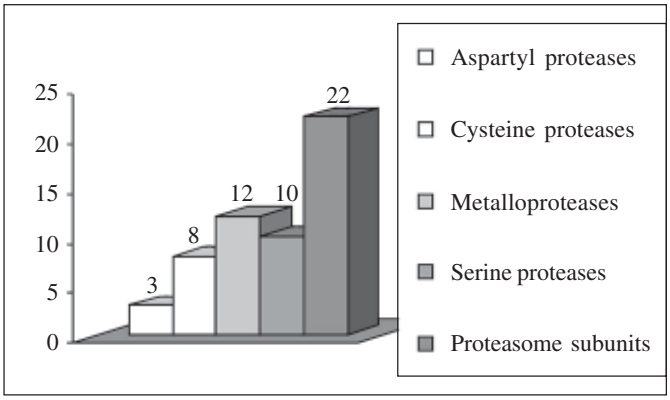

Figure 1. Classification of the *Paracoccidioides brasiliensis* proteases present in the fungus transcriptome. The sequences were obtained at (http://www.biomol.unb.br/Pb). Aspartyl, cysteine, metallo, and serine proteases were classified using the BLAST search tools in the MEROPS database (http://merops.sanger.ac.uk/). Proteasome subunits were classified by homology search, using the BLAST program at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).

Energy-independent proteases of *Paracoccidioides brasiliensis*

Exopeptidases and endopeptidases

We have annotated a total of 15 cDNAs, which encode energy-independent protease homologues in the fungus transcriptome, as shown in Table 1. From these, two were classified as exopeptidases and the remaining 13 as endopeptidases. The energy-independent proteases are distributed as following: three aspartyl, two cysteine, eight metallo, and two serine proteases.

Table 1. ATP-independent proteases of *Paracoccidioides brasiliensis.*

Product name	Best Blast Hits in NCBI database	Classification in MEROPS database*	Motifs**
Aspartyl protease (GenBank accession number AY278218)[1]	e^{-144}	A01[3]	DTG
Aspartyl protease[1]	$7e^{-18}$	A[3]	
Cathepsin D[1]	$1e^{-15}$	A[3]	
Zinc metalloprotease[1]	$2e^{-33}$	M12B[5]	HEXXH
O-sialoglycoprotein endopeptidase[1]	$5e^{-24}$	M22[5]	
Glycoprotein endopeptidase[1]	$9e^{-72}$	M22[5]	
Proline aminopeptidase[2]	$3e^{-60}$	M24B[5]	HXXE
Prenyl protease[1]	$8e^{-64}$	M48A[5]	HEXXH
Zinc metalloprotease[1]	$5e^{-39}$	M[5]	HEXXH
Metallopeptidase[1]	$8e^{-31}$	M[5]	HEXXH
Zinc protease[1]	$2e^{-48}$	M[5]	
Aminopeptidase C[2]	$1e^{-55}$	C01B[4]	QC
Caspase[1]	$3e^{-11}$	C[4]	
Serine protease (GenBank accession number AY319300)[1]	e^{-165}	S08A[6]	DHS
Kex2 endoprotease[1]	$9e^{-54}$	S08B[6]	

[1]-Endopeptidases; [2]-Exopeptidases; [3]-Aspartyl protease; [4]-Cysteine protease; [5]-Metalloprotease; [6]-Serine protease.
*Protease families and subfamilies described and obtained by MEROPS Blast.
**Active site residues obtained by experimental analysis and described in MEROPS database (http://merops.sanger.ac.uk).

Aspartyl proteases of Paracoccidioides brasiliensis

Three aspartyl proteases were found in the *P. brasiliensis* transcriptome (Table 1, Figure 1). Aspartyl proteases are a group of proteolytic enzymes including the pepsin family that share the same catalytic apparatus and usually function in acidic conditions (Rao et al., 1998). This fact limits the function of this class of proteases to specific locations in the cell. Aspartyl proteases are ubiquitous in nature and are involved in a myriad of biochemical processes. Well-known aspartyl proteases include pepsin and renin in humans (Davies, 1990).

Aspartyl proteases are directly dependent on aspartic acid residues for their catalytic activity and comprise three sub-families: i) family A1, related to pepsin; ii) family A2, retropepsins,

and iii) family A3, retropepsins-like. In the A1 family (clan AA) the catalytic Asp residue occurs within the motif Asp-Ser/Thr-Gly. This family contains many secreted enzymes, which are probably synthesized as propeptides with signal peptides (Dash et al., 2003). The aspartyl protease-deduced primary sequences of the *P. brasiliensis* were analyzed for the presence of the characteristic motif (Asp-Ser/Thr-Gly). Only one protease of this class presents this motif as shown in Table 1. The excerpt of the alignment of this *P. brasiliensis* aspartyl protease with related sequences is shown in Figure 2. The conserved catalytic motif of known aspartyl proteases and its active site are shown, which are conserved among the compared sequences (Figure 2). Since only the sequence (GenBank accession number AY278218) encoding the homolog of a secreted aspartyl protease of *P. brasiliensis* was completely sequenced and characterized, it is possible that complete sequencing of other aspartyl proteases found in the *P. brasiliensis* transcriptome would reveal the expected catalytic triad.

```
Pa    --IKRDTGSAAAIPINEVDIAYVTPVTIGTPPQTLMLDLDTGSSDLWVFSSLTPSNQVRG  58
Th    SLTKRQTGSANTNPSDSADDEYITSVSIGTPAQVLPLDFDTGSSDLWVFSSETPKSSASG  60
Af     -------GSAVTTP-EQYDSEYLTPVKVGG-TTLNLDFDTGSADLWVFSSELSASQSSG  50
Pb    ---------HSVLVDNFLNAQYFSEISIGTPPQTFKVVLDTGSSNLWVPSSQCSSIACYL  51
An    ---------HDVLVDNFLNAQYFSEIELGTPPQKFKVVLDTGSSNLWVPSSECSSIACYL  51
                  .   :  :  *.: : :*     : : :****::*** **  .

Pa    QEIYSPTKSSTSKLLSGHTWSIRYGDGSGSRGTVYTDNFTIGGLEVKSQA---------- 108
Th    HALYSPSKSSTAKK---------------------------------------------- 74
Af    HAIYKP-SANAQKLNGYTWKIQYGDGSSASGDGYKDTVTVGGVTAQSQAVEAASHISSQ 108
Pb    HSKYDSSASSTHRK-NGTEFAIRYGSGS-LSGFVSQDVLRIGDMTVESQDFAEATSEPGL 109
An    HNKYDSSASSTYHK-NGSEFAIKYGSGS-LSGFVSQDTLKIGDLKVKGQDFAEATNEPGL 109
      :  *..  *:. :
```

Figure 2. Alignment of the deduced amino acid sequence of *Paracoccidioides brasiliensis* aspartyl protease (AY278218), family A01, and related sequences. Black box represents the putative conserved active site, with the catalytic triad evidenced in bold type letters. The sequences aligned were obtained in MEROPS database: *Pa*, *Podospora anserina* (O13340); *Th*, *Trichoderma harzianum* (Q9HDT6); *Af*, *Aspergillus fumigatus* (P41748); *Pb*, *Paracoccidioides brasiliensis* aminopeptidase; *An*, *Aspergillus niger* (Q00070).

Metalloproteases of Paracoccidioides brasiliensis

Zinc-containing metalloproteases are widely distributed in prokaryotic and eukaryotic organisms and are classified into four groups comprehending DD-carboxypeptidases, carboxypeptidases, zincins, and inverzincins (Miyoshi and Shinoda, 2000). One of the most prominent group comprehends the proteins possessing the HEXXH zinc-binding motif, belonging to the zincins superfamily (Miyoshi and Shinoda, 2000). From the eight identified energy-independent zinc metalloproteases in the *P. brasiliensis* transcriptome, four presented the consensus motif HEXXH which define those proteases as members of the zincins family, as shown in Table 1. The production of such proteases by *P. brasiliensis* should be of special note, since evidence has been presented identifying the zincins as pathogenic factors in other microorganisms (Klimpel et al., 1994; Matthews et al., 1998). The motif present in carboxypeptidases (HXXE) was also found in the predicted metalloproteases of *P. brasiliensis* (Table 1).

Cysteine proteases of Paracoccidioides brasiliensis

Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 subfamilies of cysteine proteases have been recognized. A detailed analysis of the *P. brasiliensis* transcriptome database reveled two ESTs encoding cysteine proteases ATP independent (Figure 1, Table 1).

Active site residues Q, C, H, N obtained by experimental evidence are described in cysteine families in MEROPS database (http://merops.sanger.ac.uk). Two residues (Q and C) were found in one ORF encoding an aminopeptidase of the cysteine family of *P. brasiliensis* (Table 1). An excerpt of the alignment of this EST with related sequences is shown in Figure 3 and presents those conserved active site residues, which were present in all considered homologues.

Among the cysteine proteases, a caspase homolog was detected (Table 1) suggesting that the programmed cell death in *P. brasiliensis* in its initiation and execution phases could be proteolytically regulated by this class of molecules, as described in other systems (Shi, 2002).

```
Ll   -----CLDLTKDPVTNQKQSGRCWMFAALNTFRHKFINEFKTEDFEFSQA  45
Lm   ------LDLTKDPVTNQKQSGRCWMFAALNTFRHKFINEFKTEDFEFSQA  44
Sp   ------LDLTKDPVTNQKQSGRCWMFAALNTFRHKFINEFKTEDFEFSQA  44
Pb   ----IKIPLEGAPITNQRSSGRCWLFAMTNVFRVALMKLYNVKNFELSQA  46
Sc   RVFNTVVSTDSTPVTNQKSSGRCWLFAATNQLRLNVLSELNLKEFELSQA  50
            :     *:***:.*****:**  * :*  .:.  : ::**:***

Ll   YTFFWDKYEKSNWFMEQIIG--DVAMDDRRLKFLLQTPQQDGGQWDMMVA  93
Lm   YTFFWDKYEKSNWFMEQIIG--DVAMDDRRLKFLLQTPQQDGGQWDMMVA  92
Sp   YTFFWDKYEKSNWFMEQIIG--DVAMDDRRLKFLLQTPQQDGGQWDMMVA  92
Pb   YPFFWDKIEKANWFLEQVIDTAEKELDSRLVQSLMSGPVSDGGQWDMAAN  96
Sc   YLFFYDKLEKANYFLDQIVSSADQDIDSRLVQYLLAAPTEDGGQYSMFLN 100
     * **:** **:*:*::*::.  :  :*.* :: *:  * .****:.*

Ll   IFDKYGIV 101
Lm   IFDKYGIV 100
Sp   IFDKYGIV 100
Pb   LVRK---- 100
Sc   --------
```

Figure 3. Alignment of the deduced amino acid sequence of *Paracoccidioides brasiliensis* aminopeptidase C (cysteine peptidase), family C01B and related sequences. Black boxes and bold letters represent the putative conserved active site residues obtained in MEROPS database: *Ll*, *Lactococcus lactis* (Q04723); *Lm*, *Listeria monocytogenes* (O69192); *Sp*, *Streptococcus pyogenes* (Q99YL0); *Pb*, *Paracoccidioides brasiliensis* aminopeptidase; *Sc*, *Saccharomyces cerevisiae* (Q01532).

Serine proteases of Paracoccidioides brasiliensis

Serine proteases are a family of enzymes that utilize a uniquely activated serine residue in the substrate-binding site to catalytically hydrolyze peptide bonds (Schultz and Liebman, 1997). They are numerous and widespread among virus, bacteria and eukaryotes, suggesting that they are vital to the organisms. Owing to the expanding roles for serine proteases, including a diverse

array of physiological functions (Henderson et al., 1992; Froelich et al., 1993), there has been increasing interest in the identification, structural and functional characterization of members of this family. In terms of absolute numbers we identified two energy independent serine proteases in the *P. brasiliensis* transcriptome (Figure 1, Table 1). The essential amino acid residues forming the catalytic triad (DHS) were detected in one of the deduced ORFs encoding the serine protease of *P. brasiliensis* (GenBank accession number AY319300).

Figure 4 presents the alignment of the deduced amino acid sequence encoding this serine protease, family S08A of *P. brasiliensis* with related sequences present in MEROPS database. The catalytic triad is conserved among the sequences.

Among the identified serine proteases a Kex2 endoprotease was identified (Table 1). The Kex2 endoprotease presented the highest identity to the Kex2 gene of *P. brasiliensis* described elsewhere (Venancio et al., 2002).

```
Sc   YDDDAGRGVTSYVIDTGVNINHKDFEKRAIWGKTIPLNDEDLDGNGHGTHCAGTIASKHY  60
Pb   ------------------------------ATIPDGDGDEDGNGHGTHCSGTIAGKKY  28
Sp   YNETAGEGVTAYVIDTGINIEHQDFQGRATWGATIPTGEGEVDDHGHGTHVAGTIAGKTF  60
Af   ---NGGEGTYAYVVDIGINVDHEEFEGRASL-AYHAAGGQHVDGVGHGTHVSGTIGGKTY  57
                                       . .  . *. ***** :***..* :

Sc   GVAKNANVVAVKVLRSNGSGTMSDVVKGVEYAAKAHQK------EAQEKKKGFKGSTANM 114
Pb   GVAKKSHIYAVKVLRSNGSGTIGDVIKGVEFVATSHTKNVEAAKAGKSNKKGFKGSVANM  88
Sp   GVSKNAKLVAVKVMRADGTGTVSDIIKGIEFAFKQSKK----------DKESIASVVNM 109
Af   GVAKKANLLSVKVFVGESS-STSIILDGFNWAANDIVS----------KKRTGKAAINM 105
     **:*:::: :***: .:.: : . ::.*.::. .   .           .*    :. **

Sc   SLGGGKSPALDLAVNAAVEVGIHFAVAAGNENQDACNTSPASADKAITVGASTLSDDRAY 174
Pb   SLGGSRSHALDYTVNSAVETGVHFAVAAGNDNSNACYYSPAAAAQAVTVGASTLADERAF 148
Sp   SIGGDASTALDLAVNAAIAGGLFFAVAAGNDAEDACGTSPARVSNAMTVGASTWNDQIAS 169
Af   SLGGGYSKAFNDAVENAFNEGVLSIVAAGNENTDASRTSPASAPDAFTVAAINVNNTRAY 165
     *:**. * *:: :*: *.  *:   *****:  :*.  *** . .*.**.* .  :  *

Sc   FSNWGKCVDVFAPGLNILSTYIGSDDATATLSGTSMASPHVAGLLT----------     220
Pb   FSNYGMCLDVFGPGLNVMSTWIGGKYAVNTISGTSMASPHVAGLLAYFLSLQ----     200
Sp   FSNIGSCVDIFAPGSLILSDWIGSNRASMILSGTSMASPHVAGLAAYFISL-----     220
Af   FSNYGSVVDIFAPGQNILSAWIGSNTATNTISGTSMATPHIVGLSIYLMSLEVLSS     221
     *** *  :*:*.**  ::* :**.. *   :******:**:.**
```

Figure 4. Alignment of the deduced amino sequence of *Paracoccidioides brasiliensis* serine protease (AY319300), family S08A to related sequences. Black boxes and bold letters show conserved residues of the catalytic triad DHS, well described in this family. The sequences aligned were obtained in MEROPS database: *Sc*, *Saccharomyces cerevisiae* (P09232); *Pb*, *Paracoccidioides brasiliensis* aminopeptidase; *Sp*, *Schizosaccharomyces pombe* (P78879); *Af*, *Aspergillus flavus* (P35211).

Energy-dependent protease homologues in *Paracoccidioides brasiliensis*

Lon protease

The first ATP-dependent protease to be identified in *P. brasiliensis* was the Lon protease (Barros and Puccia, 2001). The *lon* gene product is a protein of 1063 amino acids, which presents a single ATP-binding consensus and a serine catalytic site (KDGPSAG). Lon is an

endoprotease, cleaving substrates at multiple sites only in the presence of ATP, in several organisms. The protease activity of *P. brasiliensis* Lon has to be determined.

Clps proteases

Clps has been described in *P. brasiliensis* (Table 2). The first Clp protein to be described in the fungus was the ClpB (Jesuino et al., 2002). The ClpB protein of *P. brasiliensis* presents two ATP-binding domains which places the protein in the class I Clp/HSP100 family (Schirmer et al., 1996). ClpB is also a heat shock protein which is induced upon the mycelium to yeast transition in *P. brasiliensis*. Heat shock element motifs in the clpB gene promoter region, in addition to the preferential protein expression in yeast cells could suggest a role of ClpB during the temperature upshift that characterizes the infective process by *P. brasiliensis*.

ClpA is another member of the Clp family described in *P. brasiliensis* (GenBank accession number AY229978) (Table 2). ClpA is a member of proteins that includes the yeast HSP104, which is required for acute thermotolerance (Parsell et al., 1991). The cDNA sequence, which encodes a predicted protein of 927 amino acids, was obtained (Oliveira et al., 2005). The characteristic two nucleotide-binding domains were present in the deduced protein.

Other members of the Clp protease family were obtained by analysis of the *P. brasiliensis* transcriptome. In agreement to Lon, ClpB and ClpA belong to the serine protease family (Table 2). Subunits of unclassified Clps were also obtained. Another member of the AAA superfamily of protease was identified (Table 2) and includes a predicted mitochondrial product, which should be involved in mitochondrial biogenesis. This mitochondrial AAA metalloprotease contains the zinc-binding domain (HEXXH) and could be encoded by a small gene family, as described in other fungi (Shah et al., 2000). Other mitochondrial peptidases were found and listed in Table 2, including members of the inverzincins and DD-carboxypeptidases of the zinc metalloprotease family (Miyoshi and Shinoda, 2000).

Table 2. ATP-dependent proteases of *Paracoccidioides brasiliensis.*

Product name	Best Blast Hits in NCBI database	Classification in MEROPS database*	Motifs
Lon protease	e^{-104}	S16	
ClpB	$2e^{-96}$	S	
ClpA	e^{-110}	S14	
ATP-dependent protease Clp	$6e^{-16}$	S	
ATP-dependent ClpP2 protease subunit	$7e^{-46}$	S14	
ATP-dependent protease Clp, ATPase subunit	$4e^{-55}$	S	
Mitochondrial matrix AAA protease	$2e^{-85}$	M41	HEXXH
Mitochondrial processing peptidase	$3e^{-38}$	M16X	
Mitochondrial processing peptidase α subunit	$4e^{-57}$	M16X	HXH
Mitochondrial processing peptidase β subunit	$1e^{-88}$	M16B	HXXEH
Inner mitochondrial membrane protease	$7e^{-21}$	S26A	
Inner mitochondrial membrane protease subunit	$4e^{-04}$	S26A	

*Protease families and subfamilies were classified according to MEROPS database.

Ubiquitin system for protein tagging

The ubiquitin system is a highly complex enzymatic system that covalently modifies selected proteins by attachment to the 8-kDa protein ubiquitin. Selective ubiquitin-mediated proteolysis is the dominant mechanism of degradation of cytosolic and nuclear proteins in eukaryotic cells (Finley and Chau, 1991). In this process, a protein substrate is tagged with a polyubiquitin chain that mediates interaction with and degradation by the proteasome (Pickart, 2000).

Proteasomes

The proteasome is the central protease in non-lysosomal ubiquitin-dependent protein degradation, and is involved in protein quality control, antigen processing, signal transduction, cell cycle control, cell differentiation, and apoptosis (Voges et al., 1999). The 26S proteasome is a large protein machine, which is found in both, nucleus and cytoplasm. It consists of the 20S proteasome, which forms the proteolytically active core and a regulatory 19S complex (Glickman et al., 1998). High-resolution crystal structures of the 20S proteasome of the yeast *Saccharomyces cerevisiae* demonstrated that it is composed of 28 protein subunits, which are arranged into four staggered heptameric rings. Each outer ring comprises seven α-type subunits and each inner contains seven β-type subunits (Groll et al., 1997). The 20S proteasome of higher eukaryotes is also composed of seven distinct α and β subunits, respectively (Krüger et al., 2001). The α subunits are inactive whereas the β subunits build up the hydrolytic chamber. From the seven β subunits, only three are proteolytically active and autoproteolytically matured as active threonine proteases (Groll et al., 1997). The proteolytic activities of the complex in yeast and mammalian proteasomes reside in β_1, β_2, and β_5 subunits (Heinemeyer et al., 1997). By using the primary information from the *P. brasiliensis* transcriptome database we have annotated ESTs encoding all the α subunits (1 to 7) and six homologues to the 20S β subunit (1 to 6) (Table 3). Of special note is the presence of the β subunits 1, 2 and 5 suggesting that the complex is proteolytically active in *P. brasiliensis*. Recognizing the polyubiquitin proteolytic signal is one of the many tasks of the 19S complex. Studies have shown that four or more ubiquitin composing chains bind the 19S complex (Thrower et al., 2000). Components of the regulatory 19S complex were found in the *P. brasiliensis* transcriptome in a total of nine different subunits (Table 3).

Deubiquitinating proteases

The deubiquitinating enzymes are defined as a group of proteases which play an important role in the regulation of all processes involving ubiquitin from the processing of poly-ubiquitin precursors into ubiquitin monomers to the targeting or salvage of proteasomal substrates. They are grouped into two classes based on the sequence homology: ubiquitin carboxy-terminal hydrolases (UCHs) and ubiquitin processing proteases, also known as ubiquitin-specific proteases (UBPs).

The physiological functions of deubiquitinating enzymes have been elucidated. Studies involving a human gene encoding an ubiquitin-specific protease reveal that the overexpression of this gene can result in deubiquitination of a broad spectrum of cellular proteins with a growth inhibitory effect. This result suggests that this protein may play an important role in regulation of

Table 3. Proteasome subunits of *Paracoccidioides brasiliensis.*

Product name	Best Blast Hits in NCBI database
Proteasome α_1 subunit	$9e^{-73}$
Proteasome α_2 subunit	$2e^{-62}$
Proteasome α_3 subunit	$8e^{-29}$
Proteasome α_4 subunit	$8e^{-88}$
Proteasome α_5 subunit	$4e^{-70}$
Proteasome α_6 subunit	$5e^{-63}$
Proteasome α_7 subunit	$2e^{-10}$
Proteasome β_1 subunit	$3e^{-51}$
Proteasome β_2 subunit	$5e^{-74}$
Proteasome β_3 subunit	$2e^{-75}$
Proteasome β_4 subunit	$5e^{-43}$
Proteasome β_5 subunit	$3e^{-76}$
Proteasome β_6 subunit	$4e^{-50}$
Proteasome regulatory subunit	$9e^{-62}$
Proteasome regulatory subunit	e^{-154}
Proteasome regulatory subunit	$2e^{-45}$
Proteasome regulatory subunit	$2e^{-70}$
Proteasome regulatory subunit	0.0
Proteasome regulatory subunit	$5e^{-79}$
Proteasome regulatory subunit	$1e^{-94}$
Proteasome regulatory subunit	$7e^{-45}$
Proteasome regulatory subunit	$5e^{-96}$

cell growth (Gong et al., 2000). In addition, these enzymes are active in regenerating free ubiquitin after proteins have been targeted to the proteasome (Wing, 2003). A large number of genes encode deubiquitinating enzymes suggesting that many of these have highly specific and regulated functions. The proteins contain conserved motifs with critical cysteine in the active sites. In agreement the UCHs and UBPs in *P. brasiliensis* are cysteine proteases (Table 4).

In *S.* cerevisiae there is one UCH and sixteen UBPs, whereas higher organisms express an expanded group of enzymes (Yan et al., 2000). In the transcriptome of *P. brasiliensis* were found three ORFs encoding ubiquitin carboxy terminal hydrolases, a high number when compared to *S. cerevisiae*. One EST encoding an ubiquitin-specific protease was also found in the transcriptome database (Table 4).

Table 4. Deubiquitinating protease homologues of *Paracoccidioides brasiliensis.*

Product name	Best Blast Hits in NCBI database	Classification in MEROPS database*
Ubiquitin carboxyl-terminal hydrolase	$9e^{-40}$	C19
Ubiquitin carboxyl-terminal hydrolase	$6e^{-27}$	C19
Ubiquitin carboxyl-terminal hydrolase	$2e^{-80}$	C19
Ubiquitin-specific protease	$2e^{-09}$	C19

*Protease families and subfamilies described and obtained by MEROPS blast.

CONCLUDING REMARKS - PUTATIVE ROLE OF PROTEASES IN HOST-PATHOGEN INTERACTION IN *PARACOCCIDIOIDES BRASILIENSIS*

The proteases present in different parasites appear to be relevant for several aspects of host-parasite interactions, quite apart from their obvious participation in the other cellular processes. Information about the seemly putative functions and importance of ATP-independent proteases in *P. brasiliensis* interaction with host was obtained by comparing them to the described homologues in other systems for whom a function was defined. The putative role in host-fungus interaction for these proteins was deduced by generation of mutants deficient in the genes, or asserting their function as potential antigen or vaccine candidates.

Aspartyl proteases are secreted by pathogenic species of *Candida in vivo*, during infection (De Bernardis et al., 1990). More direct evidence of the implication of SAP proteins in virulence has come from studies of constructing strains harboring disruptions in a number of SAP genes. In all cases, mutants showed decreased virulence in an animal model of disseminated candidiasis (Sanglard et al., 1997; Hube et al., 1997). The SAP 2 confers immune protection against systemic candidiasis in immunized mice and has been postulated as a vaccination target (Villanova et al., 2004). Also, members of this protein family have been considered as antigenic markers of disseminated candidiasis (Morrison et al., 2003). In addition, an aspartyl protease is a component of a protective vaccine in coccidioidomycosis (Johnson et al., 2000).

Serine proteases are involved in *Aspergillus* interaction with the host. A vacuolar serine protease is a major allergen of *A. fumigatus* (Shen et al., 2003). Also the involvement of serine and cysteine protease in the fungus colonization of the host's lung tissue has been reported (Kogan et al., 2004).

Metalloproteases play different roles in the host-parasite infections. A metalloprotease is a surface antigen in *Trypanosoma cruzi* and has been postulated as a virulence factor (Cuevas et al., 2003). The protein promotes the attachment of the promastigote form to host cell surface receptors and interacts with the complement system contributing to the ability of the amastigote form of *Leishmania* spp to survive inside the macrophage (Cheng and Chang, 1986; Joshi et al., 2002). The metalloproteases from the human enteropathogenic *Vibrio cholerae* accelerate the bacterial attachment to intestinal epithelial cells through digestion of the small intestinal mucosa (Ichinose et al., 1994). Of the proteases produced by *A. fumigatus*, metalloprotease presenting the consensus zinc-binding motif (HEXXH) has been involved in the infection (Markaryan et al., 1994; Jaton-Ogay et al., 1994).

In the current study, we provided an overview of proteases in the *P. brasiliensis* transcriptome. Research will be directed towards identification of all *P. brasiliensis* proteases and their functional characterization, as well as its presumed role in the infection by *P. brasiliensis*. Proteases have enormous potential as drug targets. Perhaps the main reason for this is that protein modification by proteolytic enzymes is such a ubiquitous biological phenomenon that it is difficult to find pathways in which it does not play a part. In the area of proteases of pathogens the potential as drug targets is a great promise because it should be possible to exploit the differences between enzymes of the pathogen and host to produce effective drugs.

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Discussão

Rastreamento de ESTs codificantes para proteases expressas na fase leveduriforme de *P. brasiliensis*

Discussão e Conclusões

No presente trabalho, foram realizadas análises em banco de dados de cDNAs obtidos a partir de RNA da fase leveduriforme de *P. brasiliensis* (http://dna.biomol.ubn.br/Pb). Foram rastreados e identificados transcritos codificantes para proteases. As seqüências parciais de cDNA foram traduzidas para obtenção das seqüências preditas de proteína, que foram classificadas por homologia em banco de dados MEROPS (http://merops.sanger.ac.uk). No total, foram identificadas 53 ORFs codificantes para proteases. As proteases identificadas foram classificadas de acordo com o mecanismo catalítico: 3 proteases foram classificadas pertencentes à classe das aspartil proteases, 8 cisteíno proteases, 10 metaloproteases, 10 serino proteases e 22 proteases pertencentes às subunidades do complexo proteassoma de degradação. Dentre as proteases identificadas, 15 foram classificadas como proteases ATP - independentes e 12 foram identificadas como proteases ATP-depedentes. As 26 ORFs restantes foram classificadas pertencentes ao complexo proteassoma (22) e proteases de deubiquitinação (4). As 15 ORFs codificantes para proteases ATP - independentes foram classificadas de acordo com seu sítio de ação: 13 proteases são endopeptidases e duas são exopeptidases (Parente et al., 2005).

Dentre os transcritos identificados na fase leveduriforme de *P. brasiliensis* três deles codificam para aspartil proteases (Parente *et. al.*, 2005). Em *C. albicans*, 10 genes codificantes para aspartil proteases secretadas (SAPs) foram identificados e os produtos destes genes têm sido associados a danos teciduais do hospedeiro, facilitando a penetração através da mucosa (Naglik et al., 2003). Em *A. fumigatus*, foi possível identificar uma aspartil protease denominada aspergilopepsina secretada em grande quantidade durante a infecção fúngica em pulmão de camundongos infectados através da técnica de imunolocalização com anticorpo específico para esta protease (Lee &. Kolattukudy, 1995). Estudos de infecção de *A. fumigatus* em pneumócitos, linhagem A549, mostraram que este fungo utiliza as classes serino e cisteíno proteases para clivar proteínas de pneumócitos desorganizando o citoesqueleto de actina para facilitar a penetração nas células. A invasão aos pneumócitos utilizando-se uma linhagem de *A. fumigatus* mutada para uma serino protease alcalina não promoveu o desarranjo da rede

de actina, sugerindo que esta protease tenha importância neste processo (Kogan et al., 2004).

Duas cisteíno proteases ATP - independentes foram identificadas na fase leveduriforme de *P. brasiliensis* (Parente *et. al.*, 2005). Análises da seqüência predita de aminoácidos codificantes para uma cisteíno protease revelou a presença de resíduos conservados do sítio ativo. O parasita de peixes *Trypanoplasma borreli*, apresenta uma cisteíno protease capaz de degradar hemoglobina e imunoglobulinas do hospedeiro, contribuindo assim para patogenicidade do microorganismo (Ruszczyk *et. al.*, 2008).

Foram identificadas oito ORFs codificantes para metaloproteases ATP - independentes e duas serino proteases ATP-independentes em *P. brasiliensis* (Parente et al., 2005). No fungo dermatófito *Trichophyton rubrum* metalo- e serino proteases apresentam o perfil de expressão modulados positivamente após cultivo do fungo na presença de componentes protéicos tais como queratina, colágeno e elastina sugerindo a importância destas proteases na virulência deste fungo (Leng *et. al.* 2008).

Proteínas relacionadas ao processo de degradação de espécies protéicas também foram identificadas, como aquelas relacionadas aos processos de ubiquitinação e deubiquitinação (Parente *et. al.*, 2005). Esta classe de enzimas atua na degradação de proteínas mal-traduzidas e/ou mal-enoveladas, apresentando importância no rigoroso controle da qualidade de todas as proteínas produzidas pelas células (Voges *et al.*, 1999).

O grande número de ORFs codificantes para proteases em *P. brasiliensis* sugere que os processos metabólicos que envolvem estas enzimas são importantes para sobrevivência de *P. brasiliensis*, seja na captação de nutrientes, seja no processamento intracelular de proteínas. (Voges et al., 1999; Donofrio *et al.*, 2006). A presença de proteases pH dependentes também sugere que *P. brasiliensis* apresente proteases capazes de atuar de acordo com as condições impostas pelo meio em que o fungo se encontra. Serino proteases são geralmente ativas em pHs alcalinos enquanto aspartil proteases sofrem processo de auto-ativação em pH ácido (Rawling & Barret 1993).

Capítulo III

Rastreamento de ESTs durante a transição dimórfica

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

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

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

Introduction

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

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

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

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

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

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

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

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

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

Materials and methods

RNA extraction and preparation of the cDNA library

The cDNA library was constructed, as previously reported [21]. Briefly, *P. brasiliensis*, isolate *Pb*01 (ATCC-MYA-826), was grown in Fava-Netto's medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 4% (w/v) agar, pH 7.2], at 22°C, as mycelium. The differentiation was performed in liquid medium (Fava-Netto's medium) by changing the culture temperature from 22 to 36°C for the mycelium to yeast transition, as we previously described [13]. The cells were previously grown in liquid medium for 18 h before changing the incubation temperature, which was maintained for 22 h.

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

EST processing pipeline and annotation

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

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

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

Results and discussion

cDNA library sequence annotation

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

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

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

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

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

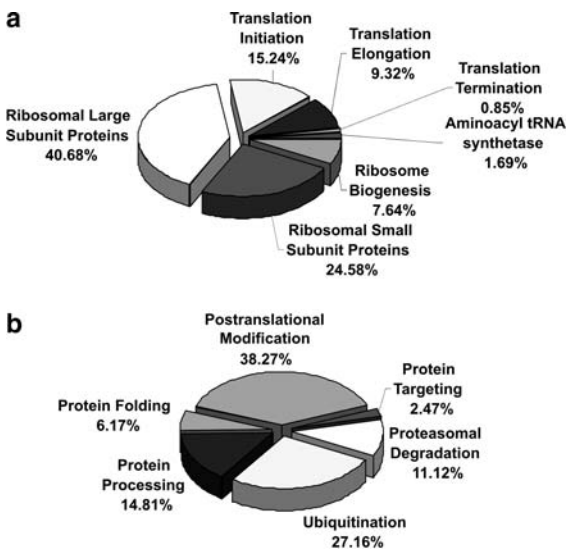

Fig. 1 Distribution of ESTs from *P.brasiliensis* mycelia ongoing transition to yeast according to their cDNA products. (**a**) Protein synthesis; (**b**) protein fate. The classification was based on *E*-value and performed according to the functional categories developed on the MIPS functional annotation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

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

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

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

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

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

Table 1 continued

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

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

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

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

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

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

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

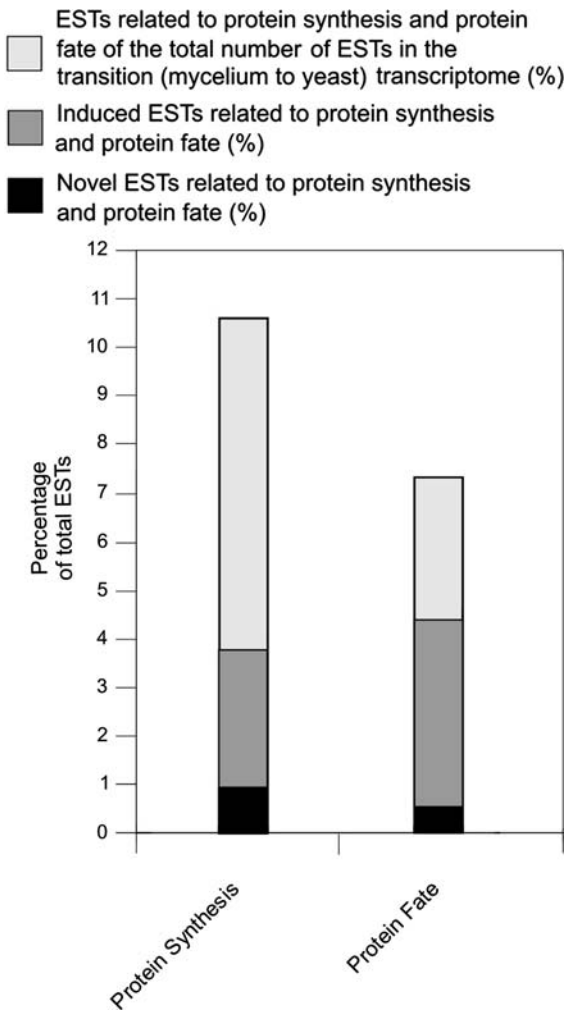

Fig. 2 Prevalence distribution of ESTs from *P. brasiliensis* mycelia ongoing transition to yeast. The percentage of ESTs related to protein synthesis and fate in the total ESTs are represented along with the percentage of over expressed and novel genes of *P. brasiliensis* in the transition library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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 3 continued

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Putative differentiation, virulence and stress tolerance factors

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

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

Concluding remarks

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

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Discussão

Rastreamento de ESTs codificantes para proteínas relacionadas à síntese e ao processamento protéico induzidas durante a transição dimórfica de *P. brasiliensis*

Discussão e Conclusões

A transição dimórfica de micélio para levedura em *P. brasiliensis* é etapa importante para o estabelecimento da infecção no hospedeiro humano (San-Blas & Nino-Veja, 2001). Sendo assim, elucidar os aspectos moleculares envolvidos nesta etapa de transição torna-se importante para melhor compreensão da patogênese de *P. brasiliensis.* Com o objetivo de ampliar os estudos das modificações transcricionais que ocorrem durante o processo de dimorfismo de *P. brasiliensis,* Bastos e colaboradores (2007) avaliaram 1107 ESTs oriundas de RNA de *P. brasiliensis* após 22 horas da indução da transição dimórfica.

O rastreamento de transcritos relacionados aos processos de síntese, enovelamento e modificações pós-traducionais de proteínas de *P. brasiliensis* foi realizado em banco de dados de transcritos de *P. brasiliensis* obtido após indução da transição de micélio para levedura por 22 horas (http://192.168.0.5/phorestwww). Um total de 200 ORFs foi identificado apresentando homologia com seqüências relacionadas aos processos de síntese e processamento de proteínas. Destas, 16 ORFs codificam para genes não descritos anteriormente em *P. brasiliensis* (Parente *et. al.*, 2008).

Do total de ESTs relacionadas à síntese protéica, 10% correspondem a cDNAs induzidos durante a transição dimórfica de *P. brasiliensis*. Várias ESTs que compõem as subunidades ribossomais, assim como fatores de iniciação transcricional foram regulados positivamente na transição dimórfica em *P. brasiliensis*, como, por exemplo, uma proteína relacionada à biogênese da subunidade 60S ribossomal, uma proteína OBG de ligação ao GTP e uma ubiquitina fusionada à proteína S27a (Parente *et. al.*, 2008). Resultados similares foram descritos durante a transição dimórfica do isolado *Pb*18 de *P. brasiliensis* e sugerem que a alteração morfológica de *P. brasiliensis* envolve um aumento da síntese de novas partículas ribossomais, o que refletiria um aumento no nível de proteínas sintetizadas requeridas neste processo (Nunes et al., 2005). Algumas proteínas ribossomais apresentam ortólogos regulados positivamente durante estágios específicos em outros organismos. Neste sentido, o produto gênico de

*rps*26 é induzido durante o estresse ambiental em plantas (Brosche et al., 1999). Da mesma forma, o produto do gene *rpl*5 de *Eimeria tenella* é detectado em esquizontes e não encontrado em oocistos, sugerindo que o transcrito codificante para esta proteína ribossomal é regulado durante as fases do desenvolvimento do microrganismo (Schaap et al., 2005).

O rastreamento em banco de dados de ESTs *P. brasiliensis* obtidas após a indução da transição dimórfica (http://192.168.0.5/phorestwww) permitiu identificar 81 ESTs codificantes para proteínas relacionadas ao processamento de proteínas. Deste total, 48 ESTs correspondem a cDNAs regulados positivamente durante o processo de transição dimórfica. Seis ESTs correspondem a genes que ainda não haviam sido descritos anteriormente para *P. brasiliensis* (Parente *et. al.*, 2008).

Trasnscritos codificantes para chaperonas foram induzidos durante a transição dimórfica de micélio para forma leveduriforme em *P. brasiliensis*, como o componente do complexo da chaperona HSP90 (*csn*1) e a subunidade épsilon da chaperona codificada pelo gene *tcp-1* (Parente *et. al.*, 2008). O complexo HSP90 é altamente conservado e abundante em vários organismos. Este complexo de chaperonas é essencial para viabilidade em *S. cerevisiae* (Marsh et al., 1998). O produto gênico de *tcp*-1 é localizado no citoplasma de eucariotos e é ortólogo ao complexo GroEL de procariotos. É descrito em *S. cerevisiae* que o produto gênico de *tcp*-1 está relacionado ao desenvolvimento e organização do citoesqueleto (Chen et al., 1994). A indução destes transcritos em *P. brasiliensis* provavelmente reflete as condições de choque térmico que ocorre durante a transição de micélio para levedura.

Outra classe de proteínas cujos transcritos foram regulados positivamente durante a transição dimórfica é a classe das glicosiltransferases (Parente *et. al.*, 2008). Estas proteínas apresentam funções na biossíntese de várias moléculas, incluindo componentes da parede celular de fungos (San Blas 1982). Em *C. albicans* mutantes para genes codificantes para manosiltransferases (*och*1 e *mnt*1) apresentam sensibilidade aos agentes perturbadores da parede celular, sugerindo o papel destas proteínas na manutenção da parede celular (Munro et al., 2005; Bates et al., 2006). Um transcrito codificantes para uma guanosina difosfatase (*gmd*1) foi detectado e descrito como um novo gene em *P. brasiliensis*. Esta proteína regula a manosilação no complexo de Golgi (Vowels & Payne 1998). A indução dos transcritos codificantes para estas proteínas durante o dimorfismo de *P. brasiliensis* pode ser associado ao remodelamento da parede celular necessário para que ocorra a transição morfológica.

Também o processo de aceleração do enovelamento de proteínas foi induzido durante a transição dimórfica de micélio para forma leveduriforme em *P. brasiliensis*. Transcritos codificantes para duas peptidil prolil cis-trans isomerases foram reguladas positivamente durante a transição dimórfica (Parente *et. al.*, 2008). O aumento na transcrição destes genes poderia refletir o aumento na produção de proteínas que ocorre nesta etapa de transição em *P. brasiliensis.* A importância desta classe de proteínas em microorganismos patogênicos não está somente na função de enovelamento de outras proteínas desde que estas isomerases são importantes para virulência de fungos, como descrito para *C. neoformans* (Wang et al., 2001).

Genes codificantes para proteínas relacionadas ao processamento protéico foram induzidos na transição de micélio para levedura. Um deles é o transcrito codificante para uma lon protease pertencente à família S16, (classe 001) regulado positivamente durante a transição dimórfica (Parente *et. al.*, 2008). Em *E. coli*, esta classe de proteases atua no controle de qualidade das proteínas produzidas (Swamy & Goldberg, 1981).

O transcrito codificante para uma metaloprotease M28 foi identificado. Este novo gene descrito para *P. brasiliensis* codifica para uma leucil aminopeptidase (Parente *et. al.*, 2008). O gene codificante para esta protease em *Thichoderma harzianum* apresenta níveis de expressão aumentados durante privação de nitrogênio, sugerindo a importância de leucil aminopeptidases em processos tais como a aquisição de aminoácidos (Suarez *et. al.* 2007).

Capítulo IV

Serino Protease de P. brasiliensis

Characterization of a secreted serine protease from *Paracoccidioides brasiliensis*

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Summary

Paracoccidioides brasiliensis is a thermodimorphic fungus, the causative agent of paracoccidioidomycose (PCM). A cDNA (*Pb*sp) encoding a secreted serine protease (*Pb*SP), was isolated from a cDNA library constructed with RNAs of fungal yeast cells recovered from liver of infected mice. Recombinant *Pb*SP was produced in *Escherichia coli*, and used to develop polyclonal antibody that was able to detect a 66 kDa protein in the *P. brasiliensis* proteome. *In vitro* deglycosylation assays with endoglycosidase H demonstrated that *Pb*SP is a *N*-glycosylated molecule. *Pb*SP was induced during nitrogen starvation both in *P. brasiliensis* proteins extracts and culture supernatants, suggesting its importance in the nitrogen acquisition. The *Pb*sp expression was higher in yeast cells compared to mycelia and in yeast cells after internalization by murine macrophages. Interactions of *Pb*SP with other *P. brasiliensis* proteins were evaluated by two-hybrid assay in the yeast *Saccharomyces cerevisiae*. *Pb*SP interacts with a peptidyl prolil cys-trans isomerase, calnexin, HSP70 and a cell wall protein PWP2

Keywords: *Paracoccidioides brasiliensis*, Secreted serine protease, *N*-glycosylation, macrophage infection, two-hybrid assay.

Introduction

Proteases are enzymes that cleave proteins catalyzing the hydrolysis of a peptide bond. Serine protease is a class of peptidases widely distributed in all domains of life that use a serine residue of that active site to cleave peptides (Rawlings & Barret 1993). Proteases are widely produced amongst fungi playing role in the nutrient cycling and post-translational processing (North, 1982).

Serine proteases are associated to virulence in many pathogens. In the human pathogen *Tiyhcophyton rubrum* seven serine proteases genes were detected, two of them encoding products able to cleave keratin, suggesting the importance of these proteases in the invasion process in the human host (Jousson *et al,* 2004). A vacuolar serine protease of the plant pathogen *Magnaporthe grisea* is induced during nitrogen starvation. This protease has been associated to virulence since the serine protease depleted mutant strain presents lower infection ability when compared to the wild type cells (Donofrio *et al,* 2006). Proteomics analysis of *Cryptococcus neoformans* culture supernatants detected several proteases able to cleave cytokines, complement components and extracellular matrix compounds facilitating tissue invasion (Eigenheer *et al* 2007).

Paracoccidioides brasiliensis is a thermally dimorphic fungus with a broad distribution in Latin America, the causative agent of the paracoccidioidomycosis (Franco *et al*, 1994). The infection is initiated by inhalation of airborne propagules of the mycelium, which reach the lungs and differentiate into the yeast parasitic phase (Lacaz, 1994). Few *P. brasiliensis* proteases have been characterized. Analysis of the ESTs in the transcriptome of mycelim and yeast cells revealed a total of 53 open reading frames (ORFs) encoding proteases in *P. brasiliensis*. The deduced amino acid sequences allowed the proteases to be classified in aspartyl, cysteine, metallo, serine proteases and proteasome subunits (Parente *et al*, 2005). Also in *P. brasiliensis* an extracellular subtilisin-like serine protease has been detected in the fungal yeast phase (Carmona *et al*., 1995). This protease is inhibited by PMSF (phenylmethyl-sulphonyl fluoride), mercury acetate and *p*-HMB (p-hidroximercurio benzoato), allowing to classify the protein as a serine-thiol protease. This serine-thiol protease was able to cleave, *in vitro*, murine laminin, human fibronectin, type IV-collagen and proteoglycans (Puccia *et al*, 1998). The serine-thiol activity of *P. brasiliensis* is

modulated by fungal extracellular galactomannan, which might act stabilizing the enzyme conformation (Matsuo *et al*, 2006). An aspartil protease has been recently characterized in *P. brasiliensis.* The cDNA encoding the aspartil protease (*Pb*sap) and the deduced amino acid sequence enconding this protease (*Pb*SAP) were identified and characterized. The recombinant protein was obtained and used to develop polyclonal antibody that detects a 66 kDa protein in the *P. brasiliensis* protein extract and culture supernatant, suggesting that *Pb*SAP is a secreted molecule. *Pb*SAP is located in the yeast cell wall by immunoelectron microscopy. Deglycosylation experiments demonstrated *N*-glycosylation of the *Pb*SAP molecule. Zymogram assays indicated the presence of aspartyl protease gelatinolytic activity in yeast cells and culture supernatant (Tacco et al., *in press*).

Transcriptome analysis of the *P. brasiliensis* yeast cells recovered from infected mice revealed a serine protease transcript positively regulated (Costa *et al*, 2007). The transcript encoding the serine protease was also induced in *P. brasiliensis* after incubation of yeast cells in human blood and plasma (Bailão et al., 2006, 2007). In the present work we sought to amplify our studies of this serine protease (*Pb*SP). The complete cDNA was isolated and characterized. The recombinant protein was obtained and used to generate polyclonal antibody in mice, which detected the serine protease in the fungal yeast cells extract as well as in fungal culture supernatants. *Pb*SP is induced during nitrogen deprivation suggesting its importance in the nitrogen acquisition. The presence of a carbohydrate chain linked to the native molecule was inferred by N-deglycosylation experiments. *Pb*sp expression was evaluated by Real Time RT-PCR in mycelium, yeast cells and yeast cells during macrophage internalization, presenting higher level in yeast cells inside macrophages. *Pb*SP interaction with other *P. brasiliensis* proteins was evaluated by two-hybrid assay in *S. cerevisiae*. *Pb*SP interactcs with proteins related to folding process such as FKBP-peptydil prolyl cis-trans isomerases and calnexin. Also, interaction of *Pb*SP with proteins related to quality control such as methionine aminopeptidase II was reported. Interaction of *Pb*SP with a mitochondrial HSP70 protein was identified, reflecting possible translocation to cell compartments. *Pb*SP also interacts with a cell wall associated periodic tryptophan protein.

Materials and methods

P. brasiliensis isolate growth conditions

P. brasiliensis isolate *Pb*01 (ATCC MYA-826) was used in all the experiments. Yeast cells were grown at 36°C in Fava-Netto's medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 1,2% (w/v) agar, pH 7.2]. For nitrogen starvation experiments, *P. brasiliensis* yeast cells were cultured in liquid MMcM minimal medium (Restrepo & Jimenez 1980) without ammonium sulfate, asparagine and cistine for eight hours. Control condition was performed by incubation of yeast cells in liquid MMcM minimal medium containing the nitrogen sources for eight hours.

Obtaining the *P. brasiliensis* serine protease cDNA and bioinformatics analysis

A complete cDNA encoding a *P. brasiliensis* homologue of the serine protease was obtained from a cDNA library of yeast cells recovered from liver of infected mice (Costa *et al*, 2007). The cDNA was sequenced on both strands by using the MegaBACE 1000 DNA sequencer (GE Healthcare) and the predicted amino acid sequence was obtained. The protease classification was performed by using the MEROPS database (http://merops.sanger.ac.uk). The entire nucleotide sequence, *Pb*sp, and the predicted amino acid sequence, *Pb*SP, have been submitted to the GenBank database under accession number AY319300.

The National Center for Biotechnology Information (NCBI) BLASTp algorithm (http://www.ncbi.nlm.nih.gov) was used to search in the non-redundant database for proteins with sequence similarities to the translated full-length *Pb*SP cDNA. The ScanProsite algorithms (http://ca.expasy.org/tools/scanprosite/) were used to search for motifs and conserved domains in the deduced protein. The presence of signal peptides was identified by using the SignalP program (http://www.cbs.dtu.dk/services/SignalP/), while the prediction of cellular localization was performed by using the PSORT II algorithm (http://psort.ims.u-tokyo.ac.jp/form2.html). The complete genomic sequence of *Pb*sp was obtained in the *Paracoccidioides brasiliensis* genomic database (http://www.broad.mit.edu/science/projects/msc/data-release-summary) and the promotor

region was analyzed by using the Promotor scan algorithms (http://www-bimas.cit.nih.gov/cgi-bin/molbio/proscan).

Cloning of *Pb*SP cDNA into expression vector

Oligonucleotide primers were designed to amplify the complete cDNA encoding the *Pb*SP. The nucleotide sequence of the sense and antisense primers were 5` - TCTGGATCCATGAAAGGCCTCTTCGC – 3' and 5` - ACACTCGAGTCCAGAGATGAAAGCGTT– 3`, which contained engineered *Bam*HI and *Xho*I restriction sites, respectively (underlined). The amplification parameters were as following: an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 90 s, annealing at 50°C for 75 s, and extension at 72°C for 2 min; final extension was at 72°C for 5 min. The PCR product was electrophoresed and a 1.5-kb amplicon was gel excised and cloned into the pGEX-4T-3 expression vector (GE Healthcare). The recombinant plasmid was used to transform the *E. coli* strain C43 competent cells by using the heat shock method (Sambrook & Russel 2001). Ampicilin-resistant transformants were cultured, and plasmid DNA was analyzed by PCR and DNA sequencing, as described above.

Heterologous expression of *Pb*SP and antibody production

Cultures of transformed *E. coli* containing pGEX-4T-3-PbSP were grown in Luria-Bertani (LB) medium supplemented with 100 µg/ml of ampicillin, at 37°C. As the cells reach the log phase (A_{600}=0.6), IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the growing culture to a final concentration of 0.5 mM to induce protein expression. After 2 h incubation, the bacterial cells were harvested by centrifugation at 5.000 *g* and ressuspended in phosphate saline buffer (PBS) 1X. *E. coli* cells transformed with pGEX-4T-3 without insert and *E. coli* without any vector were used as control. The cell extracts ressuspended in PBS 1X were electrophoresed on a 10% SDS-PAGE, followed by Coomassie brilliant blue staining. The protein species corresponding to *Pb*SP fused to glutathione S transferase (*Pb*SP-GST) was excised from the gel and 200 µg of the material was used to inoculate mice through subcutaneous injection. Animal was boosted three times, at 2 weeks intervals, with the same amount of antigen. The obtained serum,

containing anti-*Pb*SP polyclonal antibody was sampled and stored at -20°C. Preimmune serum was obtained.

Obtaining cell extracts and secreted proteins of *P. brasiliensis*

Total protein extracts from yeast and mycelium was obtained. Frozen cells (3g) were disrupted by complete grinding with a mortar and pestle in buffer (20 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$) without protease inhibitors. The mixture was centrifuged at 15.000 *g* at 4°C, for 20 min; the supernatant was sampled, and stored at -80°C. Culture supernatant of yeast cells was obtained after eight hour incubation in liquid MMcM minimal medium. The cells were separated by centrifugation at 5.000 *g* for 15 min and the supernatant was filtered in a 0,22μm filter (MilliPore). The culture supernatants were dialyzed with water during 4 h. Secreted protein fraction was concentrated with ice-cold acetone (v/v) during 16 h, centrifugated at 15.000 *g* for 15 min and the pellet was washed with 70% ice-cold acetone. Each 50 mL of culture supernatant was concentrated to 500 μL in Tris-HCl 25 mM pH 7,0. Protein concentration of all the samples was measured by using Bradford reagent (Sigma Aldrich) using BSA as standard.

Western blot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Proteins were electroblotted to a nylon membrane and transfer was checked by Pounce S staining. The membrane was blocked with 5% (w/v) non-fat dried milk in PBS 1x (pH 7.4). Serine protease was detected with the polyclonal antibody to the recombinant protein. After reaction with alkaline phosphatase anti-mouse immunoglobulin G (IgG), the reaction was developed with 5-bromo-4-cloro-3-indolylphosphate-nitroblue tetrazolium (BCIP-NBT). Negative controls were obtained with preimmune serum.

Glycosylation analysis

Total protein extract from yeast cells was incubated with recombinant endoglycosidase H (Endo H) from *Streptomyces plicatus* (Sigma-Aldrich), for 16 h at 37°C. The reaction mixture (100 μl) contained 30 μg of the protein extract and 27 mU Endo H in 60 mM sodium acetate buffer pH 5.8. Samples were analyzed by western-blot.

Azocasein assay

The azocasein assays were performed with azocasein diluted to 5mg/mL in buffer containing 25 mM Tris-HCl, 200 mM NaCl, 25 mM $CaCl_2$, 0,05% Nonidet P-40 and 0,01% NaN_3. 200 μg of *P. brasiliensis* total protein extract and 100 μL of *P. brasiliensis* culture supernatants were used in each assay. Proteinase K (Sigma Aldrich) was used as a positive control.

Infection of murine macrophage by *P. brasiliensis* yeast cells

Bone marrow-derived macrophages were obtained by flushing the femurs of 4-12 weeks old female C57BL/6 mice, as previously described (Fortier and Falk, 2007). The prepared cells were cultured at 37 ºC under 6% CO_2 in RPMI 1640 medium (Biowhittaker, Walkersville, Md.) supplemented with 10% fetal bovine serum (FBS), 1% 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 adherent cells were washed off twice with 10 mL of Hank's Balanced Salt Solution (HBSS). The cells were treated with 10ug/mL of dispase in HBSS at 37C for 5 min. Further, macrophages were removed using a cell scraper and washed in HBSS. Cells were pelleted by centrifugation at 500 *g* for 5 min, and suspended in RPMI 1640 (supplemented as describe above, minus GM-CSF) at a concentration of 1 x 10^6 cells per mL. For infection experiments, 5 x 10^6 *P. brasiliensis* yeast cells were added to 2mL of macrophage suspension plated on 6 well plates. After 24h of co-cultivation at 37º C in 6% CO_2, the non-phagocyted yeasts were discarded and the bottom cells were washed twice with HBSS to remove unattached yeasts. The RNA of infected murine macrophages was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA). RNA from uninfected macrophages and *P. brasiliensis* grown in RPMI 2640 were obtained as controls.

Quantitative real-time PCR

RNA samples were reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT)15 primer. The cDNA samples were diluted 1:5 in water, and qRT-PCR was performed using SYBR green PCR master mix (Applied

Biosystems, Foster City, CA) in the Applied Biosystems 7500 real-time PCR system (Applied Biosystems Inc.). qRT-PCR was performed in triplicate for each cDNA sample. The specificity of each primer pair for the target cDNA was confirmed by the visualization of a single PCR product in agarose gel electrophoresis. The primers and sequences were used as follows: sp sense, 5'-GGCCTCTCCACACGTTGCTG-3'; sp atsense 5'-GTTCCAGATAAGAACGTTAGC-3' and α-tubulin primers: tubulin-sense, 5'-ACAGTGCTTGGGAACTATACC-3'; tubulin-atsense, 5'-GGACATATTTGCCACTGCCA-3'. The annealing temperatures for sp and tubulin primers were 60°C and 59°C, respectively. The standard curves were generated using the target fragments cloned into pCR2.1-TOPO (Invitrogen). The plasmids were diluted 1:1000, and a standard curve was generated using 5 samples, serially diluted 1:4 from the original dilution. The relative expression levels of genes of interest were calculated using the standard curve method for relative quantification (Bookout *et al*, 2006).

Interaction of *Pb*SP with *P. brasiliensis* proteins by Two-Hybrid assay

Oligonucleotides were designed to clone the complete cDNA encoding the *Pb*SP in the pGBK-T7 (Clontech Laboratories, Inc) expression vector. The nucleotide sequence of the sense and antisense primers were 5` - CATATGATGAAAGGCCTCTTCGCCT – 3' and 5` -CTGCAGTTAAGAGATGAAAGCGTTCTTG– 3`, which contained engineered *Nde*I and *Pst*I restriction sites, respectively (underlined). The pGBK-T7 contains the TRP1 gene which allows the selection in minimal medium without tryptophan and a GAL4 DNA-binding domain. The cloned product was used to transform a *Saccharomyces cerevisiae* strain Y187 (ΔTRP1). A cDNA library was constructed with RNA from *P. brasiliensis* yeast cells and cloned in the expression vector pGADT7-Rec by using the Matchmaker™ Library Construction & Screening (Clontech Laboratories, Inc). The pGADT7-Rec vector contains LEU2 gene, allowing the selection in minimal medium without leucine and a GAL4 DNA-activation domain. The products cloned were transformed in *S. cerevisiae* strain AH109 (ΔLEU2). The Y187 strain containing pGBK-T7-*Pb*SP was used to screen the pGADT7-Rec library transformed in AH109 strain by yeast mating. The positive interactions activate the transcription of ADE2, HIS3 and MEL1 genes, which allows the selection in minimal medium without tryptophan, leucine, adenine and histidine. Minimal

medium without these amino acids and containing X-alpha-GAL also confirms the activation of the transcription of the MEL1 gene. The *Pb*SP baited clones were amplified by using AD-LD 5' and AD-LD 3' oligonucleotides for pGADT7-Rec and sequenced as described above. The positive interactions were confirmed by using the *in vitro* translation system TNT® T7 Coupled Reticulocyte Lysate Systems (Promega) with S^{35} methionine and coimmunoprecipitation of the translated proteins (Matchmaker™ Co-IP Kit, Clontech Laboratories, Inc). Briefly, the translated serine protease fused to c-myc epitope (c-myc-SP) and the translated proteins fused to hemaglutinin epitope (HA-Prey) were mixed at 25 °C for 1 h. The mixture was incubated with protein A Agarose beads and with the monoclonal c-myc antibody in PBS 1X at 25 °C for 1 h. After washing, the beads containing proteins were resuspended in SDS-loading buffer [50 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol, 2% (w/v) SDS; 0.1% (w/v) bromophenol blue; 10% (v/v) glycerol], followed by boiling at 80 °C for 5 min. The proteins were separated on a SDS-PAGE 4–12 % linear gradient. The gel was fixed with 20% (v/v) ethanol and 10 % (v/v) acetic acid for 30 min, and incubated in 20 mL of fluorographic reagent NAMP 100 (Amplify Fluorographic Reagent - GE Healthcare®). The gels were dried at 80 °C for 90 min under vacuum and autoradiography was obtained. Controls were performed. Each assay was repeated three times with a different batch of in vitro translated product to confirm the results.

Results

Analysis of the cDNA and of the deduced protein sequence

The Figure 1, supplementary material, shows the genomic and cDNA sequences, as well as the deduced protein encoding *Pb*SP. The cDNA sequence contains a 1491 bp open reading frame. The genomic sequence presents two introns and three exons. The deduced amino acid sequence presented 497 amino acids residues with a predicted molecular mass of 53 kDa and *pI* 6.12. *Pb*SP homology analysis in MEROPS database reveals homology with serine proteases from S08 family of subtilase (data not shown). Analysis of the promoter region reveals a TATA box and a 5'-GATA-3' domain, putatively related to nitrogen metabolite regulation (NMR). Also a 5'-GCCARG-3' binding motif related to pH regulation was found in the promoter region. Analysis of the deduced amino acid sequence

revealed a 16 amino acid signal peptide, suggesting that *Pb*SP is a secreted molecule. Comparisons of the predicted protein sequence with well-known serine proteases allowed us to identify three conserved amino acids residues DHS that compose the catalytic triad of the subtilase family. Six N-glycosylation sites were also predicted at positions 76-79, 98-101, 160-163, 245-248, 287-290 and 450-453 in the deduced protein sequence (Supplementary figure 1). The sequences of the serine proteases from *Ajellomyces capsulatus* and *Coccidioides immitis* showed the highest sequence identity to *Pb*SP (68%), followed by *Aspergillus fumigatus* (65%) (data not shown).

Cloning and expression of *Pb*SP in *E. coli* and antibody production

SDS-PAGE analysis of the transformants revealed that IPTG induced a dominant protein, migrating at 82 kDa (Figure 1 A, lane 2). This dominant protein was absent in the cells growing in the absence of IPTG (Figure 1 A, lane 1). The size of the induced protein is in accordance with the expected size of the *Pb*SP fused to glutathione S-transferase (GST). The polyclonal antibody produced against *Pb*SP reacted with the recombinant protein in western blot analysis (Figure 1 B, lane 2). No reaction was detected with preimmune serum (Figure 1 B, lane 1). The polyclonal antibody recognized a protein species of 66 kDa in *P. brasiliensis* protein extract (Figure 1 D, lane 1).

Deglycosylation assays

The *Pb*SP molecular mass detected in western blot analysis (Figure 1 D, lane 1) was higher in comparison to the value of the deduced protein. The probable glycosylation of the molecule was analyzed by treating total protein extract of yeast cells with endoglycosidase H. Treatment with endoglycosidase H rendered a protein species of 53 kDa (Figure 1 D, lane 2). The data support the inference that the 66 kDa protein in *P. brasliensis* yeast cells extract is the glycosylated form of the 53 kDa protein.

Analysis of proteases expression during nitrogen starvation in *P. brasiliensis*

The expression of *Pb*SP during fungal nitrogen starvation was analysed. *P. brasiliensis* yeast cells were incubated in MMcM medium without nitrogen sources as described above. Protease activity was measured by using an azocasein assay in protein

extracts and culture supernatants. The total protease activity was higher in yeast cells in the absence of nitrogen sources, both in total protein extract and in culture supernatant in comparison to the control (Figure 2, 1-4).

The *Pb*SP expression was evaluated by western blot analysis after incubation of yeast cells in MMcM medium in absence and in the presence of nitrogen sources. *Pb*SP expression was higher in the nitrogen starvation condition both in total protein extract (Figure 2 B, lane 2) and culture supernatant (Figure 2B, lane 4) in comparison to the *Pb*SP expression after incubation of yeast cells in chemically defined medium containing nitrogen sources (Figures 2 B, lanes 1 and 3).

Analysis by real-time PCR of *Pb*sp expression

The *Pb*sp expression was evaluated by using real-time PCR in mycelium, yeast cells and in yeast cells infecting mice macrophages. *Pb*sp expression was lower in mycelium compared to yeast cells. Higher *Pb*sp expression was detected during the internalization of yeast cells by macrophages (Figure 3).

Interaction of serine protease with other *P. brasiliensis* proteins

The interaction of *Pb*SP with other *P. brasiliensis* proteins was evaluated by two-hybrid system in *S. cerevisiae*. The proteins identified interacting with *Pb*SP are described in Table 1. It was detected homologues of FKBP-peptidyl prolyl cis-trans isomerase, calnexin, HSP70 and a cell wall associated periodic tryptophan protein. Protein interactions were confirmed by coimmunoprecipitation assays and are shown in Figure 4.

Discussion

The partial cDNA encoding *Pb*SP was previously characterized and the deduced protein was classified as a member of the subtilisin family S08A (Parente et al., 2005), which comprehend endopeptidases generally presenting higher activity under alkaline conditions (Barret & Rawlings, 1995). The *P. brasiliensis* serine protease cDNA here characterized encodes a protein with a N-terminal 16 amino acids with the characteristic of a leader peptide. The protein sequence corresponding to the mature *Pb*SP shows high similarity with serine proteases sequences from other fungi. Analysis of the promoter

region revealed the presence of a nitrogen metabolite repression (NMR) region binding protein, responsible for positive regulation of genes in response to nitrogen metabolite presence such as AreA proteins in *Aspergillus nidulans* (Morozov *et al*, 2001) and Nit2 protein in *Neurospora crassa* (Chiang & Marzluf; 1995). Also, a 5'-GCCARG-3'binding motif was found in the gene promotor. In *A. nidulans* the PacC protein binds to this motif activating transcription of genes expressed in alkaline condition (Tilburn *et al*, 1995). The data suggest that *Pb*SP could be a molecule regulated by the nitrogen metabolite presence and positively regulated in alkaline conditions.

The recombinant *Pb*SP was obtained fused to GST, exhibiting a molecule of 82 kDa. By using the recombinant protein, polyclonal antibody was obtained in mice. The serum, specifically, recognized the recombinant protein as well as a protein species of 66 kDa in *P. brasiliensis* yeast cells extract. Treatment of fungal protein extracts with endoglycosidase H resulted in a 53 kDa protein species, corresponding to the *Pb*SP *in silico* deduced molecular mass. The data suggest that the 13 kDa additional in the 66 kDa species is due to N-glycosylation.

The total protease activity was evaluated during fungal nitrogen starvation by incubating yeast cells in chemically defined medium without nitrogen sources. Protease activity, including *Pb*SP expression, was higher during nitrogen starvation. The result suggests that proteases can be important in the response induced by nitrogen starvation in *P. brasiliensis*. Also, the results suggest that *Pb*SP is a secreted protein involved in nitrogen acquisition, relevant in nitrogen starvation response in *P. brasiliensis*. Similar results were described to the bacteria *Natrialba magaddi,* which present higher protease activity level in chemically defined medium without nitrogen source (D'Alessandro *et al.,* 2007). Serine proteases are described as associated with nitrogen acquisition and pathogenicity in the fungal pathogen *M. grisea*. In this fungus, a serine protease is highly induced during nitrogen starvation. This protease is encoded by the *isp* gene and is associated to pathogenicity since Δ*isp* fungal cells present lower infection index in rice when compared with wild type cells (Donofrio *et al.,* 2006). The nitrogen starvation response can be important to human pathogens since neutrophil phagosome presents lower nitrogen concentration. In this way, the *S. cerevisiae* and *C. albicans* transcriptional profile caused by neutrophil internalization is most similar to that of amino acid deprivation (Rubin-

Bejerano *et al,* 2003).

The *Pb*sp expresion was evaluated by quantitative RT-PCR in mycelium, yeast cells and yeast cells during infection in macrophage. The *Pb*sp expression was higher in yeast cells during macrophage infection suggesting that this serine protease could be important in the fungal adaptative mechanism into macrophage cells. A subtilisin like serine protease from *Mycobacterium tuberculosis* is described as a cell wall-associated protein and is induced during infection of macrophages (Dave et al., 2002).

Two hybrid assays were performed to detect *P. brasiliensis* proteins interactions with *Pb*SP. *Pb*SP interacts with proteins presumably related to protein processing such as FKBP-peptidyl prolyl cis-trans isomerase, calnexin and HSP70. A calnexin homolog was identified, possible related to the retention of incorrectly folded proteins (Zhang *et al*, 1997). The calnexin of the yeast *Hansenula polymorpha* have been related to increase in the secretion of proteins (Klabunde et al., 2007). HSP70 was found interacting with *Pb*SP and possible plays role in the trafficking of serine protease into and through the compartments in the cell (Craig et al., 1989). Interactions of HSP70 proteins with proteins have been described. HSP70 interacts with the aquaporin 2 present in the principal cells of the kidney collecting duct, regulating its trafficking (Lu *et al.*, 2007).

Acknowledgments

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fungus *Paracoccidioides brasiliensis*. Yeast. 2007. 24:79-87.

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Table 1- Proteins identified interacting with *Pb*SP by using two-hybrid system in *S. cerevisiae*.

Gene Product	Best hit	e-value	Number of obtained clones
FKPB-type peptidyl prolyl cis trans isomerase	*Aspergillus clavatus* XP_001274819	$2e^{-25}$	4
Calnexin	*Paracoccidioides brasiliensis* ABB80132	$2e^{-28}$	2
Mitochondrial 70 kDa heat shock protein	*Paracoccidioides brasiliensis* AAP05987	$6e^{-83}$	2
Periodic tryptophan protein PWP2	*Ajellomyces capsulatus* XP_001543414	$2e^{-30}$	1

Figure legends:

Supplementary Figure 1: The cDNA and the genomic sequences encoding the serine protease (*Pb*SP) of *P. brasiliensis*. The nucleotide and amino acid positions are marked on the left side. Lower case letters represent the untranslated 5' region. Bold letters in nucleotide sequence represent the start and stop codons. Two introns were found in the genomic sequence and are shown in italic. Three conserved residues (marked with arrows) of amino acids (asparagine - D; histidine – H and serine – S) belonging to the active site of serine proteases from the subtilase family S08 are evidenced. Six putative N-glycosylation sites are marked in bold letters. A signal peptide formed by the first 16 amino acids is underlined. The TATA box in the promoter region is evidenced with a black box. A GATA binding region of the transcription factor AreA was found and is evidenced by a white box. A PACC protein binding domain is evidenced in a gray box.

Figure 1: Reactivity of the polyclonal antibody anti-*Pb*SP and deglycosylation assay. A: SDS-PAGE of *E. coli* extracts. 1: *E. coli* extracts; 2: *E. coli* protein extract obtained after 0.5 mM IPTG treatment. The arrow indicates the protein species corresponding to *Pb*SP fused to the GST protein. B: Western blot assay, the same extracts as in A reacted to: 1: Control mice preimmune serum. 2: Polyclonal anti-*Pb*SP. C: SDS-PAGE of *P. brasiliensis* extracts 1: Total protein extract of yeast cells from. 2: Total protein extract of yeast cells treated with endoglycosidase H for 16 h. D: Western blot using the polyclonal antibody anti-*Pb*SP reacted with the protein extracts of panel C.

Figure 2: Proteolytic activity in *P. brasiliensis* and *Pb*SP expression analysis. Yeast cells were incubated in chemically defined MMcM medium and MMcM medium without nitrogen sources (ammonium sulfate, asparagine and cystine) for 8 h. After incubation, total protein extraction was performed. The culture supernatants were precipitated with ice-cold acetone. Protease activity was obtained by using azocasein with 200 µg of total protein extract of yeast cells or the same protein amount of protein obtained from culture supernatant. Protease activity was measured at 436 nm. A: 1: Protease activity in total protein extract of yeast cells after incubation in MMcM minimal medium; 2: Protease

activity in total protein extract of yeast cells after incubation in MMcM minimal medium without nitrogen sources; 3: Protease activity in culture supernatant of yeast cells after incubation in MMcM minimal medium; 4: Protease activity in culture supernatant of yeast cells after incubation in MMcM minimal medium without nitrogen sources. B: Western blot assay using the polyclonal antibody anti-*Pb*SP of protein extracts of. 1: yeast cells cultured in MMcM medium; 2: yeast cells cultured in the same medium deprived of nitrogen; 3: culture supernatant of yeast cells in MMcM medium; 4: the same as in 3 in the absence of nitrogen.

Figure 3: *Pb*sp transcript expression analysis. Mycelium and yeast cells were grown in medium and RNA extraction was performed. Macrophage cells were grown in RPMI medium, and infected with *P. brasiliensis* yeast cells and total RNA was obtained. RNAs were used to perform quantification of *Pb*sp expression by Real Time-RT-PCR. Reactions were performed in triplicate and normalized by using α-tubulin expression. M: Expression level of *Pb*sp in mycelium. Y: Expression level of *Pb*sp in yeast cells. I: Expression level of *Pb*sp in yeast cells during macrophages infection.

Figure 4: Co-immunopreciptation of *P. brasiliensis* proteins putatively interacting with *P. brasiliensis Pb*SP. The proteins were in vitro synthesized and labeled with ^{35}S methionine. The translated serine protease molecules fused to c-myc epitope (c-myc-SP) and the translated proteins fused to hemaglutinin epitope (HA-Prey) were mixed and the mixture was incubated with protein A agarose beads and the monoclonal antibody anti-c-myc. The proteins were separated by SDS-PAGE. The gel was fixed, dried under vacuum and autoradiography was obtained. 1: Peptidyl proly cis-trans isomerase; 3:Calnexin; 5: HSP70; 7: Periodic tryptophan protein (PWP2). Negative controls for each reaction were performed and are shown in the lanes 2, 4, 6 and 8, respectively.

Supplementary figure 1

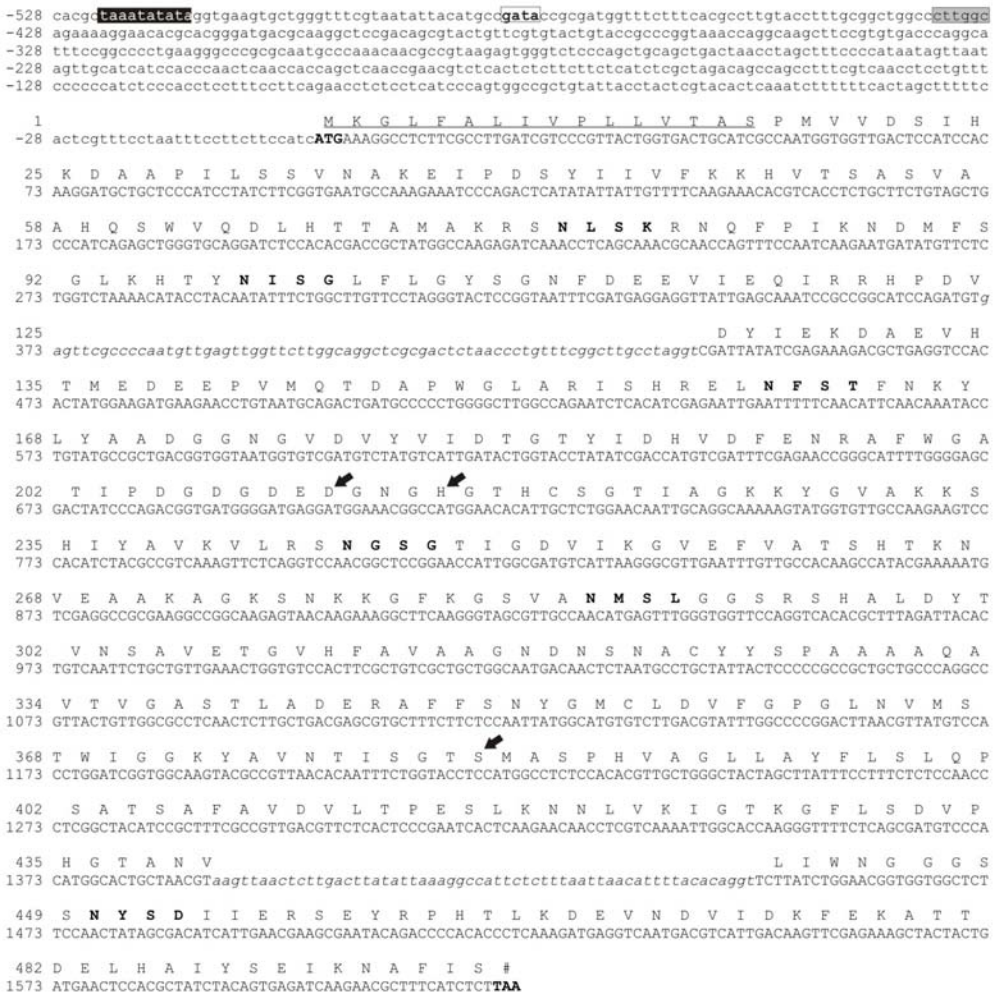


Figure 1

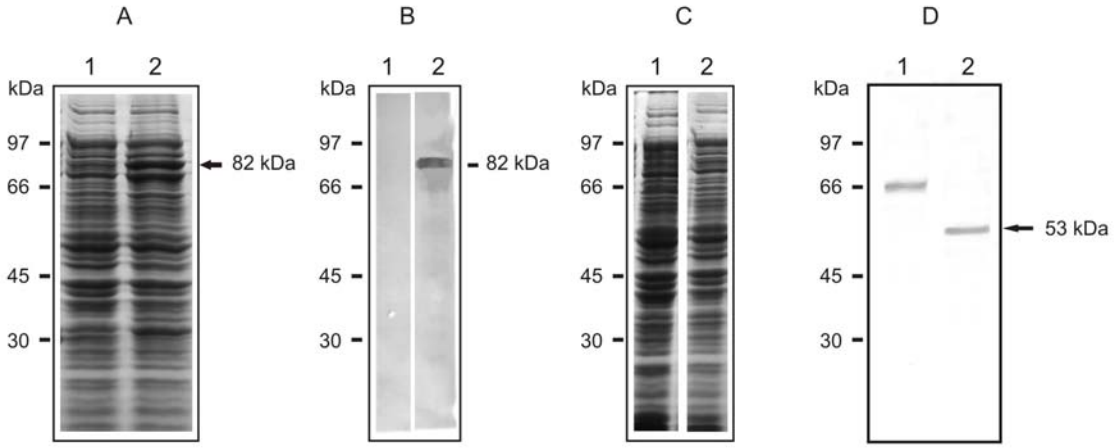


Figure 2

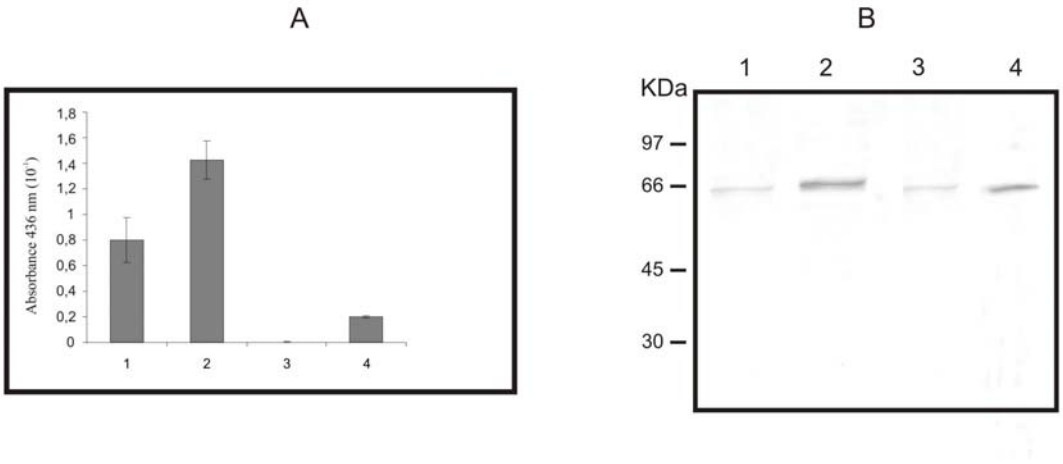


Figure 3

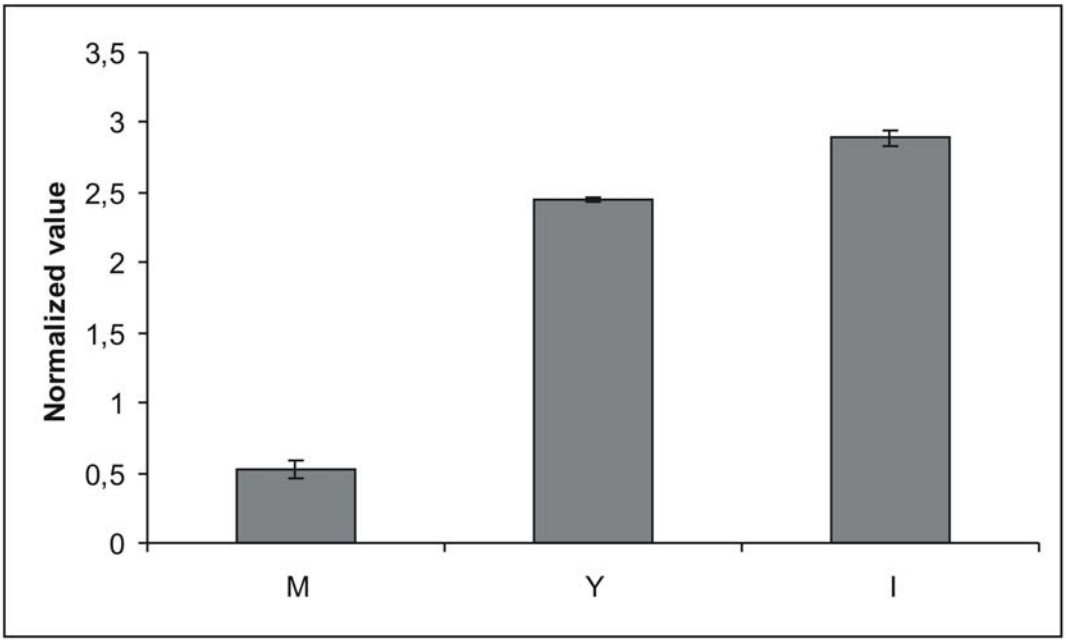
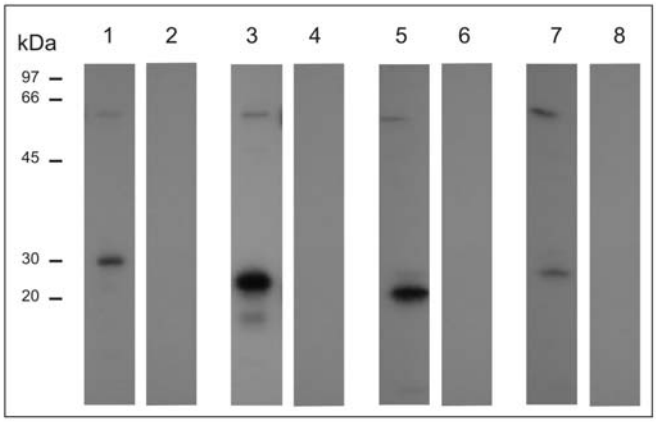


Figure 4



Discussão

Caracterização de um serino protease de *P. brasiliensis*

Discussão e Conclusões

Análises de transcritoma de células leveduriformes de *P. brasiliensis* obtidas a partir de fígado de camundongos infectados (http://www.lbm.icb.ufg.br/phorestwww/index.php) mostraram que uma serino protease S08A apresentou regulação positiva nos níveis de transcritos quando comparados com banco de dados de *P. brasiliensis* após cultivo *in vitro* (Costa et al., 2007). Esta protease também foi diferencialmente expressa em experimentos de análise representativa diferencial (RDA) de *P. brasiliensis,* células leveduriformes, após incubação com sangue e plasma humanos (Bailão et al., 2006; 2007). Serino proteases da família S08A são geralmente endopeptidases que apresentam atividade sob condições alcalinas de pH (Barret & Rawlings, 1995).

No sentido de elucidar a importância desta serino protease de *P. brasiliensis*, o cDNA completo foi obtido (*Pb*sp) e seqüenciado. A seqüência predita de aminoácidos da serino protease (*Pb*SP) foi obtida e analisada. A região promotora foi também obtida, sendo possível encontrar uma região rica em TATA, provavelmente relacionada à ligação de fatores transcricionais. A região promotora contém uma seqüência 5'-GATA-3', relacionada à regulação por metabólitos de nitrogênio (NMR). A presença desta região está relacionada à regulação positiva de genes responsáveis pela utilização de fontes alternativas de nitrogênio em resposta à ausência das fontes preferenciais de nitrogênio amônia e/ou glutamina. Em *A. nidulans*, na ausência das fontes preferenciais de nitrogênio, a proteína AreA liga-se na região NMR da região promotora de genes responsáveis pela utilização de fontes alternativas de nitrogênio (Morozov *et al*, 2001). No fungo *Neurospora crassa*, proteínas Nit1 e Nit2 se ligam na região 5'-GATA-3' regulando positivamente genes relacionados ao metabolismo de fontes alternativas de nitrogênio (Chiang & Marzluf; 1995). Em *P. brasiliensis*, há evidências da regulação positiva da glicoproteína imunogênica GP43 pela proteína Nit2, visto que a região promotora do gene codificante para GP43 possui 23 sítios de ligação à proteína Nit2. Análises da expressão do transcrito codificante para GP43 mostraram que esta proteína é regulada por fontes primárias de nitrogênio nos isolados *Pb*339, *Pb*3 e *Pb*18 (Rocha et al., 2008).

Na região promotora do gene codificante para a serino protease foi identificada uma região 5'-GCCARG-3' relacionada à ligação da proteína PacC (Parente *et. al.*, em preparação). Em *A. nidulans,* a ligação da proteína PacC nesta região regula positivamente a transcrição de genes cujos produtos gênicos são expressos em condições de pH alcalino (Tilburn *et al*, 1995).

A seqüência predita de aminoácidos de *Pb*SP foi analisada e detectou-se uma seqüência de 16 aminoácidos, característica de uma região de peptídeo sinal. Também foram identificados resíduos conservados pertencentes ao sítio ativo em serino proteases S08A (asparagina – D, histidina – H, e serina – S). Foram detectados possíveis sítios de N-glicosilação. O cDNA codificante para serino protease foi clonado em vetor de expressão para sistema bacteriano e a proteína foi obtida, exibindo massa molecular de 82 kDa quando fusionada à proteína glutationa S transferase (GST). A proteína de fusão foi utilizada para obtenção de anticorpo policlonal em camundongos (anti-*Pb*SP). O soro contendo anti-*Pb*SP reconheceu especificamente uma proteína de 66 kDa em extrato protéico e sobrenadante de cultura de células leveduriformes de *P. brasiliensis*. Visto que as massa molecular de 66 kDa era maior que aquela predita *in silico,* foi realizado tratamento do extrato protéico de *P. brasiliensis* com endoglicosidase H, para avaliar a presença de N-glicosilação. O tratamento com endoglicosidase H resultou no aparecimento de uma espécie protéica de 53 kDa, correspondendo ao tamanho predito *in silico* de *Pb*SP, sugerindo a presença de N-glicosilação na molécula de *Pb*SP (Parente *et. al.*, em preparação).

Com o objetivo de avaliar a importância das proteases durante a privação de nitrogênio, extratos protéicos e sobrenadantes de cultura foram obtidos após a incubação de células leveduriformes de *P. brasiliensis* em meio quimicamente definido na presença e na ausência de fontes de nitrogênio. Os níveis de atividade proteolítica e a expressão de *Pb*SP foram avaliados em extrato protéico total e em sobrenadantes de cultura de *P. brasiliensis*. A atividade proteolítica e a expressão de *Pb*SP tanto no extrato protéico total quanto no sobrenadante de cultura apresentou nível mais alto após a incubação das células em meio de cultura privado de fontes de nitrogênio. Este resultado sugere que as proteases, incluindo *Pb*SP, são importantes na resposta ao estresse causado pela privação de nitrogênio em *P. brasiliensis* (Parente *et. al.*, em preparação). Resultados similares são descritos para a bactéria *Natrialba magaddi,* cujo nível de atividade proteolítica aumenta em resposta à ausência de nitrogênio em meio de cultura (D'Alessandro *et al.*, 2007). Uma serino protease do fungo fitopatogênico *M.*

grisea codificada pelo gene *isp* é induzida durante privação de nitrogênio. Sugere-se que esta proteína seja importante na patogênese em *M. grisea*, visto que células mutadas para o gene *isp* (Δ*isp*) apresentam capacidade reduzida de infectar folhas de arroz quando comparadas à linhagem selvagem (Donofrio *et al.*, 2006). A resposta à privação de nitrogênio em patógenos humanos é importante visto que alguns sítios de infecção apresentam baixas concentrações deste composto. Por exemplo, o interior do fagossomo de neutrófilos parece apresentar baixa concentração de nitrogênio visto que o perfil transcricional de *S. cerevisiae* e *C. albicans* durante a internalização por neutrófilos é similar ao perfil transcricional apresentado pelas duas leveduras durante a privação de aminoácidos (Rubin-Bejerano *et al.,* 2003). O nível de expressão de *Pb*sp foi avaliado em micélio, células leveduriformes e durante a infecção de células leveduriformes em macrófagos. O menor nível de expressão de *Pb*sp ocorre em micélio, enquanto o maior nível de expressão foi detectado durante a infecção de células leveduriformes em macrófagos. Estes resultados sugerem que esta serino protease pode ser importante na resposta adaptativa de *P. brasiliensis* à internalização por macrófagos (Parente *et. al.*, em preparação). Resultados similares são encontrados para o microorganismo patogênico *Mycobacterium tuberculosis*. Para este patógeno, uma serino protease da família das subtilisinas tem níveis de expressão aumentados durante a infecção em macrófagos. Esta protease se encontra associada à parede celular e também está presente em sobrenadante de cultura (Dave et al., 2002).

Ensaio de duplo-híbrido em *S. cerevisiae* foi realizado para detectar interações de *Pb*SP com outras proteínas de *P. brasiliensis*. Foram identificadas quatro proteínas interagindo com *Pb*SP: uma peptidil prolil cis-trans isomerase do tipo FKBP, calnexina, uma proteína de choque térmico HSP70 e uma proteína de parede celular rica em triptofano PWP2 (Parente *et. al.*, em preparação). Na bactéria *E. coli*, a proteina FKBP tem a função de catalisar a maturação de outras proteínas, contribuindo para aceleração do processo de enovelamento (Arié *et al*, 2001). A proteína calnexina atua no enovelamento correto de várias proteínas (Pollock et al., 2004). Na levedura *Hansenula polymorpha*, a função da calnexina tem sido relacionada com o aumento da secreção de outras proteínas (Klabunde et al., 2007). *Pb*SP interagem também com a proteína de choque térmico HSP70, associada à translocação de outras proteínas para compartimentos celulares em células eucarióticas (Craig et al., 1989). Interações de várias proteínas com HSP70 tem sido descritas. HSP70 interage com uma aquaporina tipo 2 presente nas células do ducto coletor do rim, regulando o tráfico desta proteína

dentro da célula (Lu *et al.*, 2007). Na levedura *S. cerevisiae*, PWP2 é um complexo proteináceo associado ao citoesqueleto que atua na hidrólise da parede celular durante a divisão celular (Shafaatian *et al*, 1996).

As funções das várias proteases identificadas em *P. brasiliensis* ainda não são elucidadas. Entretanto, várias proteases apresentaram-se reguladas positivamente em processos importantes tais como transição dimórfica, infecção em modelo animal e durante a incubação com sangue e plasma humanos (Bailão et. al., 2006; 2007; Costa et al., 2007; Bastos et. al., 2007; Parente et. al., 2008). Estes dados sugerem que esta classe de enzimas tenha importância no estabelecimento da infecção e na sobrevivência de *P. brasiliensis* em condições adversas às quais este fungo é submetido no organismo do hospedeiro. Estudos funcionais tornam-se necessários para elucidar as classes de proteases envolvidas nos processos de diferenciação e infecção. Da mesma forma, ampliar os estudos de interações intermoleculares em *P. brasiliensis* pode auxiliar na compreensão dos processos nos quais as diferentes proteases possam atuar.

Capítulo IV

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Characterization of a secreted aspartyl protease of the fungal pathogen *Paracoccidioides brasiliensis*

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Paracoccidioides brasiliensis is a thermally dimorphic fungus that causes paracoccidioidomycosis, a human systemic disease prevalent in Latin America. Proteases have been described as playing an important role in the host invasion process in many pathogenic microorganisms. Here we describe the identification and characterization of a secreted aspartyl protease (*Pb*SAP), isolated from a cDNA library constructed with RNAs of mycelia transitioning to yeast cells. Recombinant *Pb*SAP was produced in *Escherichia coli*, and the purified protein was used to develop a polyclonal antibody that was able to detect a 66 kDa protein in the *P. brasiliensis* proteome. *Pb*SAP was detected in culture supernatants of *P. brasiliensis* and this data strongly suggest that it is a secreted molecule. The protein was located in the yeast cell wall, as determined by immunoelectron microscopy. *In vitro* deglycosylation assays with endoglycosidase H, and *in vivo* inhibition of the glycosylation by tunicamycin demonstrated *N*-glycosylation of the *Pb*SAP molecule. Zymogram assays indicated the presence of aspartyl protease gelatinolytic activity in yeast cells and culture supernatant.

Keywords *Paracoccidioides brasiliensis*, secreted aspartyl protease, *N*-glycosylation, gelatinolitic activity

Introduction

Paracoccidioidomycosis (PCM), caused by *Paraccocidioides brasiliensis*, is a human systemic mycosis prevalent in rural areas of Latin America. Host infection is typically initiated by inhalation of airborne fungal spores. The disease, which occurs primarily in the lungs as a granulomatous infection, can disseminate via the bloodstream and/or lymphatic system to other organs systems [1].

Aspartyl proteases constitute one of the four super families of proteolytic enzymes showing acidic optima pH for enzyme activity. They are generally similar to pepsin, which is totally inhibited by pepstatin, and show preferential specificity for cleavage at peptide bonds between hydrophobic amino acid residues [2]. The proteins share many features, including a conserved three-dimensional structure consisting of two lobes with a deep, active site cleft that contains two conserved aspartic acid residues. The protein molecule is synthesized as a large inactive precursor, which is subsequently converted into a mature enzyme by removing the N-terminal peptide from about 45 residues. In this segment, a pro peptide binds to the active site cleft and prevents undesirable degradation during intracellular transport and secretion [3,4].

Extracellular proteases from pathogenic fungi fulfill a number of specialized functions during the infective

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process in addition to the simple role of digesting molecules for nutrient acquisition. Some studies investigating the role of extracellular aspartyl proteases in pathogenesis have focused on fungi. *Candida albicans* manifests a multigene secreted aspartyl protease family (SAP), with at least 10 members identified [5]. The SAPs 1–7 are differentially expressed during the infection. SAP1 and 3 are induced in acute infection. SAPs 2, 4, 5 and 6 are the most highly expressed proteins during the infection. SAP7 is expressed when *C. albicans* is located on mucosal surfaces [6]. *C. albicans* exposure to antifungal agents increases the expression of SAP4-6, suggesting their induction may be a part of a stress-related defense mechanism in *C. albicans* [7]. The aspergillopepsin aspartic protease from *Aspergillus fumigatus* is secreted in large amounts during infection of the mouse lung [8]. An aspartyl protease associated with the cell wall was detected in *Coccidioides posadasii* and the recombinant protein was reported as a putative candidate for a new vaccine [9].

P. brasiliensis proteases are beginning to be characterized. A total of 53 open reading frames (ORFs) encoding energy-independent and -dependent proteases in *P. brasiliensis* have been described. The proteases were classified according to the domains present in the active sites, in aspartyl, cysteine, metallo and serine proteases and proteasome subunits [10]. Also, an extracellular subtilisin-like serine protease activity has been characterized in the yeast phase of *P. brasiliensis* [11]. Inhibition assays with PMSF (phenylmethylsulphonyl fluoride), mercury acetate and *p*-HMB (p-hydroxymercuri benzoate) have classified the enzyme as a serine-thiol protease that is able, *in vitro* to selectively degrade murine laminin, human fibronectin, type IV-collagen and proteoglycans[12]. This serine-thiol activity of *P. brasiliensis* is regulated by neutral polysaccharides, including a fungal extracellular galactomannan, which might help stabilize the enzyme [13].

The transcriptome analysis of the *P. brasiliensis* mycelium transition to yeast cells, revealed a positively regulated aspartyl protease transcript [14]. In order to extend the characterization of the transcript we isolated the complete cDNA encoding a homologue of aspartyl protease from *P. brasiliensis*. The recombinant protein was used to generate rabbit polyclonal antibody, which detected the aspartyl protease in the cell wall of the fungal yeast cells as well as in fungal culture supernatants. The presence of a carbohydrate chain linked to the native molecule was inferred from N-deglycosylation experiments. Those observations indicate that *Pb*SAP is a *N*-glycosylated molecule secreted by *P. brasiliensis* yeast cells. This article provides the first comprehensive survey of an aspartyl protease in *P. brasiliensis* and provides initial insights into the role of protease in the fungus.

Materials and methods

P. brasiliensis *isolate growth conditions and differentiation assays*

P. brasiliensis isolate *Pb*01 (ATCC MYA-826) was used in all the experiments. It was grown at either 22°C for the mycelium form or 36°C for yeast cells in Fava-Netto's medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 4% (w/v) agar, pH 7.2]. *P. brasiliensis* yeast cells were also cultured in Fava-Netto's liquid medium, supplemented with 8 mg of bovine serum albumin (BSA) per ml, to induce protease secretion as described previously [15].

Obtaining the P. brasiliensis *aspartyl protease cDNA and bioinformatic analysis*

A complete cDNA encoding a *P. brasiliensis* homologue of an aspartyl protease was obtained from a cDNA library constructed with the RNA of mycelia in transition to yeast cells [14]. The cDNA was sequenced on both strands by using the MegaBACE 1000 DNA sequencer (GE Healthcare, Amersham Biosciences). The Pfam database [16] and MEROPS [17] described the classification of the predicted protease. The entire nucleotide sequence, *Pb*SAP, was submitted to the GenBank database under accession number AY278218.

The BLAST algorithm [18] was used to search in the non-redundant database of the National Center for Biotechnology Information (NCBI) [19] for proteins with sequence similarities to the translated full-length *Pb*SAP cDNA. Conserved sites and motifs in the deduced protein were screened using the profile scan [20] and ScanProsite algorithms [21]. The presence of signal peptides was identified using the SignalP program [22], while the PSORT II algorithm was employed for prediction of cellular localization [23]. Multiple sequence alignments were generated using the Clustal X 1.83 software [24].

DNA extraction and Southern blot analysis

The genomic DNA of *Pb*01 yeast cells was extracted following standard procedures [25] using phenol and phenol cloroform (v/v). The RNA was removed by digestion with RNAse (10 μg/ml) for 2 h at 37°C. The genomic DNA of *P. brasiliensis* (15 μg) was digested with selected restriction endonucleases. Digestion products were fractionated on a 1.0% agarose gel and

transferred to a nylon membrane, after denaturation for 15 min in 0.5 M NaOH. The 1.2-kb cDNA insert probe was labeled and hybridization was carried out using the Gene Images Random Prime Labeling Kit (GE Healthcare). The Gene Image CDP-Star detection module (GE Healthcare) was used for hybridization detection.

Cloning of Pb*SAP cDNA into expression vector*

Oligonucleotide primers were designed to amplify the 1.2-kb cDNA containing the complete coding region of *Pb*SAP, which encodes amino acids (aa) 1–400 (predicted full length protein). The nucleotide sequence of the sense and antisense primers were 5′-ACCGAATTC TATGAAGTTCTCTCTG - 3′ and 5′-ACCCTCGAGT CACTGTCTAGCCTTCG - 3′, which contained engineered *Eco*RI and *Xho*I restriction sites, respectively (underlined). The amplification parameters were as follows; an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 90 s, annealing at 60°C for 75 s, and extension at 72°C for 2 min; final extension was at 72°C for 5 min. The PCR product was electrophoresed and the 1.2-kb amplicon was gel excised and sub cloned into the pGEX-4T-3 expression vector (GE Healthcare). Using the heat shock method [25], the recombinant plasmid was used to transform the *E. coli* strain BL21 competent cells. Ampicillin-resistant transformants were cultured, and plasmid DNA was analyzed by PCR and DNA sequencing.

Heterologous expression of Pb*SAP, recombinant protein purification and antibody production*

Cultures of transformed *E. coli* containing pGEX-4T-3-*Pb*SAP were grown in Luria-Bertani (LB) medium supplemented with 100 μg/ml of ampicillin, at 37°C. As the cells reached the log phase ($A_{600} = 0.6$), IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the growing culture to a final concentration of 0.05 mM to induce protein expression. After 12 h incubation, at 15°C, the bacterial cells were harvested by centrifugation at 5,000 *g*, ressuspended in PBS 1x and incubated with lysozyme (100 μg/ml) before three 15-min sonications. The recombinant *Pb*SAP was expressed in the soluble form by bacteria, and the protein was purified by affinity chromatography under non-denaturing conditions, as previously reported [26]. The soluble fraction of cell lysate, containing the recombinant *Pb*SAP, was applied to an affinity Glutathione Sepharose™ 4B Resin column (GE Healthcare) under non denaturing conditions. The fusion protein was cleaved following exposure to thrombin protease (50 U/ml) addition and the fusion-partner-free recombinant protein was collected after 12 h of incubation. The purified recombinant protein was electrophoresed on a 12% SDS-PAGE, followed by Coomassie brilliant blue staining.

Rabbits were subcutaneously inoculated with the purified recombinant protein (300 μg) with 2 mg of aluminum hydroxide, $Al(OH)_3$, as adjuvant. Animals were boosted twice, at 2 weeks intervals, with the same amount of antigen. The serum thus obtained, containing anti-*Pb*SAP polyclonal antibody, was sampled and stored at −20°C. Determination of the antibody title was performed by ELISA and western blot. Preimmune serum was obtained.

Obtaining cell extracts and secreted proteins of P. brasiliensis

Total protein extracts from yeast and mycelium were obtained as described [27]. Frozen cells (3g) were disrupted by complete grinding with a mortar and pestle in buffer (20 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$) containing EDTA (5 mM) and phenylmethyl-sulphonyl fluoride (PMSF) (20 mM). The mixture was centrifuged at 15,000 *g* at 4°C, for 20 min; the supernatant was sampled, and stored at −80°C.

After 6 days at 36°C under agitation (150 *g*), yeast cells supernatant (supplemented with BSA) was obtained by centrifugation at 5,000 *g* for 15 min and the secreted protein fraction was concentrated by 50% (v/v) ice-cold acetone precipitation, at −20°C.

Cell wall proteins were obtained as described [28] with modifications. After the yeast cells were disrupted, the pellet obtained by centrifugation at 12,000 *g* was washed five times, sequentially, with the following ice-cold solutions: 5% NaCl, 2% NaCl, 1% NaCl and 1 mM PMSF. Cell wall proteins were extracted by boiling in SDS extraction buffer (50 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 2% SDS, 10 mM DTT) for 10 min. The treatment was carried out twice and the supernatant, which is identified as SDS-extract throughout the text, was analyzed by SDS-PAGE. The protein concentrations of all the samples were measured.

Western blot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) [29]. Proteins were electroblotted to a nylon membrane and checked by Ponceau S to access loading of equal amounts of protein. The membrane was blocked with

5% (w/v) non-fat dried milk in PBS 1 × (pH 7.4). Aspartyl protease was detected with the polyclonal antibody to the recombinant protein. After reaction with alkaline phosphatase anti-rabbit immunoglobulin G (IgG), the reaction was developed with 5-bromo-4-cloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT). Negative controls were obtained with rabbit preimmune serum.

Immunocytochemistry of the aspartyl protease

Yeast cells of *P. brasiliensis*, isolate *Pb*01, were fixed overnight at 4°C in a solution containing 2% (v/v) glutaraldehyde, 2% (w/v) paraformaldehyde, and 3% (w/v) sucrose in 0.1 M sodium cacodylate buffer at pH 7.2. The yeast cells were rinsed in the same buffer and postfixed for 1 h in a solution containing 1% (w/v) osmium tetroxide, 0.8% (w/v) potassium ferricyanide, and 5 mM $CaCl_2$ in sodium cacodylate buffer, pH 7.2. The material was dehydrated in a series of ascending acetones (30 to 100%) (v/v) and embedded in Spurr resin (Electron Microscopy Sciences, Washington, Pa.). Ultrathin sections were stained with 3% (w/v) uranyl acetate and lead citrate. The material was observed with a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan).

For ultrastructural immunocytochemistry studies, yeast cells were fixed in a mixture containing 4% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde, and 0.2% (w/v) picric acid in 0.1 M sodium cacodylate buffer at pH 7.2 for 24 h at 4°C. The cells were rinsed several times using the same buffer, and free aldehyde groups were quenched with 50 mM ammonium chloride for 1 h, followed by block staining in a solution containing 2% (w/v) uranyl acetate in 15% (v/v) acetone for 2 h at 4°C. The material was dehydrated in a series of ascending concentrations of acetone (30 to 100%) (v/v) and embedded in LR Gold resin (Electron Microscopy Sciences, Fort Washington, PA).

The ultrathin sections were collected on nickel grids, preincubated in 10 mM PBS containing 1.5% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20, (PBS-BSA-T), and subsequently incubated for 1 h with the polyclonal antibody against the recombinant aspartyl protease (diluted 1:100). After washing with PBS-BSA-T, the grids were incubated for 1 h with the labeled secondary antibody (anti-rabbit IgG, Au conjugated, 10 nm; diluted 1:20). Subsequently, the grids were washed with distilled water, stained with 3% (w/v) uranyl acetate, , and lead citrate and observed with a Jeol 1011 transmission electron microscope (Jeol, Tokyo, Japan). Controls were incubated with rabbit preimmune serum at 1:100, followed by incubation with the labeled secondary antibody.

Glycosylation analysis

Total protein extract from yeast cells was incubated with recombinant endoglycosidase H (Endo H) from *Streptomyces plicatus* (Sigma-Aldrich), for 16 h at 37°C. The reaction mixture (100 µl) contained 30 µg of the protein extract and 27 mU Endo H in 30 mM $CaCl_2$, 3 mM NaN_3, 1.2 mM PMSF, and 60 mM sodium acetate buffer pH 5.8. Control reactions were also incubated for 16 h at 37°C. For the tunicamycin assay, inhibition of cell growth was preliminary tested. Yeast cells (1×10^6 fungal cells/ml) in liquid Fava-Netto's medium were incubated with different concentrations of tunicamycin for 7 days at 36°C. Culture growth was monitored daily by counting the cells. The higher tunicamycin concentration that presented no cell growth inhibition was 20 µg/ml and this condition was used in the assays. The cells were harvested and subjected to total protein extraction, as previously described. The samples were analyzed by western blot.

Zymogram

Zymograms were used to search for native aspartyl protease activity in *P. brasiliensis* extracts. Total protein extract of yeast cells was resuspended in buffer containing Tris-HCl (20 mM pH 8.8) $CaCl_2$ (2 mM) PMSF (20 mM) and EDTA (5 mM); the secreted protein fraction was concentrated using ice-cold acetone, as described. The proteins were subjected to 8% SDS-PAGE- gelatin (sodium dodecyl sulfate polyacrylamide gel electrophoresis, co-polymerized with 0.15% gelatin) [30]. After protein fractionation the gel was washed three times, for 15 min each time, in 25 mM Tris-HCl pH 7.0, following by incubation at 37°C for 12 h in developing buffer (0.1 M Na_2HPO_4 adjusted to pH 4.0) and stained with Coomassie brilliant blue. Enzyme inhibition assays were performed by incubating the same samples in buffer containing Tris-HCl (20 mM pH 8.8) $CaCl_2$ (2 mM) and pepstatin A (10 µM) in reaction mixtures containing 5 µg of total or secreted proteins during 15 min at room temperature. In addition, the relevance of glycosylation to the activity of *P. brasiliensis* aspartyl proteases was evaluated by incubating 30 µg of the total protein extract of yeast cells with 54 mU Endo H at 37°C for 15 min in the conditions described above.

Results

Structural features of the cDNA and the deduced aspartyl protease

The cDNA sequence of 1361 bp contained an open reading frame of 1200 bp. The deduced amino acid sequence was 400 residues with a predicted molecular mass of 44 kDa and *pI* 5.27. Analysis of the N-terminal amino acid region revealed a 19-amino-acid signal peptide as well as a cleavage-signal sequence, which is consistent with an extracellular location for the *Pb*SAP (Supplementary Fig. 1 – online version only).

Comparisons of the entire amino acid sequence with those of well-known aspartyl proteases allowed us to recognize amino acid residues necessary for enzyme activity. The protein sequence contains two conserved domains that compose the aspartyl protease active site identified by the PROSITE algorithm at $\underline{D}^{104}XG^{106}XS^{108}XXW^{111}V^{112}$ and $\underline{D}^{288}T^{289}G^{290}$ (D is the active residue). Three N-glycosylation sites were also predicted at positions 139-142, 252–255 and 339–342 in the deduced protein sequence (Fig. 1A). The sequences of the aspartyl proteases from *Coccidioides posadasii* showed the highest sequence identity to *Pb*SAP (88%), followed by *Aspergillus clavatus* (87%) and *Aspergillus terreus* (87%). The similarity of *Pb*SAP to *C. albicans* SAPs 1–10 was from 40–47% (data not shown).

Southern blot analysis

Southern blot hybridization was performed to estimate the genomic organization of *Pb*SAP. The specific 1.2 kb probe was able to detect a single DNA copy in the *P. brasiliensis* genomic DNA, as demonstrated by specific hybridization profiles of DNA digested with the restriction enzymes (Fig. 1B). This finding is supported by computational analysis of the restriction sites in the *Pb*SAP cDNA sequence. The presence of one gene encoding *Pb*SAP in the fungus genome was confirmed by search analysis at the *Paracoccidioides brasiliensis* Genomic Database [31]. Deduced *Pb*SAP, excluding the pre-propeptide region, was 90% identical with isolate *Pb*03 and 91% identical with *Pb*18 (data not shown).

Expression of Pb*SAP in* E. coli *and antibody production*

SDS-PAGE analysis of the lysate of the transformants revealed that IPTG-induced a dominant protein, migrating at 72 kDa (Fig. 2A, lane 3). This dominant protein was absent in the cells growing in the absence of IPTG (Fig. 2A, lane 2), as well as in control cells (Fig. 2A, lane 1). Lysis of bacterial cells was followed by purification of the fusion protein using a glutathione-sepharose 4B column (Fig. 2A, lane 4), which was subsequently cleaved by thrombin protease. The cleaved purified recombinant protein migrated as a single species of 44 kDa (Fig. 2A, lane 5). The polyclonal antibody produced from *Pb*SAP reacted to the purified recombinant protein in western blot analysis (Fig. 2B, lane 2). No reaction was detected with rabbit pre-immune serum (Fig. 2B, lane 1).

Identification of the aspartic protease in fungal phases, in the extracellular culture fluid and in the SDS-extracts

To identify the protein that represents the aspartic protease, western blot analysis was performed. Total protein extracts from isolate *Pb*01, yeast and mycelium, were electrophoresed in 12% SDS-PAGE and stained with Coomassie brilliant blue (Fig. 3A, lanes 1 and 2, respectively). Western blot analysis showed only one cross-reacting protein species, with a molecular mass of 66 kDa, in both samples. This was more abundant in yeast cells (Fig. 3B, lane 1). By using the extracellular culture fluid and the SDS-extracted cell wall protein fraction (Fig. 3C, lanes 1 and 2, respectively), the 66 kDa protein species was identified in both samples (Fig. 3D, lanes 1 and 2, respectively). No reactivity was detected with the culture medium employed for fungal growth (data not shown). Preimmune serum was used as a control for all samples (Fig. 3E).

Immunogold localization of the aspartic proteinase of P. brasiliensis

Immunocytochemistry experiments were performed to define the cellular localization of the aspartyl protein in yeast cells of isolate *Pb*01. Gold particles were detected predominantly in the cell wall (Fig. 4A). Control samples obtained by incubation of the yeast cells with the rabbit preimmune serum were free of label (Fig. 4B).

Deglycosylation assays

The greater molecular mass of the aspartyl protease of *P. brasiliensis*, compared to the expected value of the deduced molecule, could be due to post-translational modification, such as glycosylation. This possibility was explored by treating samples with endoglycosidase H (Fig. 5, lane 2) and including tunicamycin in the yeast-culturing medium (Fig. 5, lane 3). Analysis was performed by immunobloting. Treatment with endoglycosidase H produced a protein species of 44 kDa (Fig. 5, lane 2). The tunicamycin treatment was also observed to generate a protein of 44 kDa (Fig. 5, lane 3). The data support the inference that the 66 kDa is the glycosylated form of the 44 kDa protein.

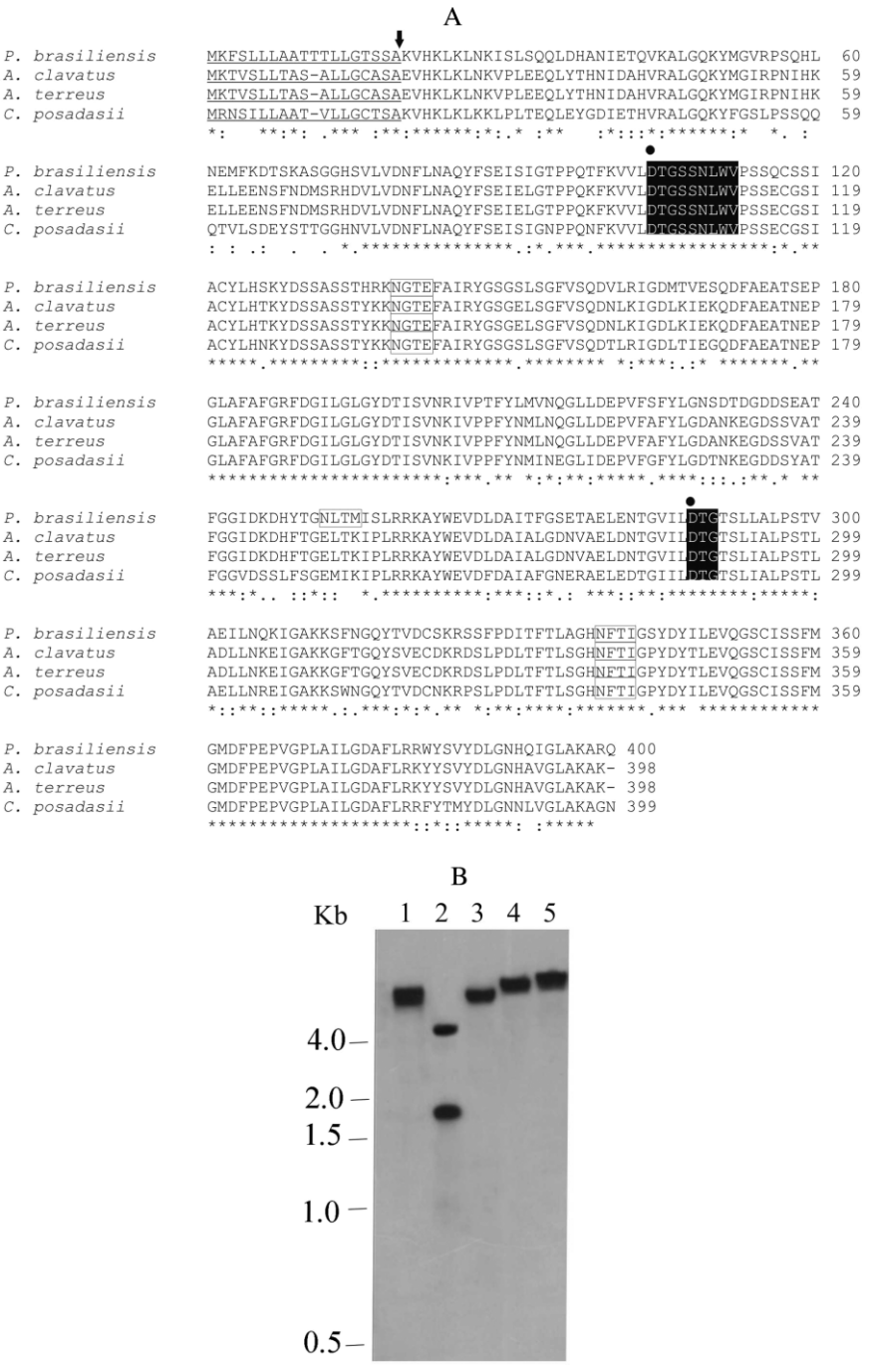

Fig. 1 Clustal X (1.83) multiple sequence alignment and *Paracoccidioides brasiliensis* aspartyl protease genomic organization. (A) Comparison of the predicted amino acid sequence of *Pb*SAP with the selected sequences of the fungal aspartyl proteases corresponding to *Aspergillus clavatus* (XM_001271140), *Aspergillus terreus* (XM_001213854) and *Coccidioides posadasii* (DQ164306). Conserved amino acids are marked by asterisks under the letters. The prepeptide sequence (signal peptide) is underlined and the arrow indicates the putative cleavage site. Amino acid sequences in black boxes represent the two conserved domains important to the active site. A black ball indicates the aspartic acid residue (D). The boxed sequences depicted the putative glycosylation sites. (B) Analysis of *P. brasiliensis* aspartyl protease genomic organization by Southern blot analysis. Total genomic DNA of *P. brasiliensis* (15 μg) was digested with the selected restriction endonucleases: (1) *Eco*RV, (2) *Bgl*II, (3) *Dra*I, (4) *Eco*RI and (5) *Sac*I. The DNA size marker is on the left.

Gelatinolytic activity of P. brasiliensis *native aspartyl proteases*

In order to investigate the activity of aspartyl proteases in total proteins and in extracellular culture fluid, a gelatin-incorporated SDS-PAGE zymogram was performed. The total protein extract of *P. brasiliensis* yeast cells and extracellular culture fluid showed a clear zone of proteolytic activity in the region of 66 kDa (Fig. 6A, lanes 1 and 2). The presence of aspartyl proteases in the gelatin degradation region was confirmed by the inhibition assay with pepstatin (Fig. 6A, lanes 3 and 4). Western blot assays with the specific antibody showed the presence of the *Pb*SAP protease (Fig. 6B,

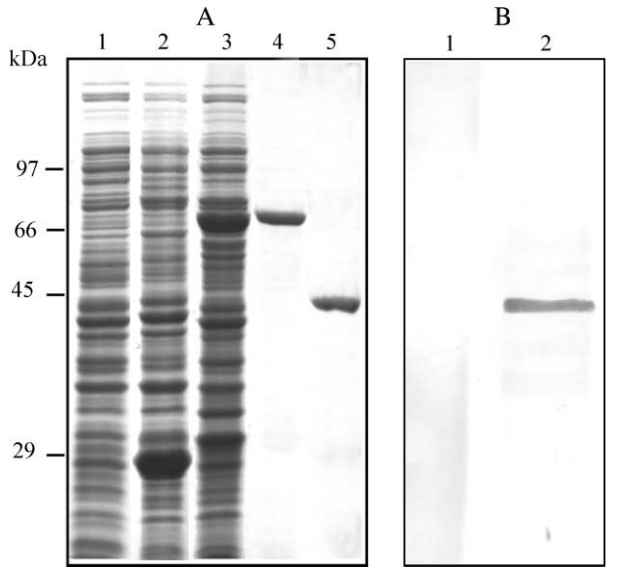

Fig. 2 SDS-PAGE and immunoblot analysis of the recombinant *Pb*SAP. (A) Profile of the Coomassie brilliant blue stained gel (12% SDS-PAGE) of *E. coli* expressing the recombinant aspartyl protease. The lanes are as follows: 1 – Control, *E. coli* extracts; 2 – Extracts of *E. coli* cells containing the pGEX-4T-3, after addition of 0.05 mM IPTG; 3 – Extracts of *E. coli* cells containing the expression vector pGEX-4T-3-*Pb*SAP, after addition of 0.05 mM IPTG; 4 – Recombinant *Pb*SAP fusion protein purified by affinity chromatography to Glutathione Sepharose™; 5 – Recombinant aspartyl protease cleaved by trombin protease. Protein molecular markers are indicated. (B) Western blot analysis. The purified *Pb*SAP cleaved by thrombin protease was reacted to: 1 – Control rabbit preimmune serum. 2 – Rabbit polyclonal antibody.

lanes 1 and 2). A control reaction was performed by using the preimmune serum in the total yeast protein extract and in the extracellular culture fluid (Fig. 6C, lanes 1 and 2, respectively).

Protease activity was inhibited in yeast cell protein extracts treated with endoglycosidase H (Fig. 6D, lanes 1 and 2) suggesting that glycosylation is essential for protein function.

Discussion

The *Pb*SAP characterized here was previously classified [10] as a member of the pepsin family (A1), which contains many enzymes that enter the secretory pathway. These proteins are synthesized as inactive zymogens activated by the self-cleavage of an N-terminal propeptide under acidic conditions [4]. The *P. brasiliensis* aspartyl protease cDNA encodes a protein that contains 19 amino acids at the N-terminal that are characteristic of a leader peptide. Computational analysis indicates that the protein must be synthesized as a precursor containing a 70-amino-acid prepropeptide at the N terminus of the mature protein. Alignment of sequences closely related to *Pb*SAP showed that, in addition of identical residues, they share important structural features such as signal peptide positions and active sites location. The protein sequence corresponding to the mature *Pb*SAP shows great similarity to the selected aspartyl proteases sequences.

The recombinant *Pb*SAP was generated and the purified protein was 44kDa, as assessed by SDS-PAGE. These data are in accordance with the predicted size of the deduced protein *Pb*SAP. Using the recombinant purified protein, high titers of rabbit polyclonal antibody were raised. The serum specifically recognized the recombinant purified protein in the western blot assays. In total yeast and mycelium protein extracts a protein of 66 kDa was detected. This molecule was more abundant in yeast cells. Treatments of protein extracts with endoglycosidase H or inclusion of tunicamycin in the culture medium resulted in the disappearance and or decrease of the 66 kDa protein species and the appearance of the 44 kDa, corresponding to the size of *Pb*SAP with the prepropeptides. These data suggest that the extra 22 kDa in the 66 kDa is due to N-glycosylation. Although the significance of glycosylation in the aspartyl protease family is not well known, it has been suggested that it stabilizes protein conformation leading to a higher thermostability [32]. Our data suggest that *Pb*SAP can be secreted as a precursor molecule. Interestingly, studies have demonstrated that aspartyl proteases precursors can be secreted in the extracellular medium where, under low pH conditions, they undergo autocatalytic activation, forming a mature enzyme [33].

The *Pb*SAP is a cell wall molecule of *P. brasiliensis*. Aspartyl proteases on the cell surface have been reported for many eukaryotes. In *C. posadasii* an aspartyl protease was found as a component of the cell wall extract [9]. Aspartyl proteases have been described as important to cell wall integrity and adherence to mammalian cells in *C. glabrata* [34]. In *C. albicans* SAP9 and SAP10 are glycosylphosphatidylinositol-anchored and located in the cell membrane or in the cell wall [35].

Gelatinolytic activity of aspartyl proteases in the yeast protein extract and in the secreted protein fraction was analyzed by zymogram. In both samples, gelatin degradation was observed in the 66 kDa region, suggesting *Pb*SAP protease activity. The extracts were prepared in buffer containing PMSF (serine proteases inhibitor) and EDTA (metalloproteases inhibitor) and the use of developing buffer in acidic conditions activated the enzyme. The treatment of yeast cells extract with Endo H resulted in the loss of protease

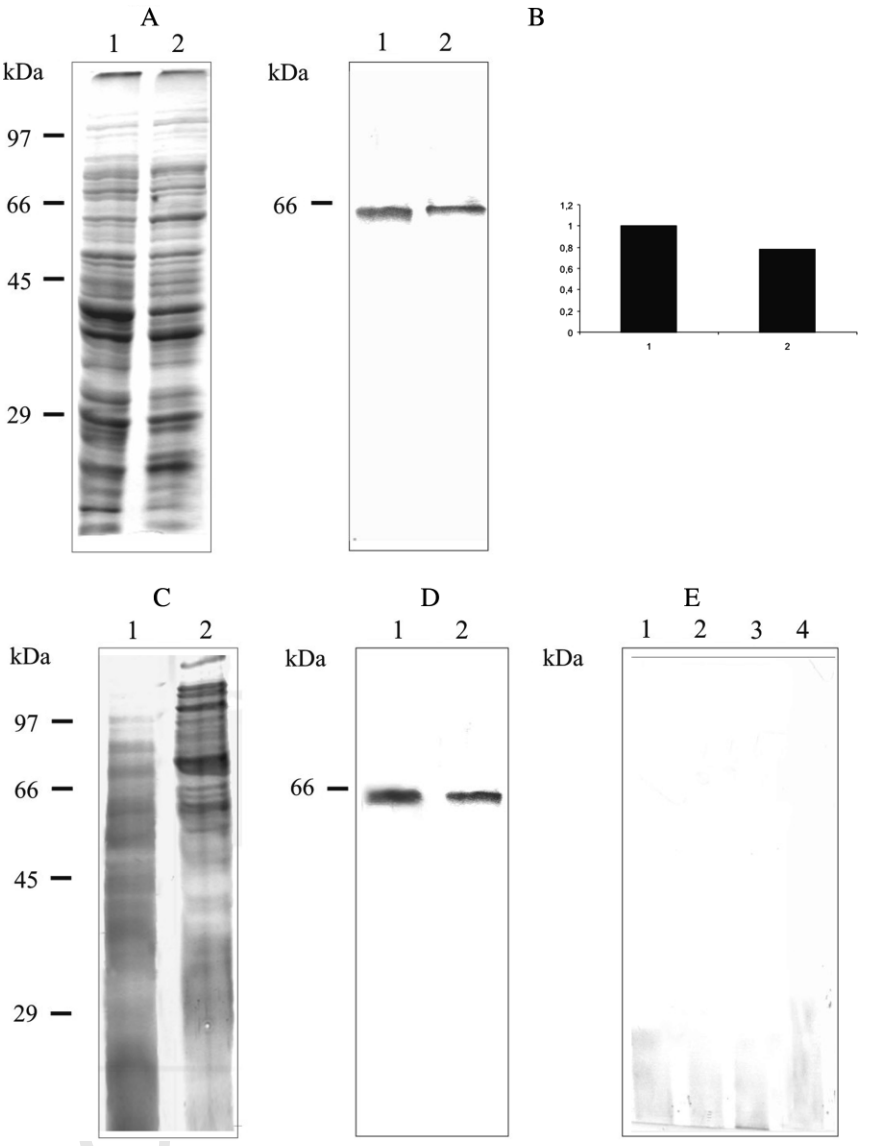

Fig. 3 Immunodetection of aspartyl protease by western blot analysis. (A) SDS-PAGE (12%) of total protein extract (30 μg) of *P. brasiliensis* yeast cells (lane 1) and mycelium (lane 2), stained with *Coomassie* brilliant blue. (B) The same *P. brasiliensis* extracts were used and aspartyl protease was visualized by western blot. The *Pb*SAP expression was quantified using the program Scion Image for Windows (http://www.scioncorp.com). (C) Electrophoretic analysis of the secreted protein fraction of *P. brasiliensis* yeast cultures (lane 1) and of the SDS-extracted fraction from the fungus cell wall (lane 2). (D) Western blot analysis with rabbit polyclonal antibody of samples from C. (E) Western blot analysis with preimmune rabbit serum of: 1 – total protein extract from yeast cells, 2 – total protein extract from mycelium, 3 – secreted proteins from yeast cells and 4-SDS-extracted fraction from yeast cell wall. After reaction with the anti-rabbit Ig-G alkaline phosphatase-coupled antibody (diluted 1:2000), the reaction was developed with BCIP/NBT. Protein molecular markers are indicated.

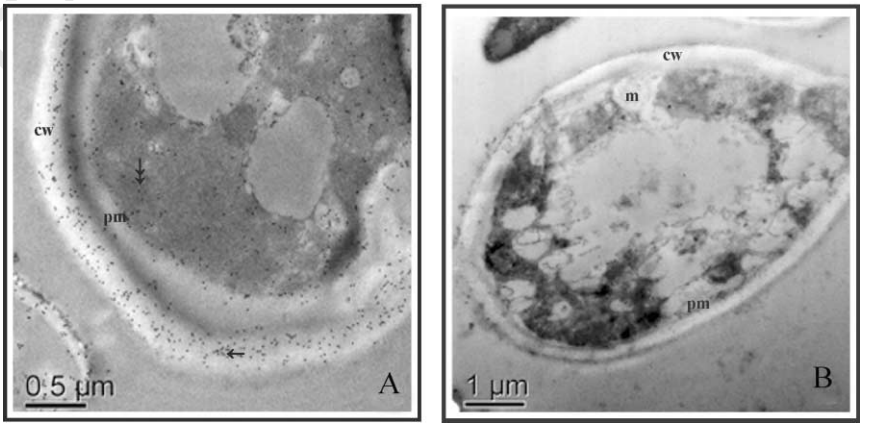

Fig. 4 Immunogold localization of *Pb*SAP in *Paracoccidioides brasiliensis* yeast cells. (A) The gold particles conjugated to the secondary antibody were numerous in the cell wall (arrows) and sparse in the cytoplasm (double arrows). (B) Negative control was obtained using rabbit preimmune serum. Typical fungal cell structures: (cw) cell wall, (m) mitochondria and (pm) plasma membrane.

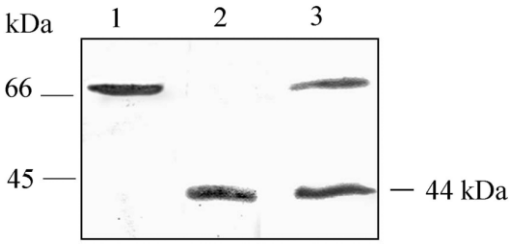

Fig. 5 *Paracoccidioides brasiliensis* aspartyl protease glycosylation studies and immunoblot assays. Glycoslylation was investigated by treating protein extracts with endoglycosidase H or by including tunicamycin in the culture medium followed by immunoblot analysis. Lane 1: Control; total protein extract of yeast cells (30 μg). Lane 2: Total protein extract from yeast cells (30 μg) after endoglycosidase H treatment. Lane 3: *P. brasiliensis* yeast cells extract (30 μg) obtained after growth of yeast cells in the presence of tunicamycin.

activity, suggesting that glycosylation is relevant for aspartyl protease activity in *P. brasiliensis*. Numerous functions have been attributed to aspartyl proteases in microorganisms. These range from nutrient degradation to the activation of signaling molecules. Aspartyl proteases from fungi serve to activate other zymogens such as alkaline phosphatase, chitin synthase, and other proteases [36–38]. Aspartyl proteases from *Schistosoma* species are known to be responsible for host-specific proteolytic degradation of mammalian hemoglobin [39]. Also, the degradative properties of secreted proteases have attracted much attention as potential mediators of fungal invasion in infected tissue [5,15]. In *A. fumigatus*, an aspartyl protease is important for the invasion process in the lung, facilitating fungus penetration [8]. The role of aspartyl protease in *P. brasiliensis* remains unclear. The fact that the protein is more abundant in yeast cells, would point to its importance in the pathogenesis of the organism. Future work will focus on this subject.

In conclusion, a novel aspartic protease, *Pb*SAP, has been identified and characterized in the pathogenic fungus *P. brasiliensis*. Recombinant *Pb*SAP expression was determinant to this work as an important tool to obtain specific polyclonal antibody. Secretion of the native protein was detected in yeast cell culture by immunoassays, and the presence of the protein was also detected in the fungal cell wall. The glycosylation of *Pb*SAP was investigated, providing fundamental information about the structure of *Pb*SAP.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

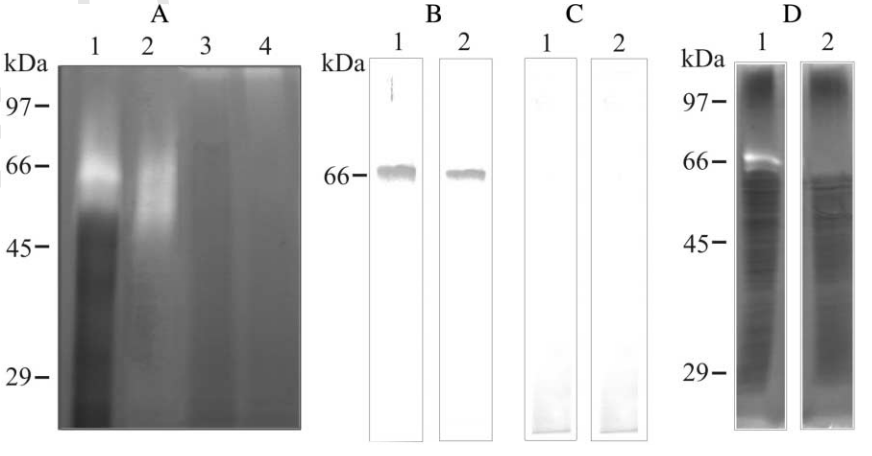

Fig. 6 Analysis of gelatinolytic activity and immunoblot assays. (A) Total protein from yeast cells (lane 1) and secreted protein fraction (lane 2) were subjected to SDS-PAGE-gelatin, under non-denaturing conditions. The same extracts were assayed to protein specific inhibition using pepstatin (10 μM) (lanes 3 and 4). After electrophoresis, the gel was washed and incubated in developing buffer. Hydrolysis of gelatin was visualized by gel staining with Coomassie Brilliant Blue. Protein molecular markers are indicated. (B) Immunoassay of the samples from A using *Pb*SAP specific serum; lane 1: Total protein from yeast cells and lane 2-secreted protein from yeast cells. (C) Reaction to the preimmune serum, lanes 1 and 2, the same samples as in B. (D) Total protein from yeast cells was incubated with endoglycosidase H for 15 min (lane 2). Control was performed (lane 1) by incubating total protein extracts without the enzyme for 15 min. The gelatinolytic activity was assayed.

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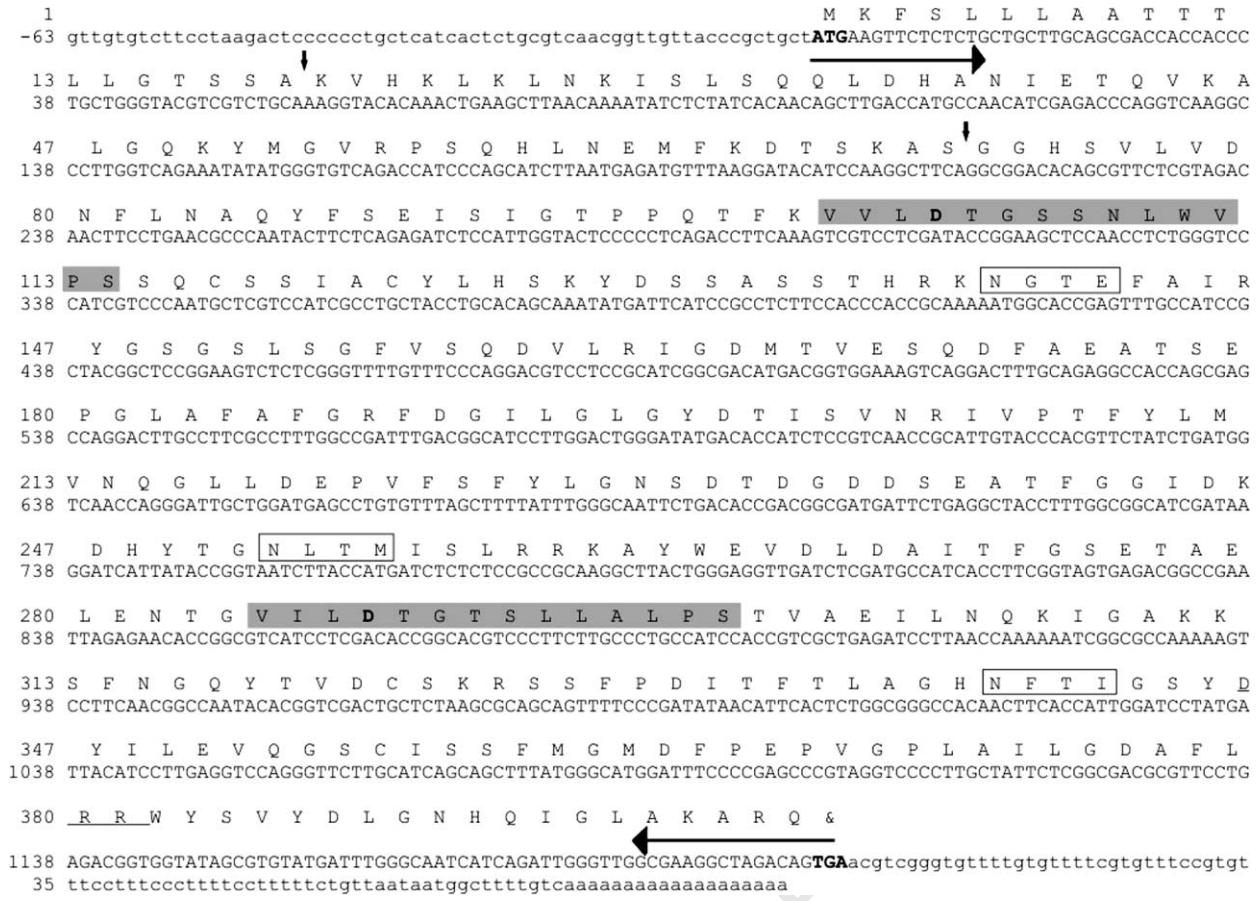

Supplementary Fig. 1 Nucleotide and deduced amino acid sequence of the *P. brasiliensis* aspartyl protease cDNA. The nucleotide and amino acid positions are marked on the left side. Lower-case letters represent the untranslated 5′ and 3′ regions. Bold letters in the nucleotide sequence represent the start and stop codons. In the amino acid sequence, the putative cleavage site that removes the signal peptide and the propeptide are indicated with vertical arrows. Two conserved aspartyl protease domains of the active site are shown in gray. In these two regions, the residues of aspartic acid (D is the active site residue) are in bold letters. Three predicted N-glycosylation sites are shown in rectangles. Primers used in this work to amplify the *Pb*SAP cDNA are marked by horizontal arrows.

Capítulo V

Artigos em colaboração

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

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***Paracoccidioides brasiliensis* is the causative agent of paracoccidioidomycosis, a disease that affects 10 million individuals in Latin America. This report depicts the results of the analysis of 6,022 assembled groups from mycelium and yeast phase expressed sequence tags, covering about 80% of the estimated genome of this dimorphic, thermo-regulated fungus. The data provide a comprehensive view of the fungal metabolism, including overexpressed transcripts, stage-specific genes, and also those that are up- or down-regulated as assessed by *in silico* electronic subtraction and cDNA microarrays. Also, a significant differential expression pattern in mycelium and yeast cells was detected, which was confirmed by Northern blot analysis, providing insights into differential metabolic adaptations. The overall transcriptome analysis provided information about sequences related to the cell cycle, stress response, drug resistance, and signal transduction pathways of the pathogen. Novel *P. brasiliensis* genes have been identified, probably corresponding to proteins that should be addressed as virulence factor candidates and potential new drug targets.**

The dimorphic human pathogenic fungus *Paracoccidioides brasiliensis* is the etiological agent of paracoccidioidomycosis (PCM)[1] (1), a major health problem in Latin America. High positive skin tests (75%) in the adult population reinforce the importance of the mycosis in endemic rural areas, where it has been estimated to affect around 10 million individuals, 2% of whom will develop the fatal acute or chronic disease (2). The acute form of PCM chiefly compromises the reticuloendothelial system; the chronic form mainly affects adult males with a high frequency of pulmonary and/or mucocutaneous involvement (1). Chronic severe multifocal PCM may also cause granulomatous lesions in the central nervous system (3). Regardless of the affected organ, PCM usually evolves to the formation of fibrotic sequelae, permanently hindering the patient's health.

P. brasiliensis Undergoes a Dimorphic Process in Vivo—It is assumed that the fungus exists as a soil saprophyte, producing propagules that can infect humans and produce disease after transition to the pathogenic yeast form (4). Pathogenicity has been intimately associated with this process, since *P. brasiliensis* strains unable to differentiate into the yeast form are avirulent (5). Mammalian estrogens inhibit dimorphism, explaining the lower incidence of disease in females (6). The mycelium-to-yeast transition in *P. brasiliensis* is governed by the rise in temperature that occurs upon contact of mycelia or conidia with the human host. *In vitro*, it can be reversibly reproduced by shifting the growth temperature between 22 and 36 °C. Molecular events related to genes that control signal transduction, cell wall synthesis, and integrity are likely to be involved in this dimorphic transition.

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

RESULTS

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

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

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

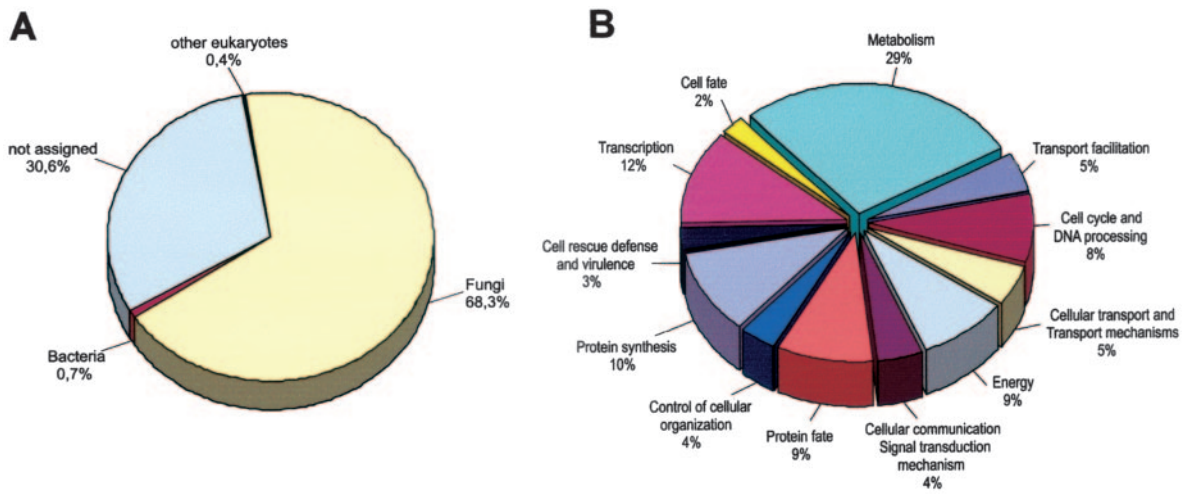

FIG. 1. ***P. brasiliensis* transcriptome characterization.** *A*, distribution of blast best hit among organisms. Each PbAEST was tested against the GenBank™ nr data base, and the best hit organism was computed. A PbAEST was considered as not assigned when the best hit exceed an *E* value of 10^{-10}.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

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

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

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

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

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

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

TABLE I

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

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

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

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

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

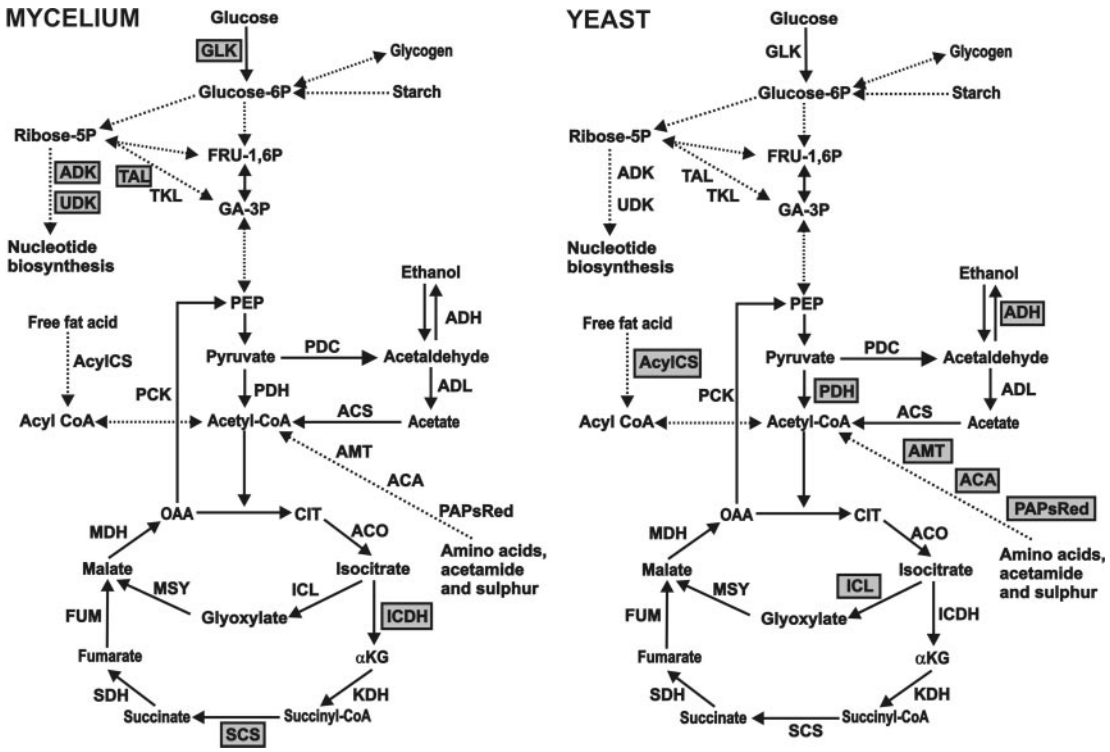

FIG. 2. **Comparison of the expression pattern of genes encoding for enzymes in mycelium-to-yeast cell differentiation of *P. brasiliensis*.** For the detailed metabolic comparison between mycelium and yeast metabolism, see Supplemental Table IV, since we have presented in this figure only the central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

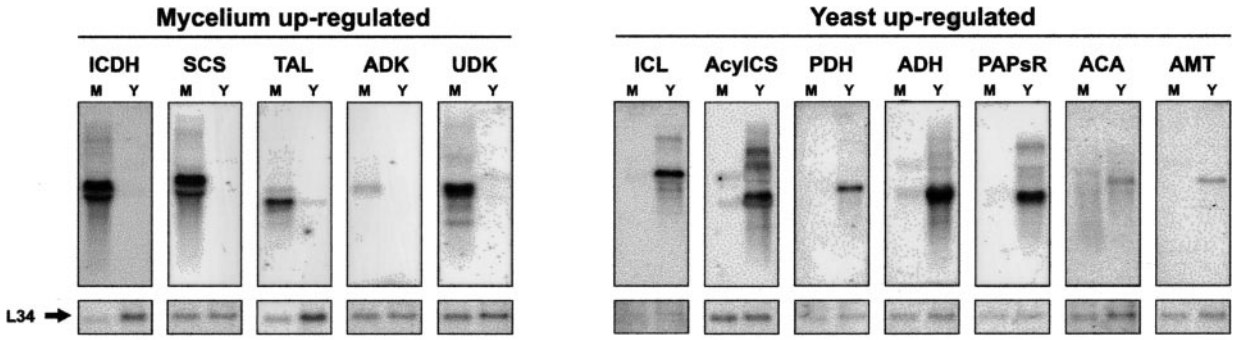

FIG. 3. **Northern blot analysis of mycelium and yeast up-regulated genes of *P. brasiliensis*.** Total RNA samples from both mycelium (*M*) and yeast (*Y*) were blotted onto nylon membranes and hybridized against gene-specific radiolabeled probes. *ICDH*, isocitrate dehydrogenase; *SCS*, β-succinyl-CoA-synthetase,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

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

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

Cell Cycle and Genetic Information—The main genes in-

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

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

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

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

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

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

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

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

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

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

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

Noteworthy is the finding of glyoxylate cycle genes in

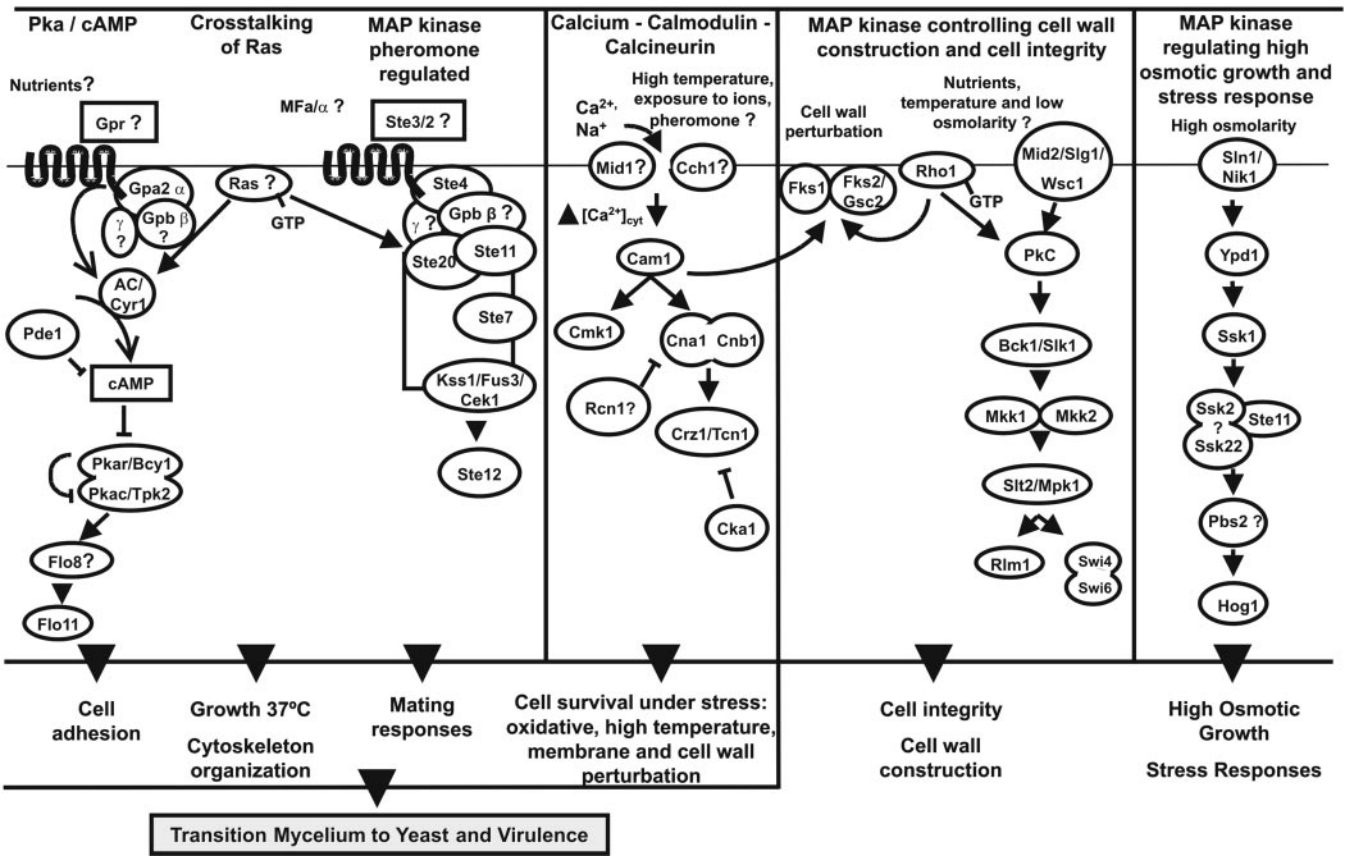

FIG. 4. **Signaling pathways in *P. brasiliensis*.** Shown are cAMP/protein kinase A regulating fungal development and virulence; MAPK signaling for cell integrity, cell wall construction, pheromone/mating, and osmoregulation; calcium-calmodulin-calcineurin controlling cell survival under stress conditions;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

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

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

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

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

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

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

TABLE II

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

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

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

TABLE III

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

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

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

DISCUSSION

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

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

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

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

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

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Research Article

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

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Abstract

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

Key words: Mycoplasma, kinases, genomes.

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Introduction

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

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

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

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

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

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

Methods

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

Results and Discussion

Mycoplasma kinases

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

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

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

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

Kinase classification

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

Nucleotide metabolism and kinases

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

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

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

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

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

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

Kinases involved in the metabolism of carbohydrates

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

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

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

Riboflavin metabolism and kinases

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

Amino acid sequence relationships

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

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

Concluding Remarks

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

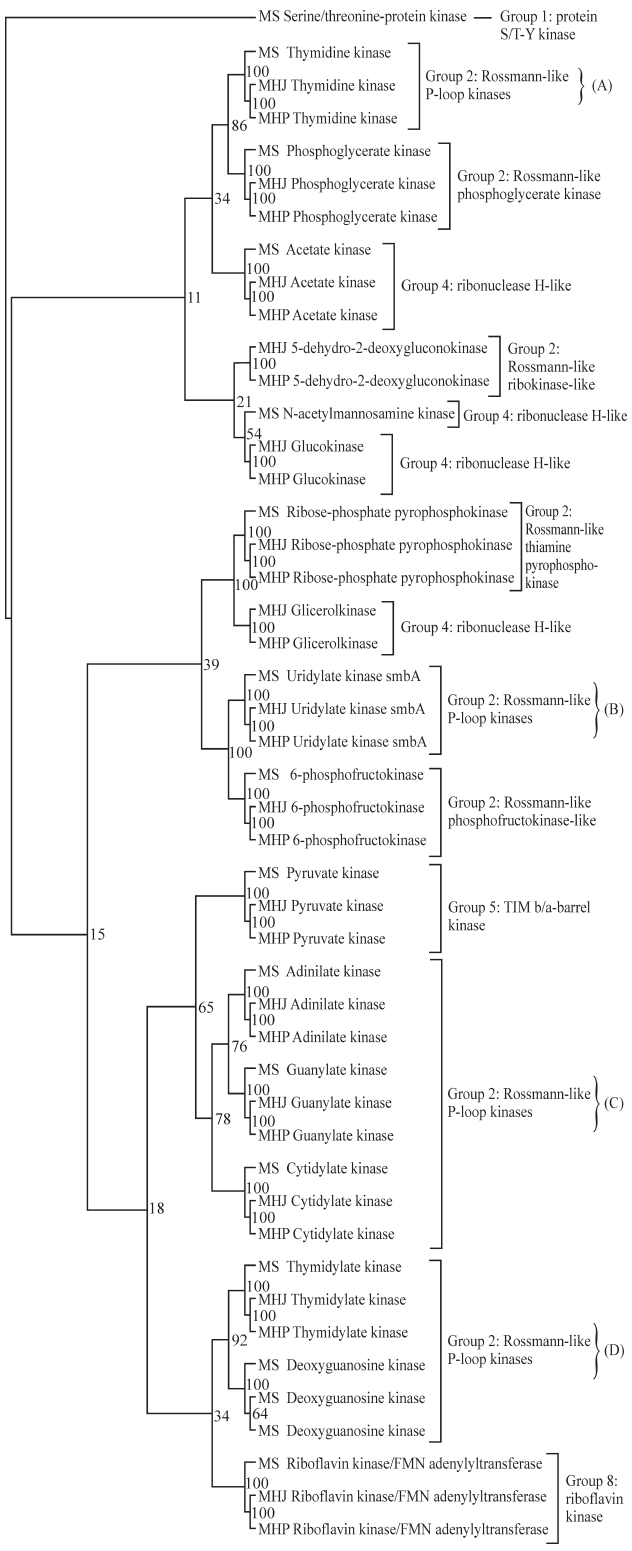

Figure 1 - Phylogenetic tree obtained from kinase amino acid sequence relationships. The kinase fold groups and families are shown in brackets on the right side. The Group 2: Rossmann-like P-loop kinases were clustered into four sub-groups (A, B, C and D). The numbers on the branches are bootstrap values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

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

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Internet Resources

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

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

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

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

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

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Research Article

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

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Abstract

Mycoplasmas are the smallest known prokaryotes with self-replication ability. They are obligate parasites, taking up many molecules of their hosts and acting as pathogens in men, animals, birds and plants. *Mycoplasma hyopneumoniae* is the infective agent of swine mycoplasmosis and *Mycoplasma synoviae* is responsible for subclinical upper respiratory infections that may result in airsacculitis and synovitis in chickens and turkeys. These highly infectious organisms present a worldwide distribution and are responsible for major economic problems. Proteins of the GTPase superfamily occur in all domains of life, regulating functions such as protein synthesis, cell cycle and differentiation. Despite their functional diversity, all GTPases are believed to have evolved from a single common ancestor. In this work we have identified mycoplasma GTPases by searching the complete genome databases of *Mycoplasma synoviae* and *Mycoplasma hyopneumoniae,* J (non-pathogenic) and 7448 (pathogenic) strains. Fifteen ORFs encoding predicted GTPases were found in *M. synoviae* and in the two strains of *M. hyopneumoniae*. Searches for conserved G domains in GTPases were performed and the sequences were classified into families. The GTPase phylogenetic analysis showed that the subfamilies were well resolved into clades. The presence of GTPases in the three strains suggests the importance of GTPases in `minimalist' genomes.

Key words: Mycoplasma, GTPase superfamily, genome.

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Introduction

Mycoplasmas are a genus of obligate parasites belonging to the Mollicutes class, the smallest known prokaryotes with self-replication ability (Razin *et al.*, 1998). They present a very small genome evolved to the minimalist status by losing non-essential genes, including those involved in cell wall synthesis, as well those related to catabolic and metabolic pathways (Himmelreich *et al.*, 1996). The two species, *Mycoplasma hyopneumoniae* and *Mycoplasma synoviae*, are responsible for significant economic impact on animal production. *M. hyopneumoniae* is the infective agent of swine mycoplasmosis (DeBey and Ross, 1994), which increases the susceptibility to secondary infections (Ciprian *et al.*, 1988). *M. synoviae* is responsible for subclinical upper respiratory infections, but may also result in airsacculitis and synovitis in chickens and turkeys (Kleven, 1997; Allen *et al.*, 2005).

Many crucial functions for life are provided by a single versatile mechanism that has evolved to fulfill many roles. A prime example is the GTPase superfamily of proteins that occurs in all domains of life, regulating functions such as protein synthesis, cell cycle and differentiation (Bourne *et al.*, 1990). Despite this extraordinary functional diversity, all GTPases are believed to have evolved from a single common ancestor, a fact which resulted in the conservation of their action mechanism, of the core structure and of sequence motifs (Bourne, 1995).

GTPases are often described as molecular switch proteins because of their particular mode of action. Each GTPase specifically binds and hydrolyzes GTP in a cyclic mechanism that activates and inactivates the GTPase protein (Bourne *et al.*, 1991). In this cycle, a GTPase passes through three conformational states. Initially, the GTPase is inactive and is not bound to any nucleotide. After binding GTP, the protein becomes active and changes its conformation, and as such its affinity to effector molecules or other enzymes. GTP is then hydrolyzed simultaneously, with an effect being generated in the GTPase target. Subsequently, GDP is released from the inactive GTPase, returning the protein to the empty state. This cycle allows the active GTPase to interact periodically with a target and, in this

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way, to act as a timed switch in the cell (Bourne *et al.*, 1990).

That cyclic reaction usually involves several other factors that either catalyze the hydrolysis step of the GTPase cycle or catalyze the release of bound GDP from the inactive state of the GTPase (Bourne, 1995). Each GTPase cycle appears to be unique. The rate of switch turnover is dependent on specific interaction factors, as well as on the intrinsic properties of each GTPase. Additionally, some GTPases interact with many different effectors and targets and, in that way, can coordinate cellular responses (Bourne *et al.*, 1990; Bourne, 1995). A core domain that is able to bind either GTP or GDP confers the characteristic switch mechanism of GTPases. The folding of this domain is a defining feature of GTPases (Jurnak *et al.*, 1990). In fact, X-ray crystallography of diverse GTPases shows that the folding of this G-domain is nearly invariant throughout the GTPase superfamily. GTPases can consist solely of the G-domain or may have additional domains on the amino- and carboxyl-terminal ends of the proteins (Sprang, 1997).

Due to the importance of the mycoplasmas, complete genome projects have been reported in the last years (Himmelreich *et al.*, 1996; Hutchison *et al.* 1999; Glass *et al.*, 2000; Chambaud *et al.*, 2001; Papazisi *et al.*, 2003; Sasaki *et al.*, 2002; Jaffe *et al.*, 2004; Minion *et al.*, 2004; Westberg *et al.*, 2004). Complete genomes of *M. synoviae* (strain 53), *M. hyopneumoniae* pathogenic strain (7448) and non-pathogenic strain (J [ATCC25934]) were recently described (Vasconcelos, *et al.*, 2005) and the data are available in databases. The objective of this work is the identification and classification of the GTPase superfamily in the three complete genomes of *M. synoviae* strain 53 and *M. hyopneumoniae* (strains J and 7448).

Material and Methods

By using data from the complete genome of *M. synoviae* and *M. hyopneumoniae,* strains J and 7448 associated to BLAST search tools we have identified 15 ORFs encoding GTPase superfamily homologs in *M. synoviae*, as well as 15 ORFs in both strains of *M. hyopneumoniae*. Classification of the GTPase families and their putative function has been performed by using Pfam interface and InterPro homepage. Search for G-domains in mycoplasma GTPases was performed by alignment of described *Escherichia coli* GTPases sequences (Caldon *et al.*, 2001) with those of *M. synoviae* and *M. hyopneumoniae* (strains J and 7448). Multiple sequence alignments were generated using the ClustalX 1.81 software (Thompson *et al.*, 1997).

The phylogenetic relationships within the GTPase superfamily were inferred from all 33 sequences from *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448. A phylogenetic tree was constructed by multiple sequence alignments using the Clustal X program and visualized by using the Tree View software. Trees were constructed by using the neighbor-joining method (Saitou and Nei, 1987). Robustness of branches was estimated by using 100 bootstrap replicates.

Results and Discussion

Structural analysis of the GTPases superfamily

Searches for GTPases performed on *M. synoviae* and *M. hyopneomoniae* strains J and 7448 genome databases revealed the presence of 15 GTPase orthologs. These GTPases were classified into subfamilies, and the results are shown in Table 1. ORFs were classified as belonging to the Elongation factor, the Era, the FtsY/Ffh and the Obg/YchF subfamilies, or were annotated as unclassified proteins related to GTPases or GTP binding proteins.

Searches for the G-domain, described in all GTPase subfamilies, was performed by using the deduced protein sequences encoded by the identified ORFs presented in Table 1. Figure 1 presents the alignment of the G1-G4 motifs of the cited GTPases. The G-domain is divided into four G motifs: G1 (G/AXXXXGKT/S), G2 (not conserved), G3 (DXXG) and G4 (NKXD) sequence motifs, where X denotes any amino acid (Caldon, *et al.*, 2001). The G1, G2 and G3 motifs were found in all mycoplasma GTPase subfamilies (Figure 1). The G4 motif was found in the EF-G, EF-Tu, IL-2, LepA, Era, EngA, ThdF/TmE, and OBG subfamilies. In the YchF, FtsY and Ffh subfamilies, the region of the G4 motif, although present, was not well conserved (Figure 1).

Functions ascribed to G-motifs include the mediation of interactions with the guanine nucleotides and effector proteins. It has been suggested that G1, G3 and G4 motifs could have evolved to bind and hydrolyze guanosine triphosphate and also for interacting with the cofactor mg^{2+} (Bourne *et al.*, 1991). The non conserved G2 motif is described as the effector domain that undergoes a conformational change necessary for GTPase function (Bourne, *et al.*, 1995, Sprang, 1997).

Elongation factor subfamily

The elongation factor subfamily (EF) is composed of the Elongation factor - G (EF-G), Elongation factor-TU (EF-TU), Initiation factor-2 (IF-2) and GTP-binding protein LepA (LepA), (Caldon, *et al.* 2001). The EF family from bacteria is composed of multidomain GTPases with essential functions in the elongation and initiation phases of translation. EF-Tu catalyzes binding of aminoacyl-tRNA to the ribosomal A-site, while EF-G catalyses the translocation of peptidyl-tRNA from the A-site to the P-site (Rodnina *et al.*, 2000; Nilsson and Nissen, 2005). The initiation factor-2 (IF-2) may be involved in introducing the initiator tRNA into the translation machinery and in performing the first step in the peptide chain elongation cycle (Kyrpides and Woese, 1998). ORFs encoding all elongation factor members were present in *M. synoviae* and *M. hyopneumoniae* J and 7448 (Table 1). All G1-4 motifs were

Table 1 - ORFs encoding GTPases and GTP binding proteins from *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448, with putative functions.

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

found in the ORFs encoding EF GTPases from both mycoplasma species (Figure 1), suggesting that the proteins can be functional in these organisms. Two truncated hypothetical EF-G proteins were also found in the *M. synoviae* genome. The ORFs present high homology to the 3' region of the complete EF-G ORF found in this organism, suggesting that they are not functional genes, in accordance with the 'minimal genome' characteristic of mycoplasmas.

Era subfamily

This family is comprised of the GTP binding protein ERA (ERA), the GTP binding protein EngA (EngA), as well as the Thiophene and furam oxidation protein (ThdF). Both *M. synoviae* and *M. hypneumoniae* (J and 7448) present ORFs related to the Era subfamily. The Era member of the Era subfamily is an essential GTPase that probably regulates the cell cycle (Gollop and March, 1991; Britton *et al.*, 1998) and is involved in regulating carbon (Lerner and Inouye, 1991) and nitrogen (Powell *et al.*, 1995) metabolism. A second member of this group, EngA, has been suggested to be essential for growth in *Neisseria gonorrhoeae* (Mehr *et al.*, 2000). ThdF may be involved in tRNA modification and in the direct or indirect regulation of ribosome function (Caldon, *et al.*, 2001). The presence of all Era subfamily members (Table 1) with all G1-G4 motifs (Figure 1) in *M. synoviae* and *M. hyopneumoniae* (J and 7448) suggests that those ORF products are active and play biological functions in the analyzed organisms.

FtsY/Ffh subfamily

The FtsY/Ffh subfamily is represented by the cell division protein FtsY, termed FtsY, and by the signal recognition particle FFH/SRP54, termed Ffh. ORFs encoding for the two proteins of this subfamily have been reported in the *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448 (Table 1). The G1-G3 motifs were found in the deduced amino acid sequences for FtsY and Ffh of *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448, when compared with *E. coli* FtsY/Ffh sequences (Figure 1). The sequence corresponding to the G4 motif was found in the three analyzed mycoplasmas, even though this motif was not well conserved (NKXD). The amino acids K and D are present in mycoplasma FtsY and Ffh sequences in comparison to the *E. coli* ortholog predicted proteins. These proteins are described as essential to *E. coli* since Ffh/SRP mutants present a lethal phenotype and SRP subunit mutants present growth defects (Lu, *et al.*, 2001).

OBG and YchF subfamily

The comparative analysis of *M. synoviae* strain 53, *M. hyopneumoniae* (strains J and 7448) showed the presence of the same ortholog ORFs encoding OBG and YchF proteins (Table 1). G1-G3 motifs were found in all ORF products. The G4 motif was found in the OBG member, but not in the YchF ORF product (Figure 1). Similarly, this motif was also not found well conserved in the *E. coli* YchF protein.

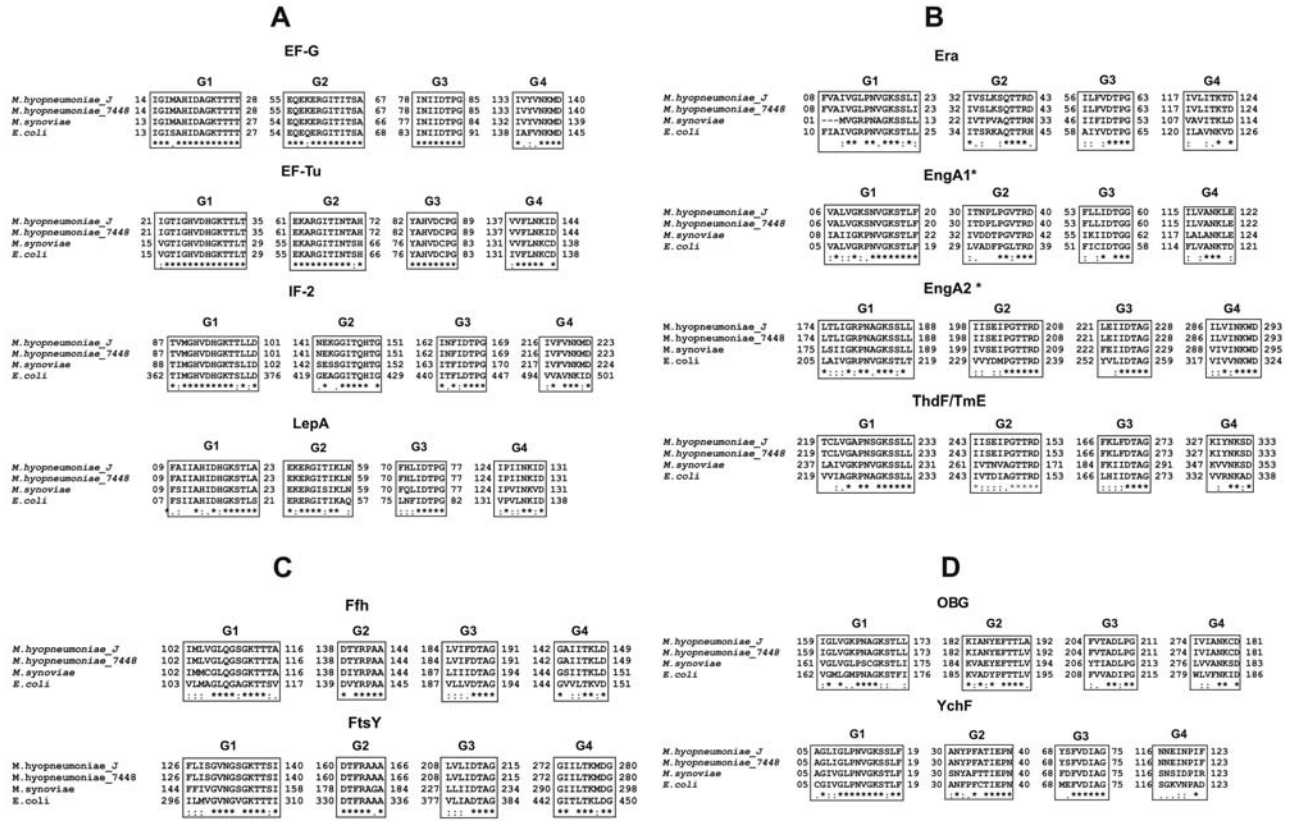

Figure 1 - Alignment of G1, G2, G3 and G4 motifs of the GTPase subfamilies. Panel A: Elongation factor subfamily. Panel B: Era subfamily. Panel C: FtsY/Ffh subfamily. Panel D: OBG YchF subfamily. The sequences used in the alignment are listed in Table 1 and were obtained from: *M. hyopneumoniae J* (*Mycoplasma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

The function of the OBG subfamily remains elusive, although there is evidence for its involvement in the initiation of chromosome replication (Kok *et al.*, 1994), in bacterial sporulation (Trach and Hoch, 1989; Vidwans *et al.*, 1995), and in the activation of a transcription factor that controls the general stress response (Scott and Haldenwang, 1999). The YchF members of the OBG/YchF subfamily are also distributed in all domains of life, (Mittenhuber, 2001), but the biological function of this protein has not been elucidated.

Unclassified GTPases

The GTPases found in the genomes of mycoplasmas which were not classified as belonging to one of the 11 universally conserved bacterial GTPases (Caldon, *et al.*, 2001) were described here as unclassified. Four ORFs from *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448 were identified in this group: EngC, YlqF, FtsZ and YihA. The *E. coli* ortholog EngC is a GTPase with a predicted role as a regulator of translation (Daigle and Brown, 2004). The putative GTP binding protein YlqF is described as necessary for growth of *Streptococcus pneumoniae* and *Staphylococcus aureus* and may be involved in ribosomal assembly (Zalacain *et al*., 2003).

The cell division protein FtsZ was also found in *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448. This protein appears to act at the earliest step in cell septation and is required at the final steps of cytokinesis (Ma, *et al.*, 1996; Jensen, *et al.*, 2005). The GTPase YihA has been described as an essential gene of the bacterial "minimal genome' , even though it seems to be dispensable in some organisms, as described for *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Treponema pallidum*, *Borrelia burgdorferi* and *Synechocystis sp.* (Dassain *et al.*, 1999).

GTPase amino acid sequence relationships

To visualize the amino acid sequence relationship of Mycoplasma GTPase subfamilies, a phylogenetic tree was constructed by using the neighbour-joining method (Saitou and Nei, 1987). A total of 33 deduced amino acid sequences encoding GTPases from *M. synoviae*, *M. hyopneumoniae* J and *M. hyopneumoniae* 7448 were aligned using the CLUSTAL X program (Thompson *et al.*, 1997). Robust-

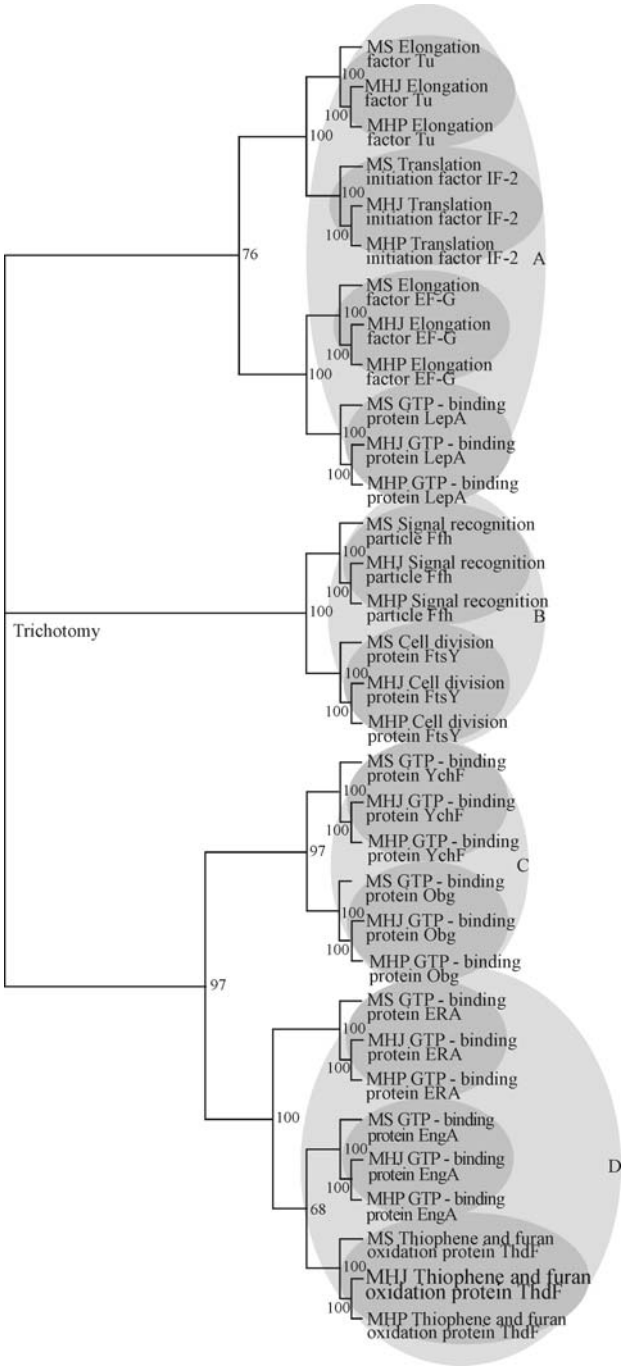

Figure 2 - Amino acid sequence relationship of the GTPase superfamily. (A) Elongation factor subfamily. (B) FtsY/Ffh subfamily. (C) OBG/YchF subfamily. (D) Era subfamily. The numbers on the branches are bootstrap values obtained with 100 replications. Members of each family are described as MS for *M.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

ness of branches was estimated by using 100 bootstrap replicates. By using the Tree View software a deduced phylogeny was visualized and is shown in Figure 2. A close relationship among amino acid sequences of proteins which belong to the same subfamily can be observed in the three Mycoplasma species. GTPases that have similar functions were clustered into the same clade, suggesting a metabolic conservation in reactions involving GTPases. The bootstrap values reveal the high homology among the subfamilies of proteins of *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448. GTPases are classified into subfamilies based on the presence of different G-domains (G1, G2, G3 and G4). Since unclassified GTPases do not present conserved G-domains, and were not classified by Caldon *et al*. (2001), they were not included in our phylogenetic analysis.

Concluding Remarks

The GTPase superfamily, present in all domains of life, is related to many functions such as protein synthesis, cell cycle and differentiation. The presence of orthologs for all the subfamily members described in prokaryotes in the complete genome of *M. synoviae* and *M. hyopneumoniae* strains J and 7448, evidences the essential functions of GTPases in these 'minimalist' organisms.

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Abbreviations

EF-G (Elongation factor G).
EF-TU (Elongation factor Tu).
IF-2 (Translation initiation factor 2).
MHJ (*Mycoplasma hyopneumoniae* strain J).
MHP (*Mycoplasma hyopneumoniae* strain 7448).
MS (*Mycoplasma synoviae* strain 53).
ThdF (Thiophene and furan oxidation protein).

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Internet Resources

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

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

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

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

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

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RESEARCH ARTICLE

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

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Paracoccidioides brasiliensis; transcription; human plasma.

Abstract

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

Introduction

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

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

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

Materials and methods

Paracoccidioides brasiliensis growth conditions

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

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

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

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

RNA extractions, subtractive hybridization and generation of subtracted libraries

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

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

Sequences, processing and EST database construction

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

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

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

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

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

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

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

Dot-blot analysis

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

Semiquantitative reverse transcriptase (RT)-PCR analysis

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

Protein extract preparation and Western blot analysis

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

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

Measurement of formamidase activity

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

SDS sensitivity tests

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

Results

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

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

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

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

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

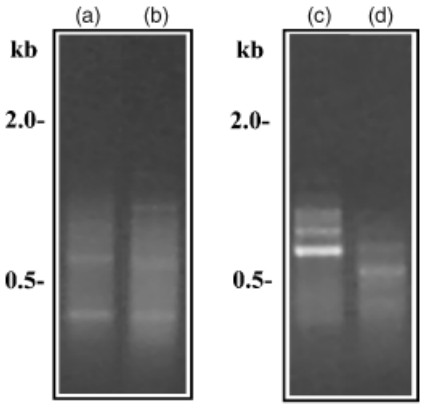

Fig. 1. Agarose gel electrophoresis of subtracted differential cDNA pools derived from *Paracoccidioides brasiliensis* yeast cells incubated with human plasma. Products of the first and second rounds of subtraction performed using as testers the cDNA obtained from RNAs of yeast cells incubated with human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

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

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

Table 1. Continued.

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

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

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

‡Novel genes detected in *P. brasiliensis*.

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

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

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

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

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

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

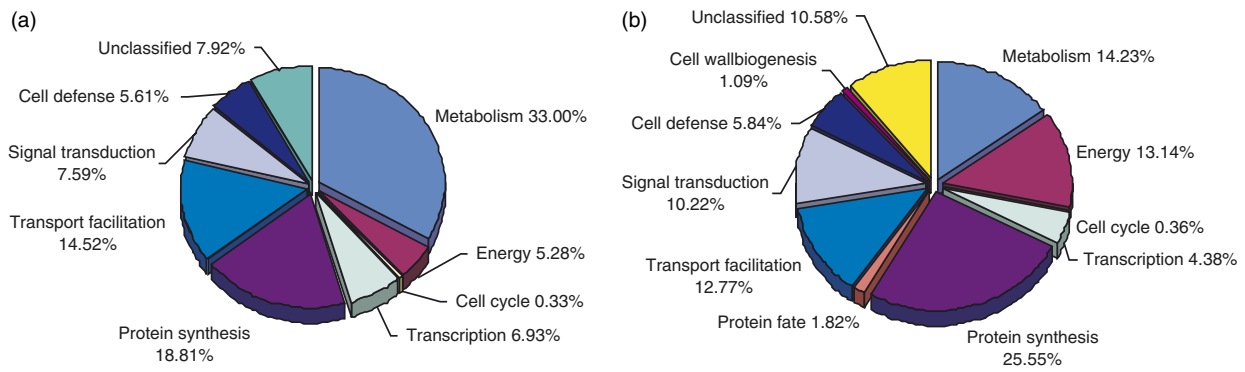

Fig. 2. Functional classification of *Paracoccidioides brasiliensis* cDNAs derived from RDA experiments using as testers the cDNAs obtained from RNA of *Paracoccidioides brasiliensis* yeast cells after incubation with human plasma for 10 min (a) or 60 min (b). The percentage of each functional category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

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

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

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

†Transcripts validated by dot blot.

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

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

¶Transcripts validated by semiquantitative RT-PCR.

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

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

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

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

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

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

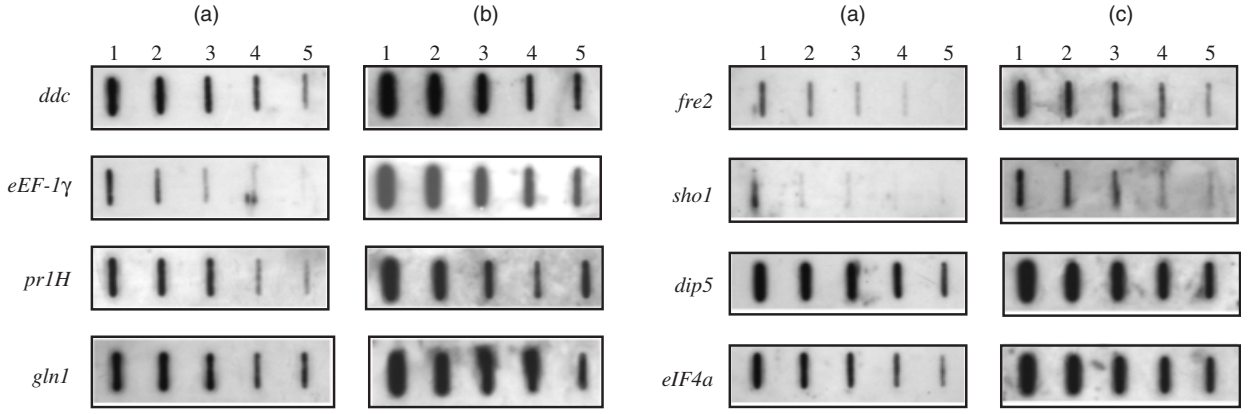

Fig. 3. Dot-blot analysis of *Paracoccidioides brasiliensis* cDNA-RDA clones. DNAs of individual clones were prepared and blotted in several dilutions (1–5). Individual clones were blotted and hybridized to the labeled cDNAs obtained from the control yeast cells (a), and labeled cDNAs obtained from *Paracoccidioides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

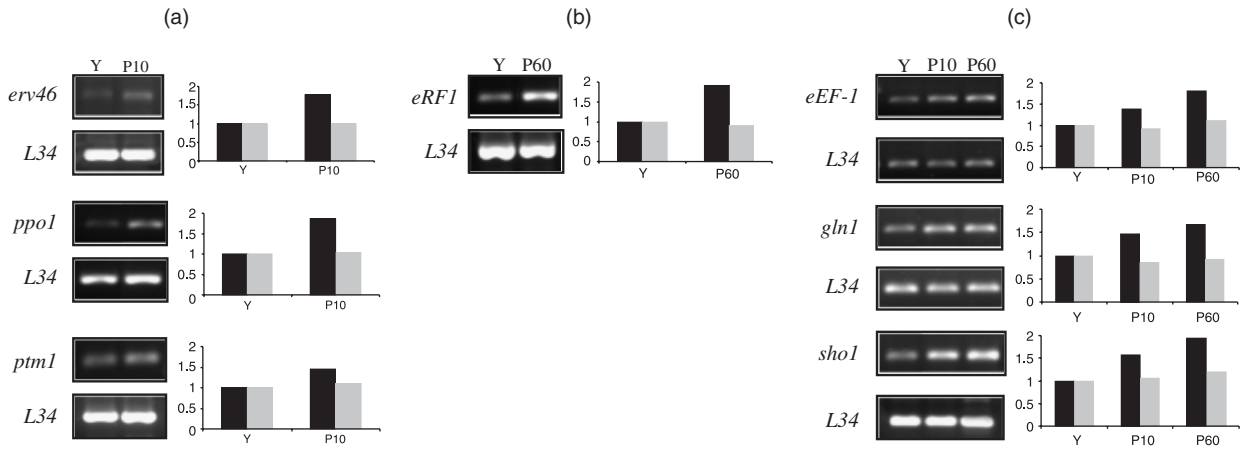

Fig. 4. Validation of RDA results by semiquantitative RT-PCR of RNAs obtained from yeast cells during incubation with human plasma. Semiquantitative RT-PCR analysis was carried out with specific primers, as described. Numbers associated with the bars indicate fold differences relative to the data for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

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

Sensitivity of yeast cells to SDS after incubation with human plasma

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

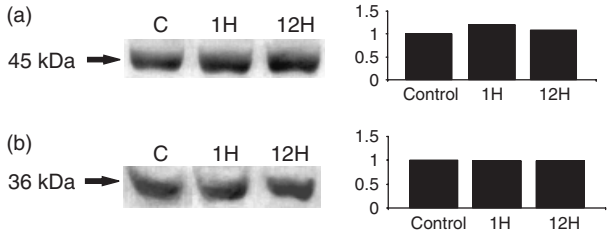

Fig. 5. Validation of the RDA results by Western blot. Total cellular extracts were obtained from yeast cells incubated with human plasma for 1 and 12 h. The proteins (25 μg) were electrophoretically transferred to a nylon membrane and checked by Ponceau S to determine equal loading. The samples were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

Table 3. Formamidase activity of yeast cell protein extracts

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

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

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

Discussion

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

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

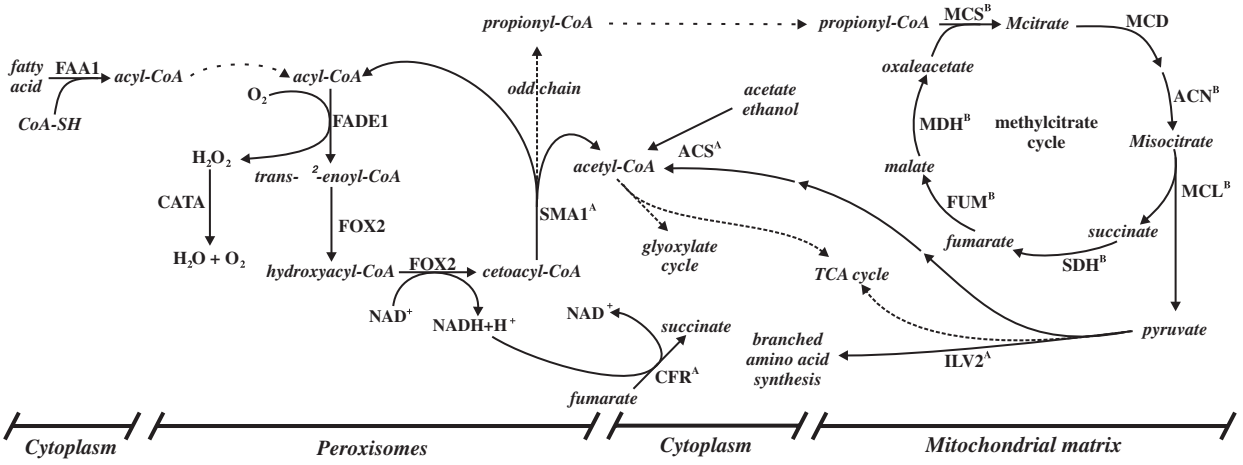

Fig. 6. Some metabolic pathways that are overexpressed during *Paracoccidioides brasiliensis* yeast cell incubation with human plasma. [(A)]Transcripts that are not overexpressed during *Paracoccidioides brasiliensis* treatment with human blood. [(B)]Transcripts present in database. FAA1, long-chain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

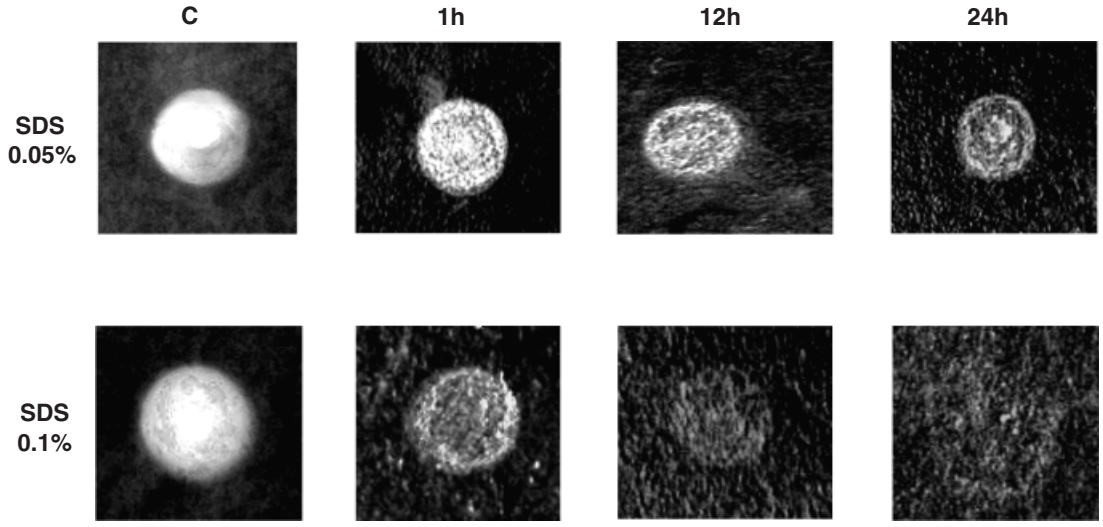

Fig. 7. Phenotypic analysis of *Paracoccidioides brasiliensis* yeast cells after incubation in human plasma for different time periods. Approximately 10^2 cells were spotted onto Fava-Neto's medium plates containing the indicated concentrations of SDS. Plates were incubated at 36 °C for 7 days. Experiments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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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ARTICLE

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

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Abstract Group A rotaviruses (RVA) are the main causing agents of acute gastroenteritis worldwide, having a great impact on childhood mortality in developing countries. The objective of this study was to identify RVA-positive fecal samples with mixed P genotypes by hemi-nested reverse transcriptase-polymerase chain reaction (RT-PCR), followed by sequencing confirmation. Our results showed that, from the 81 RVA-positive samples, 25 were positive for more than one P genotype by hemi-nested RT-PCR. Of these 25 samples, 12 (48%) had their mixed P genotypes confirmed by sequencing and, from these, 10 were identified as P[6]P[8], one as P[4]P[6], and one as P[4]P[6]P[8]. Our results confirm the occurrence of RVA mixed infections among children in Brazil and reinforce the importance of the constant monitoring of RVA circulating strains for the efficacy of control/prevention against these agents.

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Introduction

Gastroenteritis is an important cause of morbidity and childhood mortality, especially in developing countries, where it is estimated that 1.5 billion cases occur per year in children less than five years of age, with about three million deaths [1]. Group A rotaviruses (RVA) belong to the *Reoviridae* family, genus *Rotavirus*, and are the main etiological agents for acute viral gastroenteritis in children [2]. They are responsible, annually, for approximately 111 million episodes of gastroenteritis, 2 million hospitalizations, and 440 thousand deaths of children up to five years of age [1]. The RVA capsid is formed by three concentrical protein layers that surround the viral genome, composed by 11 segments of double-stranded RNA (dsRNA). The external capsid layer is formed by proteins VP7 and VP4, which are both immunogenic and define the G and P genotypes, respectively. Currently, there are at least 15 G and 27 P genotypes described for RVA [2–8]. The VP4 and VP7 genes segregate independently, resulting in several G and P combinations [9, 10], with P[8]G1, P[4]G2, P[8]G3, P[8]G4, and P[8]G9 being the most commonly found worldwide [11, 12].

The segmented nature of the rotavirus genome allows for genomic reassortment, which may result in mixed infec-

tions by uncommon G and P combinations, such as P[4]P[6], G2G8 and P[4]P[6], G2G9. Natural genomic reassortment usually occurs after the same cell is co-infected by samples of common occurrence, such as P[6]G8 with P[4]G2 and P[6]G9 with P[4]G2 [13]. From all of the control/prevention measures against RVA, vaccination has the most potential to succeed, and because RVA immunity seems to be type-specific [14], knowledge about the circulating G and P genotypes/serotypes before, during, and after the vaccination period is highly important. Mixed infections can also have an impact on vaccination effectiveness. This study presents novel information about the occurrence of RVA mixed P genotypes infections in children living in the city of Goiânia in the state of Goiás, Brazil.

Materials and methods

Fecal samples

A total of 81 RVA-positive fecal samples from children, 49 from males and 32 from females, were evaluated. These samples were collected from children up to 5 years of age with acute gastroenteritis and who lived in the city of Goiânia. The samples were collected from April 1998 to August 2003, after written authorization by the parents or legal guardians was obtained. The study was approved by the Ethics in Research Committee of the Federal University of Goiás (no. 004/2000).

RVA detection

The fecal samples were first screened for RVA by immunoenzymatic assay combined for rotavirus and adenovirus (EIARA) [15] and polyacrylamide gel electrophoresis (PAGE) [16] in a previous study conducted in our laboratory.

Viral dsRNA extraction

The viral dsRNA was extracted from 20% fecal suspensions using silica and guanidinium isothiocyanate, as described by Boom et al. [17], following modifications by Cardoso et al. [18].

P genotyping

The samples were submitted to hemi-nested reverse transcriptase-polymerase chain reaction (RT-PCR) P genotyping using a pair of consensus primers (Con2 and Con3) that correspond to conserved nucleotide sequences of VP4. The resulting amplicons of 876 bp were then used as a template in a second PCR, with a mixture of genotype-specific primers (2T-1 P[4], 3T-1 P[6], 1T-1 P[8]) complementary to variable regions of the VP4 genes. The hemi-nested RT-PCR P genotyping was performed according to Gentsch et al. [19]. The primers' designation, sequence, position, and product length are shown in Table 1.

The reverse transcription and the amplification reaction were performed in one stage. The viral dsRNA was combined with dimethyl sulfoxide and incubated at 97°C for 5 min, followed by the addition of the reaction mixture, in a final volume of 100 μl: 1× PCR buffer (20 mM Tris-HCl (pH8.0) and 50 mM KCl; Invitrogen™, Life Technologies, Carlsbad, CA), 2 mM $MgCl_2$, dNTPs mix (dATP 0.8 mM, dCTP 0.8 mM, dTTP 0.8 mM, dGTP 0.8 mM), 2.5 U Taq-DNA polymerase (Invitrogen™), 200 U Reverse Transcriptase SuperScript II (Invitrogen™, Life Technologies), and the consensual primers (0.2 μM each). The cycling parameters used were: 42°C for 60 min, 99°C for 5 min, followed by 30 cycles at 94°C for 1 min, 50°C for 2 min, and 72°C for 1 min, and a final 7-min extension cycle at 72°C.

For the hemi-nested PCR, 1 μL of the product of the first amplification was added to the same reaction mixture, minus the reverse transcriptase, described above using the Con3 as consensual primer, together with the specific primers. The cycling parameters used were: 15 cycles at 94°C for 1 min, 42°C for 2 min, 72°C for 1 min, and a final 7-min extension cycle at 72°C. In all of the reactions, the Wa (human P[8] genotype) prototype sample was used as the positive control and sterile Milli-Q water was used as the negative control. All of the samples were re-tested, under the same conditions, using the specific primers separately.

The amplified product was visualized by gel electrophoresis using 1.5% agarose gel containing ethidium bromide (1 μg/mL). The 123 pb DNA ladder (Invitrogen™, Life Technologies) was used as a molecular weight standard.

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

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

DNA sequencing and phylogenetic analysis

The purification of the hemi-nested RT-PCR products was performed using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. Sequencing of the purified PCR products was performed using the primer Con3 (VP4 gene) and the DYEnamic® ET Dye Terminator Kit (Amersham Bioscences, Piscataway, NJ), by automatic sequencing using the MegaBACE 1000 DNA Sequencer (Amersham Biosciences).

The nucleotide sequences obtained were analyzed and compared with sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and aligned using the Clustal X program [20]. The phylogenetic tree was constructed by the neighbor joining method using the TreeView program.

Results

Genotyping by hemi-nested RT-PCR

After hemi-nested RT-PCR reactions using a pool of the specific primers were performed, each sample's result was confirmed in a second hemi-nested RT-PCR reaction using each of the specific primers separately (Fig. 1). From the 81 samples, 25 reacted with more than one P genotype, 22 were identified as P[6]P[8], one as P[4]P[8], one P[4]P[6], and one was reactive for P[4]P[6]P[8] (data not shown).

Sequencing of RVA-positive samples

Of the 25 RVA samples that were identified as having more than one P genotype, 21 with enough DNA concentration were sequenced. When analyzed by the BLAST program and compared with the sequences deposited in GenBank, 12 samples showed a mixed pattern of P genotypes distributed as follows: ten were P[6]P[8], one P[4]P[6], and one P[4]P[6]P[8] (Table 2). The identity values ranged from 91–100%. Two P[6]P[8] samples (19,316 and 19,608) showed homology to both genotypes, but they were not considered for further analysis because of their sequence size. From all of the samples submitted to sequencing, nine were characterized only as P[8]. Phylogenetic tree construction was performed as follows: a sequence of 143 nucleotides (194–336 nt) for P[4], 153 (111–263 nt) for P[6], and 143 (194–263 nt) for P[8], based on the sequence of the prototype samples RV-5, 1076, and Wa, respectively (Fig. 2).

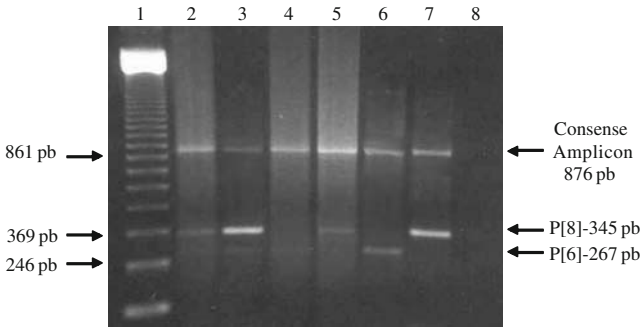

Fig. 1 1.5% agarose gel electrophoresis of hemi-nested reverse transcriptase-polymerase chain reaction (RT-PCR) using a pool of the specific primers and specific primers separately (1T-1 P[8]e, 3T-1 P[6]). Lane 1: 123 bp molecular weight ladder. Lanes 2 and 3: samples 19,318 and 17,158, respectively,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

Discussion

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

Of the 25 RVA-positive samples that were considered as having a mixed P profile by hemi-nested RT-PCR, 12 (48%) were confirmed by genome sequencing and nine (36%) were considered to be positive only for P[8]. One of the reasons why some of the samples did not have their mixed profile confirmed could be the low DNA concentration of the samples after amplification of the P[6] and P[4] fragments, resulting in sub-optimal conditions for sequencing.

In this study, the predominant combinations of P genotypes found were P[6]P[8], followed by P[4]P[6] and P[4]P[6]P[8]. Our results are similar to those of a study conducted in Guinea Bissau, where 38% of the fecal samples collected from children with acute gastroenteritis had mixed P genotypes, with P[4]P[6] being the most predominant [22]. Another study from Denmark showed that 21% of all samples analyzed had mixed P genotypes, with P[4]P[8], followed by P[4]P[6] and P[6]P[8] being the most common [23]. Similar results were also reported in Belém, Brazil, where Mascarenhas et al. [24] detected 23% of mixed P genotypes infections. Another study conducted in Rio de Janeiro, Brazil, revealed that only 16% of the

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

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

samples analyzed had mixed P genotypes, with the predominance of P[4]P[8], followed by P[6]P[8] [21].

The occurrence of mixed infections by RVA in a population may be very important when considering the potential for genetic reassortment among distinct samples, which may result in the emergence of unusual G and P genotype combinations, leading to increased genetic diversity of these agents and, in this way, it can have an impact on the vaccination effectiveness [11, 21, 25].

The G genotyping results of the 12 mixed samples used in this study were published in a previous study conducted in our laboratory [26]. In the present study, from ten of those samples that had been previously genotyped as G1, one was characterized as P[4]P[6]P[8] and nine as P[6]P[8], whereas the one sample with the G2 genotype was identified as P[4]P[6] and the G3 as P[6]P[8].

This is the first study to describe the occurrence of mixed RVA infections in the Central-West region of Brazil. Our data provide important information on the identity of the RVA circulating strains in the region, which will be useful for a better understanding of the impact of RVA mixed infections in childhood gastroenteritis. The findings can also

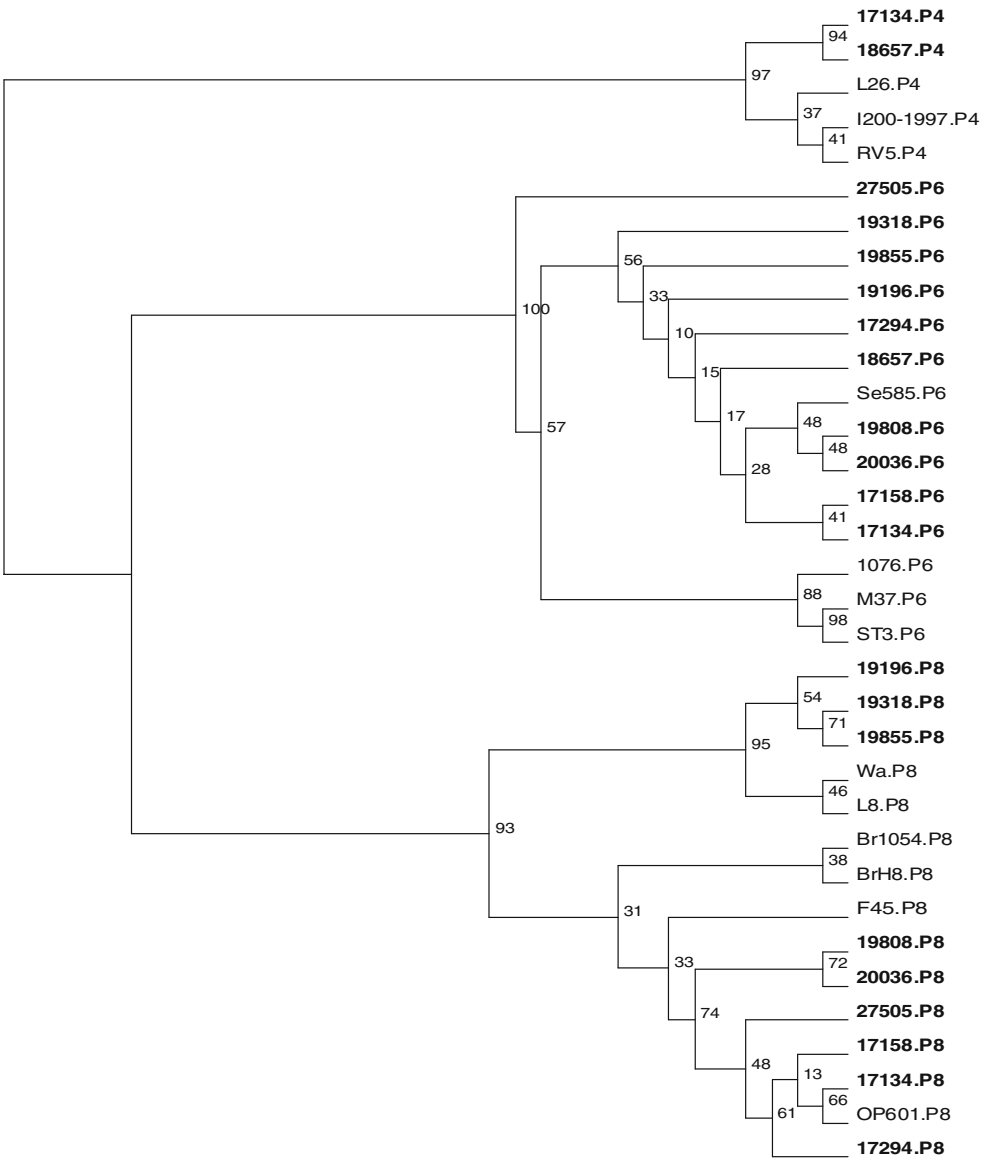

Fig. 2 Phylogenetic analysis of VP4 nucleotide sequences of genotype P mixed samples. The phylogenetic tree was constructed by the neighbor joining method using the Clustal X and TreeView programs. GenBank access numbers: P[8] [OP601 (AJ302153), F45 (U30716), BrH8 (U41006), L8 (AF061358), Wa (L34161),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

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

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

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Nonstructural protein 4 (NSP4), encoded by group A rotavirus genome segment 10, is a multifunctional protein and the first recognized virus-encoded enterotoxin. The NSP4 gene has been sequenced, and five distinct genetic groups have been described: genotypes A-E. NSP4 genotypes A, B, and C have been detected in humans. In this study, the NSP4-encoding gene of human rotavirus strains of different G and P genotypes collected from children between 1987 and 2003 in three cities of West Central region of Brazil was characterized. NSP4 gene of 153 rotavirus-positive fecal samples was amplified by reverse transcriptase-polymerase chain reaction and then sequenced. For phylogenetic analysis, NSP4 nucleotide sequences of these samples were compared to nucleotide sequences of reference strains available in GenBank. Two distinct NSP4 genotypes could be identified: 141 (92.2%) sequences clustered with NSP4 genotype B, and 12 sequences (7.8%) clustered with NSP4 genotype A. These results reinforce that further investigations are needed to assess the validity of NSP4 as a suitable target for epidemiologic surveillance of rotavirus infections and vaccine development.

Key words: group A rotavirus - NSP4 gene - genotypes - West Central region - Brazil

Group A rotaviruses are a major cause of gastroenteritis in infants and young children throughout the world. Each year, these viruses cause approximately 111 million episodes of severe diarrhea, which results in 611,000 deaths (Parashar et al. 2006). In Brazil, the frequency of group A rotavirus infection among young children was found to be between 12 and 42% (Linhares 2000, Cardoso et al. 2003, Costa et al. 2004, Cauás et al. 2006, Munford et al. 2007), and about 80,000 children are hospitalized for the infection yearly (Linhares 2000). Viral particles consist of a non-enveloped, triple-layer protein capsid structure that surrounds a genome composed of 11 segments of double-stranded RNA. The genome encodes six structural proteins (VP1-VP4, VP6 and VP7) and six nonstructural proteins (NSP1-NSP6) (Estes & Kapikian 2007).

Variability in the genes encoding VP7 and VP4 proteins forms the basis of the current strain typing of group A rotaviruses into G and P genotypes, respectively. Studies of rotavirus infections in humans have identified distinct G and P genotypes circulating simultaneously in different parts of the world (Santos & Hoshino 2005, Estes & Kapikian 2007, Gulati et al. 2007, Martella et al. 2007, Munford et al. 2007, Matthijnssens et al. 2008). There is currently only limited information available on the detection or genetic variability of the gene that encodes nonstructural protein 4 (NSP4) (Ciarlet et al. 2000, Mori et al. 2002, Iturriza-Gómara et al. 2003, Araújo et al. 2007, Mascarenhas et al. 2007).

NSP4, encoded by segment 10, is a transmembrane glycoprotein of 175 amino acids (aa) (Estes & Kapikian 2007). NSP4 serves as an intracellular receptor for the budding of subviral double-layered particles into the endoplasmic reticulum, a step that is critical for the acquisition of a transient viral membrane and viral particle maturation (Taylor & Bellamy 2003). In addition, NSP4 has been found to have an enterotoxin-like activity that was originally mapped between aa 114 and 135. Modifications in the toxigenic activity and virulence of rotavirus have been associated with aa changes in this region (Ball et al. 1996, Zhang et al. 1998). Finally, it has been proposed that antibodies against NSP4 might reduce both the frequency and severity of diarrhea in mice. Together with studies in human infants, these data suggest that the immune response to NSP4 could modulate rotavirus-induced diarrhea in human disease (Ball et al. 1996, Yuan et al. 2004, Vizzi et al. 2005).

The NSP4 genes of animal and human rotavirus have been sequenced and compared. Sequence analyses have revealed the existence of five distinct NSP4 genotypes: A (KUN), B (Wa), C (AU-1), D (EW) and E (avian-like).

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Genotypes A, B, C and D have been determined from mammalian rotavirus strains, while genotype E has been identified from avian rotavirus strains. Genotypes A, B and C have been detected in humans (Ciarlet et al. 2000, Mori et al. 2002, Lin & Tian 2003).

In Brazil, there are a few molecular studies of the rotavirus NSP4 gene from strains of diverse origin and various G and P genotypes (Cunliffe et al. 1997, Masca-renhas et al. 2006, 2007, Araújo et al. 2007). As yet, however, there has been no investigation into the detection rate and the genetic diversity of NSP4 genes in the West Central region of Brazil. This study presents novel epidemiological data regarding the circulation of NSP4 genotypes of rotaviruses samples recovered from children in three cities of the West Central region of Brazil.

PATIENTS, MATERIALS AND METHODS

Samples - The NSP4 gene was investigated in 330 rotavirus A-positive fecal samples that were previously obtained from infected children during surveillance studies performed at the Laboratory of Virology of Universidade Federal de Goiás (UFG) in the city of Goiânia, Brazil. These samples were collected from children up to five years of age with (n = 325) or without (n = 5) acute gastroenteritis between August 1987 and September 2003 in three cities of West Central region, Brazil: Goiânia (GO), Campo Grande (CG) and Brasília (BRA). A total of 202 group A rotavirus samples were identified in GO from 1987 to 2001, 81 samples in CG between 2000 and 2003 and 47 samples in BRA in 2001 and 2002. All samples from GO and CG were collected "in nature", whereas, of the samples collected in BRA, only eight were collected "in nature" and 39 were collected by rectal swab. All of the collected samples had been previously identified as group A rotavirus (Cardoso et al. 2003, Souza et al. 2003, Costa et al. 2004, Andreasi et al. 2007) with a combined enzyme immunoassay for rotavirus and adenovirus (Pereira et al. 1985) and/or by polyacrylamide gel electrophoresis (Pereira et al. 1983).

Specimens were collected from children after signed written consent was provided by their parents or other legal guardians. This study was approved by the Ethics Committee of the Research of UFG (Protocol nº.004/ 2000).

RNA extraction - The viral dsRNA was extracted from 20% fecal suspension by the glass powder method, using guanidine isothiocyanate buffer and silica as described by Boom et al. (1990) with modifications (Cardoso et al. 2002).

Reverse transcription-polymerase chain reaction (RT-PCR) amplification - The RT-PCR followed the protocol described by Lee et al. (2000). The purified viral double-stranded RNA (dsRNA) was denatured at 97ºC for 10 min and then used as template for the RT-PCR. The RT of dsRNA was carried out with SuperScript™ (Invitrogen Carlsbad, CA, USA), and PCR amplification was performed with Taq DNA polymerase (Invitrogen Carlsbad, CA, USA). Fragments of the NSP4 gene of 725 bp were amplified using forward (10BEG16) and reverse (10END722) primers as described by Lee et al. (2000).

Sequencing reaction - The PCR products were purified using the QIAquick® PCR purification kit (Qiagen, São Paulo, Brazil). The PCR-purified products were sequenced by a MegaBACE 1000 automatic sequencer (GE Healthcare, Sunnyvale, USA), using a DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Buckinghamshire, United Kingdom). The primers used were the same as for PCR amplification. The products were further purified by ethanol precipitation and resuspended in formamide.

Sequence analysis - The sequences obtained were analyzed with PHRED/PHRAP/CONSED (http://www.phrap.org) and pre-processed using the Phred (Ewing & Green 1998) and Crossmatch (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm) programs. Only sequences with at least 100 nucleotides and Phred quality greater than or equal to 20 were considered for further analysis with the Blastn program (Altschul et al. 1990) in the National Center for Biotechnology Information Database (http://www.ncbi.nlm.nih.gov). NSP4 sequences were aligned and compared to NSP4 sequences of standard reference strains [AU-1 (D89873), AU32 (D88830), Ch-1 (AB065287), EW (U96335), KUN (D88829), RV5 (U59103), OSU (D88831), Wa (AF093199)] and Brazilian strains [rj5348 (DQ498179) and rj7363 (DQ498192)] available in GenBank using Clustal X software (Thompson et al. 1997). NSP4 genotypes were determined by phylogenetic analysis using the neighbor-joining algorithm method (Saitou & Nei 1987).

Nucleotide sequence accession numbers - The NSP4 nucleotide sequence data determined in this study were deposited into the GenBank sequence database with accession numbers: EU620072-EU620111.

Statistical analysis - The statistical analysis was performed with the Epi Info version 6 program, using the chi-squared (χ^2) test for proportion comparison. Statistical significance was assessed at a p value of < 0.05.

RESULTS

The NSP4 gene was detected in 259 (78.5%) of 330 rotavirus A-positive fecal samples. Of them, rates of 82.7% (167/202), 85.2% (69/81) and 48.9% (23/47) were observed in fecal specimens from children in GO, CG and BRA, respectively ($\chi^2 = 28.55$, $p = 0.000$).

A total of 231 samples that had an amplified NSP4 gene were submitted to sequencing. NSP4 nucleotide sequences of 153 (66.2%) samples could be analyzed and were compared to nucleotide sequences of prototype strains. Of those, 141 sequences (92.2%) clustered with NSP4 genotype B with degrees of identity ranging from 89.0% to 99.0%; and the remaining 12 sequences (7.8%) clustered with NSP4 genotype A (93.0% - 95.0% of identity). Genotype A samples were identified only in children from GO, while genotype B samples were identified in samples from all three cities (Table I). In GO, the only sample collected in 1987 was genotype B; in the 1990s, however, both genotypes A and B were detected in the 45 samples analyzed, with genotype B (73.3%) predominating. From 2000 to 2003, only genotype B samples were found to be circulating in the three cities analyzed (Table II).

TABLE I

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

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

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

TABLE II

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

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

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

Rotavirus samples P[8]G1, P[6]G1, P[8]G2, P[8]G3, P[8]G4 and P[8]G9 were more closely related to NSP4 genotype B, whereas P[6]G9 rotavirus samples were closely related to NSP4 genotype A. All of these samples that clustered into genotype A or B had human origin (Fig. 1, Table III).

NSP4 genotypes A and B were identified in children with or without acute gastroenteritis. Of the 153 samples, 151 were from children with diarrhea, and two were from children without diarrhea. No significant differences were found in the nucleotide sequences of the NSP4 genes from symptomatic and asymptomatic samples.

The deduced aa sequences of the NSP4 genes of 15 human group A rotavirus samples were aligned with aa sequences of reference prototype strains (Fig. 2). Variations between sequences of genotypes A and B were found in the H3 cytoplasmic domain (aa 63-80), amphipathic alpha-helix domain (aa 93-133), VP4 binding site (aa 112-146), interspecies variable domain (aa 131-141) and VP6 binding site (aa 156-175). Changes were also identified in aa 89, 148, 153 and 154.

Several significant aa differences were observed between distinct NSP4 genotypes, mainly in the interspecies variable domain (Fig. 2, marked in gray box) and in the VP6 binding site (Fig. 2, indicated by diamonds).

In the region proposed to be the enterotoxigenic domain (aa 114-135), the following changes were observed: (Y-H) and (K-N) at aa 131 and 133, respectively (Fig. 2, indicated by asterisks). No differences in the aa sequences of NSP4 were observed between samples from children with or without diarrhea (Fig. 2).

DISCUSSION

In this study, a detection rate of 78.5% was observed for the NSP4-encoding gene from rotavirus-positive fecal samples collected in three cities located in the West Central region of Brazil. We speculate that the NSP4 gene could not be detected in all samples due to degradation of the RNA probably as a result of: i) low number of particles present in fecal specimens from rectal swabs; ii) RNA degradation by RNAses; iii) repeated freezing and unfreezing of these samples; iv) preservation of fecal specimens at -20°C and not at -70°C; v) defective particle; presence and/or vi) eventual inhibitor persistence of the RT-PCR.

Although five rotavirus NSP4 genotypes have been identified to date (Ciarlet et al. 2000, Mori et al. 2002, Lin & Tian 2003), most of the diversity in the NSP4-encoding gene among human rotaviruses lies in genotypes A and B (Iturriza-Gómara et al. 2003). Other studies, however, have detected unusual strains. Cho et al. (2006) observed that two human rotavirus samples in Seoul had a low degree of homology with the currently described NSP4 genotypes, suggesting a possible new NSP4 genotype.

In this study, NSP4 genotypes A and B could be recognized in human group A rotavirus-positive fecal samples. These results are similar to those described by other authors in Brazil (Mascarenhas et al. 2006, 2007, Araújo et al. 2007) and in other countries (Kirkwood et al. 1999, Lee et al. 2000, Iturriza-Gómara et al. 2003, Cho et al. 2006). None of the samples analyzed in our study were closely related to the AU-1 prototype strain, which was isolated from humans and described as NSP4 genotype C (Iturriza-Gómara et al. 2003).

Our data show that genotype B was the most frequently detected (92.2%) genotype in the West Central region. Similar data were also observed in the Southeast region of Brazil (Araújo et al. 2007) as well as in other countries (Kirkwood et al. 1999, Lee et al. 2000, Iturriza-Gómara et al. 2003, Cho et al. 2006); however, different results were observed in the Northern region of Brazil, where genotype A was predominant (Mascarenhas et al. 2006, 2007). Genotype A samples were identified only in children from GO, while genotype B samples were identified in all three cities. In GO, genotype A was found only in the 1990s; however, after 2000, it seemed to be replaced by genotype B. Furthermore, the only sample identified in 1980s was genotype B, suggesting that the circulation of NSP4 genotypes changes over time. After 2000, genotype B was also found in CG and BRA. In Rio de Janeiro (RJ), it was shown that genotype B circulated during the years 1986-1988, 1990 and 2001-2004, while genotype A circulated only in 2002 (Araújo et al. 2007). Both genotypes A and B were identified during the 1990s and in 2000 from children in Belém (Mascarenhas et al. 2007).

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

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

TABLE III

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

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

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

sequences of symptomatic and asymptomatic samples. These results are consistent with other investigations (Horie et al. 1997, Lee et al. 2000, Mascarenhas et al. 2007); however, since only two samples from children without diarrhea were analyzed, the role of NSP4 as a possible pathogenic determinant of rotavirus could not be assessed in this work.

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

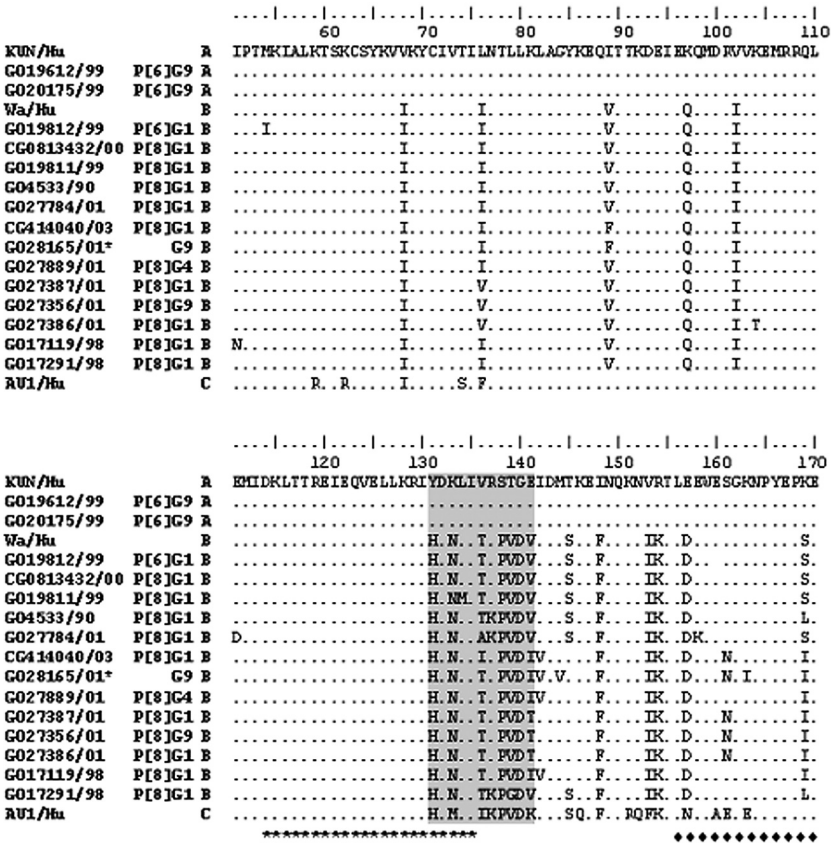

Fig. 2: multiple alignment of the partial deduced amino acid sequence of the NSP4 protein of 15 human rotaviruses samples with human prototype strains grouped in genotypes A, B and C. Dots indicate identity to the KUN/Hu strain. Gray box indicates interspecies variable domain. Diamonds indicate VP6 binding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

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

Amino acid variations between genotypes A and B were concentrated mainly in the interspecies variable domain (aa 131-141) and in the VP6 binding region (aa 156-175) (Estes & Kapikian 2007). Our data on the region of aa 131-141 are similar to a study from Brazil (Araújo et al. 2007), considering the occurrence of extensive variation in this region, as well as to a study performed in the UK (Iturriza-Gómara et al. 2003). In addition, in the present study, it was observed that aa 131 was identified as tyrosine (genotype A) or histidine (genotype B), similar to the results of Mascarenhas et al. (2007), but contrary to the common postulate that diarrheic samples have a tyrosine at this position (Ball et al. 1996). Our results are also in agreement with other investigations (Cunlife et al. 1997, Iturriza-Gómara et al. 2003, Araújo et al. 2007), and they suggest that at these NSP4 protein regions are distinct between genotypes.

The immune response to A and B genotypes of the NSP4 gene has not yet been defined, and it is still unknown whether the inclusion of this gene in rotavirus vaccination strategies is important (Araújo et al. 2007). In this context, the possible selection of NSP4 as a target for vaccine development requires further investigation (Lee et al. 2000, Araújo et al. 2007).

In this study, the nucleotide sequence of the NSP4 gene was determined for the first time in 153 human rotavirus strains belonging to genotypes of different G and P combinations recovered from children with or without diarrhea in West Central region of Brazil. The NSP4 gene

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

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Capítulo VI

Perspectivas

VI – PERSPECTIVAS

1. Análise da expressão gênica da serino protease em condições de limitação de nitrogênio e durante a infecção em diferentes sítios de infecção em camundongos através da técnica de PCR em tempo real;

2. Análise de atividade proteolítica da serino protease em gel de atividade contendo gelatina;

3. Análises proteômicas de sobrenadante de cultura de *P. brasiliensis* em condições de limitação de nitrogênio;

4. Ampliar estudos de interações intermoleculares de serino protease de *P. brasiliensis* através da técnica de duplo-híbrido em sistema *S. cerevisiae;*

5. Desenvolvimento de ferramentas genéticas para análise do papel das proteases identificadas diferencialmente expressas durante a transição dimórfica de *P. brasiliensis*.

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VIII. Anexos

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1. TACCO BACA, **PARENTE JA**, BARBOSA MS, BÁO SN, GÓES TS, PEREIRA M, SOARES CMA. Characterization of a secreted aspartyl protease of the fungal pathogen *Paracoccidioides brasiliensis*. Medical Mycology. *In press.*

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