Universidade de Brasília

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Caracterização molecular e funcional dos genes *ras1* e *ras2* do fungo dimórfico e patogênico *Paracoccidioides brasiliensis*

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DEDICATÓRIA

Dedico este trabalho a minha família que me ensinou os valores da vida, especialmente à minha mãe Amaryllis, minha avó Nadyr, minha madrinha Nair e tia Nilce e minha tia Cybele, que estiveram comigo mesmo a distância durante toda a minha caminhada. Ao meu marido, Valter, que estendeu a sua mão e foi mais que um amigo e companheiro apoiando, incentivando, e sempre acreditando que eu venceria mais esta etapa da minha vida.

"A ciência humana de maneira nenhuma nega a existência de Deus. Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável." (Galileu Galilei)

Quase
(Fernando Pessoa)

Ainda pior que a convicção do não, é a incerteza do talvez, é a desilusão de um quase!
É o quase que me incomoda, que me entristece, que me mata trazendo tudo que poderia ter sido e não foi.
Quem quase ganhou ainda joga, quem quase passou ainda estuda, quem quase amou não amou.
Basta pensar nas oportunidades que escaparam pelos dedos, nas chances que se perdem por medo, nas idéias que nunca sairão do papel por essa maldita mania de viver no outono.
Pergunto-me, às vezes, o que nos leva a escolher uma vida morna.
A resposta eu sei de cor, está estampada na distância e na frieza dos sorrisos, na frouxidão dos abraços, na indiferença dos "bom dia", quase que sussurrados.
Sobra covardia e falta coragem até para ser feliz.
A paixão queima, o amor enlouquece, o desejo trai.
Talvez esses fossem bons motivos para decidir entre a alegria e a dor.
Mas não são.
Se a virtude estivesse mesmo no meio-termo, o mar não teria ondas, os dias seriam nublados e o arco-íris em tons de cinza.
O nada não ilumina, não inspira, não aflige nem acalma, apenas amplia o vazio que cada um traz dentro de si.
Preferir a derrota prévia à dúvida da vitória é desperdiçar a oportunidade de merecer.
Para os erros há perdão, para os fracassos, chance, para os amores impossíveis, tempo.
De nada adianta cercar um coração vazio ou economizar alma.
Um romance cujo fim é instantâneo ou indolor não é romance.
Não deixe que a saudade sufoque, que a rotina acomode, que o medo impeça de tentar.
Desconfie do destino e acredite em você.
Gaste mais horas realizando que sonhando...
Fazendo que planejando...
Vivendo que esperando...
Porque, embora quem quase morre esteja vivo, quem quase vive já morreu.

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RESUMO

Paracoccidioides brasiliensis é um fungo termo-dimórfico que causa uma micose sistêmica de alta incidência na América Latina. Devido sua participação no controle de morfogênese, diferenciação e virulência em patógenos, decidiu-se caracterizar os genes *ras* em *P. brasiliensis*. Foram identificados *ras1* e *ras2* que codificam para duas proteínas diferentes com alta identidade. O padrão transcricional de *ras* também foi investigado por RT-PCR durante a transição micélio para levedura (M→Y), choque térmico a 42ºC e após internalização de leveduras em macrófagos murinos. Ambos os genes foram regulados negativamente em leveduras internalizadas em macrófagos e *ras1* foi modulado negativamente a 42ºC. Entretanto, os genes *ras* não apresentaram variação transcricional durante a transição M→Y. O fato de que as proteínas Ras são localizadas na membrana através de farnesilação, permitiu a análise *in silico* dos genes que codificam para as subunidades das preniltransferases (farnesiltransferase e geranilgeraniltransferase I): *ram1, ram2* e *cdc43*. *P. brasiliensis* apresenta em seu genoma todos os genes necessários para maquinaria de prenilação. Um inibidor de farnesiltransferase foi utilizado para investigar a importância desse processo no crescimento vegetativo e transição dimórfica. O bloqueio da farnesilação interferiu com o crescimento vegetativo de leveduras e estimulou a produção de tubos germinativos mesmo a 37ºC. Durante a transição Y→M, o inibidor aumentou a filamentação de maneira dose-dependente, indicando que o bloqueio da farnesilação favorece a forma miceliana de *P. brasiliensis*. Os resultados sugerem que os genes *ras* devem ter um papel no dimorfismo, resposta a choque térmico e na interação patógeno-hospedeiro. Uma estratégia para estudar a função de *ras1* em *P. brasiliensis* também foi desenhada através de interferência no RNA. Cassetes de silenciamento com *ras1* senso e antisenso foram construídos para futura investigação detalhada do papel dos genes *ras* na patobiologia deste fungo.

ABSTRACT

Paracoccidioides brasiliensis is a thermo-dimorphic fungus that causes a human systemic mycosis with high incidence in Latin America. Due to their participation in the control of pathogen morphogenesis, differentiation and virulence the characterization of *ras* genes in *P. brasiliensis* was done. It was identified *ras1* and *ras2* coding for two different proteins with high identity. The *ras* transcriptional pattern was investigated by RT-PCR during mycelium-to-yeast (M→Y) transition, heat shock at 42ºC and after internalization of yeast cells by murine macrophages. Both genes were down regulated inside macrophages and *ras1*, at 42ºC. In contrast, the *ras* genes did not show any transcriptional variation during the M→Y transition. The fact that Ras proteins are attached to the membrane via farnesylation prompted the search on Pb gene database for the genes coding the subunits of the prenyltransferases (farnesyltransferase and geranilgeraniltransferase I): *ram1, ram2* and *cdc43*. *P. brasiliensis* has all genes necessary to the prenylation machinary. Also, a farnesyltransferase inhibitor was used to investigate the importance that process to vegetative growth and dimorphic transition. Farnesylation inhibition interfered with vegetative growth of yeast cells and stimulated germinative tube production even at 37ºC. During Y→M transition the inhibitor increased filamentation in a dose-dependent manner, indicating that impairement of farnesylation favors the mycelium form of *P. brasiliensis.* The results suggest that *ras* genes might have a role in dimorphism, heat shock response and in host-pathogen interaction. A strategy to study the *ras1* function in *P. brasiliensis* via RNA interference was designed. Cassettes with *ras1* sense and antisense were constructed for further investigate in detail the possible roles of *ras* genes in this fungal pathogen

1 - Introdução

1.1) O fungo e a doença

Paracoccidioides brasiliensis pertence ao grupo dos fungos dimórficos, que apresentam ampla distribuição geográfica e causam doenças significativas podendo variar de uma infecção transitória a uma micose sistêmica de tratamento prolongado. Juntamente com *P. brasiliensis*, outros fungos dimórficos destacam-se como sendo de importância médica: *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides sp*., *Sporothrix schenckii* e *Penicillium marneffei* (Rappley & Goldman, 2006). Todos estes fungos apresentam-se como micélio no ambiente (temperatura de 22-25ºC) onde crescem como saprófitas, porém quando entram em contato com o hospedeiro (temperatura de 35-37ºC), pela inalação de propágulos (conídios ou artroconídios), estes se diferenciam e dão origem a células leveduriformes que é a forma patogênica de tais organismos. Dessa forma o mecanismo de transição dimórfica dependente de temperatura mostra-se fundamental para o estabelecimento da infecção, sendo desencadeado por uma série de fatores relacionados à interação patógeno – hospedeiro. As alterações morfológicas observadas nesses organismos durante o processo infectivo contribuem para a patogênese, sendo um aspecto de grande relevância para a virulência do patógeno (San-Blas & San-Blas, 1994; Odds *et al.*, 2001; Gow *et al.,* 2002; revisto por Rooney & Klein, 2002). Diferentemente de outros fungos ascomicetos oportunistas como *Aspergillus sp* e *Candida sp*, os fungos dimórficos são capazes de infectar e causar doença em pessoas imunocompetentes, como evidenciados em surtos nas regiões endêmicas (Rappley & Goldman, 2006).

P. brasiliensis é o agente etiológico da Paracoccidioidomicose (PCM), a micose sistêmica de maior prevalência na América Latina. Estima-se que mais de 10 milhões de pessoas estejam infectadas com este patógeno, sendo que apenas 2% desenvolvem a doença (Franco, 1987; McEwen *et al.*, 1995; Restrepo *et al*., 2001). Dentre as áreas de maior incidência da PCM estão o Brasil (80% dos casos), Venezuela e Colômbia (Brummer *et al*., 1993; McEwen *et al*., 1995; Restrepo *et al*., 2001) e as regiões endêmicas que se estendem do México à Argentina. Tais regiões apresentam características climáticas favoráveis ao crescimento do fungo como temperaturas que variam entre 18°C e 24°C, altos índices pluviométricos, e altitudes entre 1300 e 1700 m (Brummer *et al*., 1993; revisto por San-Blas & Niño-Vega, 2001).

Uma vez inalados os propágulos de *P. brasiliensis*, estes podem atingir os pulmões, e sofrer a transição para a forma de levedura, fundamental para o estabelecimento da infecção (revisto por Rooney & Klein, 2002). A levedura, por sua vez pode disseminar-se para os demais tecidos do hospedeiro, tornar-se latente, ou ser erradicada, dependendo da resposta imunológica do hospedeiro e da virulência do fungo (Franco, 1987; San-Blas *et al.*, 2002). A infecção pode dar-se ainda por trauma na mucosa oral, anal ou cutânea (Restrepo, 1978).

A maioria dos indivíduos infectados por *P. brasiliensis* desenvolve apenas infecção assintomática, definida como PCM-infecção, a qual acomete principalmente indivíduos sadios que vivem em áreas endêmicas. Nos indivíduos que desenvolvem a doença, as manifestações clínicas são variadas, sendo caracterizadas principalmente por granulomas crônicos com alta freqüência de lesões pulmonares e muco-cutâneas (Franco, 1987; Franco *et al*., 1993). A PCM-doença apresenta-se como duas formas que se diferenciam quanto às características clínicas, imunológicas e histopatológicas: a forma aguda (tipo juvenil) ou a forma crônica (tipo adulto). A PCM aguda é geralmente de caráter bastante severo e acomete principalmente jovens de ambos os sexos. Essa forma da doença atinge primariamente os pulmões, progredindo rapidamente por disseminação linfática e sangüínea, e comprometendo outras partes do organismo. A forma crônica é mais comum (90% dos casos), apresentando lesões restritas a alguns órgãos, e de progressão mais lenta. Pode acarretar seqüelas pulmonares e subcutâneas e, em casos mais graves, ou na ausência de tratamento adequado, pode vir a causar a morte do indivíduo infectado. Os pacientes que desenvolvem esse tipo de manifestação clínica da doença são geralmente adultos do sexo masculino (Franco, 1987; Brummer *et al.*, 1993; Camargo & Franco, 2000).

A incidência da PCM predomina em adultos do sexo masculino (80 a 90% dos casos), chegando a uma taxa homem: mulher de 78:1 casos, porém esses dados não apresentam relação com o grau de exposição dos mesmos, uma vez que estudos indicam que ambos os sexos apresentam contato similar com o patógeno (Restrepo *et al.*, 1984). Estes autores sugeriram o papel protetor do hormônio feminino β-estradiol, uma vez que este hormônio inibe a transição *in vitro* de *P. brasiliensis* de micélio ou conídios para levedura (Restrepo *et al*., 1984; Salazar *et al*., 1988).

A PCM caracteriza-se como uma doença de padrão granulomatoso. O granuloma, a lesão fundamental desta doença, é resultante de uma reação de hipersensibilidade tardia (DTH) contra antígenos do agente infeccioso que permite a contenção do patógeno e impede assim sua disseminação pelo organismo (Romani, 1997). Plasmócitos, eosinófilos e fibroblastos estão presentes no granuloma (de Brito & Franco, 1994), no entanto, a principal célula constituinte é o macrófago, que tem como funções a liberação de substâncias microbicidas (óxido nítrico, radicais superóxidos e peróxido de hidrogênio); a apresentação de antígenos e recrutamento de linfócitos T para produção de citocinas que variam de acordo com a susceptibilidade do hospedeiro. A atividade de linfócitos T e a resposta granulomatosa são características das formas localizadas da infecção, enquanto que nas formas disseminadas não se observa formação de granulomas epitelióides (Murphy, 1998, Camargo & Franco, 2000).

Através de estudos de Benard *et al*. (2001) pode-se observar que existe uma variação muito grande no padrão de citocinas liberadas por indivíduos acometidos pelas formas crônica e aguda da PCM, bem como indivíduos sadios sensibilizados por *P. brasiliensis*. Os indivíduos sadios previamente sensibilizados por *P. brasiliensis* demonstraram uma produção substancial de IL-2, IFN-γ e IL-10, enquanto que os pacientes que desenvolveram a doença apresentaram baixos níveis de IL-2 e IFN-γ e níveis elevados de IL-10. Fornari *et al*. (2001) verificaram que o soro de pacientes com a doença crônica apresentava altos níveis de IL-10, TNF-α e IL-2 e pequena produção de IFN-γ. Tais dados sugerem que a resposta imunológica celular, caracterizada pela produção de citocinas do tipo 1 (IFN-γ, IL-2 e IL-12) parece ser o principal mecanismo de defesa do hospedeiro contra *P. brasiliensis*. (Cano *et al.*, 1998; Souto *et al.*, 2000; Arruda *et al.*, 2002).

A resposta imune celular e formação do granuloma fornecem uma resposta protetora máxima (migração, diferenciação e ativação de macrófagos) contra patógenos intracelulares (Rumbley & Phillips, 1999). Esse aspecto é particularmente importante para *P. brasiliensis*, uma vez que esse fungo pode apresentar-se como um patógeno intracelular facultativo, capaz de sobreviver e se replicar no interior de células epiteliais e de macrófagos murinos e humanos não ativados (Brummer *et al.*, 1989; Moscardi-Bacchi *et al.*, 1994).

Estudos recentes demonstraram que *P. brasiliensis* também é capaz de desenvolver a doença em organismos diferentes de humanos. Ricci *et al*. (2004) relataram um caso de infecção de um cão doméstico adulto no qual foi observada a formação de granuloma epitelióide, característica da manifestação clínica da PCM. Bagagli *et al.* (1998) reportaram a infecção de tatus (*Dasypus novemcinctus*) capturados em áreas cuja incidência da PCM é endêmica, com a presença de granuloma em torno do fungo em diversos órgãos como pulmão, baço e fígado, sugerindo que esses animais sejam também possíveis hospedeiros de *P. brasiliensis*. O fato de a PCM atingir predominantemente trabalhadores rurais ou pessoas que diretamente lidam com a terra, vem sendo associado aos hábitos de vida e ao nicho ecológico do tatu (Brummer *et al.*, 1993; Restrepo *et al.*, 2001). Acredita-se que a contaminação de homens e tatus por *P. brasiliensis* possa ser ocasionada devido a atividades provavelmente relacionadas ao habitat do fungo (Wanke & Londero, 1994). O habitat e o ciclo biológico de *P. brasiliensis* ainda não foram determinados com precisão, sendo provável, com base nas informações acumuladas até o presente momento, que este apresente uma fase saprofítica no solo e/ou em vegetais, além de já ter sido isolado de fezes de alguns animais silvestres (Restrepo *et al*., 2001).

Apesar de não se conhecer o estado teleomórfico (sexual) de *P. brasiliensis*, estudos filogenéticos baseados em seqüências de nucleotídeos do RNA ribossomal e análises morfológicas, propõem a classificação deste patógeno como um ascomiceto (filo Ascomycota), provável membro da ordem Onygenales, família Onygenaceae (Leclerc *et al*., 1994; Bialek *et al*., 2000).

Desde a sua descrição em 1908 por Adolfo Lutz, o fungo *P. brasiliensis* é considerado como uma espécie única (revisto por Franco *et al*., 1994), no entanto existe uma grande diversidade genética entre diferentes isolados de *P. brasiliensis* (Matute *et al.*, 2006), e uma alta variação nos níveis de virulência desses isolados em modelos experimentais estabelecidos (Singer-Vermes *et al.*, 1989). Recentemente, Matute *et al.* (2006) propuseram com base em estudos filogenéticos de 65 isolados de *P. brasiliensis*, a existência de três diferentes espécies crípticas decorrentes de dois eventos independentes de especiação, correlacionados ao padrão de distribuição dessas potenciais espécies.

A ploidia de *P.brasiliensis* ainda não é conhecida exatamente. Cano *et al.* (1998) e Montoya *et al*, (1999) sugerem que é um organismo diplóide, com 4 ou 5

cromossomos de tamanhos que variam de 2 a 10Mb, com tamanho do genoma estimado entre 20 a 23 Mb. Feitosa *et al*. (2003) utilizando as técnicas de eletroforese em campo pulsado e hibridização do DNA compararam o cariótipo de 12 isolados de *P. brasiliensis* de diferentes regiões geográficas, sendo que os resultados sugerem a existência de isolados haplóides, diplóides e ainda aneuplóides, indicando a grande variabilidade genética deste fungo. Recentemente, Almeida *et al*. (2007) avaliaram a ploidia e o tamanho do genoma de 10 isolados de *P. brasiliensis* utilizando citometria de fluxo. Estes autores relataram um genoma de tamanho estimado de 26 a 35Mb sendo que, ao contrário do observado por Feitosa *et al.* (2003), todos os isolados analisados apresentaram-se haplóides ou aneuplóides.

1.2) As vias de sinalização em *P. brasiliensis*

A virulência fúngica é um evento altamente complexo e multifatorial resultante da regulação de diversas vias de sinalização que culminam na ativação de um conjunto de genes em diferentes estágios da infecção e cuja conseqüência está fortemente associada ao estabelecimento da patogênese. As etapas de adesão e a sobrevivência do patógeno no interior do hospedeiro mostram-se essenciais no estabelecimento da patogênese. Em *P. brasiliensis*, as proteínas envolvidas na interação e adesão do fungo com as células do hospedeiro têm sido correlacionadas à virulência e patogenicidade, dentre estas proteínas de adesão, destacam-se a glicoproteína 43 (gp43) (Gesztesi *et al.*, 1996), uma adesina de 30kDa (Andreotti *et al*., 2005) e a gliceraldeído-3-fosfato desidrogenase (Barbosa *et al.,* 2006). Nesse contexto, fatores importantes para o estabelecimento da infecção por fungos patogênicos vêm sendo descritos embora apenas recentemente os mecanismos genéticos e moleculares envolvidos na expressão de genes de virulência começaram a ser investigados (Kwon-Chung, 1998; Odds *et al*., 2001; Yang, 2003). Análise *in silico* do transcriptoma de *P. brasiliensis* revelou a presença de vários genes ortólogos envolvidos em virulência de outros patógenos. Estes genes foram agrupados de acordo com a função que desempenham na célula: metabolismo (enzimas que compõem o ciclo do glioxalato), síntese e estabilização da parede celular (α- e β-glucana sintases, quitina sintase e manosiltransferase), eliminação de substancias tóxicas

(superoxido dismutase, tiol peroxidase e oxidase alternativa) e fatores secretados (fosfolipases e urease) (Tavares *et al.,* 2005).

Microrganismos patogênicos precisam adaptar-se às mudanças dramáticas quando em contato com o hospedeiro. Nesses organismos foi demonstrado que as vias de sinalização, utilizadas para detecção de mudanças ambientais, também são responsáveis pelo controle da expressão de genes relacionados à virulência e patogênese, que por sua vez, culminam na infecção do hospedeiro. Assim, patógenos são excelentes modelos para o estudo genético do controle da adaptação e desenvolvimento bem como na definição de mecanismos de patogênese (Waugh *et al.,* 2002).

Em paralelo a este trabalho de tese, foi desenvolvido pelo nosso grupo o "Projeto Genoma Funcional e Diferencial de *P. brasiliensis*" (Felipe *et al.*, 2003 e 2005), através do qual foi possível realizar a identificação e categorização de genes ortólogos descritos para outros fungos patogênicos como componentes das vias de sinalização celular, virulência, resistência à drogas, alvos para drogas, dimorfismo, entre outros. O projeto teve como objetivo principal o mapeamento do transcriptoma na condição de cultivo *in vitro* das formas de micélio e levedura de *P. brasiliensis*. Foram geradas um total de 19.718 ESTs, sendo 16.351 agrupadas em 2.655 "contigs' e o restante descrito como "singlets" ou seqüências únicas, constituindo um total de 6.022 PbAEST (*P. brasiliensis* Assembled Expressed Sequence Tags)". Do total de PbAESTs obtidas, 69,4% mostraram similaridade com seqüências já descritas de outros organismos, depositadas em bancos de dados. Análises utilizando-se ferramentas de bioinformática permitiram categorizá-las em 12 grupos, de acordo com a provável função, como mostrado na **Figura 1** (Felipe *et al.*, 2003 e 2005). Devido à grande conservação dos componentes das vias de sinalização celular em eucariotos, Fernandes *et al*. (2005) realizaram em paralelo a este trabalho de tese, a comparação *in silico* das PbAESTs encontradas no transcriptoma de *P. brasiliensis* e os genes ortólogos já descritos nos fungos *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Candida albicans* e *Aspergillus fumigatus.* Através dessa análise foi possível identificar várias cascatas de sinalização, sendo as mais importantes: (I) MAPKinases envolvida em integridade celular, construção da parede celular, acasalamento e regulação osmótica, (II) via AMPc/PKA que regula desenvolvimento celular e virulência fúngica, (III) Ras-GTPases - que em outros

fungos permitem uma interação cruzada entre as vias MAPKinases e AMPc/PKA, (IV) via Cálcio-calmodulina-calcineurina que controla sobrevivência celular em condições de estresse oxidativo e/ou térmico e responde a perturbações da parede celular e (V) via TOR que, por sua vez, controla crescimento e proliferação celular (Fernandes *et al.,* 2005). Na **Tabela 1** estão descritas e categorizadas todas as PbAESTs envolvidas nas diferentes vias de sinalização encontradas em *P. brasiliensis* (Fernandes *et al*., 2005).

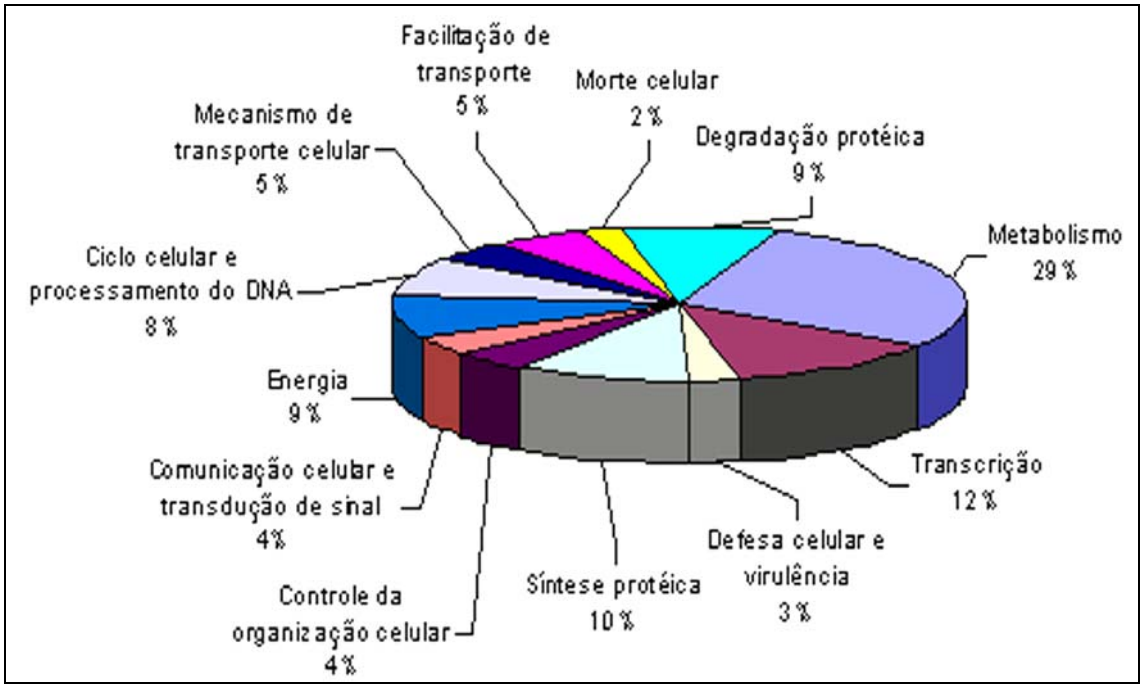

Figura 1: Principais categorias estabelecidas para os transcritos identificados pelo Projeto Genoma Funcional e Diferencial de *P. brasiliensis* (Felipe *et al.,* 2003 e 2005). As PbAESTs que apresentaram similaridade com seqüências depositadas em bancos de dados foram categorizadas em 12 grupos de acordo com suas prováveis funções, incluindo os de sinalização celular que correspondem a 4% das PbAESTs.

Tabela 1: Componentes das vias de sinalização celular anotados pela análise do transcriptoma de *P. brasiliensis* (Fernandes *et al*., 2005.)

Via de sinalização	*PbAEST*	*Nome*	*Número de acesso/ Organismo*	*E-Value*
Ca^{2+}/Calmodulina/ Calcineurina	1629	*camd1*	AF156028 *Emericella nidulans* (calmodulin).	$3.1e^{-40}$
	2381	*cna1*	AF071751 *Neurospora crassa* (calcineurin B, catalytic subunit).	$8e^{-54}$
	2252	*cnb1*	AL513466 *Neurospora crassa* (calcineurin B, regulatory subunit).	$5e^{-84}$
	2171	*cmk1*	NP_587941.1 *Saccharomyces cerevisiae* (Calcium calmodulin dependent protein kinase).	$1e^{-165}$
	2415	*crz1*	NP_014371.1 *Saccharomyces cerevisiae* (calcineurin responsive zinc-finger).	4e-41
	5082	*cka1*	NP_593642.1 *Arabidopsis thaliana* (casein kinase II alpha subunit).	$8.3e^{-26}$
Ras/AMPc/PKA	595	*gpa2*	gi|6323816 *Saccharomyces cerevisiae* (guanine nucleotide-binding regulatory protein).	0.0042
	2208	*gpb*	AF176775_1 *Aspergillus nidulans* (G protein β-subunit).	$5e^{-88}$
	3426	*cyr1*	NP_194335.1 *Arabidopsis thaliana* (adenylate cyclase).	$1e^{-15}$
	5256	*pde1*	NP_011266.1 *Saccharomyces cerevisiae* (low affinity cAMP phosphodiesterase).	$1e^{-10}$

Cont. tabela 1: Componentes das vias de sinalização celular anotados pela análise do transcriptoma de *P. brasiliensis* (Fernandes *et al*., 2005.)

Ras/AMPc/PKA	*947*	*pkar/ bcy1*	*AF401202_1 Aspergillus fumigatus (regulatory subunit of PKA).*	$2e^{-74}$
	1562	*pkac/tpk1*	NP_012371 *Saccharomyces cerevisiae* (catalytic subunit of cAMP-dependent protein kinase)	$1.8e^{-36}$
	3488	*pkac/ tpk2*	P34099 *Saccharomyces cerevisiae* (catalytic subunit of PKA).	$1e^{-60}$
	3241	*flo11/ muc1*	NP_012284.1 *Saccharomyces cerevisiae* (flocculin: extracellular alpha-1, 4-glucan glucosidase).	$2.6e^{-06}$
	983	*ras2*	NP_014301.1 *Saccharomyces cerevisiae* (Ras proto-oncogene homolog)	$1.4e^{-10}$
MAP kinase regulada por ferormônio	2475	*mat1-1*	AF100925 *Gibberella fujikuroi* (mating type protein MAT1-1).	$1e^{-16}$
	4188	*mat1-2*	AF508279_1 *Emericella nidulans* (mating-type protein MAT1-2)	$4e^{-20}$
	2208	*ste4*	AF176775 *Aspergillus nidulans* (GTP-binding protein beta subunit of the pheromone pathway). L04655 *Saccharomyces cerevisiae*	$5e^{-88}$
	266	*ste20*	L04655 *Saccharomyces cerevisiae* (ser/thr protein kinase of the pheromone pathway).	$3e^{-51}$
	659	*ste11*	AF034090 *Neurospora crassa* (ser/thr protein kinase of the MEKK family).	e^{-160}
	989	*ste7*	AJ304830 *Blumeria graminis* (ser/thr/tyr protein kinase of MAP kinase kinase family).	$8e^{-29}$

Cont. tabela 1: Componentes das vias de sinalização celular anotados pela análise do transcriptoma de *P. brasiliensis* (Fernandes *et al*., 2005.)

MAP kinase regulada por ferormônio	*1960*	*kss1/fus3/cek1*	*AF268070 Ustilago maydis (mitogen-activated protein kinase: MAP kinase).*	$5e^{-21}$
	4523	*ste12*	AF080600 *Emericella nidulans* (transcriptional activator).	$4e^{-37}$
MAP kinase regulando integridade celular e construção da parede	1346	mid2/slg1/*wsc1*	NP_014650.1 *Saccharomyces cerevisiae* (cell surface sensors for cell integrity signaling during vegetative growth).	$4e^{-11}$
	157	*pkC*	NC_001148 *Saccharomyces cerevisiae* (ser/thr protein kinase).	$2e^{-17}$
	659	bck1/*slk1*	AF034090 *Neurospora crassa* (ser/thr protein kinase of the MEKK family).	e^{-160}
	4403	*mkk1*	NC_001148 *Saccharomyces cerevisiae* (ser/thr protein kinase).	$2e^{-60}$
	989	*mkk2*	AJ304830 *Blumeria graminis* (protein kinase of the map kinase kinase (MEK) family).	$8e^{-29}$
	365	slt2/*mpk1*	AJ304831 *Blumeria graminis* (ser/thr protein kinase of MAP kinase family).	e^{-110}
	1259	*rlm1*	NP_015236 *Saccharomyces cerevisiae* (transcription factor of the MADS box family).	$9e^{-26}$
	3256	*swi4*	AL513463 *Neurospora crassa* (transcription factor).	$4e^{-08}$
	3615	*swi6*	NP_594464.*1* *Schizosaccharomyces pombe* (transcription factor).	1.6

Cont. tabela 1: Componentes das vias de sinalização celular anotados pela análise do transcriptoma de *P. brasiliensis* (Fernandes *et al*., 2005.)

MAP kinase regulando integridade celular e construção da parede	*1518*	*rho1*	*AY007297 Aspergillus fumigatus (GTP-binding protein of the rho subfamily of ras-like proteins).*	$8e^{-53}$
	3665	*fks1*	AF148715 *Paracoccidioides brasiliensis* (1,3-beta-D-glucan synthase, catalytic subunit).	$2e^{-81}$
	4433	*fks2*	AF148715 *Paracoccidioides brasiliensis* (1,3-beta-D-glucan synthase subunit).	e^{-108}
	985	*sln1/nik1*	AF435964 *Botryotinia fuckeliana* (putative sensory transduction histidine-kinase).	$5e^{-50}$
MAP Kinase regulando crescimento em alta osmolaridade e resposta ao estresse	2744	*ypd1*	MPR1_SCHPO *Schizosaccharomyces pombe* (phosphorelay intermediate between Sln1p and Ssk1p).	$1e^{-04}$
	913	*ssk1*	AF084608_1 *Candida albicans* (two-component signal transducer).	0.046
	659	*ssk2*	P53599 *Saccharomyces cerevisiae (*MAPKKK - Suppressor of sensor kinase 2)	$1e^{-38}$
	365	*pbs2*	NP_012407 *Saccharomyces cerevisiae (*mitogen-activated protein kinase kinase – MEK)	$3e^{-31}$
	3218	*sho1*	CAC81238 *Candida albicans* (protein osmosensor)	$2e^{-05}$
	1960	*ste50*	EAL02925 *Candida albicans (*mitogen-activated protein kinase kinase kinase *MAPKKK)*	$2e^{-15}$
	356	*hog1*	AF184980_1 *Magnaporthe grisea* (osmotic sensitivity MAP Kinase)	$1e^{-73}$

Cont. tabela 1: Componentes das vias de sinalização celular anotados pela análise do transcriptoma de *P. brasiliensis* (Fernandes *et al*., 2005.)

Via TOR	*3215*	tor2	*CAA50548.1 Saccharomyces cerevisiae (protein/phosphatidylinositol kinase)*	$1e^{-15}$
	4664	*rom2*	T41524 *Schizosaccharomyces pombe* (rho1 gdp-gtp exchange protein 1).	$7e^{-35}$
	3501	*sac7*	CAA20323.1 S*chizosaccharomyces pombe* (RhoGAP GTPase activating protein).	$4e^{-37}$
	70	*cdc55*	AAD15987.1 *Neurospora crassa* (protein phosphatase 2A regulatory B subunit).	e^{-105}
	5565	*tpd3*	AAB03670.1 *Dictyostelium discoideum* (Protein phosphatase 2A regulatory subunit A).	$2e^{-35}$
	1737	*sit4*	CAB98214.2 *Neurospora crassa* (probable cell shape control protein phosphatase ppe1).	$2e^{-99}$
	1318	*gap1*	CAD21063.1 *Neurospora crassa* (amino acid permease NAAP1).	0.0
	PbAEST367	*mkk1*	CAC19662.1 *Blumeria graminis* (mitogen-activated protein kinase kinase).	e^{-110}
	PbAEST358	*mpk1*	AAF09475.1 *Magnaporthe grisea* (osmotic sensitivity MAP Kinase: Hog1p)	$1e^{-73}$
	PbAEST2132	*npr1*	CAA18998.1 *Schizosaccharomyces pombe* (nitrogen permease reactivator/protein kinase).	$1e^{-40}$
	PbAEST1432	*fpr1*	CAA06962.1 *Neurospora crassa* (FKBP-type peptidyl-prolyl cis-trans isomerases).	$8e^{-38}$

Paris & Duran (1985) demonstraram o envolvimento de AMPc no dimorfismo de *P. brasiliensis*. Observou-se inibição da transição dimórfica de micélio para levedura na presença de AMPc exógeno, embora tanto a forma leveduriforme como filamentosa não tenham apresentado variação significativa da concentração endógena de AMPc. Apenas recentemente os componentes envolvidos nas vias de sinalização celular começaram a ser estudados em detalhe neste patógeno. Chen *et al*. (2007) reportaram a clonagem dos genes que codificam alguns dos componentes da via de sinalização AMPc/PKA em *P. brasiliensis*: Gpa1-3 (subunidade α da proteína G), Gpb1 (subunidade β), Gpg1 (subunidade γ) e Cyr (adenilato ciclase), além de terem avaliado a interação entre estas subunidades e o perfil transcricional destes genes durante o dimorfismo celular. Ensaios de duplo híbrido demonstraram a interação entre Gpa1 e Gpb1 e adenilato ciclase. Além disso, estes autores observaram uma variação do nível de transcrição de *cyr1, gpa1, gpb1* e *gpg1* ao longo das 240h de diferenciação celular, sendo que *cyr1, gpa1* e *gpg1* apresentaram seus transcritos em maior abundância na forma leveduriforme, enquanto que *gpb1* não apresentou diferenças significativas entre as formas de micélio e levedura de *P. brasiliensis*. Diferentemente do que se observou anteriormente por Paris & Duran (1985), Chen *et al.* (2007) observaram um aumento do nível de AMPc endógeno na fase leveduriforme de *P. brasiliensis*, correlacionado com o alto número de transcritos de *cyr1* também nesta fase. Além destas observações, Chen *et al.* (2007) identificaram um atraso na transição morfológica de micélio para levedura, ao invés de inibição (observado por Paris & Duran, 1985) quando adicionado AMPc exógeno. Apesar da divergência entre os resultados obtidos anteriormente e aqueles propostos por Chen *et al*. (2007), ambos os trabalhos contribuíram por demonstrarem o envolvimento de AMPc e da via regulada por esta molécula durante o processo de diferenciação de *P. brasiliensis.*

De Carvalho *et al*. (2003) caracterizaram molecularmente o gene que codifica a calmodulina de *P. brasiliensis*, envolvida na via Ca^{2+}-calmodulina-calcineurina, e através do uso de inibidores de kinases dependentes de Ca^{2+}-calmodulina foi possível determinar o envolvimento desta via de sinalização na transição de micélio para levedura deste fungo, visto que tais inibidores bloquearam o processo de transição dimórfica.

Apesar de ainda pouco se conhecer sobre os mecanismos das vias de sinalização em *P. brasiliensis*, os primeiros trabalhos, ainda que isolados, deram um importante passo, o que certamente contribuirá para o melhor entendimento dos mecanismos de virulência e patogenicidade deste fungo.

1.3) As pequenas GTPases

As pequenas GTPases compreendem uma superfamília de proteínas de 20 a 30 KDa, componentes de vias de sinalização celular muito conservadas em eucariotos. Estão envolvidas no sensoriamento de alterações ambientais e adaptação a estas mudanças como: regulação do citoesqueleto celular, tráfico pela membrana (exocitose), regulação transcricional, controle do crescimento e desenvolvimento celular. São consideradas "comutadores" moleculares, pois alternam entre o estado ativo, ligado a GTP e inativo, ligado a GDP (Van Aelst & D'Souza-Schorey, 1997).

Essas pequenas GTPases, Ras, Rac, Cdc42, Rho e Rab, foram estudadas em diferentes organismos, dentre eles *S. cerevisiae* (Mösch *et al.,* 1996), *Schizosaccharomyces pombe* (Danjoh & Fujiyama, 1999*)*, *C. neoformans* (Waugh *et al*., 2002; Alspaugh *et al.,* 2000), *Wangiella (Exophiala) dermatitidis* (Ye & Szaniszlo, 2000), *Ashbya gossypii* (Wendland & Philippsen, 2001), *Ustilago maydis* (Krüger *et al*., 1998) entre outros, sendo que os mecanismos utilizados por tais proteínas para mediar todas as atividades ainda são objetos de estudos (Park & Bi, 2007).

1.4) As modificações pós-traducionais das pequenas GTPases

As proteínas preniltransferases catalisam a adição pós-traducional de um grupo lipídico prenil à região C-terminal de uma grande variedade de proteínas, incluindo as pequenas GTPases (Zhang & Casey, 1996). Essas modificações são necessárias para que tais proteínas se tornem mais hidrofóbicas, o que proporciona a sua correta localização na membrana celular onde irão desenvolver suas funções.

Existem três preniltransferases já caracterizadas tanto em mamíferos como em diferentes fungos: a Farnesiltransferase (FTase), a Geranilgeranil transferase (GGTase I) e a Geranilgeraniltransferase II (GGTase II) (Zhang & Casey, 1996). Essas enzimas apresentam algumas características comuns; são heterodiméricas

e dependentes de íons divalentes, como zinco e magnésio, porém, diferem quanto aos substratos e especificidades.

A Ftase tem como substrato o grupo farnesil (15 carbonos), um intermediário da biossíntese do colesterol (mamíferos) / ergosterol (fungos), e transfere esse grupamento lipídico para proteínas como Ras e Rheb na região C-terminal contendo o motivo CAAX, onde "C" é uma cisteína, "A" é um aminoácido alifático e "X" é preferencialmente alanina, cisteína, metionina ou serina. Já a GGTase I tem como substrato um grupamento geranil (20 carbonos) e reconhece proteínas em que no motivo CAAX, "X" é uma leucina ou em alguns casos fenilalanina ou metionina, destacando-se Rho1, Rac1 e Cdc42 (Zhang & Casey, 1996). A GGTase II também transfere grupamento geranil no entanto, apenas a proteínas contendo o motivo C-terminal com duas cisteínas (CC), por exemplo as proteínas Rab. Apesar das diferenças de especificidade entre estas enzimas, tem sido relatada em mamíferos e *S. cerevisiae* a farnesilação cruzada de substratos contendo o motivo "CAAL" tanto *in vivo* quanto *in vitro* (Caplin *et al*. 1994*,* Trueblood *et al*., 1993). Na ausência de FTase, também foi observada a prenilação pela GGTase I em substratos específicos para FTase (Whyte *et al*., 1997).

As três proteínas preniltransferases são constituídas de subunidades α e β, sendo que a subunidade β contém os sítios de ligação a FPP (farnesilpirofosfato) ou GPP (geranilpirofosfato), ao substrato protéico e ao zinco. A subunidade α apresenta a função catalítica e é necessária para estabilização da subunidade β (Andres *et al*., 1993). Em *C. albicans*, FTase e GGTase I apresentam a subunidade α em comum, a qual é codificada pelo gene *RAM2*. A subunidade β em FTase e GGTase I é codificada pelos genes *RAM1* e *CDC43*, respectivamente. Os genes *BET4* e *BET2* codificam para as subunidades α e β, respectivamente, de GGTase II (Song & White, 2003). Os genes que codificam ambas as subunidades das preniltransferases foram identificados em diferentes fungos entre eles *C. albicans* (Kelly *et al.,* 2000; Song & White, 2003), *S. pombe* (Yang *et al.,* 2000), *S. cerevisiae* (Adams *et al*., 1990; He *et a*l., 1991; Mayer *et al.,* 1992), *C. neoformans* (Vallim *et al*., 2004) e, *in silico*, em *P. brasiliensis* (Amaral *et al.*, 2005), trabalho este realizado em paralelo a esta tese. Além disso, *P.*

brasiliensis apresenta os genes *bet2* e *bet4* que codificam para as subunidades β e α respectivamente de GGTase II **(Tabela 2)**.

Tabela 2: Análise dos genes ortólogos de *P. brasiliensis* que codificam as subunidades α e β de FTase e GGTase (Amaral *et al.,* 2005)

Proteína		***Gene***	***Número de acesso/ Organismo***	***% Identidade protéica***	***E-value***
Farnesiltransferase (FTase) Geranilgeraniltransferase I (GGTase I)	Subunidade α	*ram2*	NP_593518.1/ *S.pombe*	40,722%	$5.7e^{-18}$
Farnesiltransferase (FTase)	Subunidade β	*ram1*	NP_594251.1 *S.pombe*	40,288%	$6.7e^{-16}$
Geranilgeraniltransferase I (GGTase I)	Subunidade β	*cdc43*	NP_594142.1 *S.pombe*	51,471%	$8.6e^{-11}$
Geranilgeraniltransferase II (GGTase II)	Subunidade α	*bet2*	NP_015502.1/ *S. cerevisiae*	50,190%	$4.2e^{-36}$
Geranilgeraniltransferase II (GGTase II)	Subunidade β	*bet4*	NP_588463.1/ *S.pombe*	37,349%	$2.5e^{-12}$

Muitos estudos estão sendo realizados na tentativa de desenvolver e identificar possíveis inibidores de preniltransferases motivados pela correlação direta entre o mecanismo alterado de farnesilação de Ras e o desenvolvimento de câncer em humanos (Basso *et al.,* 2006). Testes pré-clínicos indicam que a inibição do processo de prenilação poderia resultar em redução da taxa de crescimento de alguns tipos de tumores (Omer & Kohl, 1997). Baseados nos dados de que as proteínas Ras controlam mecanismos centrais do desenvolvimento, diferenciação e patogenicidade de fungos, tais inibidores foram testados *in vitro* em *C. albicans* e *C. neoformans* (McGeady *et al*., 2002; Vallim *et al*., 2004). Os inibidores de FTase foram capazes de bloquear a transição dimórfica soro-induzida de levedura para hifas no fungo oportunista *C. albicans* e o crescimento haplóide em *C. neoformans* (McGeady *et al*., 2002; Vallim *et al*., 2004). A ação de inibidores de FTase em *P.brasiliensis* será alvo de estudo deste trabalho de tese.

A baixa identidade (30%) de seqüência de resíduos de aminoácidos entre a subunidade β de GGTase I de *C. albicans* e aquela encontrada em humanos (Kelly *et al*., 2000), juntamente com o fato de que Rho1 (alvo de GGTase I) é a subunidade regulatória essencial de β-1,3-glucana sintase, enzima envolvida na constituição da parede celular de fungos, faz com que inibidores de GGTase I sejam um atrativo em uma terapia antifúngica mais seletiva. Baseado nestes dados, trabalhos recentes identificaram e caracterizaram inibidores seletivos de GGTase I que apresentam atividade antifúngica (Nishimura *et al*., 2003; Murthi *et al*., 2003).

1.5) O papel de Ras em diferentes organismos

Ras-GTPases são proteínas reguladoras da via conservada de transdução de sinais, promovendo alterações adaptativas, como desenvolvimento celular e morfogênese (Waugh *et al*., 2002). Em células humanas, esta classe de proteínas tem papel importante na transdução de sinais de crescimento via receptor tirosina-quinase localizado na membrana, culminando na ativação da transcrição de diferentes genes-alvo. Algumas mutações já bem caracterizadas nestas proteínas são associadas com transformação malígna e crescimento celular desordenado, uma vez que Ras são responsáveis pelo controle da proliferação celular (Wendland & Philippsen, 2001).

Em microrganismos, proteínas Ras apresentam-se similarmente envolvidas na regulação do crescimento e desenvolvimento celular (Waugh *et al*., 2002). Em *C. neoformans*, Ras1 está envolvida em filamentação, acasalamento e crescimento em altas temperaturas (37ºC). Mutantes *ras1* são avirulentos em modelos animais de meningite, e apresentam defeito de crescimento a 37ºC, permanecendo como células grandes sem brotamentos, actina despolarizada e perda da simetria do citoesqueleto, além de serem incapazes de produzir filamentos, visto que esta proteína atua sobre a via das MAPKinases que responde a ferormônio. (Alspaugh *et al.,* 2000). Waugh *et al.* (2002) demonstraram ainda, a existência de dois genes *RAS* que codificam duas proteínas, Ras1 e Ras2 em *C. neoformans.* A proteína Ras2 é expressa em níveis muito baixos quando comparados com Ras1, e mutantes *ras2* não apresentam os defeitos no crescimento, diferenciação ou expressão de fatores de virulência observados nos mutantes *ras1*. No entanto, o duplo mutante *ras1 ras2*, mostrou-

se viável, porém com baixa taxa de crescimento em todas as temperaturas. A super-expressão de *RAS2* no mutante *ras1* suprimiu os defeitos de acasalamento e parcialmente suprimiu o defeito de crescimento a 37ºC sugerindo assim que Ras1 e Ras2 parecem ter efeito aditivo e/ou redundante sobre uma mesma via ou atuam em vias de sinalização paralelas em *C. neoformans*.

Em *U. maydis,* mutantes *ras2* apresentaram morfologia celular alterada, defeitos na fusão e/ou produção de filamentos no acasalamento. Dessa forma, Ras2 parece estar envolvida em vários processos celulares incluindo morfogênese, acasalamento, virulência e patogenicidade deste patógeno de planta (Lee & Kronstad, 2002). Estes estudos em *U. maydis* sugerem a existência de uma interação cruzada influenciada por Ras2 entre as vias AMPc/PKA e MAPKinases. Nielsen *et al.* (1992), por sua vez, demonstraram em *S. pombe* que mutantes *ras1* não apresentaram defeito no crescimento vegetativo, porém, as células mostraram-se incapazes de se acasalarem e com formato anormal. Ras1 em *S. pombe* parece não estar envolvida na homeostase do AMPc, porém controla a atividade da via que responde a ferormônio e acasalamento.

Em *S. cerevisiae*, duas proteínas Ras (Ras1 e Ras2*)*, também foram identificadas, sendo que, diferentemente de *C. neoformans*, a proteína Ras*2* tem um papel muito mais importante que Ras1 na regulação da diferenciação e produção de AMPc, além de ter maior nível de expressão quando submetida a diferentes condições, é responsável pela regulação do crescimento invasivo haplóide mediado por ambas as vias efetoras: MAPKinases e AMPc/PKA. Mutantes *ras2* apresentaram defeitos profundos na polarização do citoesqueleto em condições moderadas de estresse causado por temperatura (Ho & Bretscher, 2001). O duplo mutante *ras1 ras2* apresentou fenótipo letal, diferente do observado em *C. neoformans*. A super-expressão de *RAS1* restaurou o defeito no crescimento haplóide invasivo do mutante *ras2* de *S. cerevisiae* (Mösch *et al*., 1999). Dessa forma, Ras2 é responsável pela indução do crescimento filamentoso haplóide invasivo de *S. cerevisiae* através da regulação da via das MAPKinases (Mosh *et al.,*1996) e AMPc/PKA (Mosh *et al.*, 1999). Como ambas as vias convergem para o promotor do gene *FLO11* (efetor) e apresentam mesmo elemento regulatório (*RAS2*), sugere-se que estas vias estejam interligadas. Interações entre as vias AMPc/PKA e MAPKinases também foram sugeridas em *U. maydis* (Krüger *et al*., 1998, Lee & Kronstad, 2002), *C. neoformans* (Alspaugh

et al., 2000) e *C. albicans* (Leberer *et al*., 2001). Ras2 também está envolvida na manutenção da expectativa de vida de *S. cerevisiae*. Através da via AMPc/PKA, Ras2 parece atuar na renovação do crescimento e divisão celular quando as células leveduriformes são submetidas a condições de choque térmico (Shama, *et al*., 1998). Em *S. cerevisiae* as proteínas Ras também estão envolvidas na resposta ao estresse incluindo choque térmico e no controle do estado nutricional da célula (Engelberg *et al*., 1994, Breviario *et al*., 1986, Wang *et al*., 2004).

No fungo oportunista *C. albicans* foi encontrado apenas um gene *RAS*, denominado *CaRAS1* (Feng *et al*., 1999 e Leberer *et al*., 2001). Embora não seja um gene essencial, a deleção de ambos os alelos produz mutantes com virulência atenuada, incapazes de sofrer o processo de transição de levedura para hifas (característico de virulência deste patógeno) tanto *in vitro* como *in vivo* em modelo de camundongos para candidíase sistêmica. Leberer *et al.* (2001) sugerem ainda que CaRas1 seja necessária para regulação das vias de sinalização MAPKinases e AMPc/PKA em resposta a fatores ambientais para a transição morfológica da forma leveduriforme para a forma de hifas neste patógeno.

Fortwendel *et al.* (2004) identificaram *rasA* e *rasB* (ortólogos de *RAS1* e *RAS2* de *C. neoformans*, respectivamente) no fungo filamentoso e patogênico *A. fumigatus*. Através de mutações dirigidas demonstrou-se que tais genes apresentam funções diferentes, porém sobrepostas no crescimento vegetativo e desenvolvimento assexual deste fungo. Posteriormente, Fortwendel *et al.* (2005) relataram que mutantes *rasB* apresentavam diminuição de germinação e taxa de crescimento, defeitos morfológicos nas hifas e virulência atenuada.

Em *P. marneffei*, um fungo dimórfico dependente de temperatura, *Boyce et al*. (2005) identificaram a presença das GTPases Cdc42 (CflA), Rac (CflB) e Ras (RasA). Além disso, através de experimentos com mutantes estes autores sugeriram um modelo de sinalização em que RasA controla o início do desenvolvimento assexual e atua sobre Cdc42 (CflA) para a regulação da germinação de esporos e crescimento polarizado tanto de hifas quanto de leveduras, septação e ramificação.

Assim, as funções similares de Ras entre diferentes microrganismos demonstram que essas moléculas de sinalização desempenham papéis centrais no crescimento e desenvolvimento de fungos. Estas vias encontram-se envolvidas na adaptação microbiana e infecção do hospedeiro **(Figura 2).** Visto que ainda

não se conhece o papel de Ras em *P. brasiliensis*, torna-se relevante a elucidação da sua participação no mecanismo molecular de patogenicidade deste organismo.

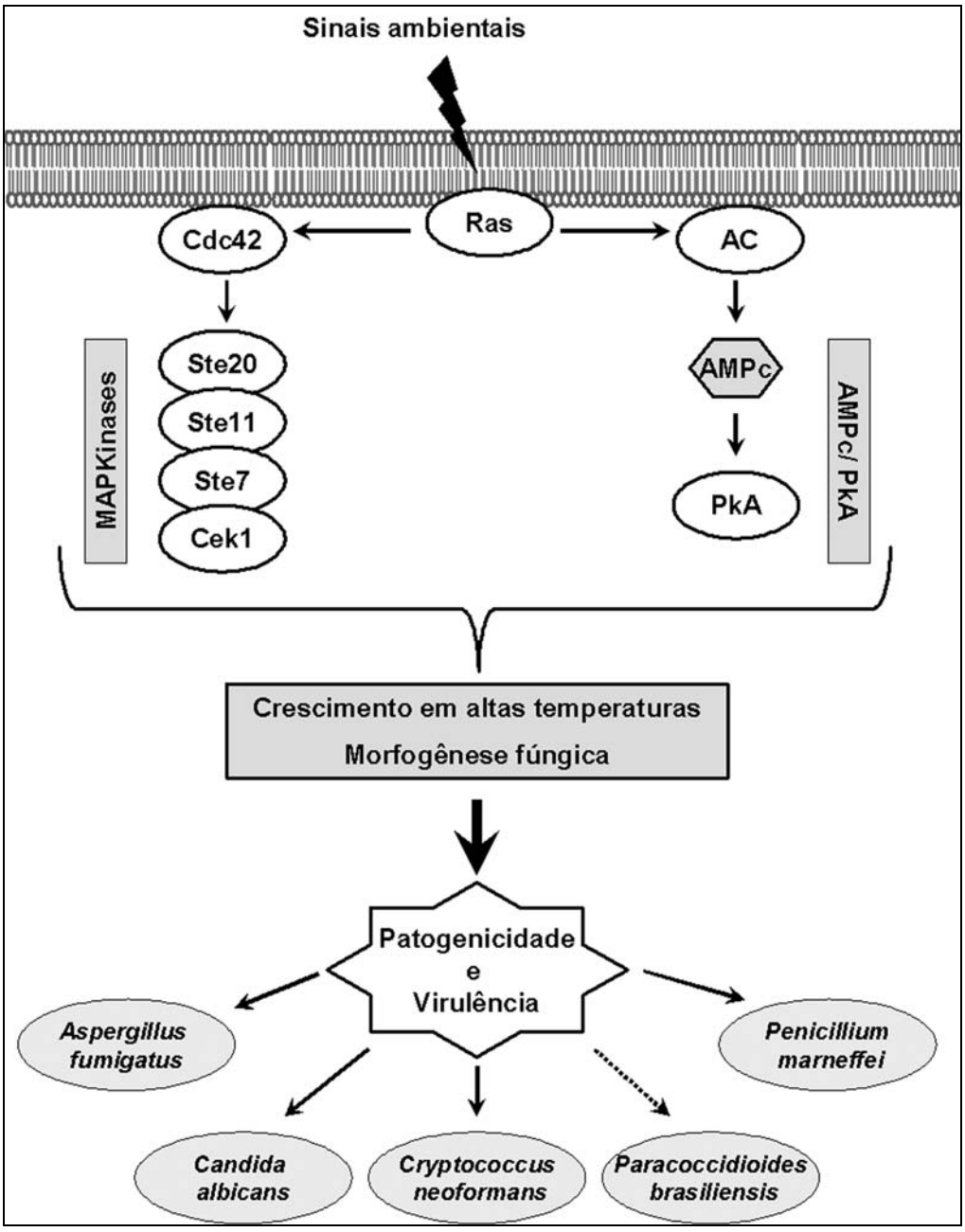

Figura 2: Mecanismo proposto de interação cruzada entre as vias AMPc/PKA e MAPKinases por Ras-GTPases em fungos patogênicos que regulam morfogênese, crescimento em altas temperaturas e virulência. Em *P. brasiliensis* as linhas pontilhadas indicam que o mecanismo ainda não foi demonstrado, no entanto, todos os componentes listados foram encontrados pela anotação *in silico* do transcriptoma.

1.6) Sistemas de transformação em fungos

A manipulação genética é uma ferramenta importante para o entendimento da biologia, evolução e patogenicidade, bem como para a identificação de novos alvos terapêuticos em fungos patogênicos (Kwon-Chung *et al*., 1998). Sistemas de transformação foram desenvolvidos para uma grande variedade de organismos patogênicos destacando-se *Candida sp*. (Kurtz *et al.,* 1986), *C. neoformans* (Edman & Kwon-Chung, 1990*), H. capsulatum* (Worsham & Goldman, 1990), *B. dermatitidis* (Hogan & Klein,1997), *A. fumigatus* (Monod *et al*., 1993) e *W. dermatitidis* (Peng *et al*., 1995) entre outros. Para organismos como *Candida sp* e *A. fumigatus*, métodos eficientes de transformação foram adaptados a partir de protocolos previamente estabelecidos para *S. cerevisiae* e *A. nidulans*, respectivamente, devido à similaridade entre tais organismos. Porém as características peculiares de cada organismo fazem do desenvolvimento de sistemas de transformação para fungos patogênicos um processo lento e laborioso.

Métodos convencionais de transformação genética, como produção de esferoplastos e permeabilização celular, requerem protocolos longos e que muitas vezes não produzem boa eficiência (Kwon-Chung *et al*., 1998). Recentemente, tais métodos foram substituídos com sucesso por técnicas mais simples, rápidas e eficientes como eletrotransformação ou eletroporação, biobalística e transformação mediada por *Agrobacterium tumefaciens* (Kwon-Chung *et al*., 1998). Em *S. cerevisiae*, a transformação por biobalística foi descrita em 1990 (Armaleo *et al*., 1990), posteriormente em outros fungos tais como: *C. neoformans* (Toffaletti *et al*., 1993), *Trichoderma harzianum* (Lorito *et al*., 1993), *Botryotinia fuckeliana* (Hilber *et al*., 1994), *A. nidulans* (Barcellos *et al*., 1998). A eletrotransformação já muito utilizada em diversos organismos foi primeiramente padronizada em *H. capsulatum,* dentre os fungos dimórficos (Woods *et al*., 1998). Tanto a eletrotransformação quanto a biobalística produz transformantes estáveis com integração genômica, principalmente em sítios ectópicos, sendo a recombinação homóloga um evento raro (Kwon-Chung *et al.*, 1998).

Atualmente, o sistema de transformação mediado por *A. tumefaciens* é muito utilizado, pois esta bactéria é capaz de transferir, através de um T-DNA, o DNA de interesse ao genoma de diversos organismos, incluindo plantas (Nishimura *et al.*, 2006; Ishida *et al.,* 2007), células de mamíferos (Ziemienowicz

et al., 1999) e fungos. Dentre os fungos, este sistema já foi testado em células leveduriformes e micélio, destacando-se os patógenos humanos *A. fumigatus* (Sugui, *et al*., 2005), *C. immitis* (Abuodeh *et al.,* 2000), *B. dermatitidis* (Sullivan *et al*., 2002), *H. capsulatum* (Sullivan *et al*., 2002), *C. neoformans* (McClelland *et al.,* 2005) e os não patogênicos *S. cerevisiae* (Piers *et al*., 1996), *Agaricus bisporus* (Chen *et al.,* 2000), *Kluyveromyces lactis* (Bundock *et al.,* 1999), *Suillus bovinus* (Hanif *et al.,* 2002), *Magnaporthe grisea* (Rho *et al*. 2001). Esta técnica apresentou-se muito versátil já que pode ser utilizada em qualquer forma celular, sejam conídios, fragmentos de micélio ou leveduras, além de produzir eficiência aumentada de transformação quando comparada aos métodos convencionais e transformantes estáveis após várias passagens em meios não seletivos. Este sistema representa ainda uma ferramenta poderosa para estudos de genes através da mutagênese insercional aleatória e genética reversa (Idnurm *et al*., 2004; Sugui *et al*., 2005; Michielse *et al*., 2005; Zhong *et al*., 2007).

No modelo *P. brasiliensis*, dois métodos de transformação genética foram testados, a eletroporação (Soares *et al.,* 2005) e o sistema mediado por *A. tumefaciens* (Leal *et al.,* 2004). Ambos os métodos empregaram como gene marcador que codifica resistência a higromicina B, no entanto, ambos os métodos demonstraram eficiência de transformação muito baixa, porém os transformantes apresentaram-se mais estáveis quando utilizado o sistema de *A. tumefaciens*. Como existe uma grande variabilidade quanto à morfologia celular, ploidia e número de núcleos por célula entre os isolados de *P. brasiliensis*, faz-se necessário escolher um isolado de fácil manipulação, que apresente crescimento rápido, seja haplóide e apresente o menor número de núcleos por célula. Ajustes nos protocolos primeiramente testados por Soares *et al.* (2005) e Leal *et al.* (2004), poderiam refletir um aumento na eficiência de transformação. Recentemente, Almeida *et al*. (2007), ao avaliar vários parâmetros importantes que influenciam a transformação mediada por *A. tumefaciens* tais como as condições de co-cultivo e susceptibilidade das leveduras de *P. brasiliensis*, relataram um aumento significativo da eficiência de transformação quando comparado com o protocolo de Leal *et al.* (2004). Dessa forma, através dessas ferramentas moleculares hoje disponíveis, tornou-se possível iniciar os estudos de função de genes candidatos à virulência e determinação da importância individual de cada um na patobiologia de *P. brasiliensis*, além de potencialmente catalisar o

desenvolvimento de drogas contra os possíveis produtos gênicos ou seus receptores.

1.7) Deleção gênica *versus* silenciamento gênico

Durante muitas décadas o estudo da função de um gene foi baseado em três estratégias principais (I) deleção gênica pela substituição do gene de interesse por um gene marcador (II) rompimento gênico, quando um gene marcador é inserido no gene de interesse causando uma proteína truncada não funcional e (III) mutações puntuais que alteram a fase de leitura do DNA. Os dois primeiros casos dependem de integração do DNA exógeno no genoma e ainda uma recombinação homóloga no *locus* do gene estudado, duas etapas bastante difíceis de serem alcançadas quando se trabalha com organismos de ploidia desconhecida e com número de núcleos variados em um mesmo citoplasma, como é o caso de *P. brasiliensis* (Woods, 2005).

Recentemente, o fenômeno de silenciamento gênico por interferência no RNA (RNAi) surgiu como uma estratégia alternativa para o estudo da função gênica em organismos de difícil manipulação genética como os fungos dimórficos *P. brasiliensis, H. capsulatum, B. dermatitidis, C. immitis* e *P. marneffei* (Woods, 2005). RNAi é um fenômeno de silenciamento gênico pós-transcricional mediado por uma série de enzimas em que um RNA dupla fita (dsRNA) leva à degradação do RNA cognato de maneira seqüência-específica, ou seja, apenas os RNAs mensageiros maduros com similaridade à seqüência do dsRNA é que serão degradados **(Figura 3).** Esse mecanismo é apontado como uma poderosa ferramenta para genômica funcional em vários eucariotos, incluindo *Drosophila melanogaster* (Boutros *et al*., 2004, Kennerdell & Carthew, 2000), *Caenorhabditis elegans* (Kamath *et al.,* 2003) e plantas (Wang & Waterhouse, 2000). Em fungos, o fenômeno de RNAi foi primeiramente descrito em *N. crassa* por Romano e Macino, em 1992, e denominado de “quelling”, do inglês “apagar”. Desde então, vários pesquisadores utilizam o sistema de RNAi para estudar função gênica em diversos fungos, incluindo *Cladosporium fulvum* (Hamada & Spanu, 1998); *C. neoformans* (Liu *et al.,* 2002; Sommer *et al.,* 2003; Reese & Doering, 2003); *Magnaporthe oryzae* (Kadotani *et al*., 2003; Nakayashiki *et al.,* 2005), *Mucor circinelloides* (Nicolas *et al.,* 2003), *A. fumigatus* (Mouyna *et al.,* 2004; Tsitsigiannis *et al.,* 2005), *H. capsulatum* (Rappleye *et al.,* 2004), *Venturia*

inaequalis (Fitzgerald *et al.,* 2004), *Dictyostelium discoideum* (Morita *et al.,* 2005), *S. cerevisiae* (Chen *et al.,* 2005) e *Neotyphodium uncinatum* (Spiering *et al.,* 2005).

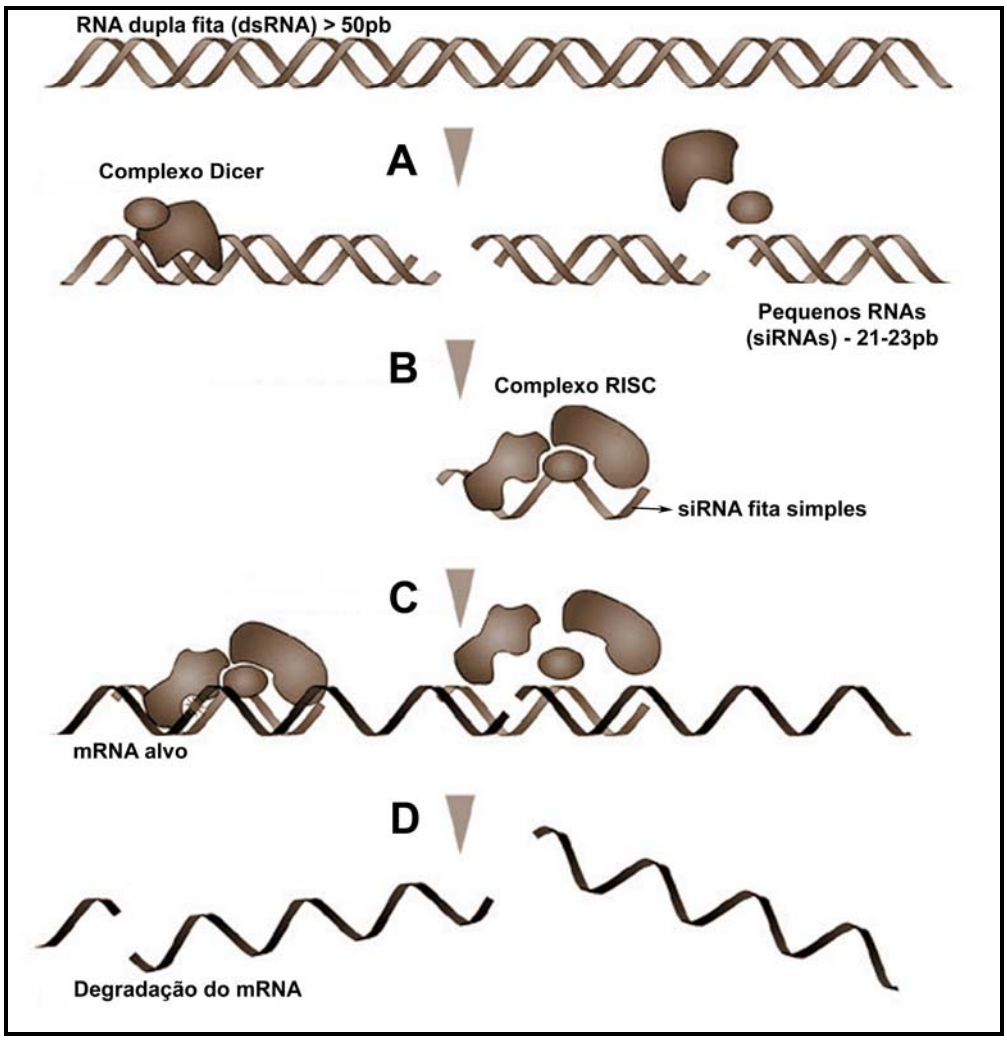

Figura 3: Mecanismo geral de silenciamento de RNA em fungos. (A) Ao entrar na célula, moléculas de RNA dupla fita (dsRNA) (no caso de fungos estes RNAs são resultantes da transcrição de um cassete de DNA em orientações opostas que *in vivo* toma a conformação de um "hairpin") elicitam o mecanismo de silenciamento de RNA. **(B)** Primeiramente, o dsRNA é reconhecido pela DICER, uma enzima RNAse III formando o complexo DICER que por sua vez promove a quebra do dsRNA em pequenos RNAs (siRNAs) com tamanhos de 21 a 23 pares de bases. Este proceso é dependente de ATP. **(C)** Os siRNAs são recrutados pelo complexo RISC (RNA-inducing silencing complex) que contém a proteína argonauta, como um dos componentes principais e que é responsável pela quebra e seleção da fita anti-senso do siRNA dupla fita, o que por sua vez leva a ativação do complexo RISC. **(D)** O complexo RISC ativo captura o RNA mensageiro maduro que apresenta complementaridade com a fita anti-senso do siRNA, promovendo a clivagem endonucleolítica do RNA alvo e consequentemente impedindo a produção da proteína.

Em *P. brasiliensis*, alguns genes ortólogos àqueles de *N. crassa* que codificam a maquinaria de RNAi foram identificados pela análise *in silico* do transcriptoma (Albuquerque *et al.,* 2005), são eles: *sms-2*, *qde-2*, *dcl-2*, *rrp-3*, indicando que, provavelmente, este fenômeno é funcional neste organismo.

Baseado na descoberta deste fenômeno biológico, vários grupos desenvolveram vetores para a construção de cassetes visando o silenciamento gênico ("knock-down") (Wesley *et al.,* 2001). No caso de fungos, Nakayashiki *et al.* (2005) desenvolveram um vetor denominado pSilent-1 para ser utilizado em fungos filamentosos. O vetor pSilent-1 **(Figura 4)** apresenta como marcador seletivo o gene *hph* que codifica resistência a higromicina B, flanqueado pelo promotor e terminador de *trpC* de *A. nidulans*. Além disso, esse vetor contém vários sítios de restrição para clonagem dos fragmentos senso e antisenso de genes alvos posicionados estrategicamente entre uma região espaçadora, que por sua vez contém um íntron do gene da cutinase de *M. oryzae,* que funcionará como "loop" favorecendo a formação do hairpin *in vivo*. O cassete para silenciamento gênico é controlado pelo promotor e terminador de *trpC* de *A. nidulans* (Nakayashiki *et al.,* 2005).

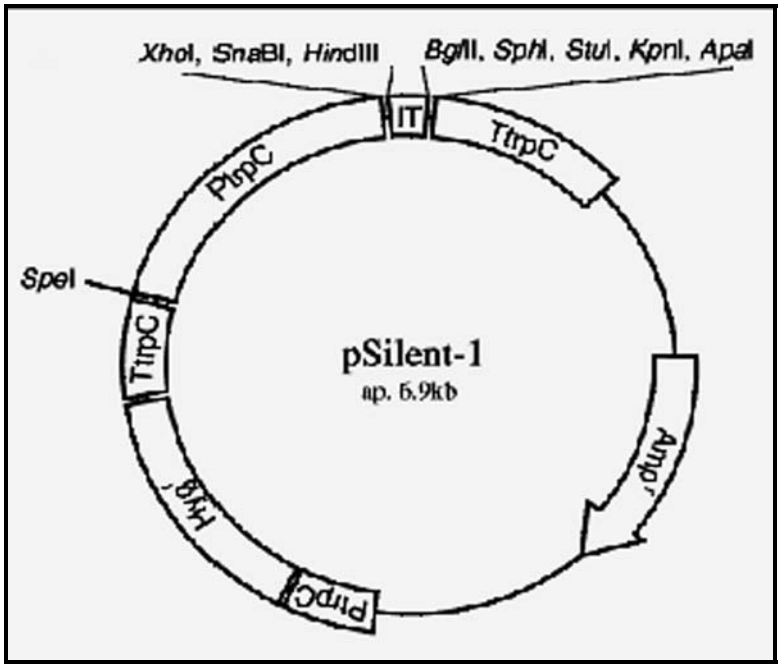

Figura 4: Representação esquemática de pSilent-1. (Extraída de Nakayashiki *et al.,* 2005)

O uso do sistema de RNAi para o silenciamento gênico apresenta vantagens e desvantagens sobre a técnica de deleção gênica. Dentre as vantagens estão a sua grande versatilidade que permite sua aplicação em

organismos multicelulares e/ou multinucleados com ploidia desconhecida, não necessitando de construção de vetores com grandes regiões de homologia necessárias para recombinação homóloga como no caso de deleção gênica; a flexibilidade em experimentos de inativação gênica, já que este fenômeno induz uma supressão gênica, seqüência-especifica e não *locus* específica, facilitando muito o trabalho, já que não há necessidade de se conhecer a seqüência completa do gene e, por último, é possível a análise simultânea de genes homólogos utilizando seqüências conservadas de uma mesma família (Allen *et al.,* 2004; Baulcombe, 1999; Thierry & Vaucheret, 1996) ou no caso de genes heterólogos utilizando vetores quiméricos (aqueles que apresentam mais de um gene com seqüências invertidas) (Fitzgerald *et al.,* 2004; Tsitsigiannis *et al.,* 2005; Liu *et al*., 2002). Além dessas vantagens, o uso de promotores controlados (induzíveis) permite o estudo da expressão gênica em estágios específicos, por exemplo, na diferenciação, desenvolvimento, infecção, entre outros, pois no caso de deleção gênica a eliminação da função gênica é permanente e não transiente como no RNAi. Dessa forma, é possível estudar os genes essenciais que no caso de deleção gênica observa-se um fenótipo letal, enquanto que por RNAi é possível obter silenciamento parcial do gene resultando na expressão diminuída e não completamente abolida para o estudo da função gênica.

Apesar de RNAi operar de maneira seqüência–específica, alguns estudos demonstram que esta especificidade não é absoluta, o que gera alterações não esperadas no padrão de expressão gênica o chamado efeito “off-target” (Fraser *et al.,* 2000), o que caracteriza uma das desvantagens da técnica. Além disso, muitas vezes o silenciamento gera resultados difíceis de serem interpretados causados por mutações incompletas e/ou reversíveis, além da supressão gênica parcial gerando um fenótipo diferente daquele observado na deleção gênica. Pelo fato de ser um fenômeno descrito recentemente, há necessidade de se obter maiores informações sobre o mecanismo de RNAi em fungos. Ainda não existem sistemas de RNAi para estudo da expressão/ função gênica em larga escala em fungos, como já foram desenvolvidos para os eucariotos superiores como *C. elegans* (Kamath *et al.,* 2003) e plantas (Ruiz *et al.,* 1998).

2 - Justificativa

P. brasiliensis é um patógeno humano, que apresenta como principal característica a capacidade de diferenciar células micelianas em leveduriformes, mecanismo este desencadeado pela mudança de temperatura de 22ºC para 37ºC. Apesar de o transcriptoma ter sido seqüenciado e analisado, o que avançou e gerou informações relevantes neste patógeno, os mecanismos moleculares precisos envolvidos na transição dimórfica, na virulência e morfogênese ainda não foram demonstrados. Baseado na necessidade de se conhecer não só os genes envolvidos nestes processos, mas também na busca de informações sobre as possíveis funções dos componentes envolvidos na sinalização celular, foi escolhido como objeto deste estudo o gene *ras* de *P. brasiliensis*, que codifica uma pequena GTPase. Este gene, em outros fungos, desempenha papel central nas vias de sinalização que convergem para o controle de virulência, morfogênese e crescimento em altas temperaturas.

3 - Objetivos

3.1) Objetivo geral

Análisar molecular e funcionalmente os genes *ras1* e *ras2* de *P. brasiliensis* que codificam duas pequenas GTPases, potencialmente envolvidas em morfogênese, diferenciação e no processo infectivo.

3.2) Objetivos específicos

1) Identificar e caracterizar as seqüências dos genes *ras1* e *ras2*.
2) Avaliar o número de cópias no genoma de *P. brasiliensis* dos genes *ras1* e *ras2* por Southern-blot.
3) Analisar a expressão relativa de *ras1* e *ras2* nas seguintes condições experimentais: diferenciação celular de micélio para levedura, choque térmico à 42ºC e, *ex vivo*, no interior de macrófagos.
4) Analisar as PbAESTs correspondentes aos genes que codificam para as subunidades α e β das enzimas GGTase I e FTase I envolvidas nas modificações pós-traducionais das proteínas Ras.
5) Bloquear farmacológicamente *in vivo* o processo de farnesilação das proteínas Ras em *P. brasiliensis* através do uso do inibidor: FPT Inhibitor III (Calbiochem).
6) Padronizar o sistema de transformação via *A. tumefaciens* para o isolado Pb113.
7) Desenvolver uma estratégia para construção de vetores para RNAi, visando o silenciamento de *ras1* em *P. brasiliensis.*
8) Construir os vetores para o silenciamento de *ras1.*

4 - Materiais

4.1) Linhagens

a) *P. brasiliensis*

- **Pb01** (ATCC MYA-826) – isolado de um paciente brasileiro do estado de Goiás apresentando PCM aguda. Este isolado foi obtido da coleção Silva do Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brasil.
- **Pb113 -** isolado por Fava-Netto em 1971 de um paciente brasileiro do estado de São Paulo apresentando PCM crônica. Este isolado foi obtido da micoteca da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil.
- **Pb192 –** isolado clínico obtido da micoteca da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil.

b) *Escherichia coli*

- **DH5α** - F- φ80*lac*Z.M15 .(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-

c) *A. tumefaciens*

- **B25 -** EHA105 (pEHA105 - pTiBo542DT-DNA) contendo o plasmídio pAD1625 (Abuodeh *et al.,* 2000).

d) *Candida sp.*

- *C. albicans* – ATCC24433.
- *C. parapsilosis* – ATCC22019.

e) Macrófagos peritoniais

- Linhagem J774.1 (ATCC TIB-67)

4.2) Meios de Cultivo

a) Para cultivo de *E.coli* (*DH5α*).

- **Meio L**

Extrato de levedura	0,5% (p/v)
NaCl	1,0% (p/v)
Peptona de caseína	1,0% (p/v)

Ajustar o pH para 7,0. Para o preparo de meio L sólido, acrescenta-se 1,5% de ágar bacteriológico. Autoclavar 120ºC/15 min.

- **Meio SB**

Extrato de levedura	2,0% (p/v)
MOPS	1,0% (p/v)
Peptona de caseína	3,0% (p/v)

Ajustar o pH para 7,0. Autoclavar 120ºC/15 min.

- **Meio SOB**

Extrato de levedura	0,5% (p/v)
KCl	0,0186% (p/v)
NaCl	0,06% (p/v)
Peptona de caseína	2,0% (p/v)

Ajustar o pH para 7,0. Autoclavar 120ºC/15 min.

- **Meio SOC**

2M ($MgCl_2$ + $MgSO_4$)	1,0% (v/v)
2M glicose	1,0% (v/v)
Meio SOB	98,0% (v/v)

b) Para cultivo de *A. tumefaciens*

- **Meio AB Líquido**

$FeSO_4$	0,01mM
KCl	2,0mM
NaH_2PO_4 H_2O	8,3mM
NH_4Cl	18,6mM

Após autoclavado 120ºC/15 min, adicionar para cada 100mL:

0,5M $CaCl_2$	14µL
1M $MgSO_4\,7H_2O$	125µL
Glicose 20%	1000µL

- **Meio AB sólido**

Ágar bacteriológico	1,5% (p/v)
Glicose	2,0% (p/v)

Após autoclavado 120ºC/15 min, resfriar a 65ºC e adicionar:

25X Tampão AB	5,0% (v/v)
50X Sais AB	2,0% (v/v)

Solução estoque de **50X Sais AB** é composta:

$CaCl_2$	3,50mM
$FeSO_4$	0,50mM
KCl	100mM
$MgSO_4\,7H_2O$	60mM
NH_4Cl	930mM

Autoclavar 120ºC/15 min para esterilização.

Solução estoque de **25X Tampão AB** é composta:

$NaH_2PO_4\,H_2O$	208mM
$K_2HPO_4\,3H_2O$	420mM

Autoclavar 120ºC/15 min para esterilização.

- **Meio de indução AB/MES líquido**

25X Tampão AB/MES pH 5,8	4,0% (v/v)
50X Sais AB	2,0% (v/v)
Glicose	0,2% (p/v)

Esterilizar por filtração em membranas do tipo milipore 0,2µm.

Solução estoque de **25X Tampão AB/MES pH 5,8** é composta:

$K_2HPO_4\,3H_2O$	9,6% (p/v)

MES 13,3% (p/v)

$NaH_2PO_4\ H_2O$ 2,9% (p/v)

Ajustar o pH para 5,8 com H_3PO_4 e esterilizar por filtração em membranas do tipo milipore 0,2μm.

- **Meio de indução AB/MES sólido**

Ágar bacteriológico 1,8% (p/v)

Autoclavar 120ºC/15 min, resfriar a 65ºC e adicionar:

25X Tampão AB/MES pH 5,8 4,0% (v/v)

50X Sais AB 2,0% (v/v)

Glicose 0,2% (p/v)

c) Para cultivo de *P. brasiliensis*

- **Meio YPD**

Extrato de levedura 1,0% (p/v)

Glicose 2,0% (p/v)

Peptona 2,0% (p/v)

Ajustar o pH para 7,2. Autoclavar 120ºC/15 min.

- **Meio BHI ("Brain Heart Infusion")**

BHI 0,37% (p/v)

Glicose 0,10% (p/v)

Ajustar o pH para 7,2. Autoclavar 120ºC/15 min. Adicionar 5% de fator de crescimento, 4% de soro de cavalo com complemento inativado, penicilina (0,05U/mL) e neomicina (20μg/mL). Para o preparo de meio BHI sólido, acrescentar 2,0% (p/v) de ágar bacteriológico.

- **Meio Semi-sólido Fava-Netto**

Ágar bacteriológico 1,60% (p/v)

Extrato de carne 0,05% (p/v)

Extrato de levedura 0,05% (p/v)

Glicose 0,40% (p/v)

NaCl	0,05% (p/v)
Peptona	0,10% (p/v)
Protease peptona	0,03% (p/v)

Ajustar o pH para 7,2. Autoclavar 120ºC/15 min.

- **Meio N**

Asparagina	0,125% (p/v)
Glicose	1,8% (p/v)
Neopeptona	3,0% (p/v)
Tiamina	0,009% (p/v)

Ajustar o pH para 7,2. Autoclavar 120ºC/15 min.

- **Meio MVM (McVeigh and Morton)**

$CaCl_2$ $2H_2O$	0,015% (p/v)
Glicose	1,0% (p/v)
KH_2PO_4	0,15% (p/v)
L-asparagina	0,2% (p/v)
[a]L-cistina	0,02% (p/v)
$MgSO_4$ $7H_2O$	0,05% (p/v)
$(NH_4)_2SO_4$	0,2% (p/v)

Ajustar o pH para 7,2. Autoclavar 120ºC/15 min, resfriar a 65ºC e adicionar:

Elementos traços	0,01% (v/v)
Vitaminas	0,1% (v/v)

[a] A cistina deve ser dissolvida antes de ser misturada ao restante do meio de cultura em um pequeno volume de água destilada aquecida, adicionando-se 1M NaOH até a completa dissolução.

Para o preparo de meio MVM sólido, acrescentar 1,5% (p/v) de ágar bacteriológico.

Solução estoque de **elementos traço** é composta (para 100mL):

$(NH_4)_6Mo_7O_{24}.4H_2O$	3,6mg
$CuSO_4.5H_2O$	15,7mg
$Fe(NH_4)_2(SO_4)_2.6H_2O$	140,4mg

H_3BO_3	5,7mg
$MnSO_4.14H_2O$	8,1mg
$ZnSO_4.7H_2O$	79,2mg

Esterilizar por filtração em membranas do tipo milipore 0,2μm.

Solução estoque de **vitaminas** é composta (para 100mL):

Ácido fólico	10,0mg
Biotina	0,1mg
Cloreto de colina	10,0mg
Hidrocloreto de tiamina	6,0mg
Hipocloreto de piridoxina	10,0mg
Inositol	1,0mg
Niacina	6,0mg
Pantotenato de cálcio	6,0mg
Riboflavina	1,0mg

Esterilizar por filtração em membranas do tipo milipore 0,2μm.

- **Meio Ágar Batata**

Ágar bacteriológico	1,5% (p/v)
Glicose	2,0% (p/v)
Infusão de batatas	0,4% (p/v)

Ajustar o pH para 5,6. Autoclavar 120ºC/15 min.

- **RPMI-1640**

Glicose	2% (p/v)
MOPS	0,165M
RPMI-1640	1,04% (p/v)

Ajustar o pH para 7,0. Esterilizar por filtração em membranas do tipo milipore 0,2μm.

4.3) Antibióticos, acetoseringona (indutor de *A.tumefaciens*) e fator de crescimento de *P. brasiliensis.*

- **Ampicilina**

  Solução estoque: 100mg/mL.

  Concentração final de uso: 100μg/mL

  A ampicilina é dissolvida em água bidestilada, esterilizada por filtração em membrana Millipore de 0,2μm e armazenada a -20°C.

- **Cefotaxima Sódica**

  Solução estoque: 100mM

  Concentração final de uso: 0,2mM

  A cefotaxima é diluída em água bidestilada, esterilizada por filtração em membrana Millipore de 0,2μm e armazenada a -20°C.

- **Neomicina**

  Solução estoque: 200mg/mL.

  Concentração final de uso: 20μg/mL.

  A neomicina é dissolvida em água bidestilada, esterilizada por filtração em membrana Millipore de 0,2μm e armazenada a 4 °C.

- **Penicilina**

  Solução estoque: 500U/mL.

  Concentração final de uso: 0,05U/mL

  A penicilina é dissolvida em água bidestilada, esterilizada por filtração em membrana Millipore de 0,2μm e armazenada a 4°C.

- **Tetraciclina**

  Solução estoque: 5mg/mL

  Concentração final de uso: 15μg/mL

  A tetraciclina é diluída em etanol absoluto e armazenada a -20°C.

- **Acetoseringona**

  Solução estoque: 200mM

  Concentração final de uso: 0,1mM

  A acetoseringona é diluída em dimetilformamida e armazenada a -20ºC.

- **Fator de crescimento**

Duas alçadas do isolado Pb192 crescido por 7 dias a 37ºC em meio semi-sólido Fava - Netto de *P. brasiliensis* devem ser inoculadas em 500mL de meio BHI e crescidas por 7 dias a 37°C sob agitação 150rpm. Após esse período, a cultura é centrifugada a 4.000*g* por 10 minutos. O sobrenadante deve ser coletado, armazenado à -20°C e usado como fator de crescimento (Singer-Vermes *et al.,* 1992).

4.4) Solução corante Verde-Janus

Solução estoque: 0,5% (p/v) em água destilada estéril.

Concentração final de uso: 0,05% (p/v).

A solução estoque do corante vital Verde-Janus deve ser esterilizada por filtração usando-se membranas do tipo milipore 0,2μm.

4.5) Soluções para eletroforese em géis de agarose

- **Tampão de Corrida Tris-EDTA-borato (TBE) 10X**

Ácido bórico	890mM
EDTA	20mM
Trizma base	890mM

Ajustar o pH para 8,0 com ácido bórico.

- **Tampão de Amostra para DNA/RNA (10X)**

Azul de bromofenol	0,1% (p/v)
Glicerol	50,0% (v/v)
Xileno cianol	0,1% (p/v)

- **Solução de Brometo de Etídio**

  Solução estoque: 10mg/mL em água destilada.

  Concentração final de uso: 0,5μg/mL.

4.6) Soluções para Extração de DNA genômico de *P. brasiliensis*

- **Tampão de extração**

EDTA	25mM
NaCl	250mM
SDS	0,5% (p/v)
Tris-HCl pH 8,5	200mM

- **Solução estoque de RNAse A**

  Solução estoque: 20mg/μL

  Concentração final de uso: 100μg/mL

  A solução estoque é preparada em água MilliQ, aquecida a 100ºC durante 20 min e estocada a –20ºC.

4.7) Soluções para extração de DNA plasmidial em pequena escala

- **Solução I**

EDTA pH 8,0	1mM
Glicose	50mM
Tris-HCl pH 8,0	10mM

- **Solução II**

NaOH	200mM
SDS	1% (p/v)

- **Solução III**

5M Acetato de potássio	60% (v/v)
Ácido acético glacial	11,5% (v/v)

- **TE**

EDTA pH 8,0	1mM
Tris-HCl pH 7,5	10mM

4.8) Soluções para Southern blot

- **Tampão Tris-EDTA-Acetato (TAE) 50 X**

EDTA	50mM
Tris base	2M

Ajustar o pH 8,0, utilizando-se ácido acético glacial.

- **Solução de depurinação**

HCl	125mM

- **Solução de desnaturação**

NaCl	1,5M
NaOH	500mM

- **Tampão de neutralização**

NaCl	1,5M
Tris base	500mM

Ajustar o pH 7,5 com HCl.

- **Tampão de transferência SSC 20 X**

Citrato de sódio	300mM
NaCl	3M

Ajustar o pH para 8,0.

- **Solução de hibridização**

À solução de hibridização (“hybridization buffer”) fornecida pelo kit “AlkPhos Direct labelling and detection system” (Amersham Pharmacia), acrescenta-se NaCl para uma concentração final de 500mM e reagente bloqueador para uma

concentração final de 4% (p/v). O volume de solução de hibridização deve ser de 0,125mL/cm^2 de membrana, de acordo com as recomendações do fabricante.

- **Solução para primeira lavagem**

1M $MgCl_2$	1mM
500mM Na_2PO_4 pH 7,0	50mM
NaCl	150mM
Reagente bloqueador	0,2% (p/v)
SDS	0,1% (p/v)
Uréia	2M

- **Solução estoque para segunda lavagem 20 X**

NaCl	2M
Tris base	1M

Ajustar pH 10,0 e conservar entre 2 – 8 °C.

- **Solução de uso para segunda lavagem**

A solução estoque deve ser diluída 1:20 e acrescida de 0,2% (v/v) de solução de 1M $MgCl_2$ (obtendo-se uma concentração final de 2mM de magnésio). Esta solução não pode ser estocada e deve ser preparada no momento do uso.

5 - Métodos

5.1) Crescimento e manutenção dos isolados de *P. brasiliensis*

Todos os isolados de *P brasiliensis*, Pb01, Pb113 e Pb192 foram mantidos em meio semi-sólido Fava-Netto, e repicados a cada sete dias no caso de leveduras. Já a forma de micélio foi mantida em meio sólido ágar batata e repicado a cada dois meses. Dependendo das condições estudadas, foram utilizados diferentes meios de cultura, que serão indicados em cada experimento.

5.2) Transição de micélio para levedura

O isolado Pb01 foi cultivado em meio N líquido a 22°C por 48 horas. A cultura foi transferida para banho a 37 °C sob agitação de 150rpm e as células foram coletadas nos tempos 0 (M); 0,5; 1; 2; 6; 24 horas e 15 dias (L) após a mudança de temperatura. Em seguida, as células foram utilizadas para extração de RNA.

5.3) Choque térmico

O isolado Pb01 foi cultivado em Erlenmeyers de 250mL contendo 50 mL de meio YPD, a 37°C sob agitação de 150rpm. Alíquotas de 15mL da cultura a 37ºC foram transferidas para tubos falcon de 50mL e incubadas no banho a 42°C sob agitação de 150rpm durante os tempos de 15, 30, 60, 90 e 120 minutos. A densidade celular utilizada nos tratamentos foi de 10^5 leveduras/mL. As células foram centrifugadas logo após o tratamento por 2 minutos a 3.000*g* e congeladas em nitrogênio líquido para posterior extração de RNA.

5.4) Extração de DNA total de *P. brasiliensis*

O procedimento experimental é baseado no método descrito por Raeder & Broda (1985). Resumidamente, micélio ou leveduras foram macerados com pistilo e nitrogênio líquido em almofariz de porcelana até obtenção de um pó fino. Esse pó foi transferido para tubo de 2mL (cerca de 50mg por tubo) e ressuspendido em 500μL de tampão de extração. Essa suspensão foi misturada em vortex por 15 minutos, e em seguida foram adicionados 500μL de clorofane. Misturou-se em vortex, e centrifugou-se por 1 hora a 12.000*g*. A fase aquosa foi transferida para novo tubo e tratada com RNase A numa concentração final de 100μg/mL por 15 minutos em banho a 37°C. Após esse tratamento, adicionou-se igual volume de

clorofórmio, misturou-se vigorosamente (uso de vortex) e centrifugou-se a 12.000*g* por 10 minutos. A fase aquosa foi transferida para novo tubo, foram adicionados 0,5V de isopropanol e a solução foi centrifugada a 12.000*g*, por 20 minutos a 4°C. O DNA precipitado foi lavado com etanol 70%, seco a temperatura ambiente e ressuspendido em volume apropriado de água destilada ou TE.

5.5) Extração de RNA total de culturas de *P. brasiliensis*

O RNA foi extraído com o reagente Trizol (Invitrogen) de acordo com as recomendações do fabricante. As células de *P. brasiliensis* foram coletadas por centrifugação a 3.000*g* por 2 min e lavadas duas vezes com água livre de contaminação com RNAse (5.000*g* por 30 segundos, cada lavagem). As células (3g de peso úmido) foram maceradas com pistilo e nitrogênio líquido em almofariz de porcelana até obtenção de um pó fino o qual foi transferido para um tubo e homogeneizado com 8mL de Trizol. A suspensão obtida foi centrifugada a 10.000*g* por 20 segundos e o sobrenadante transferido para um novo tubo. Esta solução foi incubada por 5 minutos a temperatura ambiente, em seguida foram adicionados 0,2mL de clorofórmio para cada 1mL de Trizol inicial. O tubo foi invertido várias vezes para homogeneização e centrifugado a 12.000*g* por 15 minutos a 4°C. A fase aquosa foi transferida para novo tubo e foram adicionados 0,5mL de isopropanol para cada 1mL de Trizol inicial. Após incubação por 10 minutos a temperatura ambiente, centrifugou-se a 12.000*g* por 10 minutos a 4°C. O RNA precipitado foi lavado uma vez com etanol 75% e deixado à temperatura ambiente para secar. O RNA foi ressuspendido em volume apropriado de água MilliQ, livre de contaminação com RNAse e estocado a -80°C.

5.6) Tratamento do RNA total com DNAse I livre de RNAses

Após quantificação por espectrofotometria, verificou-se a integridade do RNA total extraído através de eletroforese em gel de agarose 1%, em condições livre de RNAse (Sambrook & Russel, 2001). Cerca de 10μg do RNA total obtido foi submetido a tratamento com DNAse I livre de RNAse (Promega) a fim de se eliminar qualquer traço residual de contaminação por DNA genômico. As condições de tratamento com DNAse I, para um volume final de 25μL de reação, foram: Tampão da DNAse I 1X; 10U da enzima DNAse I livre de RNAse

(Promega). O tratamento foi realizado a 37ºC por 30 minutos, interropendo-se a reação pela adição de EDTA 2,5mM (concentração final) e aquecendo-se a 65°C por 10 minutos. Após precipitação padrão com etanol (Sambrook & Russel, 2001), o RNA total tratado com DNAse I livre de RNAse foi novamente quantificado e analisado por eletroforese em gel de agarose 1%, sendo todos os procedimentos realizados em condições livres de RNAse (Sambrook & Russel, 2001).

5.7) Quantificação de ácidos nucléicos

Os ácidos nucléicos extraídos foram quantificados no "GeneQuant™*pro*"(GE Healthcare) e em gel de agarose 0,8% (p/v), contendo brometo de etídio na concentração final de 0,5μg/mL. Usou-se tampão de corrida TAE 1X ou TBE 1X. Para a eletroforese de RNA, todos os reagentes e as condições de preparo do gel foram livres de contaminação com RNAse. O gel também foi utilizado com o objetivo de verificar a integridade das amostras.

5.8) Preparação de células DH5α eletrocompetentes

a) Inoculou-se uma colônia isolada de DH5α em 10mL de meio SB. Cultivou-se durante a noite sob agitação de 250rpm a 37ºC.

b) 1mL dessa cultura foi diluído em 500mL de meio SB, contendo 2,5mL de uma solução estoque de glicose 2M e 2,5mL de solução estoque de Magnésio 2M em um frasco de 1L. Incubou-se sob agitação de 250rpm a 37ºC até OD_{600nm} de 0,7 a 0,9.

c) Ao atingir a densidade óptica esperada, as células foram incubadas por 15 minutos no gelo. Em seguida as células foram centrifugadas a 3.000*g* por 20 minutos a 4ºC.

d) Em seguida, as células foram ressuspendidas em 25mL de glicerol 10% (v/v) gelado usando pipetas de vidro pré-resfriadas. Adicionou-se 75mL de glicerol 10% gelado e centrifugou-se a 3.000*g* por 20 minutos a 4ºC. Descartou-se o sobrenadante e esse processo foi repetido por mais uma vez.

e) As células foram ressuspendidas em 25 mL de glicerol 10% gelado e centrifugadas novamente a 3.000*g* por 20 minutos a 4ºC. Descartou-se o sobrenadante.

f) Ressuspendeu-se as células em 2mL de glicerol 10% gelado e foram feitas alíquotas de 100µL em tubos previamente resfriados, que por sua vez foram congelados posteriormente em nitrogênio líquido e armazenados a -80ºC.

5.9) Eletroporação de células DH5α competentes

Os seguintes parâmetros foram utilizados para eletroporação de *E. coli*: voltagem de 2,5kV, capacitância de 25µF, resistência de 200Ω e cubeta de 0,2cm.

Após o descongelamento das células competentes no gelo, o volume de DNA (no máximo 1/10 do volume total de células) a ser transformado foi adicionado às células. Em seguida ao choque, as células foram recuperadas com 2mL de SOC e incubou-se por 1h a 37ºC/250rpm.

Posteriormente a recuperação as células foram plaqueadas em meio L contendo o antibiótico adequado para seleção das colônias transformantes. As placas foram incubadas a 37ºC/12h.

5.10) Extração de DNA plasmidial em pequena escala de *E. coli*.

O clone de *E. coli* de interesse foi crescido em 5mL de meio LB com ampicilina (100µg/mL) a 37°C sob agitação de 250rpm por aproximadamente 16 horas. A cultura foi transferida para um tubo de 1,5mL e as células foram precipitadas por centrifugação a 10.000*g* por 1min. O sobrenadante foi descartado e o precipitado foi ressuspendido em 200µL de **solução I**. Acrescentou-se 360µL de solução II fresca. O tubo foi invertido várias vezes e incubado à temperatura ambiente por 5 minutos. Foram acrescentados 300µL de **solução III** gelada, o tubo foi invertido várias vezes e incubado no gelo por 5 minutos. Centrifugou-se a 10.000*g* por 5 minutos. O sobrenadante foi transferido para novo tubo e foram acrescentados 750µL de isopropanol. O tubo foi invertido várias vezes e incubado a temperatura ambiente por 5 minutos. Centrifugou-se a 12.000*g* por 5 minutos a 4°C. O precipitado foi ressuspendido em 200µL de TE e acrescentado 110µL de acetato de amônio 7,5M. Após vigorosa agitação (com uso de vortex), centrifugou-se a 10.000*g* por 10 minutos. O sobrenadante foi transferido para novo tubo e foram acrescentados 750µL de etanol 100%, invertendo-se o tubo

várias vezes. Centrifugou-se a 10.000 *x g* por 10 minutos. O precipitado foi lavado duas vezes com etanol 70%. O precipitado final obtido foi seco à temperatura ambiente e ressuspendido em volume apropriado de água contendo RNAse A na concentração final de 100µg/mL.

5.11) Obtenção das seqüências de *ras1* e *ras2* de *P. brasiliensis*

- ***ras1* de *P. brasiliensis***

Através da ferramenta de BLAST do endereço eletrônico http://www.ncbi.nih.nlm.org e a opção para análise de ESTs, a seqüência deduzida da proteína Ras1 de *C. neoformans* (AF294647) foi utilizada para procura de ESTs de *P. brasiliensis* que foram depositadas pelo grupo de Goldman *et al.* (2002) ao estudarem o transcriptoma do isolado Pb18, visto que não foi encontrada nenhuma PbAEST que apresentasse similaridade ao gene *RAS1* de *C. neoformans* no banco de dados do transcriptoma do Projeto Genoma Funcional e Diferencial de *P. brasiliensis* (isolado Pb01) (http://www.biomol.unb.br/Pb). O resultado obtido pelo BLAST de ESTs no site do NCBI foi uma EST com número de acesso BQ493380, que contém 450pb, os quais codificam para a região 3' da seqüência de *ras1* de *P. brasiliensis*, porém do isolado Pb18. Baseado nesta seqüência, oligonucleotídeos foram desenhados e utilizados para amplificação utilizando DNA genômico do isolado de estudo Pb01. O produto de PCR obtido no tamanho de 550pb foi completamente seqüenciado e foi identificada a presença de um íntron de 99pb. As seqüências obtidas dos isolados Pb01 e Pb18 foram alinhadas e não se observou nenhuma diferença entre os nucleotídeos. Utilizando a seqüência de 550pb obtida pelo PCR foram desenhados oligonucleotídeos específicos para amplificação das regiões 5' e 3´, através da técnica RACE (Rapid Amplification of cDNA ends) descrita no item seguinte **(5.12)** desta seção.

- ***ras2* de *P. brasiliensis***

O gene *ras2* de *P. brasiliensis*, isolado Pb01, foi obtido através do transcriptoma do Projeto Genoma Funcional e Diferencial de *P. brasiliensis* (Felipe *et al.*, 2003 e 2005). A seqüência do gene *RAS2* de *C. neoformans*

(AF294349) foi utilizada para realizar um BLAST no banco de PbAEST e obteve-se como resultado o contig986 correspondente a PbAEST983 com E-value igual a 1e-09. O clone correspondente à PbAEST983 foi seqüenciado e observou-se que a ORF (Open Reading Frame) de *ras2* de *P. brasiliensis* estava completa. Baseando-se na seqüência obtida, foram desenhados oligonucleotídeos para amplificação de *ras2* a partir do DNA genômico para avaliar a presença de íntrons.

5.12) RACE (Rapid Amplification of cDNA Ends) para obtenção da seqüência completa de *ras1*

Para amplificação das regiões 5' e 3' desconhecidas de *ras1* foi utilizado o kit "BD Smart TM Race cDNA Amplification Kit", BD Bioscience Clontech. Resumidamente, o procedimento é baseado na síntese da primeira fita de cDNA utilizando uma Transcriptase reversa (BD PowerScript RT) variante de MMLV RT que ao alcançar a região terminal de um RNA alvo (pode ser tanto RNA total quanto RNAm), exibe uma atividade terminal transferase adicionando de 3 a 5 resíduos (predominantemente dC) a terminação 3' da primeira fita de cDNA. O oligonucleotídeo fornecido pelo kit (BD Smart oligo) contém uma série de resíduos terminais G que anelam a cauda do cDNA rica em dC e serve como um alvo estendido para a transcriptase reversa. Após a transcrição reversa, o cDNA pode ser usado diretamente nas reações de PCR 5'- e 3'-RACE. Através dessa tecnologia, é possível utilizar um oligonucleotídeo universal e um específico do gene de interesse, sendo necessário conhecer pelo menos de 23 a 28pb da seqüência do gene de interesse para o desenho dos oligonucleotídeos específicos (GSP- Gene specific primer).

a) Desenho dos oligonucleotídeos GSP para *ras1*

O desenho dos oligonucleotídeos GSP para *ras1* foi baseado nas informações do kit, sendo que os requisitos básicos são: apresentar de 23 a 28 nucleotídeos, de 50 a 70% de conteúdo de GC, e T_m ≥65ºC. Além disso, os oligos 5' e 3' GSP devem apresentar uma região de sobreposição para que sejam utilizados como controle positivo da reação **(Figura 5).** Os oligos GSP para *ras1* estão na **Tabela 3**, sendo que apresentam uma região de sobreposição de aproximadamente 200 pb.

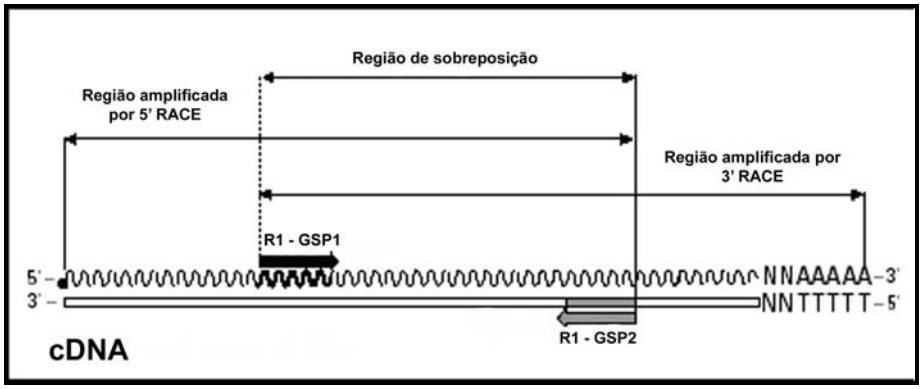

Figura 5: Relação entre os oligonucleotídeos específicos de *ras1* (GSP1 e GSP2) e as regiões amplificadas do cDNA alvo pelas reações de 5'- e 3'-RACE. A região de sobreposição correponde a 200pb. (Modificada do manual do kit BD Smart TM Race cDNA Amplification).

Tabela 3: Características dos oligonucleotídeos GSP para 5'- e 3'-RACE de *ras1* de *P. brasiliensis*

Oligos (GSP)	*Seqüência de nucleotídeos (5'→3')*	*Tamanho (mer)*	*T_m (ºC)*	*% GC*
R1-GSP1 (3'RACE)	GCGAGAACAATACATGCGCACAGGCG	26	82	57,7
R1-GSP2 (5'RACE)	GATACCACACGTTCCTTCTCCAAGTCGC	28	86	53,6

b) Síntese da primeira fita de cDNA

Utilizou-se como molde 1µg de RNA total de Pb01 extraído da fase leveduriforme e tratado com DNAse I, como descrito no itens **5.5** e **5.6** e montaram-se as seguintes reações conforme o protocolo do kit:

5' RACE-READY-cDNA	**3' RACE-READY-cDNA**
1,2µL RNA total (1µg)	1,2µL RNA total (1µg)
1µL 5'CDS primer	1µL 3'CDS primer
1µL BD Smart II oligo A	-
1,8µL água MilliQ	2,8µL água MilliQ
5,0µL Volume final	5,0µL Volume final

Incubou-se o sistema a 70ºC por 2 minutos, seguido de gelo por 2 minutos e adicionaram-se os seguintes componentes: 2µL 5X First strand Buffer; 1µL DTT

(20mM); 1µL dNTPs (10mM) e 1µL BD Power Script RT. O sistema foi então incubado novamente a 42ºC por 1,5h.

c) Reações de RACE

Para amplificação das regiões 5' e 3' de *ras1* utilizando os oligonucleotídeos específicos GSP1 e GSP2 respectivamente, os cDNAs obtidos na etapa anterior foram diluídos através da adição de 100µL do Tampão Tricina EDTA recomendado pelo fabricante. Como controle do funcionamento do kit, utilizou-se um cDNA de placenta humana e oligonucleotídeos específicos para amplificação do gene TFR que codifica o receptor de transferrina, fornecidos pelo fabricante.

As condições do PCR foram: 94ºC/30s, 72ºC/ 3 min - 5X; 94ºC/30s, 70ºC/ 30s, 72ºC/3 min - 5X; 94ºC/30s, 68ºC/ 30s, 72ºC/3 min - 25X.

Para cada PCR utilizou-se 2,5µL do cDNA diluído e os seguintes componentes:

	5' RACE ras1	***3' RACE ras1***	***Controle (+) 5' RACE***	***Controle (+) 3' RACE***
5' RACE cDNA (dil)	2,5µL	-	2,5µL	-
3' RACE cDNA (dil)	-	2,5µL	-	2,5µL
UPM 10X	5µL	5µL	-	-
***ras1*-GSP1 (10 µM)**	-	1µL	1µL	1µL
***ras1*-GSP2 (10 µM)**	1µL	-	1µL	1µL
Tampão PCR Advantage 2 10X	5µL	5µL	5µL	5µL
dNTPs (10mM)	1µL	1µL	1µL	1µL
BD Advantage 2 Polymerase Mix 50X	1µL	1µL	1µL	1µL
Água MilliQ	34,5µL	34,5µL	38,5µL	38,5µL
Volume final	50µL	50µL	50µL	50µL

d) Caracterização dos produtos obtidos após PCR

Após o PCR, 5µL do volume final da reação foram submetidos a uma eletroforese em gel de agarose 0,8% em TBE 1X. Em seguida, após análise dos fragmentos obtidos, o volume restante (45µL) foi submetido a uma eletroforese em gel de agarose 0,8% em TBE 1X para purificação dos fragmentos obtidos utilizando o kit Pure link gel extraction (Invitrogen).

e) Clonagem em pGEMT (Promega)

Os fragmentos purificados anteriormente foram clonados no vetor comercial pGEMT (Promega), transformados em DH5α eletrocompetentes , conforme descrito no item **5.9**. Os clones com inserto foram selecionados para seqüenciamento.

f) Seqüenciamento automático

O seqüenciamento foi realizado empregando o kit "DYEnamic^{TM}ET - Dye terminator" e o seqüenciador automático "MegaBACE 1000" (GE Healthcare). Foram preparadas reações em placas de 96 poços, em um volume final de 5µL contendo aproximadamente 100-200ng de DNA e 5 picomoles do iniciador universal (5'-CCCAGTCACGACGTTGTAAAACG-3') ou reverso (5'-AGCGGATAACAATTTCACACAGG-3'). Os demais componentes da reação de seqüenciamento foram adicionados, conforme protocolo padrão fornecido pelos fabricantes.

5.13) Southern blot

- **Digestões do DNA genômico**

100µg de DNA genômico de Pb01 extraído de acordo com o protocolo previamente descrito foram digeridos com 500U, proporção de 5U/ µg DNA (conforme recomendações de Sambrook & Russel, 2001) das seguintes enzimas *Bam*HI (QBiogene), *Eco*RI (Promega), *Hind*III (Promega) e *Pst*I (Promega). Todas as digestões foram incubadas por 12h a 37ºC.

- **Preparo do gel**

Após a digestão ser completada, 10µg de DNA genômico digerido com cada uma das enzimas, foram aplicados em um gel de agarose 0,8% com 1X TAE e submetidos a uma eletroforese a 5V/cm de gel.

- **Transferência para membrana**

Utilizou-se membrana Hybond N (Amersham Pharmacia) e realizou-se uma transferência neutra seguindo as seguintes etapas:

a) Tratamento do gel

O gel foi lavado por 15 minutos à temperatura ambiente com HCl 0,125M para depurinação. A segunda lavagem foi realizada à temperatura ambiente por 30 minutos com a solução de desnaturação. A terceira lavagem de neutralização também foi realizada à temperatura ambiente por 30 minutos. Entre cada uma das lavagens e após a última lavagem, o gel foi rinsado com água MilliQ.

b) Transferência capilar

O DNA foi transferido para membrana seguindo as recomendações do fabricante. Utilizou-se como solução de transferência SSC 20X, o tempo de 12h à temperatura ambiente. Após a transferência, a membrana foi aquecida a 80ºC/ 2h para fixação do DNA.

c) Pré-hibridização

A pré-hibridização foi feita seguindo o protocolo fornecido pelo kit "AlkPhos Direct labelling and detection system" (Amersham Pharmacia). Basicamente, a solução de pré-hibridização foi pré-aquecida a 55ºC e adicionada a uma garrafa contendo a membrana e incubou-se o a 55ºC por 30 minutos.

d) Preparo da sonda

As sondas para *ras1* e *ras2* foram amplificadas utilizando os oligonucleotídeos citados na **Tabela 4** e purificadas utilizando o kit Pure link gel extraction (Invitrogen). Os tamanhos das sondas de *ras1* e *ras2* são 300pb e 75pb, respectivamente. Utilizou-se a proporção de 10ng de sonda por mL de solução de pré-hibridização. Basicamente, 200ng de sonda de *ras1* ou *ras2* foram

desnaturadas a 100ºC por 5 minutos e incubada no gelo por mais 5 minutos. Em seguida adicionou-se 20µL do tampão de reação, 4µL do reagente de marcação e 20µL da solução de trabalho de "cross-linker" (10µL solução estoque de "cross-linker" acrescido de 40µL de água) todos os reagentes provenientes do kit. A reação foi incubada a 37ºC por 30 minutos. Após incubação, a sonda foi adicionada diretamente à solução de pré-hibridização.

e) Hibridização

Após a adição da sonda, a hibridização procedeu a 55ºC/ 12h.

f) Lavagens pós-hibridização

Foram realizadas duas lavagens sucessivas com o tampão primário pré-aquecido a 55ºC por 10 minutos. Em seguida, foram realizadas mais duas lavagens com tampão secundário por 5 minutos à temperatura ambiente.

g) Geração do sinal e detecção com CDP-Star

Após remoção do excesso de tampão de lavagem secundário, adicionou-se o reagente de detecção na proporção de 40µL/cm^2 de membrana e incubou-se o sistema por 5 minutos à temperatura ambiente. O excesso foi retirado e a membrana foi envolvida em papel PVC para impedir sua secagem. A exposição foi feita utilizando filme: Hyper film ECL (Amersham Pharmacia) por 4h.

h) Revelação

A revelação foi feita incubando o filme na solução reveladora (Kodak) até o aparecimento de sinal, em seguida o filme foi rinsado em água para interromper a revelação e finalmente rinsou-se no fixador por alguns minutos.

5.14) Infecção de macrófagos peritoniais J774 com leveduras de *P. brasiliensis* e extração do RNA total das leveduras internalizadas.

Os experimentos de infecção e isolamento do RNA total de leveduras de *P.brasiliensis* internalizadas em macrófagos peritoniais foram realizados de acordo com Tavares *et al.* (2007). Basicamente macrófagos peritoniais da linhagem J774.1 (ATCC TIB-67) foram cultivados em monocamadas aderentes em RPMI-1640 e infectadas com 2 × 10^6 células leveduriformes de *P. brasiliensis*

opsonizadas, com uma taxa de levedura:macrófago de 1:5. A co-cultura foi incubada por 9h a 37ºC numa atmosfera contendo 5 % CO_2. As leveduras que não foram internalizadas neste período foram removidas pela lavagem com meio RPMI-1640 (previamente aquecido a 37ºC). Em seguida os macrófagos foram lisados com solução de isotiocianato de guanidina (Monahan *et al*., 2002) e as células leveduriformes intactas foram coletadas através de uma rápida centrifugação. O RNA total das células leveduriformes foi extraído utilizando Trizol (item **5.5**) e em seguida este RNA foi amplificado utilizando o Kit MessageAmp aRNA (Ambion) para obtenção de maior quantidade de RNA. O RNA controle utilizado nos experimentos de RT-PCR foi obtido de uma cultura de células leveduriformes crescidas por 7 dias a 37ºC em meio semi-sólido Fava-Netto também foi amplificado.

5.15) RT-PCR semi-quantitativa (RT-PCRsq)

- **Síntese da Primeira Fita de cDNA**

A reação de síntese da primeira fita de cDNA foi realizada a partir de 500ng de RNA total de *P. brasiliensis* cultivado de acordo com as condições especificadas nos tratamentos e posteriormente, tratado com DNAse I livre de RNAse. A essa quantidade de RNA total foi adicionado 0,5μg de iniciador dT_{12-18}, sendo a mistura incubada a 70°C por 10 minutos, e imediatamente colocada no gelo por cerca de 1 minuto. Em seguida, para um volume final de 25μL, os seguintes reagentes foram adicionados (para as concentrações finais indicadas): tampão da transcriptase reversa 1X; DTT 8mM; dNTPs 0,4mM cada. Após incubação a 42°C por 2 minutos, 200U da enzima transcriptase reversa (SuperScript II, Invitrogen) foram adicionadas para cada sistema, seguido de incubação a 42°C por 1h. Finalmente, a enzima foi desnaturada aquecendo-se a 70°C por 20 minutos.

- **PCR**

Uma alíquota correspondente a 1/5 (5μL) da reação de síntese de cDNA foi submetida à reação de amplificação em um volume final de 25μL contendo: tampão *Taq* DNA Polimerase 1X; 1,5mM $MgCl_2$; 0,2mM dNTPs; 0,2μM de cada oligonucleotídeo (tendo sido utilizado na mesma reação um par de

oligonucleotídeos específicos para o(s) gene(s) em estudo, e o par de oligonucleotídeos referente ao controle interno *α-tub, clat* ou *l34*); 2U da enzima *Taq* DNA Polimerase (Cenbiot-RS/Brasil). As reações de PCR para amplificação dos genes de interesse para as análises de expressão nessas condições foram feitas em triplicata.

O número de ciclos foi padronizado para garantir a fase exponencial de amplificação. Como controle negativo da reação, utilizou-se RNA tratado com DNAse I para verificar uma possível contaminação por DNA genômico. Os produtos da PCR foram analisados por eletroforese em gel de agarose 1,5%.

5.16) Estabelecimento da fase exponencial de amplificação e compatibilidade entre os oligonucleotídeos: RT-PCR semi-quantitativa (RT-PCRsq)

Para análise semi-quantitativa dos níveis de expressão dos genes em estudo, determinou-se a compatibilidade entre os oligonucleotídeos e o número de ciclos correspondente à fase exponencial de amplificação. Uma vez que o número ótimo de ciclos adotado deve ser o mesmo para a amplificação do gene em estudo e do controle interno (α-tubulina; proteína ribossomal L34*;* cadeia leve da clatrina), procedeu-se a PCR utilizando-se, a partir de um mesmo molde, os pares de oligonucleotídeos para amplificação do gene de interesse (*ras*) e do gene controle interno. O molde utilizado foi uma alíquota correspondendo a 1/5 do produto da reação de transcrição reversa feita a partir de 500ng do RNA total de *P. brasiliensis* proveniente de cada tratamento a ser estudado. A compatibilidade entre os oligonucleotídeos que serão utilizados para *ras1* e *ras2* e aqueles constitutivos *l34,* α-tub e *clat* foi acessada por uma PCR utilizando os pares de oligos *ras/ l34*, *ras/α-tub* e *ras/clat* (**Tabela 4**). A reação utilizou como molde o cDNA da fase leveduriforme de *P. brasiliensis* e o número total de 30 ciclos (**Figura 6**). Em seguida, para todos os genes foram feitas PCRs variando-se o número de ciclos (24, 27, 30 e 33 ciclos) com o controle interno *l34*. O resultado da PCR foi analisado por eletroforese em gel de agarose 1,5% (**Figura 7**). Com base nestes resultados, decidiu-se utilizar o número de 24 ciclos para amplificação de todas as condições testadas, para garantir a fase exponencial da PCR. Assim, o programa de PCR consistiu: um primeiro passo de denaturação

(94ºC/2min), 24 ciclos de 94ºC/1min, 60ºC/1min e 72ºC/1min; e uma extensão final a 72ºC/10 min. As reações de PCR para amplificação dos genes de interesse (*ras1* e *ras2*) para as análises de expressão nas condições testadas foram feitas em triplicata utilizando como controle interno *l34* para choque térmico e *clat* para diferenciação celular.

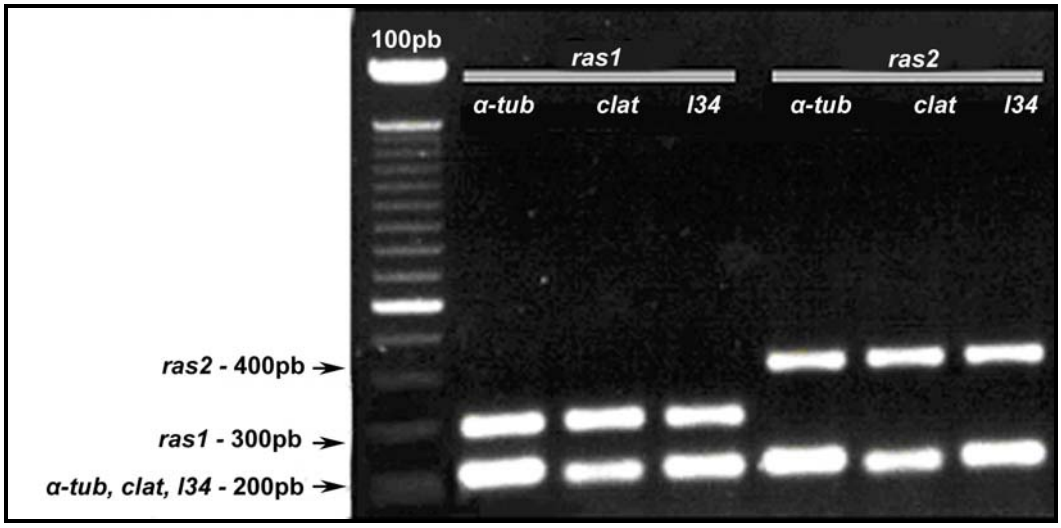

Figura 6: Compatibilidade entre os oligonucleotídeos para *ras* (*ras1* e *ras2*) e os constitutivos *α-tub*, *l34* e *clat*. Os oligonucleotídeos utilizados em cada reação de PCR estão indicados na figura bem como os tamanhos dos fragmentos obtidos. Gel de agarose 1,5%.

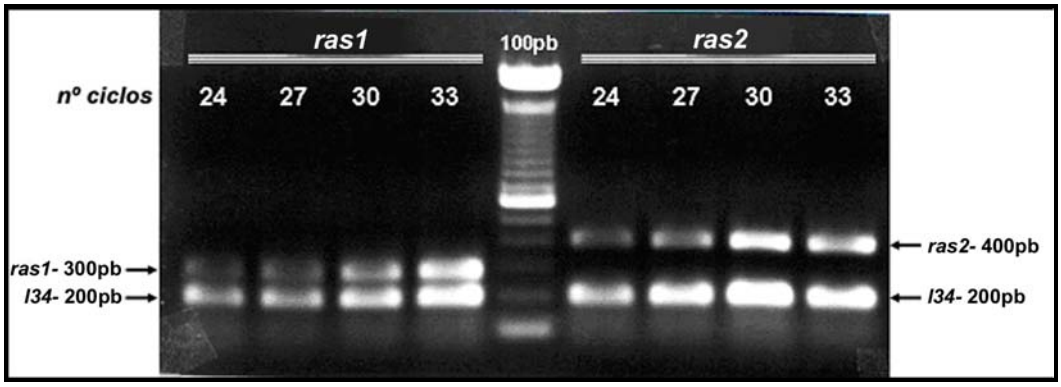

Figura 7: Padronização do número de ciclos correspondentes a fase exponencial de amplificação para posterior análise por RT-PCRsq dos níveis de expressão de *ras1* e *ras2*. Os números dos ciclos utilizados em cada reação de PCR estão indicados na figura. Utilizou-se como gene constitutivo aquele que codifica para a proteína ribossomal L34 *(l34)*. Gel de agarose 1,5%.

5.17) Análise da expressão de *ras1* e *ras2* do fungo *P. brasiliensis* recuperado de macrófagos peritoniais de camundongo

300ng de RNA amplificado extraído de leveduras internalizadas em macrófagos foram utilizados como molde nos experimentos de RT-PCRsq, com base no protocolo descrito no item **5.15**, diferenciando-se apenas quanto a utilização de Random Primers como iniciadores nas reações de síntese da primeira fita de cDNA (0,5μg / reação). Como controle dos experimentos, foi utilizado RNA de *P. brasiliensis* cultivado *in vitro* em meio Fava-Neto por 7 dias a 37ºC, e também submetido ao procedimento de amplificação. As reações de PCR para amplificação dos genes de interesse (*ras1* e *ras2*) para as análises de expressão nessas condições foram feitas em quadruplicata utilizando como controle interno *l34*.

5.18) Quantificação dos níveis de expressão gênica utilizando-se o programa "Scion Image"

A análise dos resultados obtidos visando à quantificação dos níveis de expressão dos genes de interesse foi feita por densitometria, utilizando-se o programa "Scion Image", disponível no endereço eletrônico http://www.scioncorp.com. Os níveis de expressão do gene controle interno, permitiram a normalização dos resultados. A partir dos valores obtidos por densitometria, referentes a cada fragmento de DNA amplificado, foram determinadas as razões entre o nível de expressão dos genes *ras* em relação à expressão do gene controle para cada uma das condições empregadas. Comparando-se os valores das razões obtidas (gene de interesse/gene controle) a partir da condição experimental (diferenciação celular M→Y, choque térmico ou ainda da internalização de leveduras em macrófagos peritoniais de camundongos) àquelas obtidas para a condição controle de cultivo, é possível verificar se está ocorrendo regulação positiva ou negativa dos genes em estudo quando da submissão do fungo a tais condições.

5.19) Análise estatística dos resultados

As análises estatísticas foram feitas com o auxílio do programa de computador "Mynova", versão 1.3 (S. Brooks, Copyright 1993). Os dados estão apresentados como médias +/-SEM (erro padrão da média). Para comparações

entre dois dados (condição experimental X condição controle) foi utilizado o teste-t de Student, sendo considerados os valores de $P < 0,05$ como significativamente diferentes.

5.20) Determinação da Concentração Mínima Inibitória (CMI) de FPT Inhibitor III

O procedimento foi baseado no protocolo M-27 A: "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts", NCCLS, com algumas modificações para *P. brasiliensis*. Utilizaram-se como cepas padrão *C. parapsilosis* (ATCC22019) e *C. albicans* (ATCC24433). O meio de cultura utilizado foi MVM (Hahn & Handam, 2000). Os inóculos das espécies de *Candida* foram obtidos de meio sólido Fava-Netto após 24 h a 37ºC, sendo a concentração final de 10^3 células/mL. No caso de *P. brasiliensis*, isolado Pb01, utilizou-se como inóculo, leveduras crescidas de 5 - 7 dias a 37ºC em meio Fava-Netto, e por ser um fungo de crescimento lento, a concentração final foi de 10^5 células/mL. Para contagem das células leveduriformes de *P. brasiliensis* durante a preparação do inóculo utilizou-se o corante vital Verde-Janus. Como controle dos inóculos utilizou-se o agente antifúngico Anfotericina B. As concetrações testadas para Anfotericina B variaram de 4,0 a 0,00039µg/mL, sendo que a Anfotericina foi diluída em água estéril. Já o inibidor de farnesiltransferase FPT Inhibitor III (Calbiochem) foi diluído em meio de cultura, sendo que as concentrações testadas variaram de 500 a 0,43µM. Não foi possível utilizar uma concentração acima de 500µM de FPT Inhibitor III devido à limitação da quantidade (1mg/ frasco). O experimento foi realizado em placas de 96 poços, sendo o volume final por poço de 100µL. As placas foram incubadas a 37ºC, por um período de 48 h para *C. albicans* e *C. parapsilosis* e 7 - 10 dias para *P. brasiliensis*. A inibição do crescimento foi quantificada por leitura da absorbância a 600nm.

5.21) Teste de FPT Inhibitor III na diferenciação de *P. brasiliensis.*

O inibidor de farnesiltransferase foi testado nas mesmas concentrações utilizadas para determinação da CMI, sendo que os inóculos de leveduras foram preparados da mesma maneira. Para o inóculo de micélio, fragmentos de micélio foram inoculados em meio líquido BHI e incubados sob agitação 150rpm a 22ºC por um período de 10 dias. Em seguida centrifugou-se, descartou-se o

sobrenadante e as hifas foram lavadas três vezes com solução salina (0,9%). Após as lavagens, utilizou-se uma seringa de 1mL para padronização do tamanho dos pequenos fragmentos de micélio, que posteriormente foram contados em câmara de Neubauer e ressuspendidos na concentração final de 10^5 fragmentos de micélio/mL. Após preparo das placas, estas foram mantidas por 48h a 37ºC e 22ºC, respectivamente para o inóculo de levedura e micélio. Seguidas às 48 horas de incubação, as placas foram incubadas nas temperaturas opostas para que as leveduras se diferenciem para micélio e vice-versa. A incubação nas temperaturas assinaladas acima ocorreu por 10 dias. Após esse período, observou-se em microscópio óptico, a morfologia das células que cresceram nas diferentes concentrações de FPT Inhibitor III.

5.22) Aquisição das imagens dos testes do Inibidor de Farnesyltransferase.

Todas as fotos foram tiradas utilizando a câmera digital da marca Sony DSC-W5, utilizando o zoom óptico de 3 vezes e o microscópio Nikkon.

5.23) Teste da sensibilidade de Pb01 e Pb113 a higromicina B

Foram feitas suspensões celulares de leveduras de Pb01 e Pb113, e plaqueadas em meio sólido BHI acrescido de soro de cavalo, fator de crescimento e as seguintes concentrações de higromicina B: 0, 50, 100, 150, 200, 250 e 300µg/mL. As placas foram incubadas a 37ºC por 10 a 15 dias. Após esse período o número de colônias recuperadas em cada concentração foi contado e os dados foram plotados num gráfico.

5.24) Transformação genética de *P. brasiliensis* utilizando o sistema mediado por *A. tumefaciens*

- **Inóculo de Pb113**

Após 5 dias de crescimento em meio Fava-Netto a 37ºC, duas alçadas do isolado Pb113 foram adicionadas a 50mL de meio líquido BHI (sem adição de antibióticos) e mantidos a 150rpm a 37ºC por 72h. Após incubação, a concentração foi ajustada para $1x10^7$ células/mL.

- **Inóculo de *A. tumefaciens* (B25)**

a) Plaqueou-se a cepa B25 em meio sólido BHI acrescido de tetraciclina e ampicilina. Incubação 28ºCpor 48h.

b) Pré-inóculo BHI: Inoculou-se uma colônia isolada de B25 em BHI líquido acrescido de tetraciclina e ampicilina e incubou-se a 28ºC a 200rpm por 24h.

c) Inóculo AB: Após 24h de crescimento, foi feito um novo inóculo em meio AB líquido acrescido de tetraciclina e ampicilina. Incubação a 28ºC a 200rpm por 24h. A densidade óptica a 600nm deve estar entre 1,0-1,5 no final das 24h.

d) Inóculo AB/MES – Indução de *A. tumefaciens*: Inoculou-se 1mL em 50mL do meio indutor AB/MES acrescido de tetraciclina e ampicilina. Incubação a 28ºC a 200rpm por 30 - 36h. A densidade óptica a 600nm deve estar entre 0,5-1,0 no final do período de incubação. A densidade óptica de 1,0 a 600nm equivale a $1x10^9$ bactérias/mL.

e) As células foram centrifugadas a 4500*g* por 10 min e ressuspendidas em meio de indução AB/MES acrescido tetraciclina, ampicilina e acetoseringona na concentração final de $1x10^9$ bactérias/mL.

- **Co-cultivo B25: Pb**

As proporções Pb:Agro foram ajustadas para 1:10; 1:100 e 1:200 e as células foram adicionadas num mesmo tubo de 1,5mL. Em seguida adicionou-se 500µL de meio de indução AB/MES acrescido tetraciclina, ampicilina e acetoseringona. Os co-cultivos foram espalhados em membranas Hybond N, que tiveram como suporte BHI/ MES/AS e incubados a 28ºC por 48h.

- **Pós-co-cultivo**

Após 48h de co-cultivo as membranas foram lavadas com solução salina 0,9%. Em seguida as células foram centrifugadas 4.000*g* por 5 min e plaqueadas em meio sólido BHI acrescido de soro de cavalo, fator de crescimento, cefotaxima (para impedir crescimento de *A. tumefaciens*) e 100µg/mL de higromicina B (Invitrogen). A incubação procedeu-se a 37ºC por 10-15 dias. Após esse período, adicionou-se 5mL de meio semi-sólido BHI Top Ágar (0,5%) acrescido de 100µg/mL de higromicina B a cada uma das placas para evitar crescimento de transformantes falsos- positivos.

- **Análise dos transformantes**

Os transformantes que cresceram após o co-cultivo com *A. tumefaciens* foram repicados em placas de BHI acrescido de soro de cavalo, fator de crescimento contendo 0, 100 e 200µg/mL de higromicina B e incubados a 37ºC por 10-15 dias.

- **Estabilidade mitótica**

Os transformantes que se mantiveram estáveis na concentração de 200µg/mL de higromicina B foram submetidos a 4 passagens sucessivas em meio não seletivo para avaliação da estabilidade mitótica. Foram escolhidos aleatoriamente alguns transformantes para extração de DNA genômico e confirmação por PCR utilizando oligonucleotídeos específicos hph1-5' e hph3-3' **(Tabela 4)** que amplificam 400pb do gene que confere resistência a higromicina B.

5.25) Lista de oligonucleotídeos utilizados para RT-PCRsq, sonda de *ras1* e *ras2* e confirmação dos transformantes de *P. brasiliensis*.

Os oligonucleotídeos utilizados estão listados na **Tabela 4.**

Tabela 4: Oligonucleotídeos utilizados

Oligo	*Seqüência (5'→3')*	T_m (ºC)	*Utilização*
ras1/1	CTTGCTGGTCTACTCCATCACTTC	68	RT PCR sonda *ras1*
ras1/3	CAGATGAATACG AGGACATCTC	64	RT PCR sonda *ras1*
ras2/1	CAACGGGACTCAGCTACC	58	RT PCR sonda *ras2*
ras2/4	GGAGAGCTCCATCATTTCCC	62	RT PCR
ras2/2	CCACAAGCATTACTGGAAC	58	sonda *ras2*
l34-3'	CTTGACACAGCCAGCGCAG	62	RT PCR
l34-5'	GTCCGCATCATCAAGACTCC	62	RT PCR
α-tub-5'	TCTTCATCCCAATCCGAGAC	60	RT PCR
α-tub-3'	CCGTGCTCGAGGAGGTATAG	64	RT PCR
clat-5'	CCTGGGTGAAGATGCGGATC	64	RT PCR
clat-3'	GGATGTGCCTGTGATGGTTC	62	RT PCR
hph1-5'	AGATCTATGCCTGAACTCACCGCGAC	64	Confirmação dos transformantes
hph3- 3'	AGATCTCTATTCCTTTGCCCTCGGACG	66	Confirmação dos transformantes

5.26) Análise *in silico* das seqüências

Todos os BLASTs de seqüência foram feitos através do endereço eletrônico: http://www.ncbi.nih.gov/Blast. Os alinhamentos múltiplos foram realizados pelo programa CLUSTALW no endereço eletrônico http://clustalw.genome.jp/. As análises de identidade e similaridade foram realizadas através de https://bioinformatics.org/sms/.

Foram utilizadas as seguintes seqüências de aminoácidos de diversas proteínas Ras para comparação *in silico* com Ras1 e Ras2 de *P. brasiliensis*: *A. fumigatus* RasA (AAB07703), *A. fumigatus* RasB (AAP94030), *C. albicans* Ras1(AF177670), *C. neoformans* Ras1 (AF294647), *C. neoformans* Ras2 (AF294349), Human H-Ras (AF493916), *N. crassa* Ras1 (P22126), *N. crassa*

Ras2 (BAA03708), *S. cerevisiae* Ras1 (CAA99298), *S. cerevisiae* Ras2 (AAA34959), *S. pombe* Ras (CAA27399).

As seqüências de Ram1 utilizadas foram: Ram1 *S. cerevisiae* (NP_010193), Ram1 *C. albicans* (XP_710548), Ram1 *C. neoformans* (AAN87033) e Ram1 *A. fumigatus* (XP_751793). As seqüências de Ram2 foram: Ram2 *S. cerevisiae* (NP_012906), Ram2 *C. albicans* (AAN40697), e Ram2 *A. fumigatus* (XP_752045). As seqüências de Cdc43 foram: Cdc43 *S. cerevisiae* (NP_011360), Cdc43 *C. albicans* (AF110690) e Cdc43 *A. fumigatus* (EAL88526).

5.27) Construção dos cassetes para silenciamento gênico de *ras1* de *P. brasiliensis*

A estratégia baseia-se na construção de um vetor que contenha um cassete que dará origem ao hairpin *in vivo*. Este cassete é flanqueado pelo P*cpc1* e T*trpC* de pAD1625 **(Figura 8).** Clonados entre o promotor e o terminador estão as seqüências invertidas do gene *ura3* de *P. brasiliensis*, que atuará como um marcador de contra-seleção, quando as células estiverem na presença de 5'-FOA (apenas aquelas colônias que tiverem o gene *ura3* silenciados crescerão em 5'-FOA). Utilizando sítios de restrição posicionados estrategicamente entre a seqüência senso de *ura3* e o promotor, bem como a seqüência antisenso e o terminador, foram clonados os fragmentos senso e antisenso de *ras1*, tornando-se assim um vetor quimérico. Todas essas construções foram feitas no vetor de base pBluescript KS+ (Stratagene) devido sua fácil manipulação em *E. coli* **(Figura 9)**. Após a clonagem de todos os fragmentos em pBluescript KS+, o cassete de silenciamento para RNAi pode ser facilmente retirado através das enzimas de restrição *Not*I e *Xho*I para clonagem em qualquer vetor de preferência ou ainda, o cassete pode ser amplificado com oligonucleotídeos contendo o sítio *BclI* compatível com o sitio *Bgl*II que está presente no vetor de *A. tumefaciens* pAD1625 para que seja utilizado o método de transformação mediado por *Agrobacterium*. O vetor pAD1625 apresenta como marcador seletivo o gene *hph* que confere resistência a higromicina B flanqueado pelo P*cpc1* de *N. crassa* e T*trpC* de *A. nidulans*, cassete este que já foi testado e funciona bem em *P. brasiliensis* (Leal *et al*., 2004).

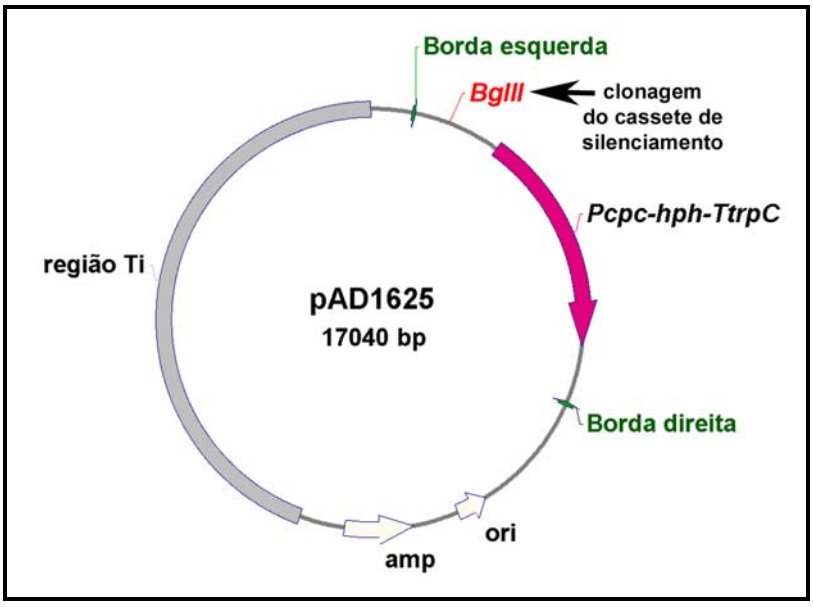

Figura 8: Mapa do vetor pAD1625 utilizado para transfomação fúngica via *Agrobacterium*. A região **Ti** em cinza, corresponde aos genes de virulência de *A. tumefaciens*, **amp** é a marca de seleção para resistência a ampicilina, **ori** é a origem de replicação bacteriana, a borda direita e a borda esquerda delimitam a região do T-DNA (DNA quer será transferido ao genoma do fungo), o cassete ***Pcpc-1-hph-TtpcC*** em rosa corresponde ao cassete de resistência a higromicina B, sendo **P*cpc-1*** o promotor de *cpc-1* de *N. crassa*, *hph*, o gene higromicina fosfotransferase de *E. coli* e T*trpC*, o terminador de *trpC* de *A. nidulans* (Abuodeh *et al.,* 2000). O sítio de restrição *Bgl*II em vermelho será utilizado para as clonagens dos cassetes de silenciamento de *ras1.*

Goldoni *et al.* (2004) e Nakayashiki *et al.* (2005) em seus trabalhos utilizando os fungos *N. crassa* e *M. oryzae* respectivamente, demonstraram que a existência de um íntron no loop que dará origem ao "hairpin" aumenta a eficiência de silenciamento, possivelmente por favorecer a formação do "hairpin" *in vivo*. Com base nestes dados, decidiu-se optar por três estratégias diferentes para construção dos vetores de acordo com os "loops": **(1)** o primeiro vetor contendo um "loop" de 100pb e um íntron de 163pb, **(2)** o segundo contendo apenas o "loop" de 100pb sem o íntron, e o último **(3)** contendo apenas o íntron que ao sofrer processamento *in vivo* restará apenas poucos nucleotídeos. O íntron I do gene *ura3* de *P. brasiliensis* (163pb) foi utilizado, e o "loop" corresponde a 100pb da seqüência de *ura3* que não apresenta nenhuma similaridade com os fragmentos senso ou antisenso de *ura3* escolhidos para clonagem em pBluescriptKS+ **(Figura 10).**

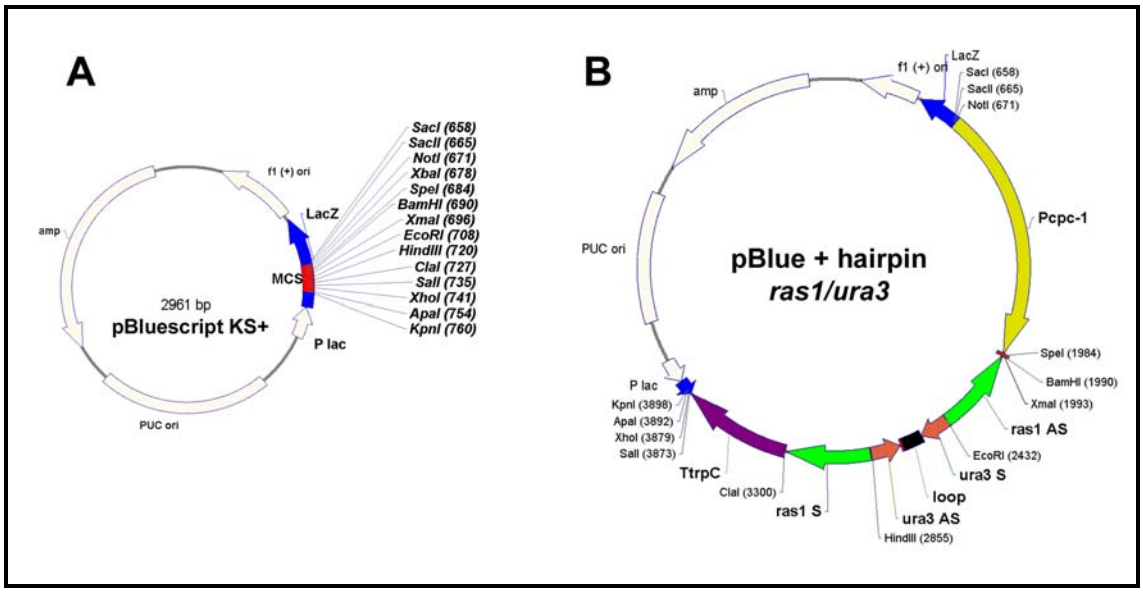

Figura 9: Representação dos vetores pBluescriptKS+ e pBlue + hairpin *ras1/ura3*. Em **A** a região em vermelho correponde aos sítios de clonagem do vetor pBluescriptKS+, sendo que os sítios de restrição estão indicados. No painel **B**, esta a representação geral das construções para silenciamento de *ras1*. Os fragmentos clonados em pBluescriptKS+, bem como os sítios de restrição utilizados nas clonagens estão indicados na figura.

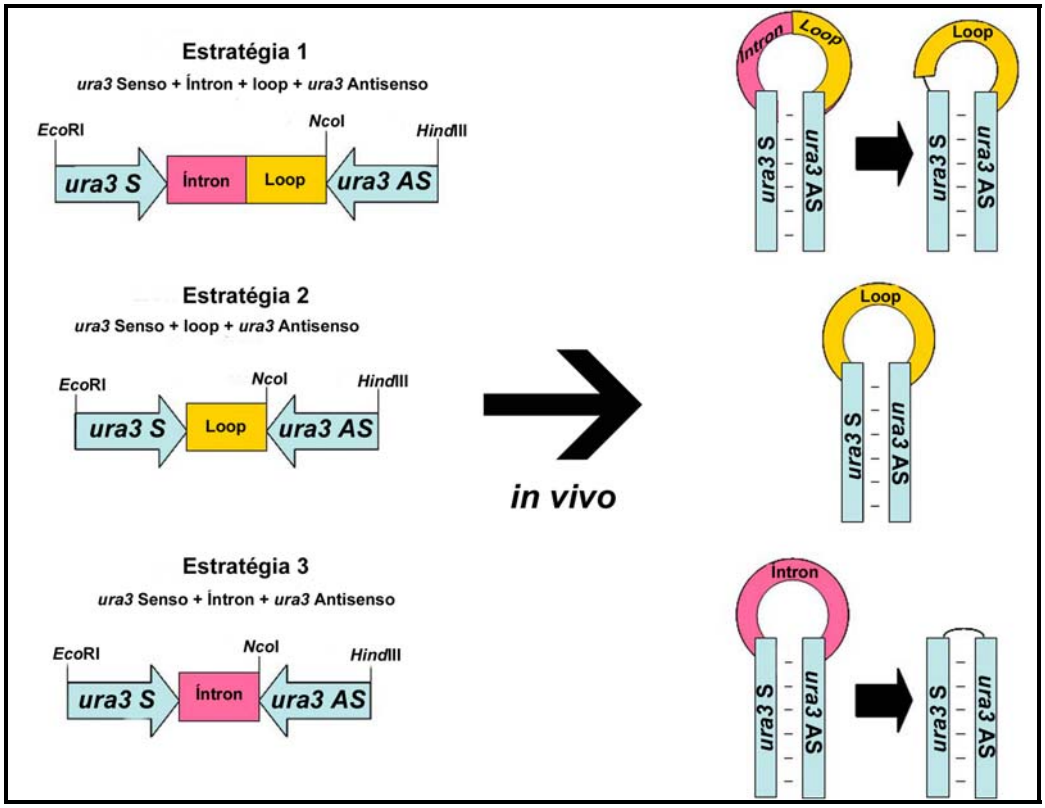

Figura 10: Representação esquemática das estratégias de clonagens dos fragmentos referentes ao loop e as regiões senso e antisenso do marcador de contra seleção *ura3*. Estão representadas também as conformações que cada construção atingirá *in vivo* após o processamento do íntron. Os sítios utilizados nas clonagens estão indicados na figura.

- **Escolha da região de *ras1* de *P. brasiliensis* para ser utilizada na construção dos cassetes**

A região de *ras1* de *P. brasiliensis* utilizada para a construção do cassete que dará origem ao hairpin *in vivo* e que direcionará o silenciamento foi escolhida através do programa "siRNA Target Designer" disponível no endereço eletrônico http://www.promega.com/siRNADesigner/. Este programa é capaz de identificar na seqüência de um gene as regiões que apresentam maior número de siRNAs ("Small interfering RNA"). Além disso, o programa apresenta quais os siRNAs da seqüência analisada com maior probabilidade de silenciar o gene em estudo. Através dessa ferramenta é possível escolher qual a região do gene mais rica em siRNAs para a construção do hairpin, no caso de *ras1* escolheu-se a região que compreende os nucleotídeos de 1 a 438 da ORF de *ras1* por apresentar um número considerável de siRNAs **(Figura 11).**

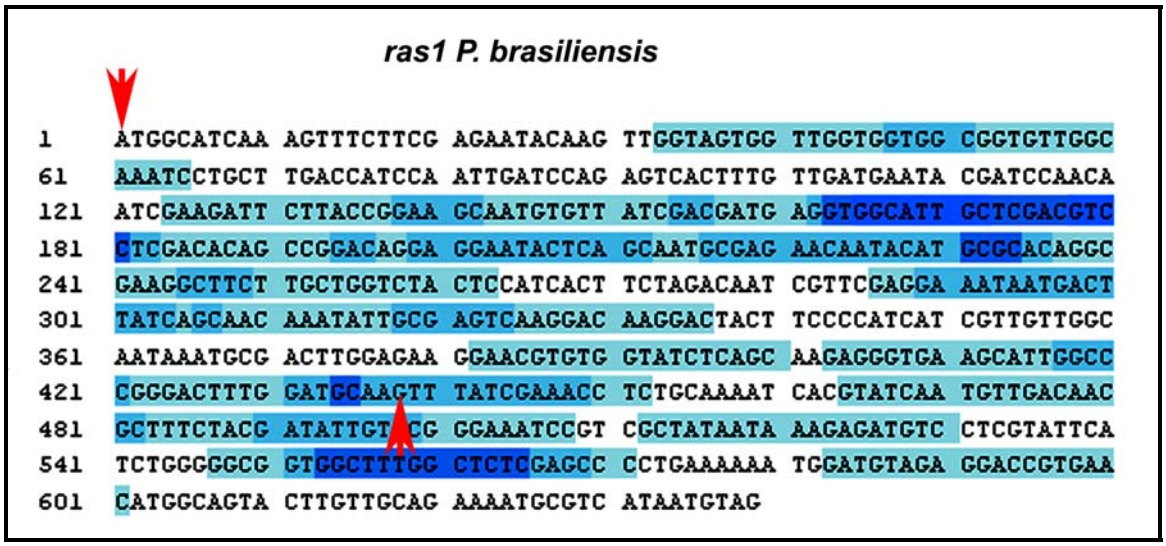

Figura 11: **Análise da seqüência de *ras1* de *P. brasiliensis* para escolha da região utilizada para construção do cassete de silenciamento no vetor pBluescript KS+.** As regiões marcadas em azul claro indicam a presença de siRNAs, as regiões em azul representam dois possíveis siRNAs e aquelas regiões com mais de dois siRNAs estão na cor azul escuro. As flechas em vermelho delimitam a região amplificada de *ras1* e utilizada na montagem do cassete para silenciamento. O programa "siRNA Target Designer" está disponível no endereço eletrônico http://www.promega.com/siRNADesigner/ .

- **Etapas de trabalho**

Para a construção dos vetores quiméricos os seguintes passos foram realizados:

- Desenho e síntese dos oligonucleotídeos **(Tabela 5);**
- Amplificação dos fragmentos: Todas as amplificações utilizaram *Taq* DNA Polimerase da marca Cenbiot-RS/Brasil, sendo que para cada fragmento foram padronizadas diferentes condições dentre elas, a concentração de magnésio e o programa do PCR.
- Purificação dos fragmentos amplificados utilizando o kit Pure link gel extraction (Invitrogen).
- Ligação dos fragmentos amplificados em pGEMT (Promega), seguindo as recomendações do kit;
- Digestão dos fragmentos clonados em PGEMT, de acordo com enzimas específicas para clonagem em pBluescript KS+ ;
- Ligação dos fragmentos em pBluescript KS+ utilizando a enzima T4 Ligase (Invitrogen). Todas as ligações foram feitas seguindo a proporção de 5:1 (inserto: vetor) e incubadas a 16ºC por 12h. Em seguida as ligações foram transformadas em células *E. coli* DH5α eletrocompetentes para seleção dos clones positivos e plaqueadas em meio L acrescido de 100µg/mL de ampicilina.
- Todos os três cassetes para silenciamento (**cassete 1**: *Pcpc1/ras1/ura3*/loop+íntron/T*trpC*; **cassete 2:** *Pcpc1*/*ras1/ura3*/loop/ T*trpC* e **cassete 3:** *Pcpc1*/*ras1/ura3*/íntron/T*trpC*) foram amplificados com os oligonucleotídeos BclI-5'P*cpc1* (5' CAACTCGAG**<u>TGATCA</u>**GCTTTGAAGTTGCTGCAAGCTGGC 3', Tm = 74ºC) e BclI-3'T*trpC* (5' CTCTGGGCCC**<u>TGATCA</u>**GTCTAGAAAGAAGGATTACCTCTAAAC3' Tm=74ºC) utilizando Vent Polymerase (Biolabs). Os moldes para os PCRs foram os plasmídios contendo os cassetes clonados diluídos 1:20. O programa consistiu de uma etapa de denaturação a 94ºC/2min, 35 ciclos a 94ºC/1mim, 68ºC/1mim, 72ºC/3,5min e uma extensão final a 72ºC/5min.

Tabela 5: Características dos oligonucleotídeos utilizados na amplificação dos fragmentos para construção do cassete para silenciamento de *ras1* e *ura3* de *P. brasiliensis*

Oligonucleotídeo	Seqüência (5'→3')	T_m (°C)	Fragmento Amplificado	Tamanho (pb)	Molde
*Xba*I - 5' P*cpc1*	G**TCTAGA**GCTTTGAAGTTGCTGCAAG	58	Promotor *cpc-1*	1300	pAD1625
*Spe*I - 3' P*cpc1*	C**ACTAGT**TGTTGCCCTGCTTTCC	58			
*Cla*I - *Nhe*I - 5' T*trpC*	C**ATCGAT*GCTAG***CGATTTAATAGCTCCATGTC	54	Terminador *trpC*	565	pAD1625
*Sal*I – 3' T*trpC*	C**GTCGAC**GTCTAGAAGAAGGATTACC	56			
*Eco*RI - 5' *ura3* senso	C**GAATTC**ACAATCTCACATCCCGTAC	56	*ura3* senso+ íntron+loop	421	DNA genômico Pb01
*Nco*I - 3' ura3 senso Loop	G**CCATGG**AAGCGATGTTTGCTTGCGAG	56			
*Eco*RI - 5' *ura3* senso	C**GAATTC**ACAATCTCACATCCCGTAC	56	*ura3* senso +loop	258	cDNA Pb01
*Nco*I - 3' *ura3* senso Loop	G**CCATGG**AAGCGATGTTTGCTTGCGAG	56			
*Eco*RI - 5' *ura3* senso	C**GAATTC**ACAATCTCACATCCCGTAC	56	*ura3* senso + íntron	316	DNA genômico Pb01
*Nco*I - 3' *ura3* senso Íntron	C**CCATGG**TCCTAGGCCTGTTTGGAC	56			
*Hind*III- 5' *ura3* antisenso	C**AAGCTT**CACAATCTCACATCCCGTAC	56	*ura3* antisenso	153	DNA genômico Pb01
*Nco*I - 3' *ura3* antisenso	C**CCATGG**ATCGGCCAGGTCTAGCAGC	62			
*Hind*III – 5' *ras1* senso	C**AAGCTT**CCAATTCAATCATCATGGCATC	62	*ras1* senso	438	cDNA Pb01
*Cla*I – 3' *ras1* senso	C**ATCGAT**GCCAATGCTTCACCCTCTTG	62			
*Eco*RI – 5' *ras1* antisenso	C**GAATTC**CCAATTCAATCATCATGGCATC	62	*ras1* antisenso	438	cDNA Pb01
3' *ras1* antisenso	GATAAGGAGAATGAGCTTGCCCTG	68			

5.28) Clonagem de *ras1* senso e *ras1* antisenso no vetor comercial pSilent-1

O vetor pSilent-1 descrito por Nakayashiki *et al.* (2005), apresenta como sítios únicos de restrição flanqueando o Promotor de *trpC* de *A. nidulans Xho*I, *SnaB*I e *Hind*III e enquanto que flanqueando o Terminador (*trpC*) estão os sítios *Bgl*II, *Sph*I, *Stu*I, *Kpn*I e *Apa*I **(ver Figura 4).** Oligonucleotídeos para *ras1* contendo nas extremidades sítios de restrição para amplificação dos fragmentos senso e antisenso foram desenhados **(Tabela 6).**

Tabela 6: Características dos oligonucleotídeos utilizados na amplificação de *ras1* de *P. brasiliensis* para clonagem em pSilent – 1

Oligonucleotídeo	*Seqüência (5'→3')*	T_m *(ºC)*	*Fragmento Amplificado*
*Bgl*II - 5' *ras1* senso	C**AGATCT**CCAATTCAATCATCATGGCATC	62	*ras1* senso
*Sph*I - 3' *ras1* senso	CGCATGCGAGAGCCAAAGCCACCGC	60	
*Hind*III - 5' *ras1* antisenso	C**AAGCTT**CCAATTCAATCATCATGGCATC	62	*ras1* antisenso
3' *ras1* antisenso (*Xho*I)	GATAAGGAGAATGAGCTTGCCCTG	68	

Os fragmentos senso e antisenso de *ras1* com tamanho de 560pb foram clonados em pGEMT (Promega) para posterior digestão com as enzimas selecionadas (*ras1* senso: *Bgl*II e *Sph*I /; *ras1* antisenso: *Hind*III e *Xho*I). As bandas digeridas foram purificadas e clonadas no vetor pSilent-1 digerido com as mesmas enzimas acima mencionadas.

5.29) Eletrotransformação de leveduras de *P. brasiliensis.*

a) Uma alçada de Pb01 ou Pb113 crescidos por 7 dias em meio semi-sólido Fava-Netto a 37ºC foi inoculada em 50mL de meio BHI líquido. Incubou-se a 37ºC por 5 dias a 150rpm.

b) As células foram centrifugadas a 4000rpm/ 5 min e lavadas três vezes com manitol 10% (p/v) + 1mM hepes.

c) Após a última lavagem as células foram ressuspendidas em 1 x 10^7 leveduras/mL.

d) A cada cubeta de eletroporação adicionou-se 200µL de leveduras (1 x 10^6 células) e 5µg do plasmídio pSilent-1 ou pSilent-1/*ras1*. As células foram mantidas no gelo até o momento do choque.

e) As condições da eletroporação foram: 5kV/cm (1.000V), 600Ω, 25µF, e cubeta de 0,2cm.

f) Após cada choque as células foram recuperadas utilizando manitol 10% (p/v) acrescido de 1mM hepes e plaqueadas imediatamente em membranas de Nylon com suporte de meio BHI + 4% soro de cavalo + 5% fator crescimento, sem marcador seletivo e incubadas a 37ºC por 24h para recuperação das células.

g) As membranas foram lavadas com solução salina estéril (0,9% p/v) e centrifugadas a 4.000rpm/ 5 min. As células precipitadas foram então plaqueadas em meio BHI + 4% soro de cavalo + 5% fator crescimento contendo 100µg/mL de higromicina B. As placas foram vedadas e incubadas a 37ºC/ 30 dias.

6 – Resultados e Discussão

6.1) Caracterização das seqüências de *ras1* e *ras2* de *P. brasiliensis*

- ***ras1* de *P. brasiliensis***

A reação de 5'-RACE para *ras1* de *P. brasiliensis* resultou em um fragmento de aproximadamente 500pb que foi clonado e seqüenciado posteriormente. Já a reação de 3'-RACE gerou os fragmentos de 1.800pb, 700pb, 600pb e 500pb (**Figura 12**), sendo que optou-se pela clonagem do fragmento de 500pb devido a sua maior nitidez no gel e por apresentaram tamanho próximo ao resultado esperado baseando-se em seqüências de *ras1* de outros fungos. Dessa forma, o seqüenciamento dos fragmentos obtidos da reação de 5'-RACE de 500pb e aquele da reação de 3'-RACE também de 500pb, foi possível obter a seqüência completa do gene *ras1* de *P. brasiliensis.*

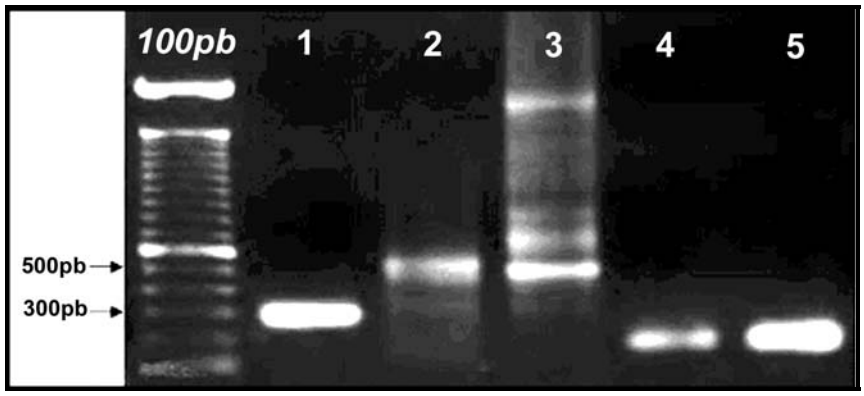

Figura 12: 5'- e 3'-RACE de *ras1* de *P. brasiliensis*. 1) controle (+) do kit TFR, **2)** 5'-RACE *ras1*, **3)** 3'-RACE *ras1*, **4)** controle (+) 5'-RACE *ras1* e **5)** controle (+) 3'- RACE *ras1*.

A seqüência completa de *ras1* de *P. brasiliensis* (isolado Pb01) foi depositada no GenBank (http:// www.ncbi.nih.nlm.org) sob número de acesso **DQ157363**. Basicamente, *ras1* apresenta uma ORF de 636 nucleotídeos que codificam uma proteína Ras1 de 212 aminoácidos. Determinou-se a presença de um único íntron de 99pb; 192pb correspondem a região 5'-UTR e 57pb são referentes à região 3'-UTR. A análise da seqüência deduzida da proteína Ras1 de *P. brasiliensis* revelou a presença de quatro domínios conservados que se ligam ao GTP, um domínio efetor e o domínio contendo o sítio de prenilação "CVIM", em que é adicionado o grupo farnesil (**Figura 13**).

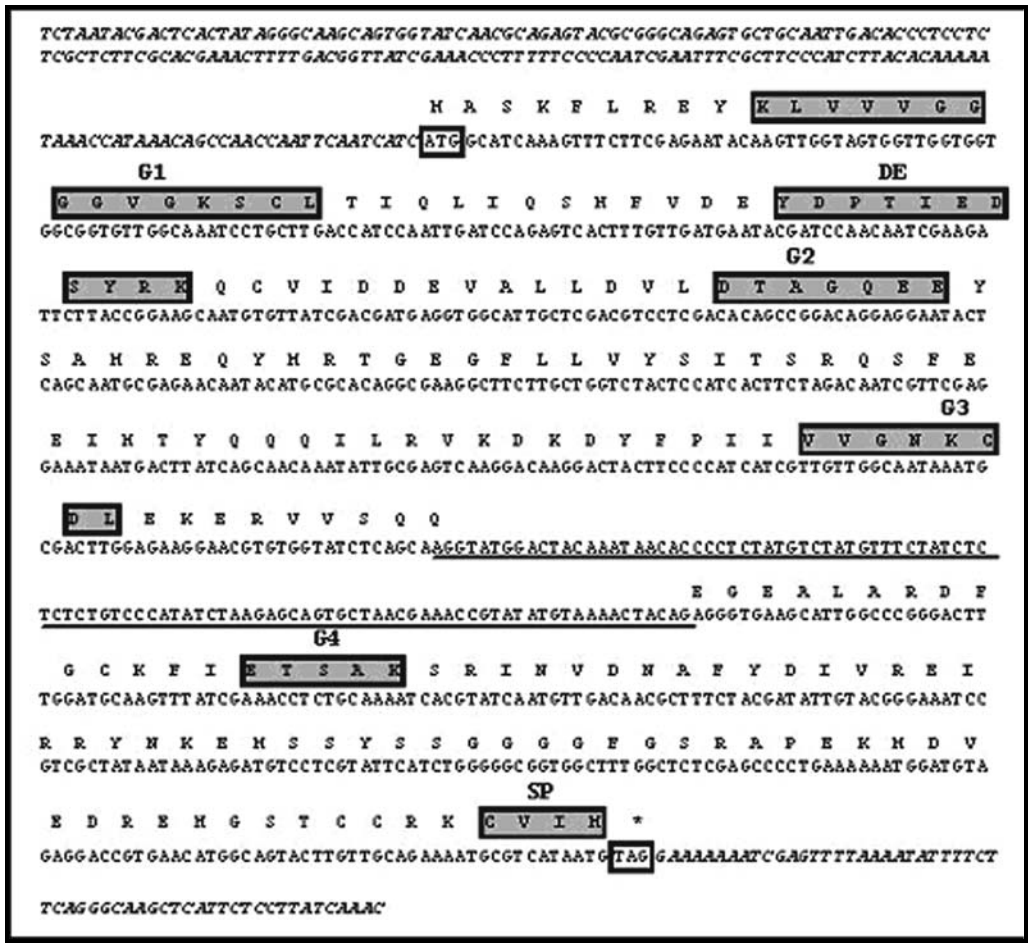

Figura 13: Seqüência completa de *ras1* de *P. brasiliensis* (Pb01), depositada no GenBank sob número de acesso DQ157363. Os nucleotídeos em itálico correspondem as regiões 5'- e 3'-UTR; os nucleotídeos sublinhados representam o íntron, os códons de iniciação e terminação ATG e TAG, respectivamente estão indicados através de caixa branca. A seqüência deduzida da proteína Ras1 está mostrada acima da seqüência de nucleotídeos, sendo que os domínios conservados de Ras1: G1, G2, G3 e G4 correspondentes aos domínios de ligação ao GTP, o domínio efetor (DE) e SP que corresponde ao sítio de prenilação estão indicados através de caixa cinza.

- ***ras2* de *P. brasiliensis***

Baseando-se na seqüência obtida do Transcriptoma do isolado Pb01, foram desenhados oligonucleotídeos para amplificação de *ras2* a partir do DNA genômico. Após o seqüenciamento do clone genômico foi possível determinar a presença de um íntron de 89pb, uma ORF contendo 714pb que codificam uma proteína de 238 aminoácidos. As regiões 5'- e 3'-UTR seqüenciadas apresentam respectivamente 181pb e 76pb. A seqüência completa de *ras2* foi depositada no GenBank sob número de acesso **AY910576**. A análise da seqüência deduzida da

proteína Ras2 de *P. brasiliensis* também revelou a presença dos quatro domínios conservados que se ligam ao GTP, um domínio efetor e o domínio que contém o sítio de prenilação “CLIL” (**Figura 14**).

Recentemente, Chen *et al.* (2007) identificaram e clonaram *ras2* (AY547438) do isolado ATCC90659 de *P. brasiliensis*, no entanto, estes autores não reportaram a existência de um segundo gene *ras* no genoma deste patógeno.

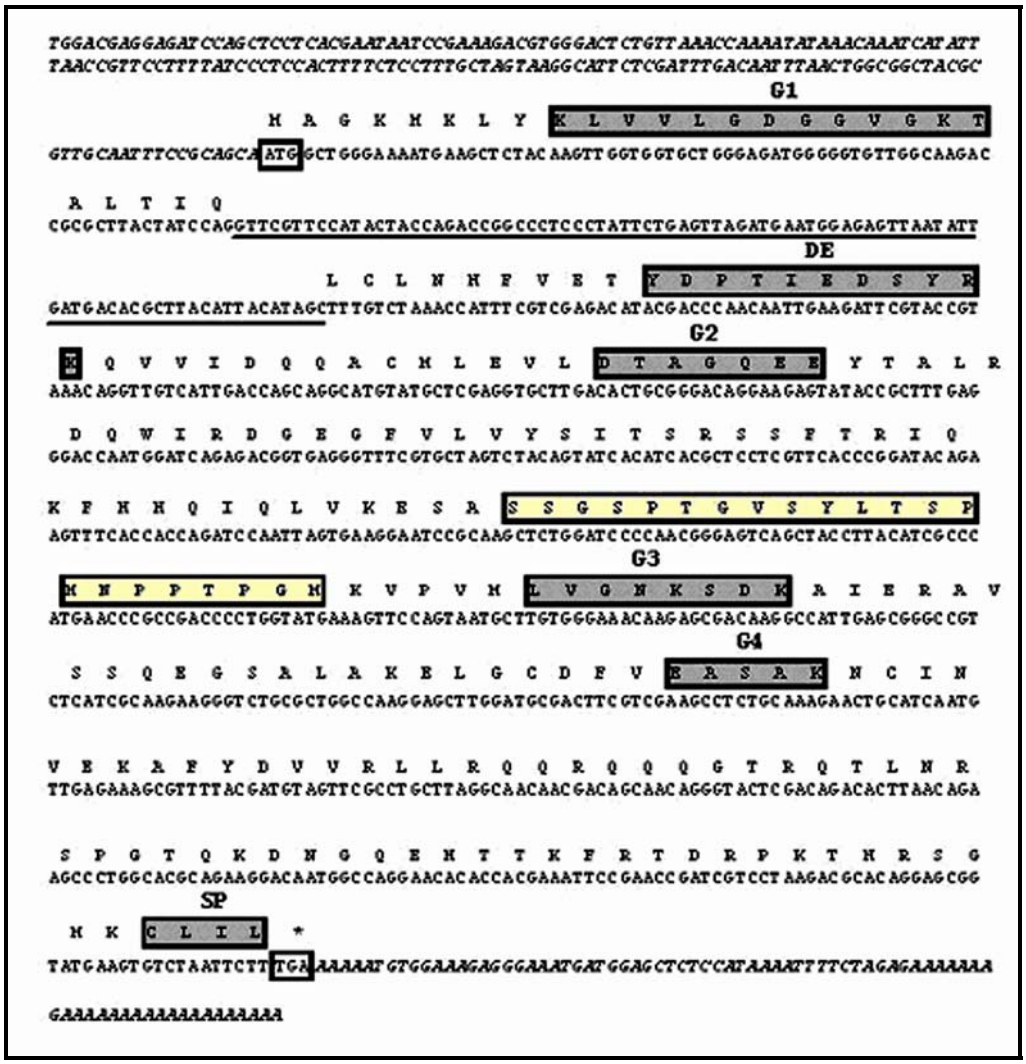

Figura 14: Seqüência completa de *ras2* de *P. brasiliensis* (Pb01), depositada no GenBank sob número de acesso AY910576. Os nucleotídeos em itálico correspondem as regiões 5’ e 3’ UTR; os nucleotídeos sublinhados representam o íntron, os códons de iniciação e terminação ATG e TGA, respectivamente estão indicados através de caixa branca. A seqüência deduzida da proteína Ras2 está mostrada acima da seqüência de nucleotídeos, sendo que os domínios conservados de Ras2: G1, G2, G3 e G4 correspondentes aos domínios de ligação ao GTP, o domínio efetor (DE) e SP que corresponde ao sítio de prenilação estão indicados através de caixa cinza. Os 24 aminoácidos indicados pela caixa amarela são aqueles encontrados apenas em Ras2 e ausentes em Ras1.

6.2) Análise *in silico* das seqüências deduzidas das proteínas Ras1 e Ras2 de *P. brasiliensis*

Através de análises *in silico*, as seqüências deduzidas das proteínas Ras1 e Ras2 de *P. brasiliensis* foram comparadas com aquelas de outros organismos, revelando uma alta similaridade que variou de 45,7 a 79,3% para Ras1 e 39 a 82% para Ras2. A identidade entre as proteínas Ras de Pb e de outros organismos também foi alta de 34,9 a 90,1% para Ras1 e 30,5 a 77,5% para Ras2 **(Tabela 7)**. A similaridade e identidade entre as proteínas Ras1 e Ras2 de *P. brasiliensis* foi de 41,3 e 51,4%, e massa molecular estimada de 24,19KDa e 26,51KDa respectivamente, característica que atribui a denominação de pequenas GTPases. É importante ressaltar a conservação entre todas as seqüências protéicas de Ras analisadas, destacando-se os quatro domínios de ligação ao GTP, o domínio efetor e o sítio de prenilação (**Figura 15**).

Tabela 7: Porcentagens de identidade e similaridade de Ras1 e Ras2 de *P. brasliensis* em relação a proteínas Ras de diversos organismos.

	PbRas1	***PbRas2***
AfRasA	90,1 / 93,4	42,9 / 51,4
AfRasB	42,7 / 51,2	77,5 / 82,0
CaRas1	43,4 / 47,8	34,0 / 42,5
CnRas1	64,0 / 72,0	40,3 / 47,6
CnRas2	34,9 / 45,7	40,2 / 54,0
Human H-Ras	53,1 / 60,1	39,1 / 47,7
PbRas1	-	41,3 / 51,4
PbRas2	41,3 / 51,4	-
NcRas1	70,0 / 79,3	41,4 / 52,0
NcRas2	43,0 / 50,4	65,8 / 72,5
ScRas1	39,8 / 46,0	30,5 / 39,6
ScRas2	39,1 / 45,8	30,8 / 39,0
SpRas	61,3 / 69,8	42,8 / 51,0

Af : A. fumigatus, Ca: C.albicans, Cn: C. neoformans, Pb: P. brasiliensis, Nc: N. crassa, Sc: S. cerevisiae, Sp: S. pombe.

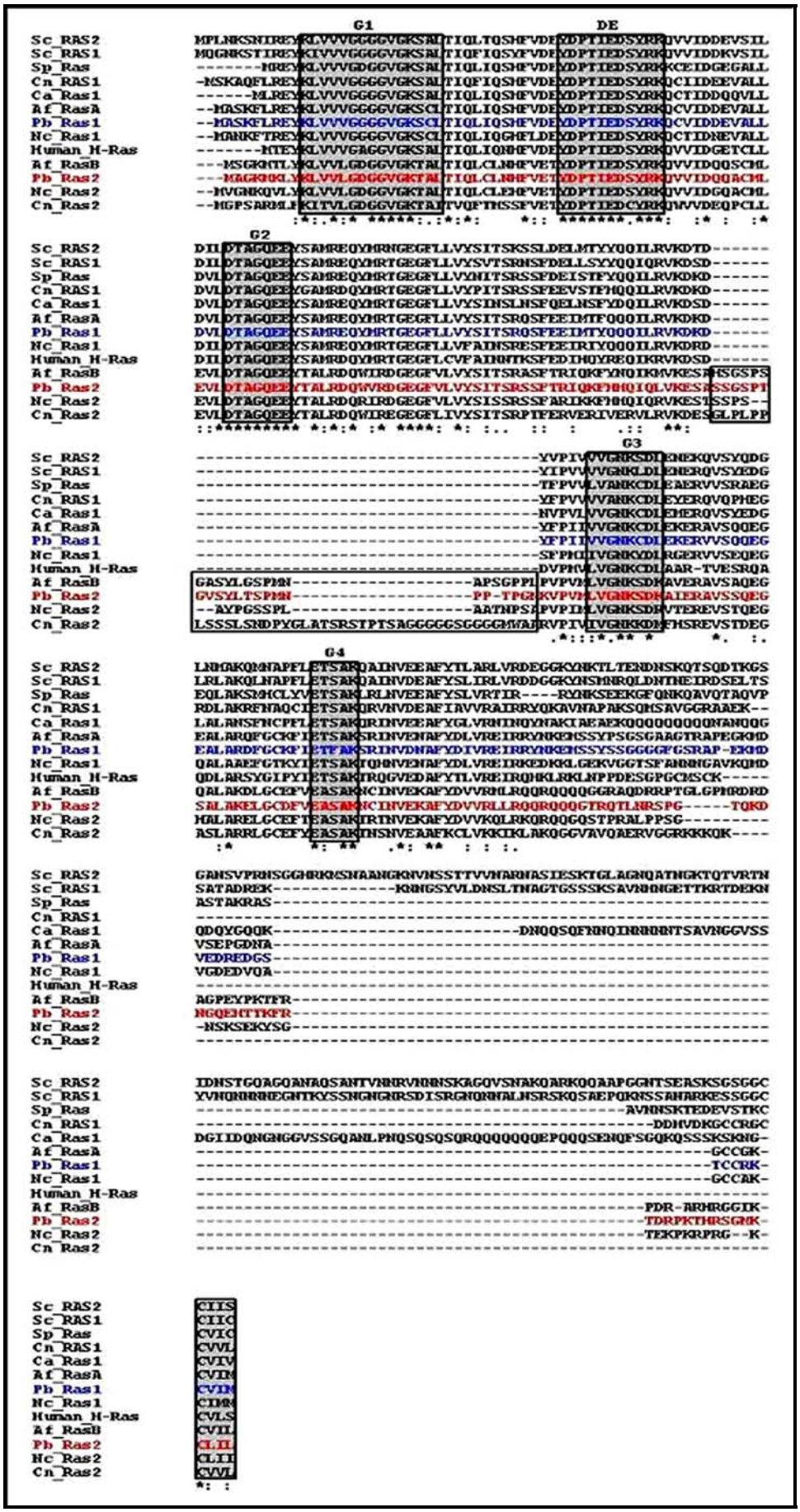
G1
DE
G2
G3
G4
Sc_RAS2
Sc_RAS1
Sp_Ras
Cn_RAS1
Ca_Ras1
Af_RasA
Pb_Ras1
Nc_Ras1
Human_H-Ras
Af_RasB
Pb_Ras2
Nc_Ras2
Cn_Ras2

Figura 15: Alinhamento múltiplo das seqüências protéicas de Ras1 e Ras2 de *P. brasiliensis* com diferentes organismos. Os domínios conservados: G1, G2, G3 e G4 que correspondem aos domínios de ligação a GTP, o domínio efetor (DE) e SP correspondente ao sítio de prenilação estão indicados através de caixa cinza. A região de aproximadamente 20 aminoácidos indicados pela caixa branca é encontrada apenas em Ras2. As seqüências de Ras1 e Ras2 de *P. brasiliensis* estão em azul e vermelho, respectivamente. "*****" indica que o mesmo aminoácido está presente em todas as seqüências,"**:**" substituições conservadas, "**.**"substituições semi-conservadas. Af: *A. fumigatus*, Ca: *C. albicans*, Cn: *C. neoformans*, Nc: *N. crassa*, Pb: *P. brasiliensis*, Sc: *S. cerevisiae* e Sp: *S. pombe.*

A comparação entre as proteínas Ras1 e Ras2 de diferentes organismos demonstra que são seqüências diferentes, que conservam os domínios de ligação ao GTP e o domínio efetor, no entanto, o sítio de prenilação é variável. É interessante notar que as proteínas Ras2 de *P. brasiliensis, C. neoformans* e *A. fumigatus* apresentam na região carboxi-terminal no motivo "CAAX", o resíduo "X" como sendo uma leucina, aminoácido favorável à adição de grupo geranil e não farnesil como é esperado para as proteínas da família Ras. Outra característica importante é que Ras2 apresenta uma região de aproximadamente 20 aminoácidos localizada entre G2 e G3 (domínios de ligação ao GTP) que não está presente nas seqüências de Ras1 (**Figura 15**). A seqüência de nucleotídeos que codifica esta região foi deletada no fungo filamentoso *A. fumigatus* e os mutantes apresentaram defeitos na morfologia celular idênticos aos defeitos observados quando o gene inteiro que codifica para RasB (homólogo de Ras2 em *A. fumigatus*) é deletado do mesmo organismo, indicando assim que essa região é essencial para a função de RasB em *A.fumigatus* (Fortwendel *et al*., 2005). Fortwendel *et al*. (2005) sugerem ainda que a perda da função de RasB após deleção desta região que codifica aproximadamente 20 aminoácidos pode ser decorrente de alteração da estrutura da proteína Ras2.

Através da comparação das seqüências deduzidas das proteínas Ras foi possível gerar uma árvore filogenética baseada na homologia entre as seqüências. É possível observar a formação de dois grupos distintos para Ras1 e Ras2. Ras1 de *P. brasiliensis* agrupou-se na mesma chave de RasA de *A. fumigatus* enquanto que Ras2 de *P. brasiliensis* é mais próximo de RasB também de *A. fumigatus*, o que sugere uma grande proximidade entre estes fungos. As proteínas Ras1 e Ras2 formaram grandes chaves distintas, exceto H-Ras que

formou uma chave independente e Ras2 de *S. cerevisiae* que está no grupo das seqüências de Ras1 **(Figura 16).**

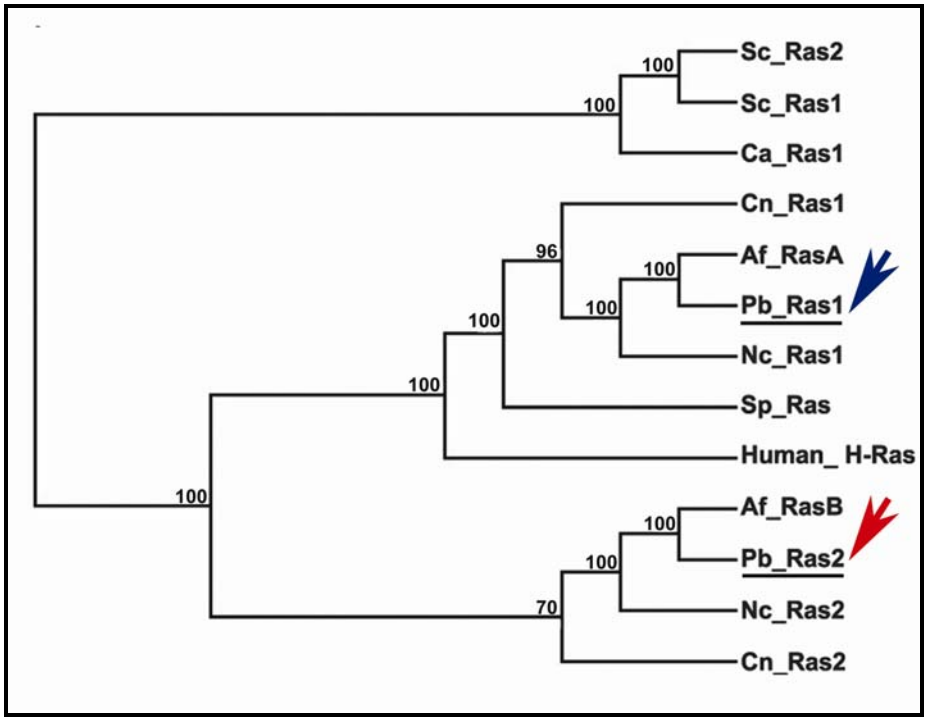

Figura 16: Análise filogenética baseado no algoritmo de agrupamento para avaliar a relação das proteínas deduzidas Ras de *P. brasiliensis* com aquelas de outros fungos. Os valores de "bootstrap" estão indicados em cada ramo da árvore filogenética. Pb_Ras1 está indicado pela seta azul e Pb_Ras2 pela seta vermelha. Af_RasA: *A. fumigatus* RasA, Af_RasB: *A. fumigatus* RasB, Ca_Ras1: *C. albicans* Ras1, Cn_Ras1: *C. neoformans* Ras1, Cn_Ras2: *C. neoformans* Ras, Human H-Ras, Nc_Ras1: *N. crassa* Ras1, Nc_Ras2: *N. crassa* Ras2, Pb_Ras1: *P. brasiliensis* Ras1 , Pb_Ras2: *P. brasiliensis* Ras2 , Sc_Ras1: *S. cerevisiae* Ras1 , Sc_Ras2: *S. cerevisiae* Ras2 , Sp_Ras: *S. pombe* Ras.

6.3) Análise do número de cópias dos genes *ras1* e *ras2* de *P. brasiliensis*

Experimentos de Southern-blot foram realizados com o objetivo de confirmar a presença e determinar o número de cópias dos dois genes *ras1* e *ras2* no genoma de *P. brasiliensis*. Com base no perfil de restrição das seqüências de nucleotídeos de *ras1* e *ras2*, foram escolhidas as enzimas de restrição: *Eco*RI, *Hind*III, *Pst*I que não possuem nenhum sítio de restrição nas respectivas seqüências, e *BamH*I que apresenta um sítio em *ras2* e nenhum sítio em *ras1*. É importante mencionar que a sonda utilizada para *ras2* é constituída por 75 nucleotídeos que codificam para a região de 22 aminoácidos que está

presente apenas em *ras2*. Para a sonda de *ras1* foram utilizados 300 nucleotídeos correspondentes a região 3' que é menos conservada entre as seqüências de *ras* (**Figuras 17 e 18**).

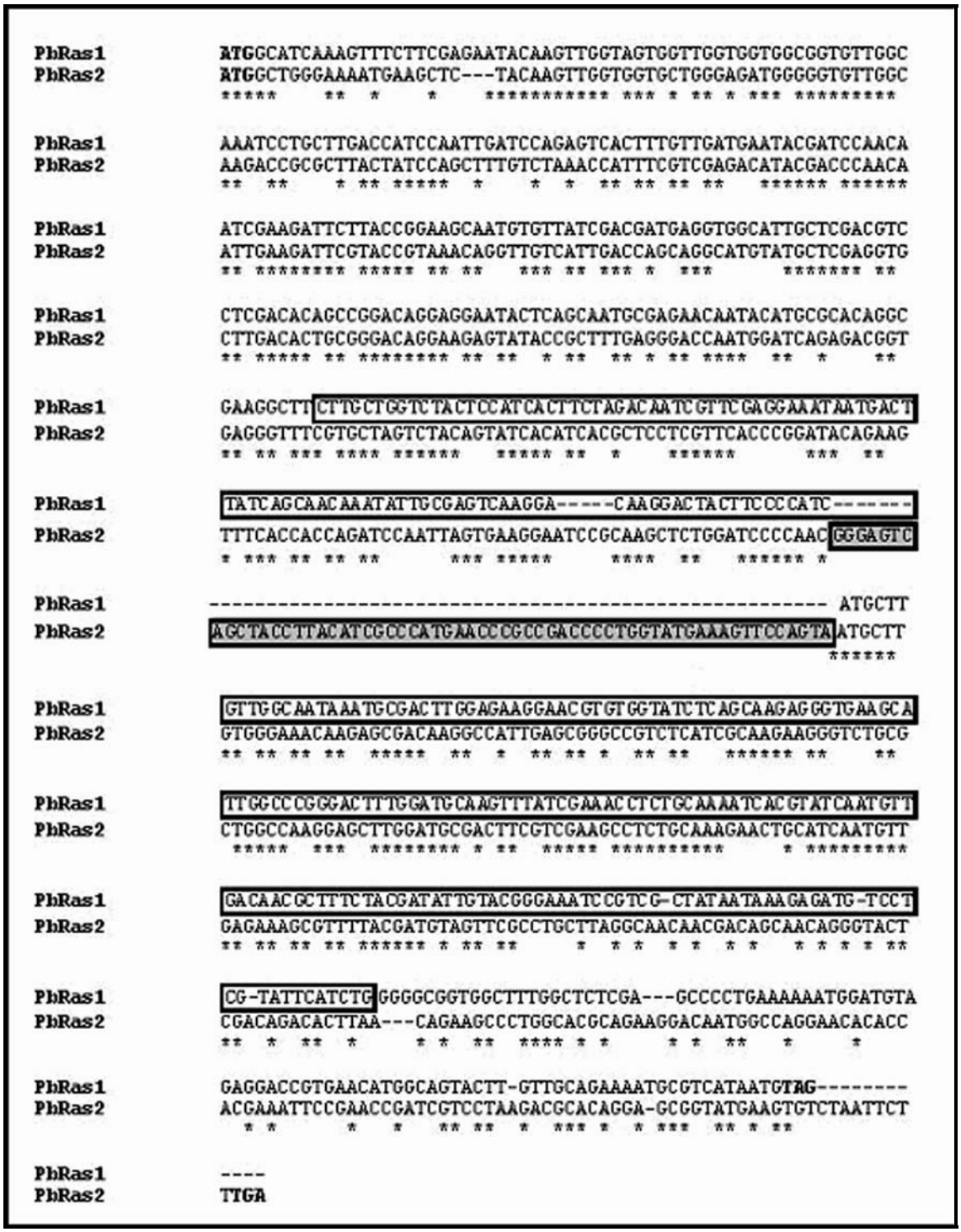

Figura 17: Alinhamento das seqüências de nucleotídeos das ORFs dos genes *ras1* e *ras2* de *P. brasiliensis*. "*" indicam os nucleotídeos idênticos entre as duas seqüências. As regiões destacadas em caixas branca e cinza correspondem às sondas utilizadas para Southern Blot para *ras1* e *ras2*, respectivamente.

Os resultados das hibridizações demonstraram a presença de apenas uma banda em todas as digestões analisadas quando utilizadas ambas as sondas para *ras1* e *ras2*, **(Figura 19).** Os resultados demonstram pela primeira vez a existência de dois genes*, ras1* e *ras2,* no genoma de *P. brasiliensis* contendo apenas uma única cópia de cada, como observado para maioria dos outros fungos estudados, exceto para *C. albicans* e *S. pombe*, que apresentam apenas um único gene *RAS* identificado em seus genomas (Leberer *et al*., 2001; Fukui & Kaziro, 1985).

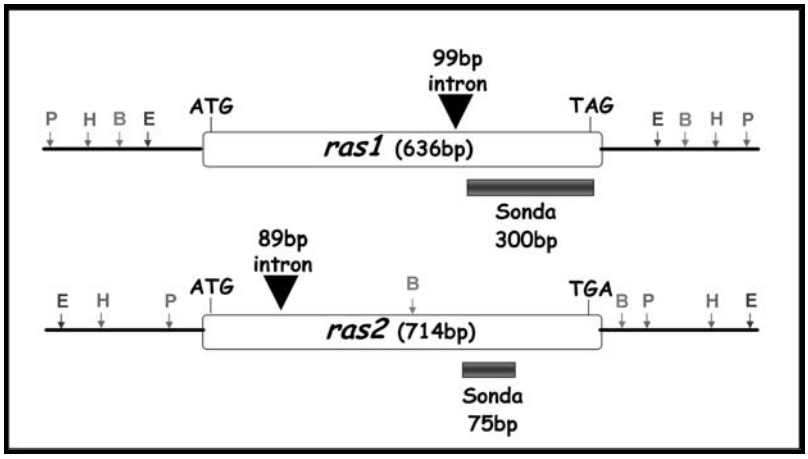

Figura 18: Representação esquemática dos genes *ras1* e *ras2* de *P. brasiliensis*. Em cinza destacam-se as sondas utilizadas para o experimento de Southern blot. Os códons de iniciação (ATG), terminação (TGA e TAG) e os íntrons de cada gene estão indicados na figura. B: *Bam*HI, E: *Eco*RI, H: *Hind*III, P: *Pst*I

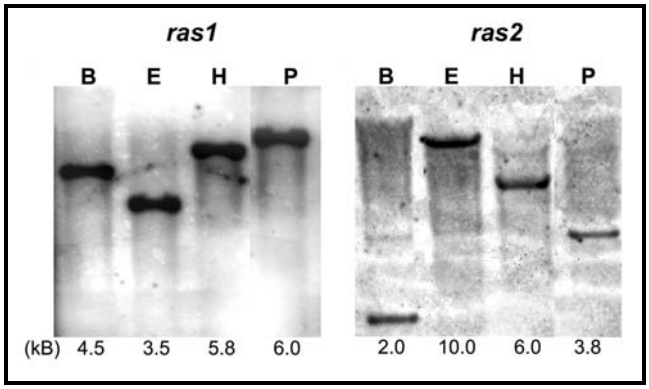

Figura 19: Análise do número de cópias de *ras1* e *ras2* por Southern blot. 10 µg de DNA total digerido com as enzimas de *Bam*HI (B), *Eco*RI (E), *Hind*III (H) e *Pst*I (P) foram separados por eletroforese em gel de agarose 0,8%, transferidos para membrana de nylon e hibridizados contra as sondas de *ras1* e *ras2* marcadas quimicamente com fosfatase alcalina (Gene Images Alkphos direct labelling and detection system, Amersham Biosciences). Os tamanhos aproximados em kilobases (Kb) das bandas obtidas estão indicados abaixo de cada painel.

6.4) Análise do perfil transcricional de *ras1* e *ras2* de *P. brasiliensis* em diferentes condições

Com o objetivo de investigar o padrão transcricional dos genes *ras*, primeiramente foi realizado um experimento de northern-blot utilizando RNAs das formas de micélio e levedura de *P. brasiliensis,* no entanto não foi possível detectar nenhum transcrito de ambos os genes (dados não apresentados). Essa impossibilidade de detecção de *ras* por esta técnica foi relatada previamente em *C. neoformans* (Waugh *et al*., 2002), *A. fumigatus* (Fortwendel *et al.,* 2004) e *P. marnefffei* (Boyce *et al.,* 2005). Devido a esta limitação, decidiu-se utilizar a técnica de RT-PCR semi-quantitativa (RT-PCRsq). Dessa forma, avaliou-se o padrão transcricional de *ras1* e *ras2* nas seguintes condições: transição dimórfica dependente de temperatura de micélio para levedura, choque térmico a 42ºC e durante a internalização de leveduras em macrófagos murinos, mimetizando a infecção do hospedeiro. Como *ras1* e *ras2* são genes distintos e pouco se conhece a respeito de suas funções em *P. brasilienses*, a análise de suas expressões em cada uma destas situações permite uma avaliação geral dos possíveis eventos celulares em que estas proteínas estariam envolvidas.

a) Expressão *in vitro* de *ras1* e *ras2* de *P. brasiliensis* durante o dimorfismo de micélio para levedura e em resposta ao choque térmico a 42ºC.

Como Ras são proteínas chaves que controlam vias de transdução de sinais muito conservadas em outros fungos que estão envolvidas em processos de diferenciação como formação de pseudo-hifas em *C. albicans* (Feng *et al*., 1999; Leberer *et al*., 2001) e frutificação haplóide em *C. neoformans* (Alspaugh *et al*., 2000; Waugh *et al*., 2002), decidiu-se avaliar se os genes *ras* de *P. brasiliensis* sofrem algum tipo de regulação transcricional durante as primeiras 24h da transição dimórfica de micélio para levedura de *P. brasiliensis*. Foi utilizado como controle interno das reações de RT-PCRsq o gene que codifica a cadeia leve da clatrina *(clat),* uma proteína que participa da organização estrutural de vesículas membranares de células eucariontes (Maldonado-Baez & Wendland, 2006), já que suas PbAESTs apresentaram-se igualmente distribuídas entre as formas de micélio e levedura do transcriptoma de Pb01 (Felipe *et al*., 2003 e Felipe *et al*., 2005). Este fato foi confirmado por RT-PCRsq indicando que o gene

clat não apresenta modulação de sua expressão durante a transição de micélio para levedura (dados não mostrados), podendo ser usado como um controle interno de expressão constitutiva durante o processo de diferenciação de *P. brasiliensis*.

RNA total de cada ponto da diferenciação de micélio para levedura (0 - micélio, 0,5h, 1h, 2h, 6h, 24h e levedura) foi usado nos experimentos de RT-PCRsq com oligonucleotídeos específicos para *ras1* e *ras2* e o controle interno *clat*. Os produtos amplificados correspondentes aos transcritos de *ras1*, *ras2* e *clat* foram quantificados por análise densitométrica. A **Figura 20A** mostra as quantidades relativas de *ras1* e *ras2* durante a transição dimórfica dependente de temperatura de micélio para levedura de *P. brasiliensis*. Após análise estatística dos resultados, não foram observadas diferenças significativas nos níveis de expressão de *ras1* ou *ras2* nas primeiras 24h de diferenciação de micélio para levedura. Este trabalho mostra que a transcrição dos genes *ras1* e *ras2* de *P. brasiliensis* não é modulada pela mudança de temperatura (22ºC para 37ºC), apresentando-se como genes constitutivos durante a transição dimórfica deste patógeno, evidenciando que, provavelmente, a influência dos genes *ras1* e *ras2* na diferenciação celular deste patógeno pode ser decorrente da modulação no nível de tradução ou pós-traducional. Chen *et al.* (2007) utilizando a técnica de PCR em tempo real e α-tubulina como controle interno, relataram uma leve oscilação da expressão de *ras2* ao longo da diferenciação (0h, 10h, 24h, 48h, 72, 120h e 240h) de micélio para levedura do isolado ATCC90659 de *P. brasiliensis.* A diferença dos resultados deste trabalho e aqueles obtidos por Chen *et al*., (2007) podem ser devido à escolha do controle interno (α-tubulina ou clatrina) ou ainda da variabilidade entre os isolados de *P. brasiliensis*.

O processo de diferenciação celular dependente de temperatura em fungos dimórficos é um evento em que muitos genes devem ser regulados. Nunes *et al.* (2005) identificaram 2583 genes com a expressão modulada durante o processo de diferenciação de *P. brasiliensis* (isolado Pb18), por análise de microarranjos. Dentre os genes positivamente regulados na direção M→Y estão os que codificam para proteína kinase A (PKA) e proteínas G-subunidades α e β. Além disso, Chen *et al*. (2007) também detectaram a modulação dos genes que codificam para as proteínas G (*gpa1*, *gpb1* e *gpg1*) e adenilado ciclase (*cyr1*) durante o processo dimórfico de *P. brasiliensis.* Estas proteínas são componentes

comuns das vias de sinalização controladas por Ras **(ver Figura 2)** que regulam morfogênese e diferenciação celular via AMPc/PKA, integridade celular, crescimento em altas temperaturas e ainda virulência de patógenos (*C. neoformans, C. albicans*).

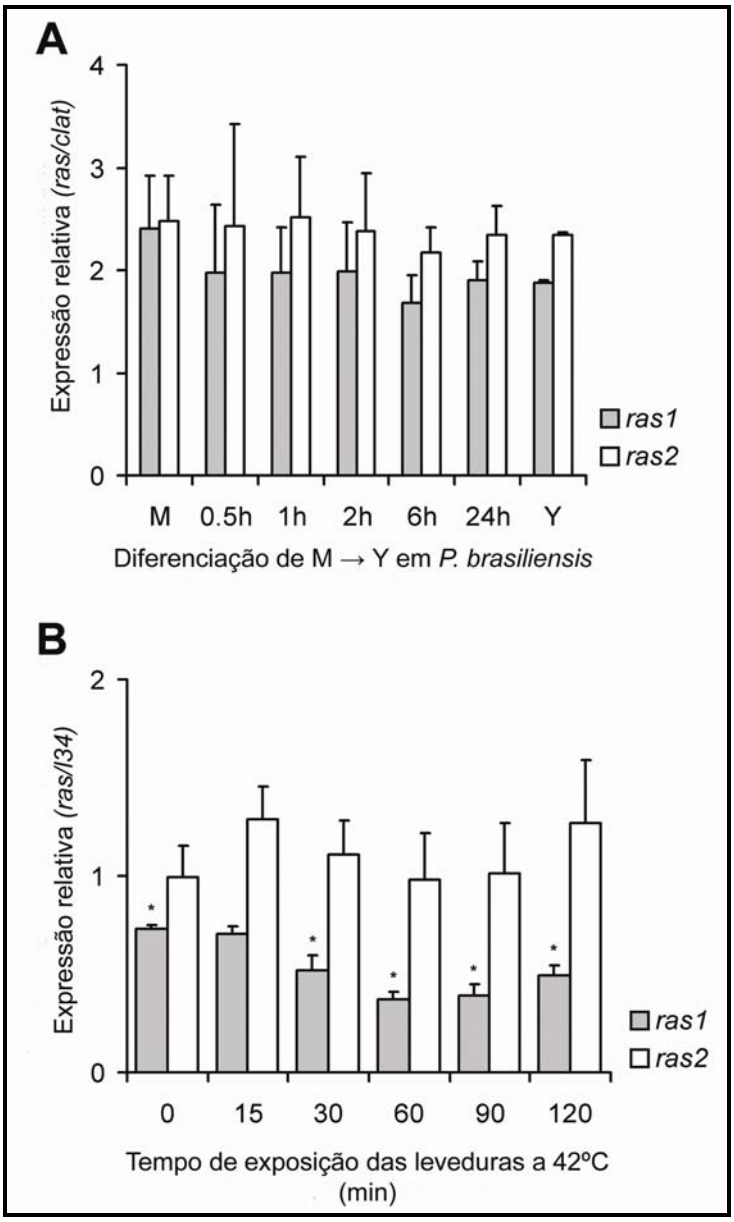

Figura 20: Análise da expressão de *ras1* e *ras2* durante a transição dimórfica M→Y (A) e em resposta a choque térmico a 42ºC. O **painel A** representa a quantificação relativa dos transcritos de *ras1* e *ras2* obtidos dos experimentos de RT-PCRsq realizados em triplicata em vários pontos da transição dimórfica (0,5h; 1h; 2h; 6h e 24h), além das formas vegetativas de micélio (M) a 22ºC e levedura (Y) a 37ºC. O **painel B** representa as quantificações dos transcritos de RNA mensageiro dos genes *ras* de leveduras de *P. brasiliensis* submetidas a choque térmico por 0; 15, 30, 60, 90 ou 120 minutos de exposição a 42ºC. As análises semi-quantitativas foram realizadas por densitometria. Os níveis de *ras1* e *ras2* foram calculados em relação ao controle interno *l34*. As colunas cinza correspondem aos transcritos de *ras1* e as colunas brancas aos de *ras2*. Os experimentos de RT-PCRsq foram realizados em triplicata, sendo que as barras representam o erro padrão. Os asteriscos indicam diferença significativa, com $P < 0,05$.

O envolvimento das proteínas Ras no controle de vias de sinalização em resposta a choque térmico foi previamente relatado por Engelberg *et al.* (1994). Esses autores investigaram a interação da via Ras/AMPc/PKA com as HSPs (Proteínas de choque térmico) em mamíferos e na levedura *S. cerevisiae*. Mutantes de *S. cerevisiae* incapazes de produzir AMPc apresentavam células resistentes a choque térmico. No entanto, mutantes em que Ras apresentava-se dominantemente ativo, o fenótipo observado é exatamente o oposto, as células apresentavam-se muito sensíveis ao choque térmico. Estes estudos demonstraram que independente de HSF (fator de transcrição que responde a choque térmico) havia indução das proteínas de choque térmico (HSPs) quando a via Ras/AMPc/PKA estava bloqueada nas células leveduriformes expostas a condições de choque térmico, resultando assim em um fenótipo termo-tolerante.

Para analisar se os genes *ras* de *P. brasiliensis* sofrem alguma modulação transcricional em resposta ao choque térmico a 42ºC, células leveduriformes foram submetidas à mudança de temperatura de 37ºC para 42ºC por 0, 15, 30, 60, e 120 minutos. Os resultados demonstram que a transcrição de *ras1* é modulada pelo choque térmico a 42ºC. Após o tempo de 30 minutos até 120 minutos de exposição, a expressão de *ras1* diminuiu significativamente chegando ao menor nível no tempo de 60 minutos comparando-se com o controle (T=0 min) **(Figura 20B).** Contrariamente, *ras2* de *P. brasiliensis* não é induzido ou reprimido em resposta ao choque térmico. Como as proteínas Ras são moléculas sinalizadoras necessárias para diversas respostas a diferentes estímulos, pode-se sugerir que as leveduras de *P. brasiliensis* apresentem um gene *ras*, no caso *ras2*, com expressão constitutiva para responder imediatamente a um estímulo qualquer, diferente de mudança de temperatura, enquanto que o outro gene, *ras1*, seria o responsável especificamente pela resposta ao choque térmico, neste caso a 42ºC.

b) Expressão *ex vivo* dos genes *ras1* e *ras2* após internalização de leveduras de *P. brasiliensis* em macrófagos murinos.

No momento em que *P. brasiliensis* infecta as células hospedeiras, a primeira linha de defesa contra o patógeno são os macrófagos pulmonares. Dentro dos macrófagos os propágulos fúngicos têm de se adaptar as condições extremas do ambiente do fagossomo para que possam diferenciar-se para a

forma patogênica (leveduriforme), deste fungo. As células leveduriformes por sua vez devem desenvolver um estado de latência para permitir sua sobrevivência até que as condições ambientais favoreçam a replicação e disseminação do patógeno. Esta capacidade de sobrevivência é critica para o estabelecimento e progressão da infecção em hospedeiros susceptíveis. De acordo com Tavares *et al*. (2007) esta sobrevivência do patógeno parece ser conseqüência de uma reprogramação genética do fungo. Tavares *et al.* (2007) demonstraram que leveduras de *P. brasiliensis* respondem ao microambiente do hospedeiro adaptando-se principalmente ao estresse nutricional e oxidativo gerado pelo fagossomo.

Com o objetivo de estudar o perfil transcricional de *ras* na interação patógeno-hospedeiro, utilizou-se RNA obtido de leveduras de *P. brasiliensis* internalizadas por 9h em macrófagos murinos da linhagem J774. A análise por RT-PCRsq revelou que os genes *ras1* e *ras2* são altamente reprimidos, 6 e 3 vezes, respectivamente, quando comparados com células leveduriformes crescidas *in vitro* em meio semi-sólido Fava Netto (controle) **(Figura 21).** Esta é a primeira vez que a modulação da expressão de *ras* de um fungo é detectada no microambiente de macrófagos.

Para a quantificação dos níveis de transcritos *ras1* e *ras2* utilizando RNA de leveduras internalizadas nos macrófagos incubados em RPMI foi utilizado como controle RNA extraído de leveduras crescidas em meio semi-sólido Fava Netto, e a modulação da expressão de *ras* poderia estar ocorrendo em função das diferenças de incubação, tensão de CO_2 e meio de cultura. Em função disto, foi também realizada a comparação da expressão de *ras1* e *ras2* com leveduras cultivadas em meio Fava Netto, como descrito anteriormente e, submetidas ou não às mesmas condições de incubação em RPMI. Pode-se observar na **Figura 22** que não há diferença de expressão de *ras2* entre as duas condições de cultivo (meio Fava Netto e meio Fava Netto + RPMI-1640), confirmando os dados de repressão *ex vivo* de *ras2*. O gene *ras1* apresenta-se até mais expresso nesta última condição (meio Fava Netto + RPMI-1640), o que reforça os dados deste trabalho de repressão de *ras1* e *ras2* na interação patógeno-hospedeiro.

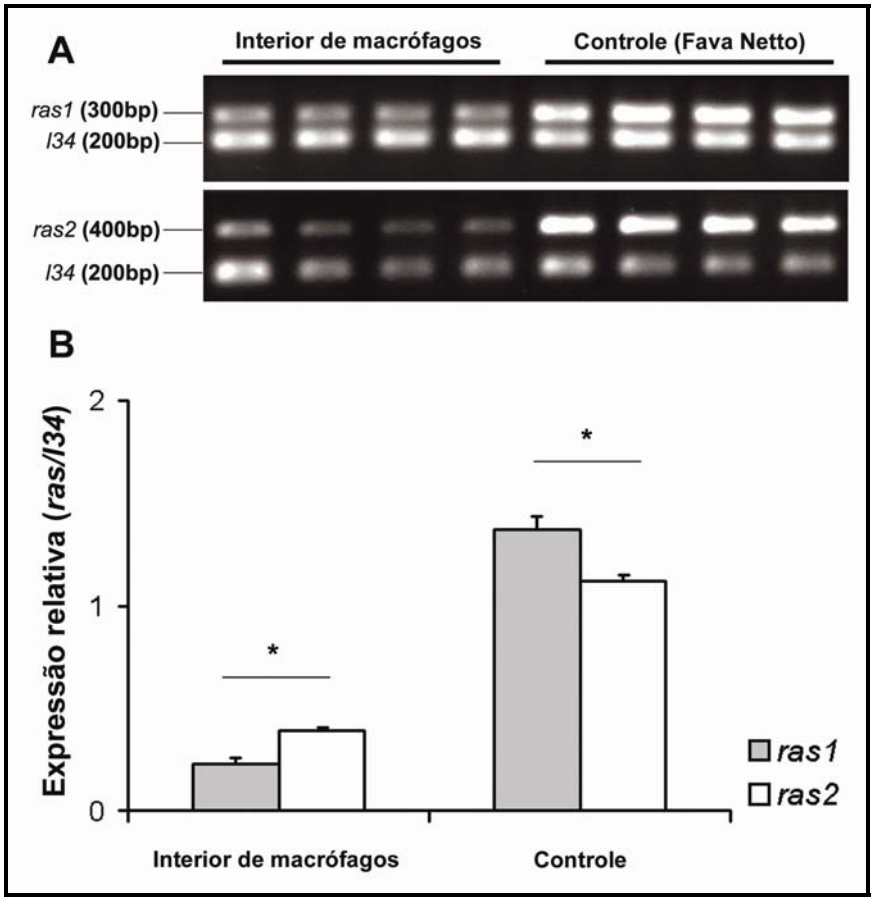

Figura 21: Perfil transcricional dos genes *ras1* e *ras2* após internalização de leveduras por 9h em macrófagos murinos. Células leveduriformes de *P. brasiliensis* foram expostas a macrófagos murinos (J774) por 9h. RNA total das leveduras internalizadas foi extraído e utilizado nos experimentos de RT-PCRsq, como descrito em métodos. O controle utilizado foi RNA total extraído de leveduras crescidas por 7 dias a 37ºC em meio Fava Netto. O **painel A** mostra a análise em gel de agarose 1,5% dos transcritos de *ras1* e *ras2* obtidos por RT-PCRsq em cada uma das condições testadas (no interior de macrófagos e controle). O **painel B** representa a análise semi-quantitativa dos níveis de RNA mensageiro de *ras1* e *ras2* de *P. brasiliensis* em cada uma das condições testadas. Os níveis de *ras1* e *ras2* foram calculados em relação ao controle interno *l34*. As colunas cinza correspondem aos transcritos de *ras1* e as colunas brancas aos de *ras2*. As análises semi-quantitativas foram realizadas exatamente como descritas na seção métodos. Os experimentos de RT-PCRsq foram realizados em quadruplicata, sendo que as barras representam o erro padrão. Os asteriscos indicam diferença significativa, com $P < 0,05$.

Os resultados da análise dos genes *ras* na interação patógeno hospedeiro levam a hipótese de que a modulação da expressão de *ras1* e *ras2* no ambiente do macrófago é uma resposta à limitação nutricional. O nosso grupo já relatou a repressão da via glicolítica (Tavares *et al.,* 2007) acompanhada de indução do ciclo do glioxilato e da via gliconeogênica (Derengowski *et al.*, 2007, em

impressão) em leveduras de *P. brasiliensis* internalizadas em macrófagos. Corroborando tais dados, Breviário *et al.* (1988) demonstraram que os transcritos de *RAS1* e *RAS2* estavam reprimidos quando as células leveduriformes de *S. cerevisiae* foram submetidas à limitação de glicose. Em contraste, Wang *et al.* (2004) ao analisar o padrão transcricional de células de *S. cerevisiae* quando *RAS2* estava super-expresso, relataram uma repressão do processo de gliconeogênese, fenômeno este observado quando a célula encontra-se na presença de glicose.

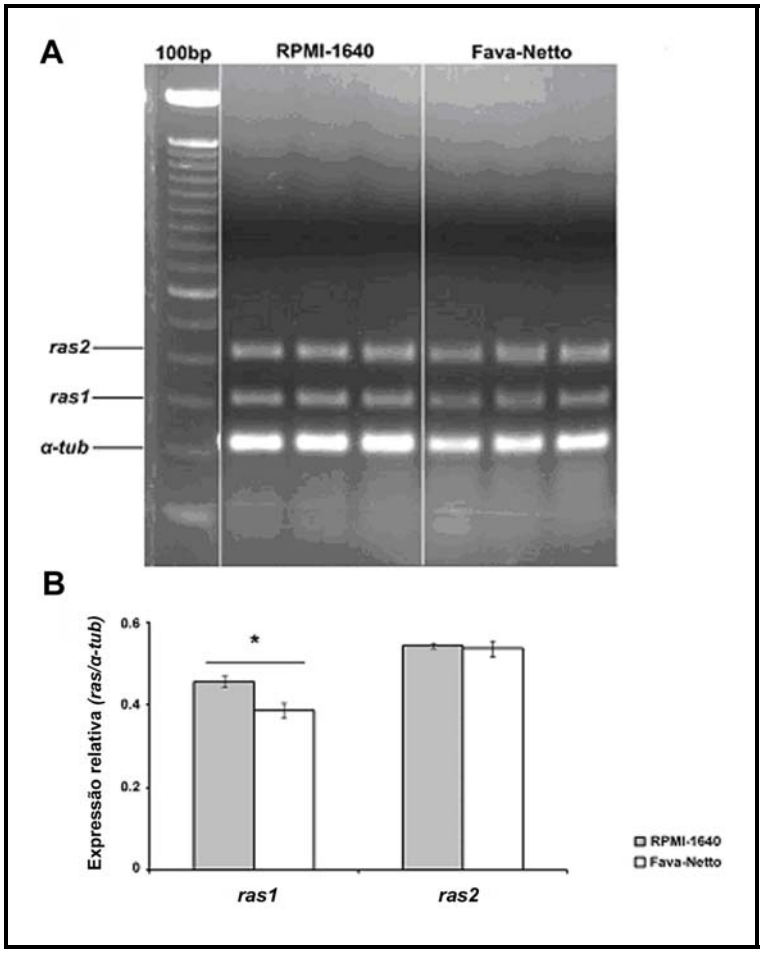

Figura 22: Análise da expressão dos genes *ras* de *P. brasiliensis* em resposta a incubação de leveduras em meio Fava Netto por 7 dias a 37ºC seguida ou não de incubação por 9h em meio RPMI-1640. Painel A apresenta os resultados dos experimentos de RT-PCRsq realizados em triplicata. Os transcritos correspondentes a *ras1* (300pb), *ras2* (400pb) e *α- tub* (200pb) estão mostrados no gel de agarose 1,5% em cada uma das condições testadas (Fava-Netto ou Fava-Netto + RPM-1640). O **painel B** mostra a análise semi-quantitativa da expressão dos genes *ras* realizada por densitometria. Os níveis de *ras* foram calculados em relação ao controle interno *α-tub*, para cada uma das condições testadas. As barras representam os erros padrões dos experimentos em triplicata, sendo os dados significativos, com *P <0,05.

De fato, o fagossomo é um ambiente nutricionalmente pobre, com um pH extremamente baixo e rico em espécies reativas de oxigênio produzidas para limitar a disseminação do patógeno. As células leveduriformes de *P. brasiliensis* respondem a esta agressão modulando o aparato transcricional para sobreviverem na presença das defesas do macrófago. Assim, a latência do patógeno, parece ser a melhor estratégia para evasão do sistema imune do hospedeiro. Baseado neste fato, a regulação negativa dos genes *ras* é completamente plausível já que a situação em que as leveduras se encontram no fagossomo é desfavorável para proliferação ou diferenciação, provavelmente parando o seu ciclo celular na fase Go.

6.5) Análise das PbAESTs geradas pelo transcriptoma de Pb01 que codificam as subunidades regulatória e catalítica de FTase e GGTase I

Com o objetivo de avaliar a presença das preniltransferases em *P. brasiliensis*, decidiu-se utilizar as PbAESTs que representam os genes *ram1* (subunidade β de FTase), *cdc43* (subunidade β de GGTase I) e *ram2* (subunidade α comum a FTase e GGTase I), (**ver Tabela 2**) para uma análise mais detalhada da seqüência de nucleotídeos (total ou parcial), da seqüência deduzida de aminoácidos e realizar uma comparação com as seqüências dessas proteínas de diferentes fungos. Todas as três seqüências analisadas apresentam-se parciais, no entanto, foi possível determinar os domínios e motivos conservados. Nas seqüências deduzidas de Ram1 e Cdc43, que codificam as subunidades β de FTase e GGTase I respectivamente, foi possível determinar os aminoácidos que compõem o sítio catalítico, os aminoácidos que coordenam a ligação ao íon Zn^{2+}, e os aminoácidos que participam da interface com a subunidade α **(Figuras 23 e 24).** Em Ram2, que codifica a subunidade α comum a Ftase e GGTase I, foi possível determinar os dois domínios conservados da família das preniltransferases **(Figura 25).**

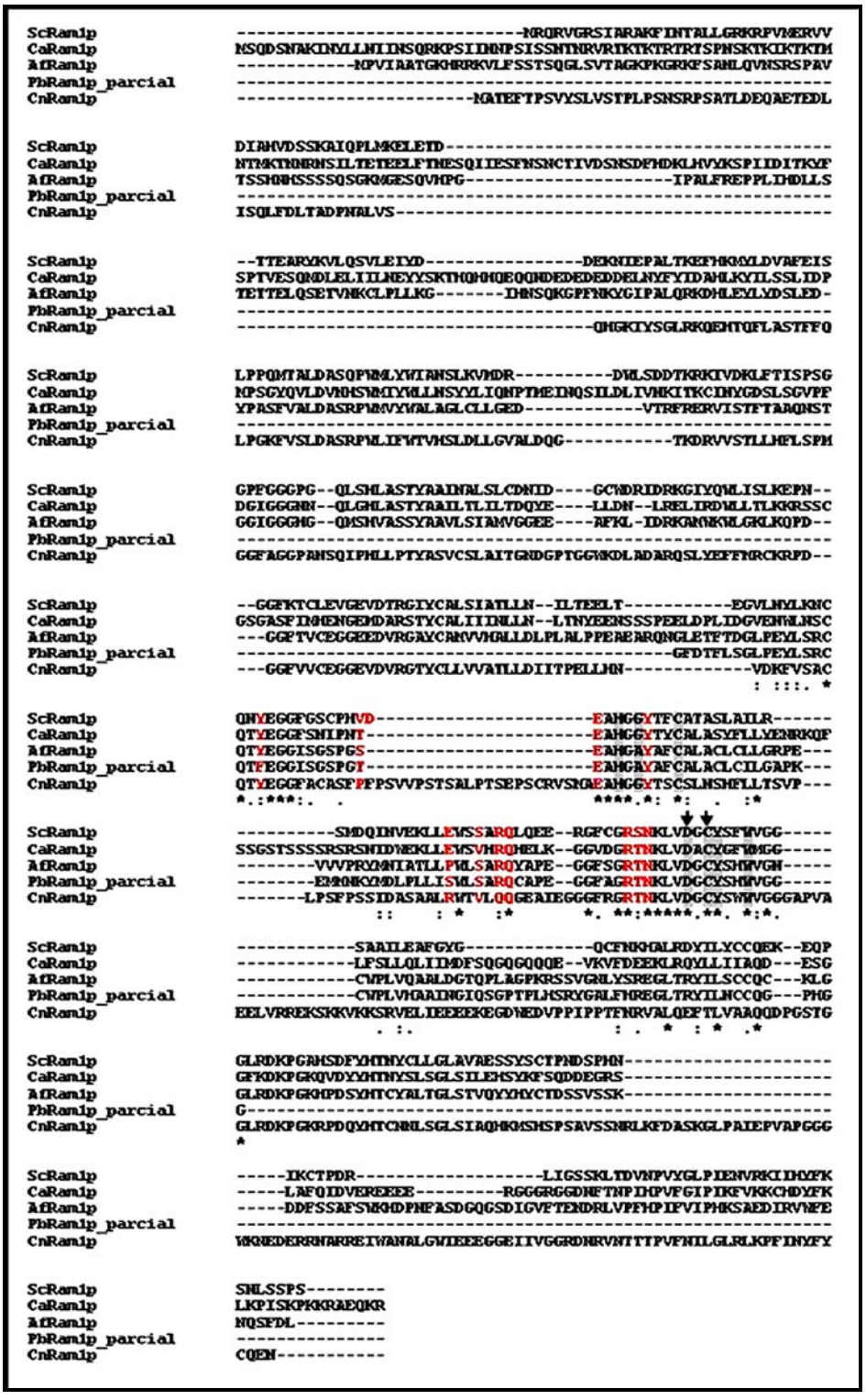

Figura 23: Alinhamento múltiplo das seqüências de Ram1 que codificam as subunidades β de FTase de diferentes organismos. Os aminoácidos destacados com caixas cinza fazem parte do sítio catalítico da enzima, os destacados em vermelho compõem a interface com a subunidade α e as setas indicam os aminoácidos de coordenação ao íon Zn^{2+} ."*" indica que o mesmo aminoácido está presente em todas as seqüências,":" indica substituições conservadas, "." indica substituições semi-conservadas. Af: *A. fumigatus*, Ca: *C. albicans*, Cn: *C. neoformans*, Pb: *P. brasiliensis* e Sc: *S. cerevisiae*.

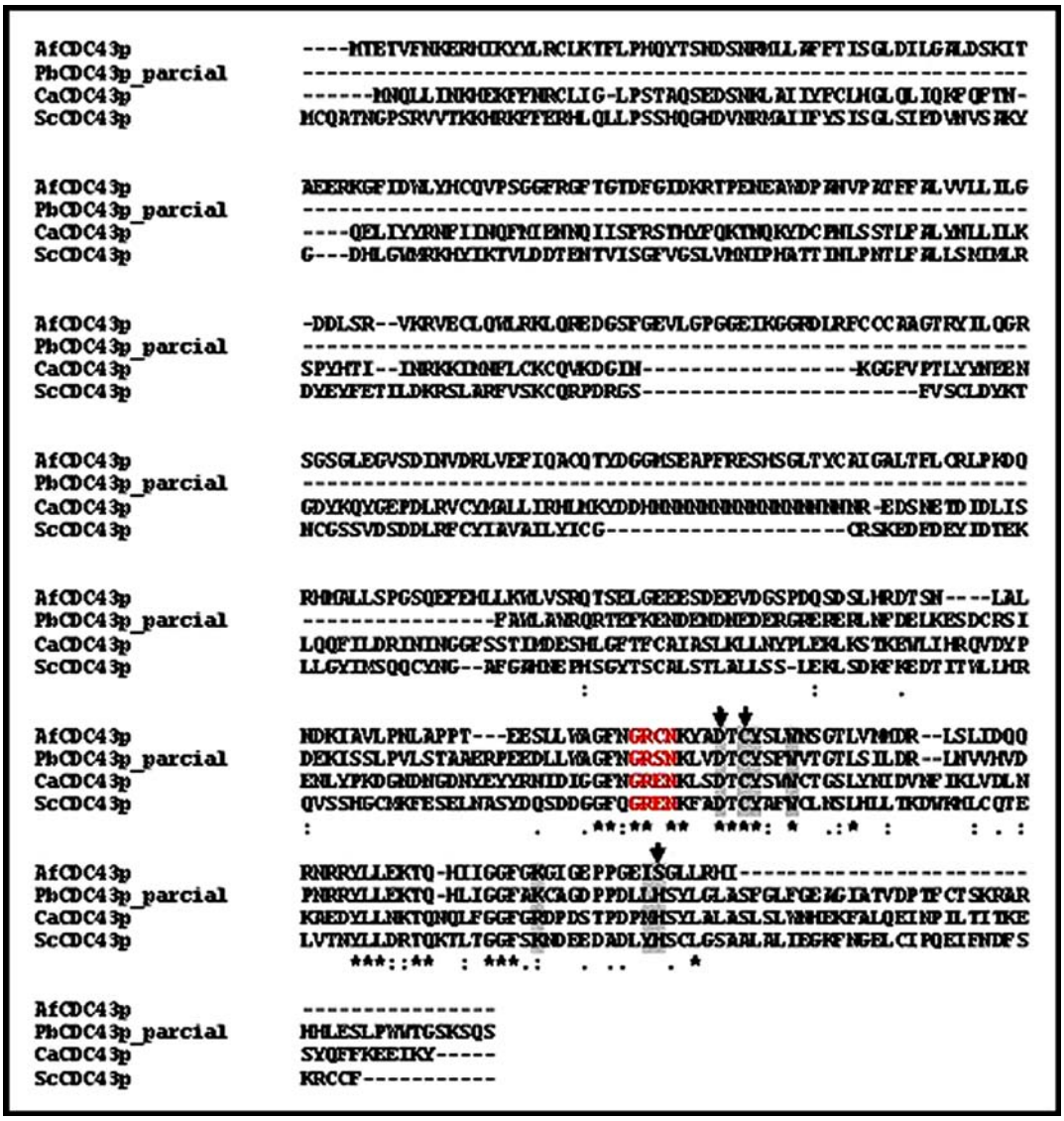

Figura 24: Alinhamento múltiplo das seqüências de Cdc43 que codificam as subunidades β de GGTase I de diferentes organismos. Os aminoácidos destacados com caixas cinza fazem parte do sítio catalítico da enzima, os destacados em vermelho compõem a interface com a subunidade α e as setas indicam os aminoácidos de coordenação ao íon Zn^{2+}.“*” indica que o mesmo aminoácido está presente em todas as seqüências,“**:**”indica substituições conservadas, “**.**” indica substituições semi-conservadas. Af: *A. fumigatus*, Ca: *C. albicans*, Pb: *P. brasiliensis* e Sc: *S. cerevisiae*.

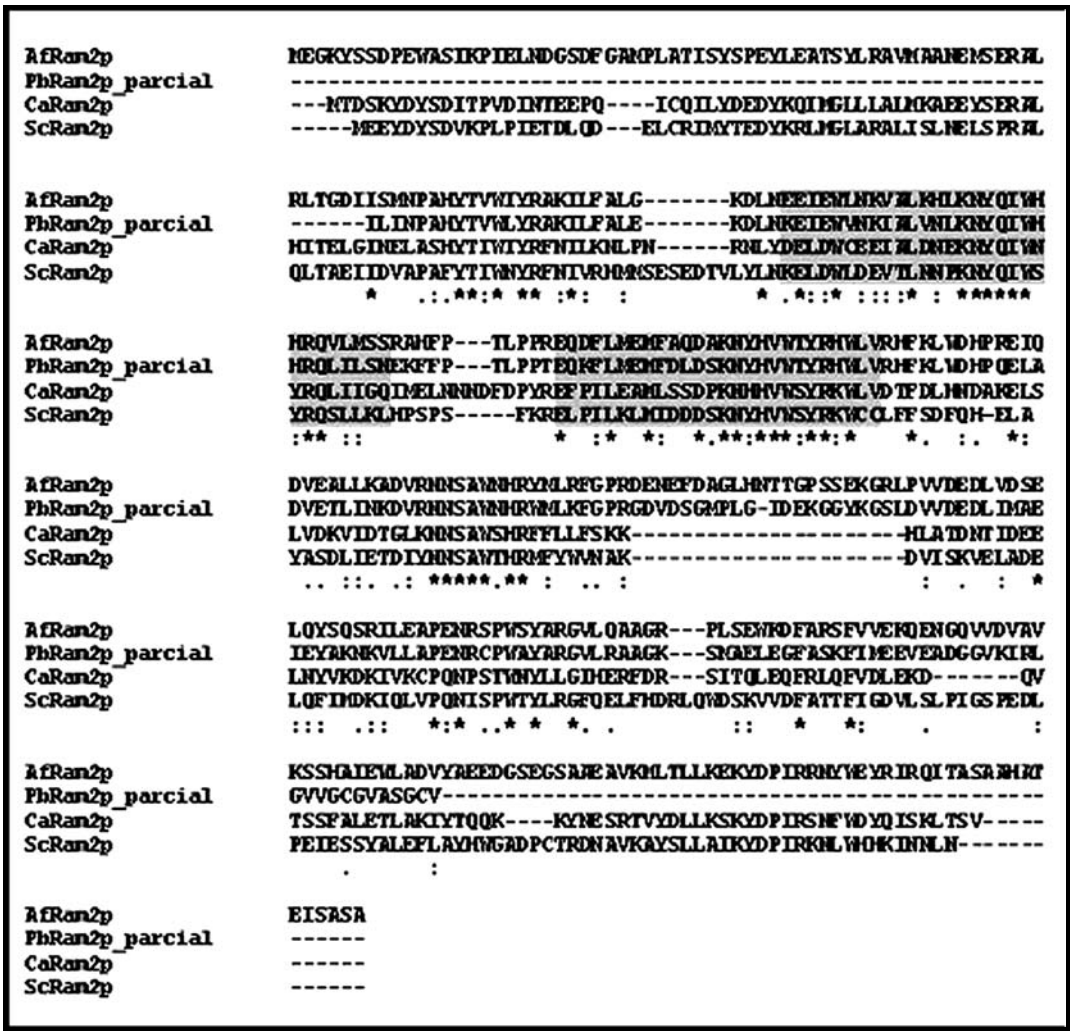

Figura 25: Alinhamento múltiplo das seqüências de Ram2 que codificam as subunidades α comum a FTase e GGTase I de diferentes organismos. Os aminoácidos destacados com caixas cinza compõem os dois domínios conservados da família das preniltransferases.“*****” indica que o mesmo aminoácido está presente em todas as seqüências,“**:**”indica substituições conservadas, “**.**” indica substituições semi-conservadas. Af: *A. fumigatus*, Ca: *C. albicans*, Pb: *P. brasiliensis* e Sc: *S. cerevisiae*.

As seqüências foram agrupadas em um dendograma e observou-se que todas elas Ram1, Ram2 e Cdc43 foram agrupadas na mesma chave das respectivas seqüências de *A. fumigatus*, indicando mais uma vez, uma alta proximidade deste organismo com *P. brasiliensis* **(Figura 26).**

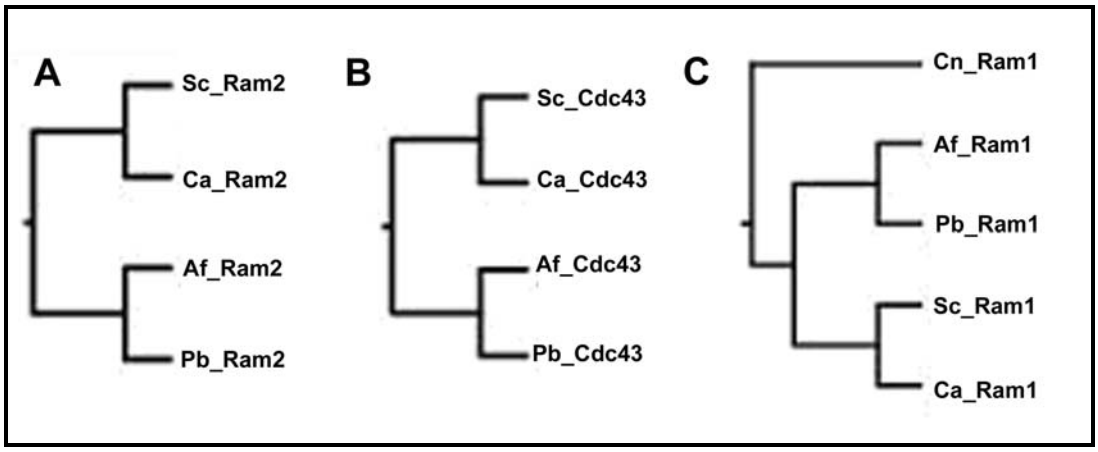

Figura 26: Dendogramas de similaridade das proteínas A) Ram2 (subunidade β de FTase), B) Cdc43 (subunidade β de GGTase I) e C) Ram1 (subunidade α comum a Ftase e GGTaseI) dos organismos: Af: *A. fumigatus*, Ca: *C. albicans*, Cn: *C. neoformans*, Pb: *P. brasiliensis* e Sc: *S. cerevisiae.*

Estes dados demonstram claramente que *P. brasiliensis* apresenta todos os genes necessários para codificar FTase e GGTase I, diferentemente de *C. neoformans* que não apresenta o gene *CDC43* que codifica a subunidade catalítica de GGTase I (Vallim *et al*., 2004). Sendo assim, *P. brasiliensis* em princípio, é capaz de promover as modificações pós-traducionais de farnesilação e geranilação nas proteínas que requerem a adição destes grupamentos hidrofóbicos.

6.6) Avaliação do envolvimento do processo de farnesilação no dimorfismo e crescimento vegetativo de *P. brasiliensis*

A alta freqüência de mutações nos genes *ras* associadas ao desenvolvimento de câncer em humanos e a elucidação do processo de farnesilação da proteína Ras para que seja posicionada corretamente na membrana têm motivado uma crescente busca por inibidores de farnesiltransferase para tratamento de células malignas (Basso *et al.,* 2006).

Para avaliar se o processo de farnesilação estaria envolvido com crescimento vegetativo e diferenciação em *P. brasiliensis,* utilizou-se o inibidor de farnesiltransferase FPT Inhibitor III (Calbiochem) **(Figura 27)**, devido a sua permeabilidade celular e também por ter sido utilizado em experimentos prévios com os fungos patogênicos *C. albicans* (McGeady *et al*., 2002) e *C. neoformans*

(Vallim *et al.,* 2004). Este composto atua inibindo a ação da farnesiltransferase por competir pela ligação de farnesilpirofosfato (FPP) no sítio ativo da enzima. Em *C. albicans* o inibidor de farnesiltransferase não gerou nenhum efeito no crescimento vegetativo, no entanto causou uma diminuição da taxa de conversão de levedura para pseudo-hifas dependente de soro (McGeady *et al*., 2002). Em *C. neoformans*, Vallim *et al.* (2004) também não observaram interferência do inibidor no crescimento vegetativo deste fungo, no entanto, o composto foi capaz de bloquear dois eventos de diferenciação celular neste patógeno, a frutificação haplóide e o acasalamento. Como as proteínas Ras devem ser farnesiladas para que sejam localizadas na membrana, decidiu-se utilizar o composto FPT Inhibitor III em *P. brasiliensis* para investigar o papel do processo de farnesilação, bem como o de Ras e possivelmente de outras proteínas que são farnesiladas neste patógeno.

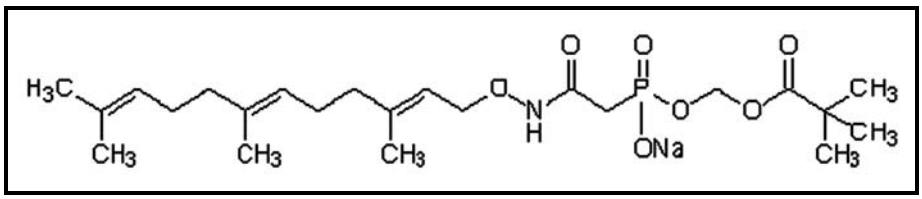

Figura 27: Estrutura química do inibidor de farnesyltransferase FPT Inhibitor III - (E,E)-[2-Oxo-2-[[(3,7,11-trimethyl-2,6,10-dodecatrienyloxyaminoethyl phosphonic Acid, (2,2-Dimethyl-1-oxopropoxymethyl Ester, Na (Calbiochem).

O composto FPT Inhibitor III foi testado no crescimento vegetativo de micélio (22ºC), levedura (37ºC) e durante a transição de micélio para levedura (M→Y) e no sentido inverso do dimorfismo Y→M. A concentração mínima inibitória (CMI) do inibidor na fase leveduriforme do isolado Pb01 de *P. brasiliensis* foi maior que 500µM (dados não mostrados). Este dado corrobora com Vallim *et al*. (2004) e McGeady *et al*. (2002), os quais relataram que apenas concentrações >500uM de FPT Inhibitor III eram capazes de bloquear o crescimento vegetativo de *C. neoformans* e *C. albicans*.

Ao avaliar o crescimento vegetativo da fase miceliana de *P. brasiliensis* a 22ºC, bem como o processo de transição dimórfica dependente de temperatura de micélio para levedura (M→Y) na presença de FPT Inhibitor III não foram detectadas alterações no crescimento vegetativo (**Figura 28A**) nem na transição

M→Y (**Figura 28B**), mesmo na maior concentração testada (500μM), quando comparados com o controle na ausência do inibidor. Em contraste, quando o crescimento vegetativo de leveduras foi avaliado em diferentes concentrações do inibidor de farnesiltransferase, observou-se que as leveduras apresentavam tubos germinativos típicos de micélio, que não foram observados no controle (**Figura 29**). É importante observar que o dimorfismo de *P. brasiliensis* é dependente da mudança na temperatura de 37ºC para 22ºC. O que pode ser observado, na Figura 29, é que com o bloqueio do processo de farnesilação, mesmo a 37ºC, ocorre uma predominância da forma filamentosa deste fungo.

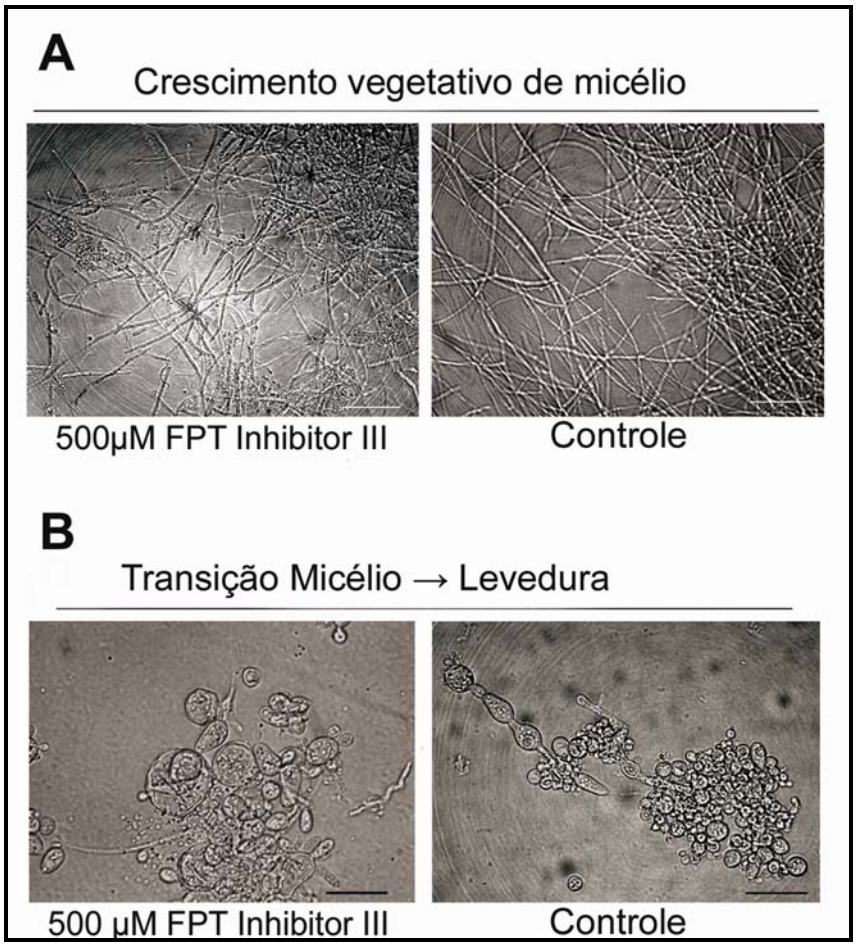

Figura 28: Avaliação do efeito do bloqueio do processo de farnesilação no crescimento vegetativo de micélio (A) e durante a transição dimórfica de micélio para levedura de *P. brasiliensis* (B). No **painel A**, fragmentos de micélio foram incubados na presença de 500μM FPT Inhibitor III em meio de cultura MVM a 22ºC por 10 dias. O controle foi crescido na ausência de inibidor. A barra representa 10μm. O painel B representa a transição dimórfica de micélio para levedura (M→Y), em que fragmentos de micélio foram incubados por 48h a 22ºC e submetidos à mudança para 37ºC por 10 dias para indução da diferenciação para células leveduriformes na presença e na ausência de 500μM FPT Inhibitor III, a barra de aumento representa 10μm.

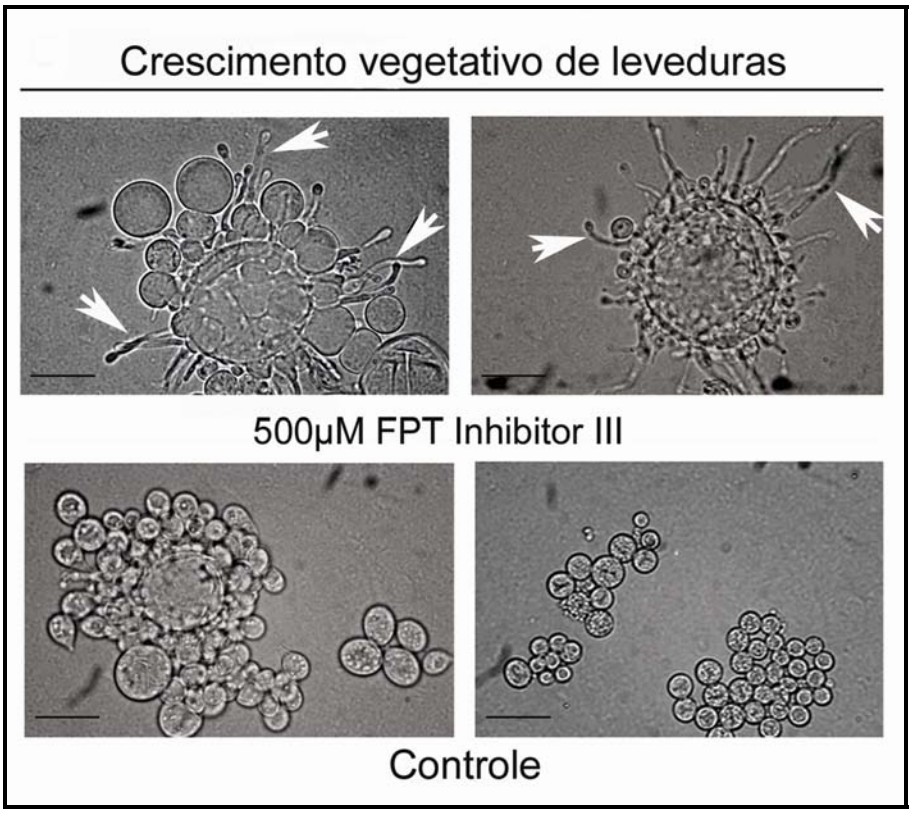

Figura 29: Alteração do crescimento vegetativo de leveduras de *P. brasiliensis* causadas pelo bloqueio do processo de farnesilação. Leveduras de *P. brasiliensis* foram incubadas na presença de 500µM FPT Inhibitor III em meio de cultura MVM a 37ºC por 7 - 10 dias (painel acima). As células leveduriformes que cresceram na ausência do inibidor correspondem ao controle (painel abaixo). As setas brancas indicam a presença de tubos germinativos emergindo das leveduras quando a farnesilação é bloqueada. A barra de aumento representa 10µm.

No experimento de transição de levedura para micélio (Y→M) de *P. brasiliensis*, as células leveduriformes foram incubadas a 22ºC na presença de 0; 125; 250 e 500µM do inibidor. Como pode ser observado na **Figura 30** as leveduras apresentam tubos germinativos característicos da transição Y→M, tanto na presença do inibidor quanto no controle. Entretanto, observa-se uma maior quantidade de leveduras com tubos germinativos quando as leveduras estão em presença do inibidor. Os dados da **Figura 31** mostram a porcentagem de células leveduriformes com tubos germinativos em relação à concentração de FPT Inhibitor III. Os dados indicam uma possível correlação direta entre a produção de tubos germinativos e a concentração do inibidor, já que na maior concentração testada (500µM) observa-se um aumento aproximado de duas vezes quando comparado ao controle. Dessa forma, a inibição do processo de farnesilação parece provocar uma profunda alteração no dimorfismo e morfogênese do

patógeno, ao favorecer a transição de Y→M de maneira dose dependente e, ao promover alteração morfológica do crescimento vegetativo das células leveduriformes, induzindo a formação de tubos germinativos mesmo a 37°C, fazendo com que este processo seja um evento independente da mudança de temperatura.

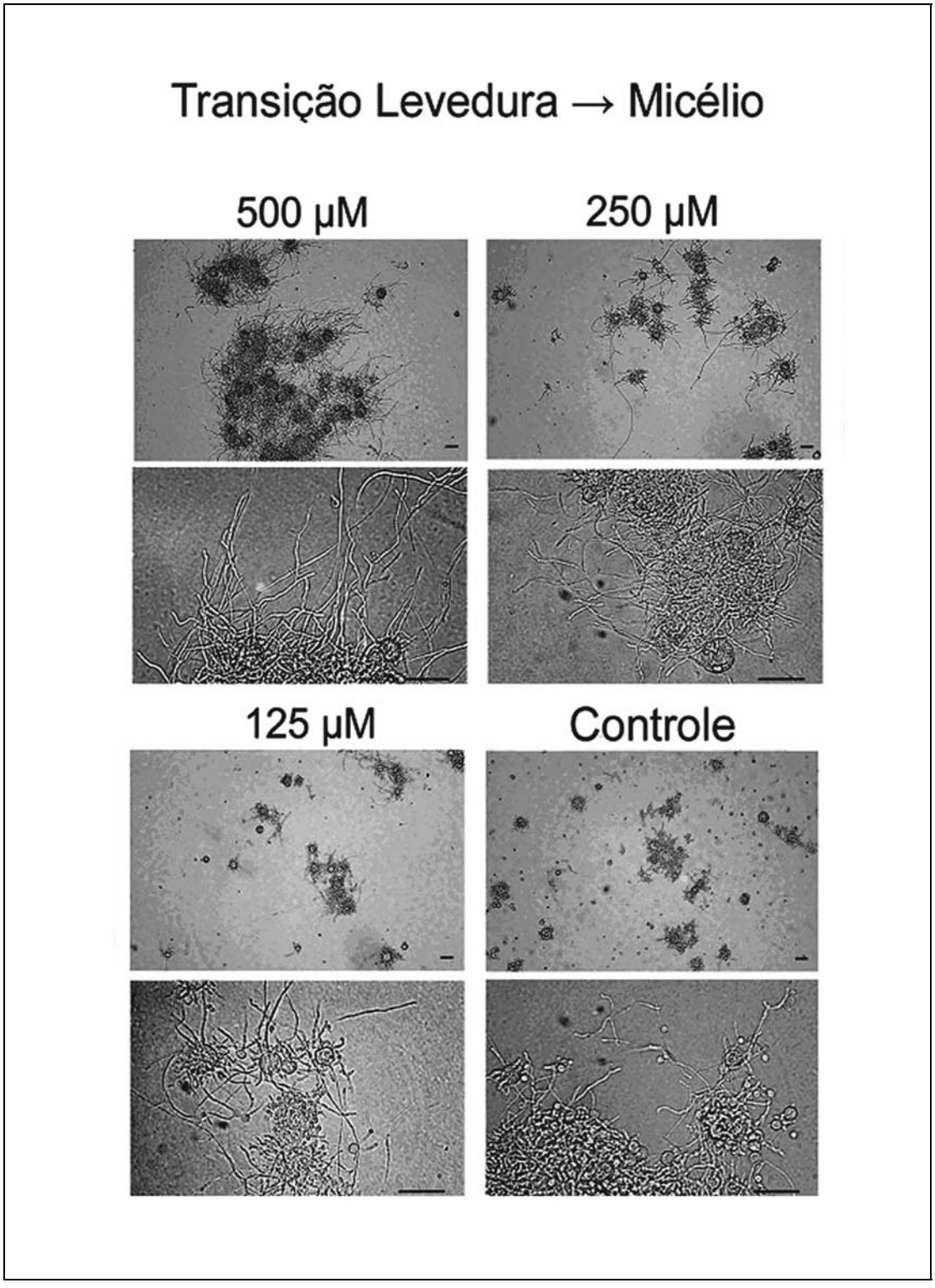

Figura 30: Transição dimórfica de levedura para micélio na presença do inibidor de farnesiltransferase. Leveduras de *P. brasiliensis* foram inoculadas em meio de cultivo MVM, incubadas por 48h a 37ºC seguida de mudança para temperatura de 22ºC por 10 dias para induzir a diferenciação para forma de micélio na presença de 500, 250 e 125µM of FPT Inhibitor III e na ausência deste composto (controle). Em todas as concentrações testadas as leveduras produziram tubo germinativos. As barras de aumento dos painés acima representam 1µm e dos painéis abaixo 10µm.

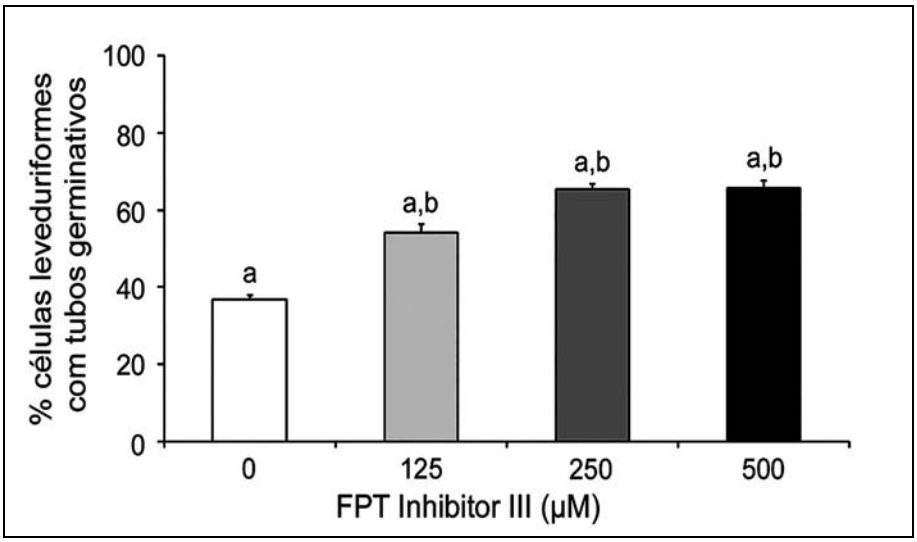

Figura 31: Quantificação das células leveduriformes com tubos germinativos em relação à concentração do inibidor de FTase. Para quantificação das diferenças nas taxas de produção de tubos germinativos em cada concentração testada, contaram-se aleatoriamente todas as leveduras (com tubos germinativos ou não) e calculou-se a proporção relativa ao total do número de células em quatro campos escolhidos aleatoriamente em três diferentes lâminas de três experimentos independentes. Os resultados foram plotados no gráfico como porcentagem de células leveduriformes com tubos germinativos *versus* a concentração do inibidor testada. As barras representam o erro padrão dos experimentos em triplicata, com *a=P < 0,001* e *b=P< 0,01*.

Como o inibidor de farnesilação não é específico para bloquear as proteínas Ras, os efeitos observados na morfologia de *P. brasiliensis* podem ser resultantes da inibição de outras proteínas que também necessitam do processo de farnesilação para realizarem suas funções. É pouco provável que FPT Inhibitor III, específico do processo de farnesilação, esteja atuando nas proteínas que pertençam a superfamília das Rho-GTPases, incluindo RhoA, Rac, Cdc42, pois são geraniladas ao invés de farnesiladas (Arellano *et al*., 1999). Dentre as 30 proteínas conhecidas por sofrerem farnesilação em células humanas (Appels *et al*., 2005) apenas duas delas, Rheb e Ras estão envolvidas em crescimento e diferenciação celular fúngica em resposta a diferentes estímulos. As proteínas

Rheb parecem ser responsáveis pela ativação da via de sinalização Tor, que controla diferenciação celular em *S. pombe* (Alvarez & Moreno, 2006; Uritani *et al.,* 2006), *C. neoformans* e *C. albicans* (Rhode & Cardenas, 2004). As proteínas Ras ativam Cdc42 para promover polaridade celular e acasalamento em *S. pombe* (Marcus *et al.,* 1995), crescimento filamentoso em *S. cerevisiae* (Mosh *et al.,* 1996), crescimento vegetativo e transição dimórfica soro-induzida em *C. albicans* (Leberer *et al.,* 2001), germinação de esporos e crescimento polarizado em *P. marneffei* (Boyce *et al.,* 2005) e ainda crescimento em alta temperatura e diferenciação celular em *C. neoformans* (Alspaugh *et al*., 2000; Waugh *et al.,* 2003).

Este trabalho demonstra que a inibição do processo de farnesilação promove profundas alterações no crescimento vegetativo de leveduras de *P. brasiliensis* e induz a transição dimórfica de levedura para micélio. Apesar disto, não é possível precisar qual proteína, se Ras ou Rheb ou ambas, estariam controlando esses eventos. O que se pode afirmar é que *P. brasiliensis* possui todos os componentes envolvidos com sinalização celular, para promover a diferenciação e morfogênese, como em outros fungos (Fernandes *et al.,* 2005), incluindo as vias controladas por Rheb (Tor) e Ras (MAPKinases ou AMPc/PKA), no entanto como elas funcionam, interagem e a quais sinais elas respondem, ainda precisam ser investigados.

6.7) Padronização do sistema de transformação via *A. tumefaciens* para o isolado Pb113

Em 2004, Leal *et al.* demonstraram que a bactéria *A. tumefaciens* é capaz de transformar o isolado ATCC-60855 do fungo dimórfico *P. brasiliensis*. Como existe uma variação muito grande entre os diferentes isolados deste patógeno, tanto no que diz respeito à variabilidade genética, morfológica e até mesmo no tempo de crescimento, decidiu-se escolher um isolado para ser manipulado geneticamente que fosse mais viável para a execução dos experimentos de transformação genética. Foi escolhido o Pb113, isolado por Fava-Netto em 1971 de um paciente brasileiro do estado de São Paulo apresentando PCM crônica. Feitosa *et al.* (2003) relataram o cariótipo de doze isolados de *P. brasiliensis* através das técnicas de eletroforese de campo pulsado e hibridização do DNA e demonstraram que o isolado Pb113 parece ser haplóide, apresentando apenas

dois núcleos por célula e um genoma estimado de 25,8Mb. Além disso, o isolado Pb 113 cresce muito mais rápido em meio de cultura quando comparado ao isolado Pb01 (dados não mostrados). Tais características foram essenciais para a escolha deste isolado para testar a transformação via *A. tumefaciens*.

Primeiramente foi realizado um experimento para medir a susceptibilidade dos isolados Pb01 e Pb113 ao antibiótico higromicina B. O experimento consistiu na contagem de unidades formadoras de colônias (UFC) oriundas de células leveduriformes dos isolados indicados, plaqueadas em meio BHI acrescido de soro de cavalo e fator de crescimento e diferentes concentrações de higromicina B (0 a 300µg/mL) (**Figura 32**). Pode-se observar no gráfico que ambos os isolados são sensíveis a concentração de 100µg/mL de higromicina B.

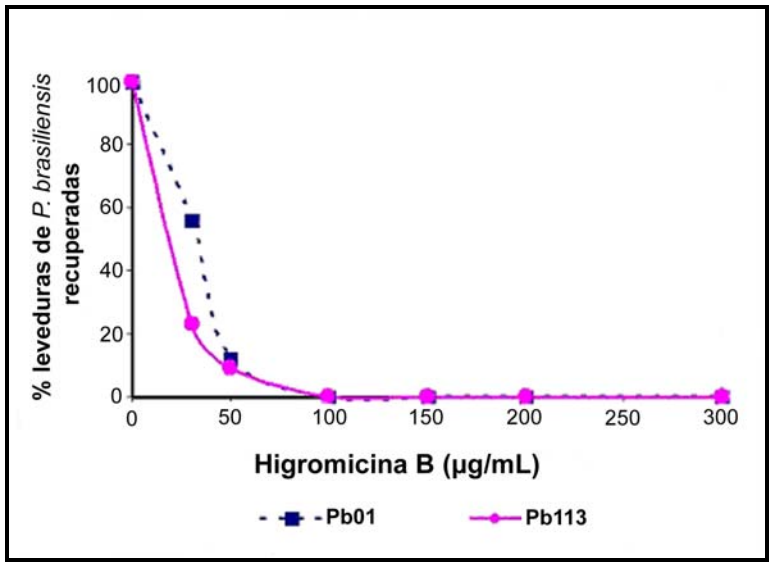

Figura 32: Curva de susceptibilidade dos isolados Pb01 e Pb113 de *P. brasiliensis* a higromicina B. As células leveduriformes foram inoculadas em placas de BHI acrescidas de 5% fator crescimento e 4% de soro de cavalo contendo higromicina B nas concentrações de 0 a 300µg/mL. As placas foram incubadas a 37ºC por 15 - 20 dias. O experimento foi feito em duplicata, sendo que as quantificações foram feitas através da contagem das colônias que cresceram nas concentrações testadas após o período de incubação. Os dados foram plotados num gráfico como % de leveduras recuperadas versus a concentração de higromicina B em µg/mL.

Conhecendo-se a concentração de higromicina B que é capaz de impedir o crescimento dos isolados Pb01 e Pb113, foram então realizados os experimentos de transformação utilizando linhagens de *A. tumefaciens* contendo o vetor pAD1625. O plasmídio pAD1625 **(ver Figura 8)** de aproximadamente 17Kb

apresenta uma região de virulência (Ti) que contém o gene virG mutante constitutivo (virGN54D), uma origem de replicação 75Δ2 e o gene *hph* de *E. coli*, que codifica para higromicina fosfotransferase que confere resistência a higromicina B. O gene *hph* é flanqueado pelo promotor de *cpc-1* de *N. crassa* (*cpc-1* codifica para a "**C**ross-**p**athway **c**ontrol protein") e o terminador de *trpC* de *A. nidulans* (Abuodeh *et al.,* 2000).

Foram testadas as proporções Pb113: Agro 1:10, 1:100 e 1:200. O número de colônias obtidas após a transformação e repique em meio BHI contendo 30µg/mL de higromicina B foi de 35, 9 e 1, respectivamente. No entanto, após repique em meio BHI contendo 100 e 200µg/mL de higromicina B, somente 25 colônias apresentaram-se viáveis, sendo que 23 são oriundas do co-cultivo 1:10 e apenas duas foram obtidas de 1:100. É interessante mencionar que duas colônias mostraram-se viáveis nas concentrações de 30 e 100µg/mL de higromicina B, porém em 200µg/mL de higromicina B foram incapazes de crescer, por serem possivelmente colônias falso-positivas (**Figura 33**). Realizou-se uma PCR para confirmação daqueles que apresentavam o gene exógeno *hph* utilizando como molde DNA genômico de colônias escolhidas aleatoriamente ainda durante a fase de seleção dos clones transformantes (placa de repique de 30µg/mL de higromicina B).

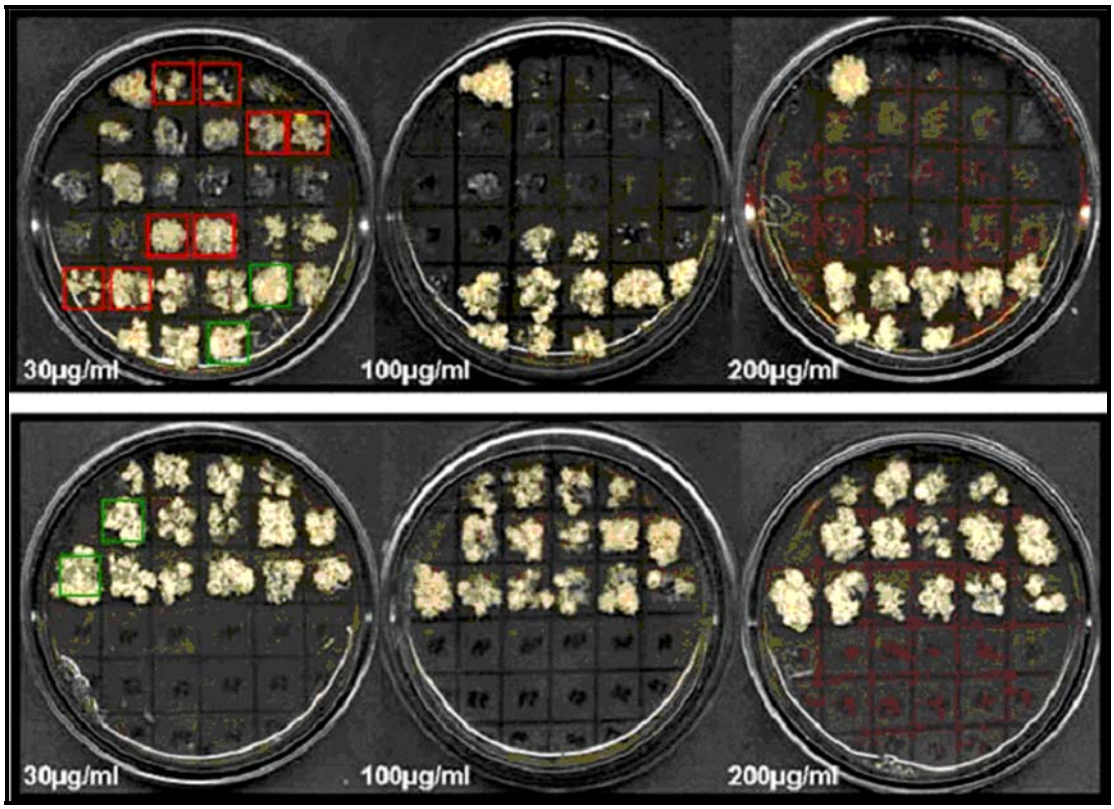

Figura 33: Transformantes do isolado Pb113 de *P. brasiliensis* obtidos do co-cultivo de *A. tumefaciens* contendo o plasmídio pAD1625 após 20 dias de crescimento em BHI acrescido de 4% de soro de cavalo e 5% de fator de crescimento a 37ºC. Na figura estão indicadas as concentrações de higromicina B utilizadas no repique. Os transformantes que estão indicados são aqueles que tiveram o DNA genômico extraído, e analisados por PCR, em verde são os positivos e em vermelho estão os falso-positivos (não apresentam o gene *hph*).

O gel de agarose revelou que das 17 colônias escolhidas aleatoriamente da placa de repique de concentração 30µg/mL de higromicina B, apenas 6 apresentavam o gene exógeno *hph* que confere resistência ao antibiótico utilizado como marcador seletivo integrado em seus genomas (**Figura 34**), o que é compreensível uma vez que as colônias ainda não tinham sido repicadas para maiores concentrações de higromicina B, sendo portanto falso-positivas.

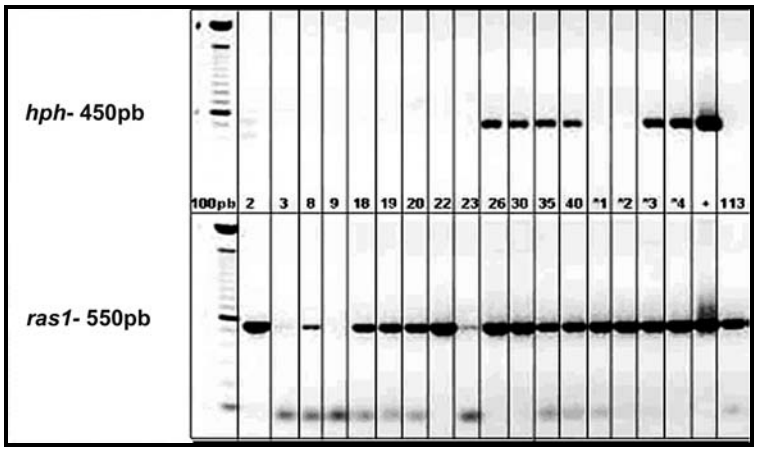

Figura 34: PCR confirmatória dos transformantes de *P. brasiliensis* isolado Pb113 obtidos via *A. tumefaciens.* Gel de agarose 0,8% dos produtos amplificados por PCRs utilizando-se como molde DNA genômico e oligonucleotídeos para o gene *hph* que confere resistência a higromicina B (painel acima) e para o gene *ras1* (controle interno) (painel abaixo). Os números das colônias que tiveram o DNA genômico extraído (2,3,8,9,18,19,20,23,26,30,35,40*1,*2,*3,*4) estão indicados na figura. O controle positivo é o plasmídio pAD1625 e o controle negativo é o isolado Pb113 não transformado. Os tamanhos dos fragmentos obtidos estão indicados na figura.

A estabilidade mitótica dos 25 transformantes que se mostraram viáveis em 200µg/mL de higromicina B foi avaliada através de quatro passagens sucessivas em meio não seletivo e posterior repique em meio seletivo (200µg/mL de higromicina B), e observou-se que os 25 transformantes eram estáveis. Não foi possível analisar o número de inserções do gene *hph* no genoma de cada um dos transformantes por Southern blot, pois sem nenhum motivo explicável (talvez, o clima seco), todos os transformantes pararam de crescer mesmo quando repicados em meio não seletivo. No entanto, os dados obtidos demonstram claramente que o sistema de *A. tumefaciens* é funcional no isolado escolhido, Pb113.

6.8) Construção do vetor quimérico para silenciamento de *ras1* de *P. brasiliensis*

Em paralelo ao estabelecimento do protocolo de transformação do *P.brasiliensis* utilizando *Agrobacterium*, foram também iniciadas as construções de vetores visando o silenciamento gênico por RNAi neste patógeno. Após a realização da amplificação e clonagem de todos os fragmentos (P*cpc-1*, T*trpC*, *ras1* senso e anti-senso, *ura3* senso e anti-senso e loop) no vetor pBluescript KS+

para construção dos 3 vetores quiméricos para silenciamento de *ras1* e *ura3* (marcador de contra-seleção), os plasmídios foram digeridos com a enzima de restrição *Nco*I para confirmar a presença dos insertos nos três cassetes de silenciamento obtidos (I) *ura3/ras1*/loop, (II) *ura3/ras1*/ íntron/ loop e (III) *ura3/ras1*/íntron **(Figura 35A).** Esta etapa demandou aproximadamente um ano de trabalho, devido à complexidade e o número de etapas de clonagem dos referidos insertos. Os resultados da digestão demonstrando a correta inserção dos fragmentos no vetor estão mostrados na **Figura 35B**, em que todos os clones analisados de cada uma das construções liberaram os fragmentos esperados. Após confirmação dos cassetes de silenciamento, decidiu-se amplificá-los utilizando-se os oligonucleotídeos BclI-5'P*cpc1* e BclI-3'T*trpC* , para inserção do sítio de *Bcl*I nas extremidades dos cassetes para posterior digestão com *Bcl*I e clonagem no sítio *Bgl*II do vetor pAD1625 (vetor de *A. tumefaciens*). As reações de PCRs foram realizadas **(Figura 36),** e os fragmentos de tamanhos de 2,8kb; 3,0kb e 2,9kb que correspondem aos cassetes completos (I) *ura3/ras1*/loop, (II) *ura3/ras1*/íntron/loop e (III) *ura3/ras1*/íntron; respectivamente foram observados no gel de agarose. Além das bandas de cerca de 3Kb esperadas na amplificação, outras inespecíficas são também amplificadas, o que mostra que as condições de PCR ainda precisam ser padronizadas para ajuste das condições adequadas. Mesmo assim, as bandas esperadas estão bem resolvidas e podem ser purificadas diretamente do gel de agarose para sua posterior clonagem no vetor pAD1625 (**Figura 36**). Esta etapa do trabalho está no momento sendo realizada em nosso laboratório para em seguida realizar os experimentos de transformação genética do isolado Pb113 visando o silenciamento do gene *ras1*, usando como marcador o gene que confere resistência à higromicina B (hph) e contra-seleção por *ura3* em presença de 5-FOA.

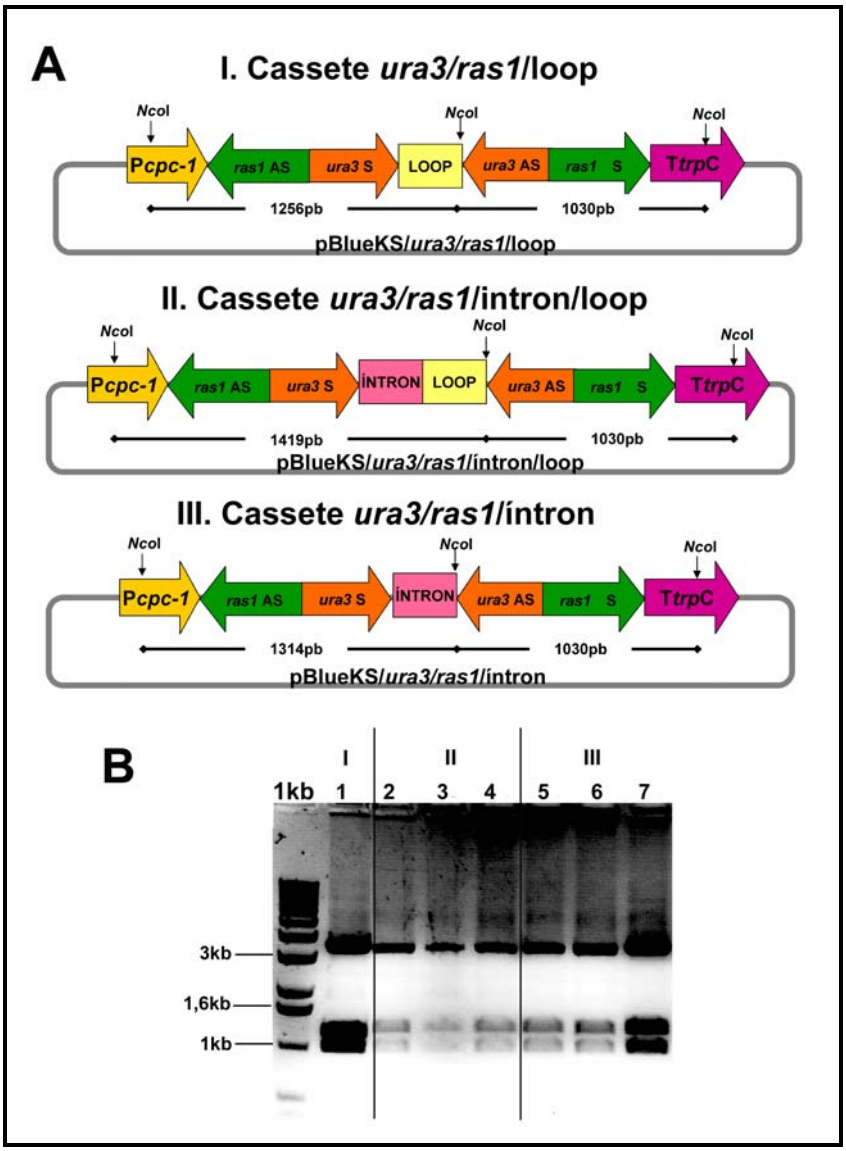

Figura 35: Digestão confirmatória dos cassetes para silenciamento de *ura3/ras1*. O **painel A** apresenta os esquemas de cada um dos cassetes de silenciamento construídos em pBluescript KS+, com a localização dos sítios de *Nco*I, bem como os tamanhos dos fragmentos obtidos em pb após digestão com *Nco*I. O **painel B** representa a análise por eletroforese em gel de agarose 0,8% das digestões dos plasmídios contendo as construções dos cassetes de silenciamento com *Nco*I. O poço 1 representa o perfil dos fragmentos de restrição de um clone contendo o cassete (I) *ura3/ras1*/loop; os poços 2, 3 e 4 representam o perfil de fragmentos de restrição três clones distintos que contém o cassete (II) *ura3/ras1*/ íntron/ loop e os poços 5, 6 e 7 representam o perfil de fragmentos de restrição três clones distintos que contém o cassete (III) *ura3/ras1*/íntron. Os tamanhos obtidos nas digestões são exatamente aqueles esperados pela análise do perfil de restrição (**painel A**).

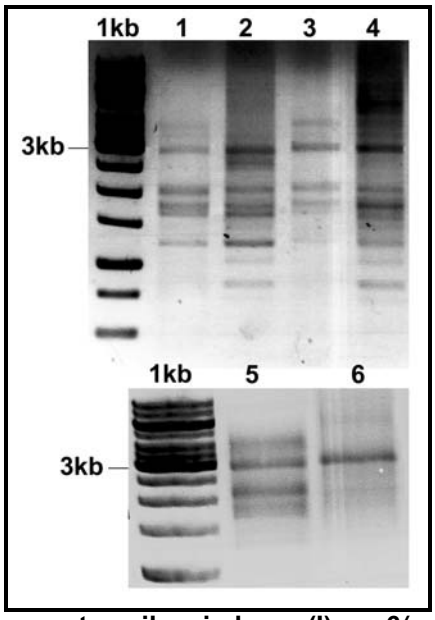

Figura 36: Amplificação dos cassetes silenciadores (I) *ura3/ras1*/loop, (II) *ura3/ras1*/ íntron/ loop e (III) *ura3/ras1*/íntron. Utilizaram-se como molde para PCR os plasmídios contendo tais cassetes e variou-se a concentração de magnésio em 2 e 4mM. Os fragmentos amplificados foram analisados em gel de agarose 0,8%, sendo que os poços 1 e 2 correspondem a amplificação do cassete (I) *ura3/ras1*/loop de tamanho 2,8Kb. Os poços 3 e 4 correspondem a amplificação do cassete (II) *ura3/ras1*/ íntron/ loop de tamanho 3,0Kb. Os poços 5 e 6 correspondem a amplificação do cassete (III) *ura3/ras1*/íntron de tamanho 2,9Kb. Os poços 1, 3 e 5 utilizou-se 2mM de magnésio na PCR, enquanto que os poços 2, 4 e 6 utilizou-se a concentração final de 4mM de magnésio.

6.9) Clonagem dos fragmentos de *ras1* senso e antisenso no vetor pSilent-1 e a transformação deste vetor em *P. brasiliensis* através de eletroporação.

Ainda em paralelo à construção dos vetores para transformação via *Agrobacterium*, os fragmentos senso e antisenso do gene *ras1* de *P. brasiliensis* foram também amplificados e clonados no vetor pSilent-1. A confirmação da correta inserção dos fragmentos senso e anti-senso foi realizada por digestão com as enzimas *Sph*I e *Xho*I (**Figura 37**). Os controles utilizados foram pSilent-1, contendo apenas o fragmento *ras1* senso e o vetor pSilent-1 vazio. O padrão de restrição obtido foi exatamente o esperado; pSilent-1 *ras1*, liberou uma banda de 1200pb correspondente a clonagem dos fragmentos senso e antisenso de *ras1*, enquanto que pSilent-1 contendo apenas o fragmento senso de *ras1* liberou uma banda de 600pb. O vetor vazio deveria ter liberado uma banda de 100pb correspondente ao loop (**Figura 37B**), no entanto, não foi possível observá-la no

gel, pois esta banda pode ter migrado juntamente com o RNA (amostra não foi tratada com RNAseA).

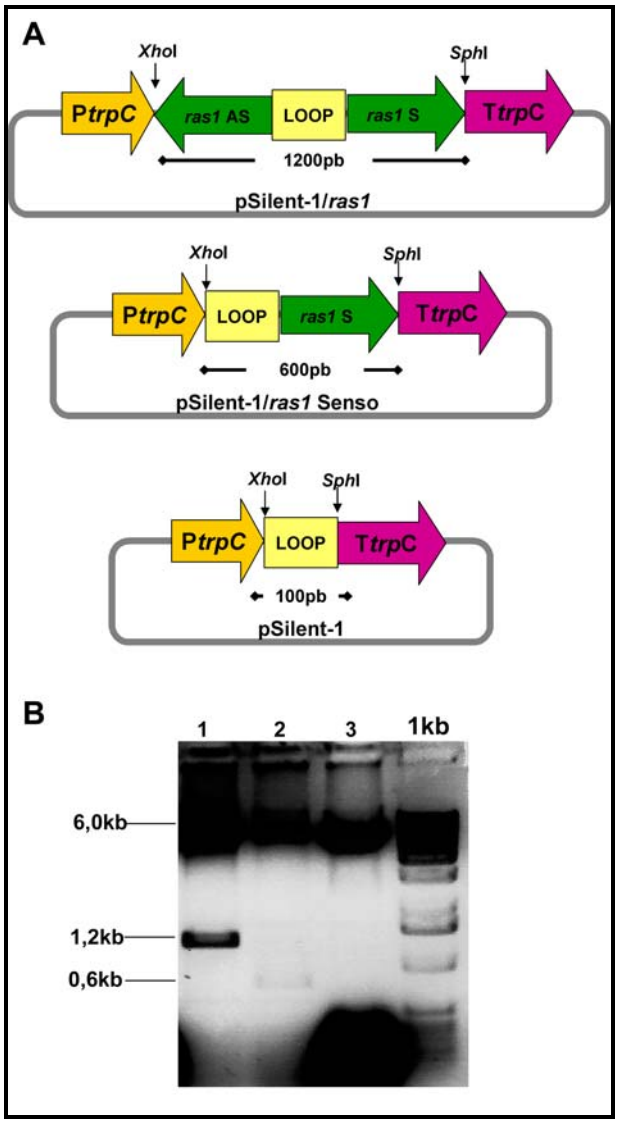

Figura 37: Digestão confirmatória do vetor pSilent-1/*ras1* contendo os fragmentos senso e antisenso de *ras1* para silenciamento. O **painel A** apresenta os esquemas de cada um dos vetores utilizados na digestão: pSilent-1/*ras1*; pSilent-1/*ras1* senso e pSilent-1, com a localização dos sítios de *Sph*I e *Xho*I, bem como os tamanhos dos fragmentos obtidos em pb após digestão com *SphI* e *Xho*I. O **painel B** representa a análise por eletroforese em gel de agarose 0,8% das digestões dos plasmídios acima mencionados. O poço 1 representa o perfil dos fragmentos de restrição do vetor pSilent-1/*ras1*, contendo *ras1* senso e *ras1* antisenso; o poço 2 representa o perfil de fragmentos de restrição de pSilent-1 contendo apenas *ras1* senso e o poço 3 representa o perfil do vetor pSilent-1 vazio Os tamanhos obtidos nas digestões são exatamente aqueles esperados pela análise do perfil de restrição (**painel A**).

Após a confirmação da construção do vetor pSilent-1/*ras1*, realizou-se a transformação do isolado Pb113 e Pb01 pelo método de eletroporação (ver seção métodos para detalhes). Após 20 dias de incubação não foram obtidas colônias do isolado Pb01. Para o isolado Pb113 foram obtidas cerca de 50 colônias que cresceram em presença de 100µg/mL de higromicina B, a seguir foram repicados para placas de BHI contendo 100 e 200µg/mL de higromicina B para confirmar se realmente representavam transformantes positivos. Após 20 dias de incubação das placas-repique nenhuma colônia foi obtida, indicando que as colônias obtidas da transformação de pSilent-1/*ras1* do isolado Pb113 eram transformantes falso-positivos ou muito instáveis (dados não mostrados). Anteriormente, Soares *et al.* (2005) demonstraram a instabilidade dos transformantes de Pb01 oriundos de eletroporação e sugeriram que esta instabilidade, se deve muitas vezes a não integração do plasmídio no genoma do fungo, permanecendo na forma episssomal, susceptível a eliminação com a pressão seletiva.

Finalizando, este trabalho permitiu a identificação e caracterização dos genes *ras1* e *ras2,* no fungo dimórfico e patogênico *P. brasiliensis.* Além disso, foi possível analisar o perfil transcricional destes genes durante o processo dimórfico de micélio para levedura, no qual não foram observadas variações significativas da modulação transcricional de *ras*, enquanto que leveduras internalizadas em macrófagos mostraram uma regulação negativa de *ras1* e *ras2*. Entretanto, apenas *ras1* responde ao choque térmico a 42ºC. Este trabalho avaliou também a maquinaria celular para prenilação, sendo que *P. brasiliensis* apresenta todos os genes necessários para o correto funcionamento das preniltransferases. Através da utilização de inibidor de farnesiltransferase, foi possível demonstrar o envolvimento deste processo no crescimento vegetativo e dimorfismo deste patógeno, já que a inibição do processo de farnesilação promoveu alterações no crescimento vegetativo de leveduras de *P. brasiliensis* e induziu a transição dimórfica de levedura para micélio. Em paralelo, este trabalho delineou uma estratégia para silenciamento gênico em *P. brasiliensis*, utilizando como instrumento o fenômeno de interferência no RNA. Foram construídos três vetores que estão sendo testados para silenciar o gene *ras1* de *P. brasiliensis*. Ainda em paralelo, foi realizada a padronização do método de transformação mediado por *A. tumefaciens* para futuramente ser utilizada na inserção dos cassetes de silenciamento no genoma de *P. brasiliensis*. Os resultados sugerem que os genes

ras devem ter um papel no dimorfismo, na resposta a choque térmico e na interação patógeno-hospedeiro, no entanto as funções de *ras* nesses processos serão avaliadas em detalhe quando os genes *ras* forem silenciados via interferência no RNA.

7 – Conclusões e Perspectivas

Este trabalho identificou e caracterizou pela primeira vez a presença de dois genes, *ras1* e *ras2,* no fungo dimórfico e patogênico *P. brasiliensis* apresentando cópias únicas no genoma, alta similaridade e identidade tanto entre as seqüências deduzidas das proteínas quanto entre as seqüências de nucleotídeos, demonstrando ainda uma alta conservação com as proteínas Ras de outros organismos.

Além disso, os genes *ras* são modulados negativamente quando leveduras deste patógeno entram em contato com células hospedeiras, no caso macrófagos peritoniais, devido principalmente à limitação nutricional e ao ambiente hostil encontrado pelo fungo. Apenas *ras1* apresentou uma regulação negativa quando leveduras de *P. brasiliensis* foram submetidas a choque térmico. Durante a transição dimórfica dependente de temperatura de micélio para levedura, nenhum dos genes *ras* se mostraram regulados transcricionalmente.

P. brasiliensis apresenta todos os genes que codificam as enzimas preniltransferases, capazes de adicionar grupamentos lipídicos em proteínas que devem se localizar na membrana plasmática para exercerem as suas funções biológicas. Dentre os genes que foram identificados e tiveram suas seqüências deduzidas de proteínas analisadas estão: *ram1* (subunidade β de FTase), *cdc43* (subunidade β de GGTase I) e *ram2* (subunidade α comum a FTase e GGTase I).

Este trabalho mostra o envolvimento do processo de farnesilação no crescimento vegetativo e dimorfismo de *P. brasiliensis*. A inibição do processo de farnesilação promoveu alterações no crescimento vegetativo de leveduras de *P. brasiliensis* e induziu a transição dimórfica de levedura para micélio. Possivelmente, proteínas como Ras e Rheb envolvidas em diferenciação e morfogênese celular estariam sendo bloqueadas, no entanto quais os componentes celulares responsáveis pelo fenótipo observado quando há o bloqueio do processo de farnesilação ainda precisam ser investigados.

Este trabalho, em paralelo, permitiu avançar no desenvolvimento da estratégia para silenciamento gênico em *P. brasiliensis*, que por ser um fungo multinucleado, com multibrotamentos e ploidia ainda não definida, a técnica de interferência no RNA parece ser uma opção estratégica para estudar função gênica neste patógeno humano. Foram construídos três vetores que estão sob testes para silenciar o gene *ras1* de *P. brasiliensis*. Além disso, a padronização do método de transformação mediado por *A. tumefaciens* foi realizada com intuito de

utilizar esta bactéria como carreadora dos cassetes de silenciamento para o *P. brasiliensis*, já que a transformação por eletroporação utilizando o vetor construído neste trabalho pSilent-1/*ras1*, para silenciamento de *ras1*, produziu apenas transformantes instáveis.

A funcionalidade dos dois genes *ras* de *P. brasiliensis* será analisada utilizando experimentos de silenciamento gênico, na tentativa de elucidar o papel biológico de *ras1* e *ras2*, e ainda verificar se os dois genes apresentam funções redundantes ou paralelas em uma mesma via de sinalização e/ou processo biológico. No entanto, a importância das proteínas Ras na patobiologia de *P. brasiliensis* foi demonstrada por este trabalho, o através dos experimentos empregando inibidores de farnesilação. Estas análises abrem novas possibilidades para identificação e caracterização dos componentes das cascatas de sinalização de *P. brasiliensis* (Ras, Rheb) que certamente muito contribuirão para o entendimento das respostas adaptativas, controle dos processos de morfogênese, dimorfismo, virulência e patogenicidade deste fungo.

Assim, como perspectivas deste trabalho, existem as seguintes etapas: padronização da PCR para amplificação dos cassetes de silenciamento, purificação dos fragmentos, clonagem no vetor pAD1625 para posterior transformação via *A. tumefaciens* do isolado Pb113 visando o silenciamento de *ras1*. A análise dos transformantes silenciados de *ras1* fornecerá um melhor entendimento das funções deste gene em *P. brasiliensis*. Além disso, as clonagens de *ras2* nos vetores gerados por este trabalho, bem como a transformação em *P. brasiliensis* para obtenção de transformantes silenciados para *ras2*, fornecerão informações valiosas quanto ao papel desempenhado por *ras2* neste patógeno. Como uma perspectiva futura desta tese de doutorado, está a análise molecular e funcional de *rheb* de *P.* brasiliensis para elucidação do envolvimento desta proteína no dimorfismo e pátobiologia deste fungo.

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9 - Anexos

9.1) Trabalhos publicados e/ou submetidos como primeira autora

- **Fernandes, L.**, Araujo, M.A., Amaral, A., Reis, V.C., Martins, N.F. and Felipe, M.S. (2005) Cell signaling pathways in *Paracoccidioides brasiliensis*--inferred from comparisons with other fungi. *Genet Mol Res*. **4**: 216-31.

- **Fernandes, L.,** Paes, H.C., Tavares, A.H., Silva, S.S., Dantas, A., Soares, C.M.A., Torres, F.A. and Felipe, M.S.S. (2007) Transcriptional profile of *ras1* and *ras2* and the potential role of farnesylation in the dimorphism of the human pathogen *Paracoccidioides brasiliensis, FEMS Yeast Research* , em impressão.

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9.2) Trabalhos publicados e/ou submetidos como co-autora

- Felipe, M.S., Andrade, R.V., Arraes, F.B., Nicola, A.M., Maranhão, A.Q., Torres, F., Silva-Pereira, I., Pocas-Fonseca, M.J., Campos, E.G., Moraes, L.M.P., Albuquerque, P., Tavares, A.H.F.P., Silva, S.S., Kyaw, C.M., Souza, D.P., PbGenome Network, Pereira, M., Jesuíno, R.S.A., Andrade, E.V., Parente, J.A., Oliveira, G.S., Barbosa, M.S., Martins, N.F., Fachin, A.L., Cardoso, R.S., Passos, G.A.S., Almeida, N.F., Walter, M.E.M.T., Soares, C.M.A., Carvalho, M.J., Brigido, M.M. and **PbGenome Network** (2005) Transcriptional Profiles of the Human Pathogenic Fungus *Paracoccidioides brasiliensis* in Mycelium and Yeast Cells. *J Biol Chem*. **280**: 24706–14.

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Cell signaling pathways in *Paracoccidioides brasiliensis*--inferred from comparisons with other fungi

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Cell signaling pathways in *Paracoccidioides brasiliensis* - inferred from comparisons with other fungi

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ABSTRACT. The human fungal pathogen *Paracoccidioides brasiliensis* is an ascomycete that displays a temperature-dependent dimorphic transition, appearing as a mycelium at 22°C and as a yeast at 37°C, this latter being the virulent form. We report on the *in silico* search made of the *P. brasiliensis* transcriptome-expressed sequence tag database for components of signaling pathways previously known to be involved in morphogenesis and virulence in other species of fungi, including *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus*. Using this approach, it was possible to identify several protein cascades in *P. brasiliensis*, such as i) mitogen-activated protein kinase signaling for cell integrity, cell wall construction, pheromone/mating, and osmo-regulation, ii) the cAMP/PKA system, which regulates fungal development and virulence, iii) the Ras protein, which allows cross-talking between cascades, iv) calcium-cal-

modulin-calcineurin, which controls cell survival under oxidative stress, high temperature, and membrane/cell wall perturbation, and v) the target of rapamycin pathway, controlling cell growth and proliferation. The ways in which *P. brasiliensis* responds to the environment and modulates the expression of genes required for its survival and virulence can be inferred through comparison with other fungi for which this type of data is already available.

Key words: Fungi, *Paracoccidioides brasiliensis*, Signaling pathways, Comparison

INTRODUCTION

Signal transduction describes a great number of biochemical events that transmit a signal from the cell exterior, through the cell membrane, and into the cytoplasm. This involves a number of molecules, including receptors, intermediate proteins, and messengers. The signaling pathways are commonly used by an extensive array of biological ligands to modulate various cell processes, such as growth, differentiation, and proliferation. These transduction pathways communicate information about the external environment to the inside of a cell. Signaling systems in fungi also regulate cell polarity, mating, and pheromone control, and hence they play a role in determining cell shape. Some of the known responses include changes in the cell cycle, polarized growth, and modifications to the transcriptional profile of the cell.

Since the 50's, signaling pathways have been investigated by genetic and biochemical experimentation. In a large series of experiments, eukaryotic organisms were studied for their nutritional limitations and for their reactions to various environmental stresses, such as heat, oxidative, osmotic, or ethylic shock (Engebrecht, 2003).

The genomic era changed the perspective of signaling pathway studies. By using a sequence database, such as GeneBank, the screening of potentially transcribed genes in a given cell led to the rapid identification of critical genes. These methods enable researchers to assess genetic diversity or find similarities among cell types.

Pathogenic organisms sense and respond to the harsh conditions imposed by the host-activating components of signaling pathways that culminate in the expression of genes responsible for the virulence and differentiation of the pathogen. Therefore, studies on signal transduction in various fungi may reveal common conserved mechanisms of signal transduction as well as the differences between these organisms. These studies could lead to drug development.

We report the *in silico* search made of the *Paracoccidioides brasiliensis* transcriptome expressed sequence tag database (Felipe et al., 2003) to evaluate the presence of cellular signaling pathway elements (Figure 1) and to compare them with the cascade components in i) the non-pathogenic fungus *Saccharomyces cerevisiae*, ii) the human opportunistic non-dimorphic fungus *Cryptococcus neoformans*, iii) the human opportunistic dimorphic fungus *Candida albicans*, and iv) the human pathogenic fungus *Aspergillus fumigatus*.

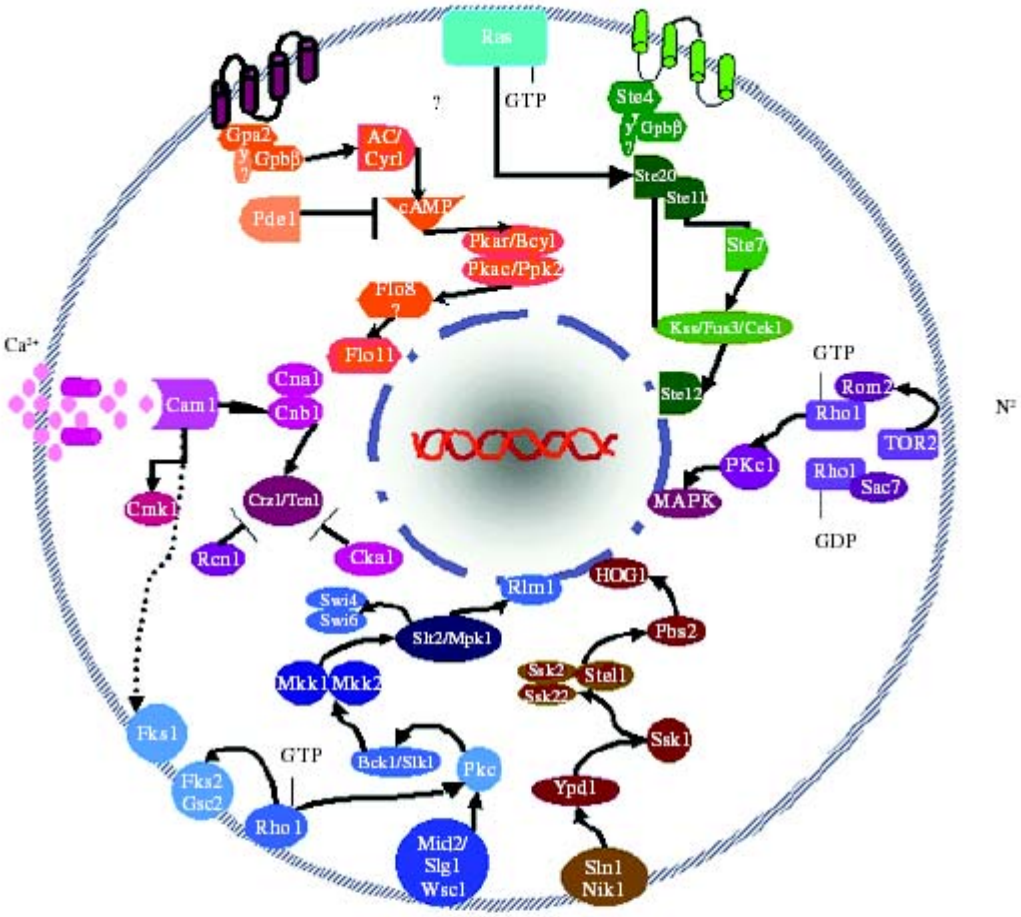

Figure 1. Signal transduction pathways in *Paracoccidioides brasiliensis*. Cell adhesion (orange), pheromone response (green), calcium/calmodulin (pink), cell integrity (blue), high osmotic growth stress response (brown), and TOR (purple) pathways are depicted.

MATERIAL AND METHODS

The identification of putative genes involved in the cellular signaling pathways was performed by the "search by key word" service provided by the bioinformatics group of the PbGenome project (Felipe et al., 2003). The classification of candidates according to the signaling category families was performed by a BLASTx (Zhang, 2003) comparison of sequences against a database with all the signaling protein sequences from Genbank (Benson et al., 2004). The analyzed clusters were assembled by CAP3 software in the sequence-processing pipeline from the PbGenome project. The multiple sequence alignment of the candidates was performed using CLUSTAL W software (Aiyar, 2000).

THE SIGNALING PATHWAYS IN *PARACOCCIDIOIDES BRASILIENSIS*

The mitogen-activated protein kinase cascade

The mitogen-activated protein kinase (MAPK) pathway is a major mechanism for controlling transcription in eukaryotes (Seger and Krebs, 1995). MAPK was originally discovered as an insulin-activated protein-serine kinase, though biochemical studies, reinforced by genetic analysis, indicate a pheromone response in budding yeast.

Fungal cells contain five MAPK cascades that orchestrate responses to different physiological stimuli. One cascade operates in cells undergoing meiosis and regulates spore formation. The other four cascades operate in vegetative, mitotically active cells. Among these cascades, two control developmental events - mating and filamentation. The pheromone response pathway, the cell integrity pathway, and the high osmolarity glycerol (HOG) pathway, can all activate their MAPKs within minutes after initial stimuli (Lengeler et al., 2000).

The pheromone response

Pheromones are ubiquitous in fungal mating systems, and even their general structure is well conserved. Although the pheromone response pathway, and that regulating filamentous growth, share a common MAPK module, their upstream regulators appear to be specific to each of them. Haploid fungal cells respond to pheromones by a MAPK cascade involved in mating.

Normal *S. cerevisiae* cells undergo a vegetative life cycle, but after binding of the appropriate mating pheromone they activate a different developmental pathway that leads to the production of mating filaments. This intercellular communication between the two mating types of cells activates a signal transduction pathway that stimulates the various physiological changes required by the process, such as induction of cell surface agglutinins, cell division arrest at G1, morphogenesis to form a conjugation tube, and cell fusion. The components of this cascade include a G-protein-coupled receptor, several protein kinases, and a pheromone-responsive transcription factor. The molecular mechanisms that transduce the pheromone signal are remarkably similar to the mechanisms of hormone signaling used in multicellular organisms (Konopka and Fields, 1992). The yeast protein kinases encoded by *STE11*, *STE7*, and *FUS3* constitute the kinase cascade in which Ste11p phosphorylates and activates Ste7p, which in turn phosphorylates the MAPK Fus3p (Neiman and Herskowitz, 1994). In this model of signaling, specificity is defined by at least four ramifications of the same signaling pathway. Briefly, Ste12p and Mcm1p activate the transcription factor of the genes that respond to pheromones in haploid cells, whereas in diploid cells, Ste12p and Tec1p activate the genes responsible for filamentation (Lengeler et al., 2000).

In *C. neoformans*, the *STE12* homologue gene, though it has conserved roles in morphogenesis, exists only in mating type (MAT)α cells, and it is directly involved in virulence (Chang et al., 2000). Recently, three genes encoding the MF pheromone were identified in the mating-type locus, and they were found to be transcriptionally induced by limiting nutrients and to co-culture with MATα cells. Overexpression of MFα pheromone enhances haploid budding and the MFα pheromone is not essential for virulence, but it contributes to overall virulence (Wang et al, 2000).

The mating process in *C. albicans* was cytologically described for the first time by Bennett et al. (2003). The cascade consists of Cst20p kinases, which phosphorylate Hst7p and

Cek1p, which are homologous to Fus3p and Kss1p (Lockhart et al., 2002).

The finding of homologue genes suggests the existence of a sexual cycle in *A. fumigatus*. Using the available incomplete genome database, Poggeler and Kuck (2002) deduced that the gene products of the receptor are putative proteins of seven transmembrane domains, which display a high-level amino acid identity with the α-factor receptor Ste3p and the α-receptor Ste2p of *S. cerevisiae*.

The complete pathway from the Skh1p protein (Ste4p), which includes the downstream Ste20p and its targets in tandem Ste11p and Ste7p, was identified in *P. brasiliensis*. The downstream components, such as Kss1p (also called Fus3p/Cek1p), which phosphorylates Ste12p, were also identified in this transcriptome. The proteins *Ste20p*, *Ste11p*, *Ste7p*, and *Kss1p* appear to be constitutively expressed in both mycelial and yeast forms. On the other hand, genes *MOT2* and *MOT3*, which encode nuclear Zn-finger proteins that attenuate the mating pheromone, were not found in the *P. brasiliensis* transcriptome. The function assigned to the whole pathway is to positively regulate growth and cell proliferation (Table 1).

Four mating-type proteins were found through the transcriptome analyses of *P. brasiliensis*: a homologue to the MAT-1 protein of *A. nidulans*, with an E-value of 1e-46, and three PbAESTs, which possibly encode a MAT-2 (Table 1). Despite the presence of the genes involved in the cascade responsive to pheromones, a conclusive observation of the sexual cycle of *P. brasiliensis* is still pending; however, we present evidence that suggests the existence of mating in this organism.

Maintenance of cell integrity

In the budding yeast, *S. cerevisiae*, the MAPK cascade responsible for cell integrity, mediates cell cycle-regulation, cell wall synthesis and responds to various signals, including temperature, changes in external osmolarity, and mating pheromone. Signaling proteins found in *P. brasiliensis* that compose this pathway include GTP binding protein Rho1p, protein kinase C homologue, Pkc1p, MEKK Bck1p (also known as Slk1p), a redundant pair of MEKs, Mkk1p and Mkk2p, MAPK Slt2p (also called Mpk1p), and transcription factor targets Rlm1p and SBF complex (Table 1); the latter is composed of the polypeptides Swi4p and Swi6p (Gustin et al., 1998). There are probably many branches into and out of this pathway.

Rho1p is a small GTP-binding protein of the *Rho* subfamily of Ras-related proteins that is required for cell growth. In *C. neoformans*, a homologue of *S. cerevisiae Mpk1/Slt2* MAPK was identified by Kraus and Heitman (2003). Their work characterized the role of Mpk1p in the maintenance of cell integrity in response to elevated growth temperature and to cell-wall-synthesis inhibitors. The protein encoded by *C. neoformans MPK1* is required for growth at 37°C *in vitro*; this growth defect is suppressed by osmotic stabilization. The cell wall is a fungus-specific dynamic structure essential to almost every aspect of the biology and pathogenicity of *C. albicans*. Its structure confers physical protection and shape to fungal cells, and as the most external part of the fungus, it mediates the interaction with the host, including adhesion to host tissues and modulation of the host anti-*Candida* immune response. Therefore, the search for potential cell wall-related targets can be envisaged as key to understanding fungal pathobiology. Moreno et al. (2003) characterized (*in silico*) a *C. albicans* gene encoding a putative transcriptional factor required for cell wall integrity. This gene codes for a Zn(II) Cys(6) transcriptional factor involved in cell wall architecture.

Table 1. PbAESTs ortholog names, accession number and BLAST e-value.

PbAEST	Ortholog name	Acession number organism	e-value
Calcium/calmodulin-calcineurin			
PbAEST 1629	Camd1	AF156028 *Emericella nidulans* (calmodulin).	3.1e-40
PbAEST 2381	Cna1	AF071751 *Neurospora crassa* (calcineurin B, catalytic subunit).	8e-54
PbAEST 2252	Cnb1	AL513466 *Neurospora crassa* (calcineurin B, regulatory subunit).	5e-84
PbAEST 2171	Cmk1	NP_587941.1 *Saccharomyces cerevisiae* (calcium calmodulin-dependent protein kinase).	1e-165
PbAEST 2415	Crz1/Tcn1	NP_014371.1 *Saccharomyces cerevisiae* (calcineurin-responsive zinc-finger).	4e-41
PbAEST 5082	Cka1	NP_593642.1 *Arabidopsis thaliana* (casein kinase II α subunit).	8.3e-26
PKA/cAMP			
PbAEST 2581	Gpr	splP19533 *Emericella nidulans* (G-protein-coupled receptor).	4e-79
PbAEST 595	Gpa2	gil6323816 *Saccharomyces cerevisiae* (guanine nucleotide-binding regulatory protein).	0.0042
PbAEST 2208	Gpb	AF176775_1 *Aspergillus nidulans* (G protein β-subunit).	5e-88
PbAEST 3426	AC/Cyr1	NP_194335.1 *Arabidopsis thaliana* (adenylate cyclase).	1e-15
PbAEST 5256	Pde1	NP_011266.1 *Saccharomyces cerevisiae* (low-affinity cAMP phosphodiesterase).	1e-10
PbAEST 947	Pkar/Bcy1	AF401202_1 *Aspergillus fumigatus* (regulatory subunit of PKA).	2e-74
PbAEST 1562	Pkac/Tpk1	NP_012371. *Saccharomyces cerevisiae* (catalytic subunit of cAMP-dependent protein kinase).	1.8e-36
PbAEST 3488	Pkac/Tpk2	P34099 *Saccharomyces cerevisiae* (catalytic subunit of PKA).	1e-60
PbAEST 3241	Flo11/Muc1	NP_012284.1 *Saccharomyces cerevisiae* (flocculin: extracellular α-1,4-glucan glucosidase).	2.6e-06
MAPK pheromone regulated			
PbAEST 2475	Mat1-1	AF100925 *Gibberella fujikuroi* (mating-type protein MAT1-1).	1e-16
PbAEST 4188	Mat1-2	AF508279_1 *Emericella nidulans* (mating-type protein MAT1-2).	4e-20
PbAEST 2208	Ste4	AF176775 *Aspergillus nidulans* (GTP-binding protein β subunit of the pheromone pathway).	5e-88
PbAEST 266	Ste20	L04655 *Saccharomyces cerevisiae* (ser/thr protein kinase of the pheromone pathway).	3e-51
PbAEST 659	Ste11	AF034090 *Neurospora crassa* (ser/thr protein kinase of the MEKK family).	e-160
PbAEST 989	Ste7	AJ304830 *Blumeria graminis* (ser/thr/tyr protein kinase of mitogen-activated protein kinase (MAPK) kinase family).	8e-29
PbAEST 1960	Kss1/ Fus3/Cek1	AF268070 *Ustilago maydis* (MAPK).	5e-21
PbAEST 4523	Ste12	AF080600 *Emericella nidulans* (transcriptional activator).	4e-37
MAPK controlling cell wall construction and cell integrity			
PbAEST 1346	Mid2/ Slg1/Wsc1	NP_014650.1 *Saccharomyces cerevisiae* (cell surface sensors for cell integrity signaling during vegetative growth).	4e-11
PbAEST 157	PKC	NC_001148 *Saccharomyces cerevisiae* (ser/thr protein kinase).	2e-17
PbAEST 659	Bck1/Slk1	AF034090 *Neurospora crassa* (ser/thr protein kinase of the MEKK family).	e-160
PbAEST 4403	Mkk1	NC_001148 *Saccharomyces cerevisiae* (ser/thr protein kinase).	2e-60

Continued on next page

Table 1. Continued.

PbAEST	Ortholog name	Acession number organism	e-value
PbAEST 989	Mkk2	AJ304830 *Blumeria graminis* (protein kinase of the MAPK kinase (MEK) family).	8e-29
PbAEST 365	Slt2/Mpk1	AJ304831 *Blumeria graminis* (ser/thr protein kinase of MAPK family).	e-110
PbAEST 1259	Rlm1	NP_015236 *Saccharomyces cerevisiae* (transcription factor of the MADS box family).	9e-26
PbAEST 3256	Swi4	AL513463 *Neurospora crassa* (transcription factor).	4e-08
PbAEST 3615	Swi6	NP_594464.1 *Schizosaccharomyces pombe* (transcription factor).	1.6
PbAEST 1518	Rho1	AY007297 *Aspergillus fumigatus* (GTP-binding protein of the rho subfamily of ras-like proteins).	8e-53
PbAEST 3665	Fks1	AF148715 *Paracoccidioides brasiliensis* (1,3-β-D-glucan synthase, catalytic subunit).	2e-81
PbAEST 4433	Fks2/GSC2	AF148715 *Paracoccidioides brasiliensis* (1,3-β-D-glucan synthase subunit).	e-108
MAPK for high osmotic growth/stress response			
PbAEST 985	Sln1/Nik1	AF435964 *Botryotinia fuckeliana* (putative sensory transduction histidine-kinase).	5e-50
PbAEST 2744	Ypd1	MPR1_SCHPO *Schizosaccharomyces pombe* (phosphorelay intermediate between Sln1p and Ssk1p).	1e-04
PbAEST 913	Ssk1	AF084608_1 *Candida albicans* (two-component signal transducer).	0.046
PbAEST 659	Ssk2	P53599 *Saccharomyces cerevisiae* (MAPK kinase kinase, MAPKKK - suppressor of sensor kinase 2).	1e-38
PbAEST 365	Pbs2	NP_012407 *Saccharomyces cerevisiae* (MAPK kinase - MEK).	3e-31
PbAEST 3218	Sho1	CAC81238 *Candida albicans* (protein osmosensor).	2e-05
PbAEST 1960	Ste50	EAL02925 *Candida albicans* (MAPKKK).	2e-15
PbAEST 356	Hog1	AF184980_1 *Magnaporthe grisea* (osmotic sensitivity MAPK).	1e-73
TOR pathway			
PbAEST 3215	Tor2	CAA50548.1 *Saccharomyces cerevisiae* (protein/ phosphatidylinositol kinase).	1e-15
PbAEST 4664	Rom2	T41524 *Schizosaccharomyces pombe* (rho1 gdp-gtp exchange protein 1).	7e-35
PbAEST 3501	Sac7	CAA20323.1 *Schizosaccharomyces pombe* (RhoGAP GTPase activating protein).	4e-37
PbAEST 70	Cdc55	AAD15987.1 *Neurospora crassa* (protein phosphatase 2A regulatory B subunit).	e-105
PbAEST 5565	Tpd3	AAB03670.1 *Dictyostelium discoideum* (protein phosphatase 2A regulatory A subunit).	2e-35
PbAEST 1737	Sit4	CAB98214.2 *Neurospora crassa* (probable cell shape control protein phosphatase ppe1).	2e-99
PbAEST 1318	Gap1	CAD21063.1 *Neurospora crassa* (amino acid permease NAAP1).	0.0
PbAEST 367	Mkk1-	CAC19662.1 *Blumeria graminis* (MAPK kinase).	e-110
PbAEST 358	Mpk1	AAF09475.1 *Magnaporthe grisea* (osmotic sensitivity MAPK: Hog1p).	1e-73
PbAEST 2132	Npr1	CAA18998.1 (nitrogen permease reactivator/protein kinase).	1e-40
PbAEST 1432	Fpr1	CAA06962.1 (FKBP-type peptidyl-prolyl cis-trans-isomerases).	8e-38

Cell wall integrity in *A. fumigatus* is less well defined, and its role in protecting fungal cells is not clearly understood. In the model yeast *S. cerevisiae*, the cell integrity *Mpk1/Slt2* MAPK and calcineurin pathways monitor the state of the cell wall and promote remodeling under stress conditions. The Rho1p of *P. brasiliensis* bears 86.9% identity with the *S. cerevisiae* counterpart. Rho1p binds to and is required for the activity of Pkc1p *in vivo*, which in turn regulates the MAPK pathway. In addition, Rho1p may participate in the synthesis of α-1,3 glucan, the main structural component of the yeast cell wall in *P. brasiliensis*. The synthesis in *S. cerevisae* occurs at the cell surface by action of two differentially expressed glucan synthases, Fks1p and Fks2p. For Fks1p to be active, Rho1p must be in its GTP-bound form (Gustin et al., 1998).

Osmoregulation by the HOG pathway

To maintain cellular homeostasis, the fungal cells need to have a way to avoid dehydration in conditions of solute excess in the extracellular environment. One strategy is the accumulation of glycerol inside the cell, a compatible substance that avoids cellular water outflow in high osmolarity conditions (Hohmann, 2002).

One system of glycerol production is the HOG pathway. It uses some proteins that are sensitive to differences in the concentration of solutes in the environment and transmit signals to the cell to promote osmoadaptation. The initialization of glycerol production by HOG can be triggered by two membrane osmosensors that detect osmotic alterations and transduce signals to a series of proteins that will activate MAPK *HOG1* (O'Rourke and Herskowitz, 2004).

Sho1p and Sln1p, two osmosensors situated in the membrane, can detect extracellular hyperosmolarity. They transfer signals from the environment through a cascade that will activate Pbs2p (MAPKK), which phosphorylates Hog1p (Marles et al., 2004).

Saccharomyces cerevisiae Sln1p is a histidine-kinase protein that contains 1,220 amino acid residues, with homologues in some fungi, such as *C. albicans* and *P. brasiliensis*. Sln1p is essential for osmoadaptation in the budding yeast *S. cerevisiae*, in which it acts as a negative regulator of the HOG pathway; its deletion in this organism is lethal (Posas et al., 1996). In *C. albicans*, Sln1p is partially required for osmoadaptation, and *sln1* mutants have defective morphogenesis and are less virulent (Nagahashi et al., 1998). At a constant osmotic pressure, Sln1p inhibits the expression of Ssk1p, which is required in one branch of this pathway. Under high extracellular osmolarity, Sln1p is inhibited and Ssk1p is activated, transmitting signals to the other MAPKKs, such as Ssk2p (O'Rourke and Herskowitz, 2004).

The second osmosensor is Sho1p, a protein with 367 amino acid residues, found mainly in polarized growth regions of *S. cerevisiae* cells. Under conditions of high osmolarity, Sho1p transfers the signal to Ste20p, a PKA that activates MAPKKK Ste11p, which is required for activation of Pbs2p.

Glycerol production is triggered when the Hog1p is phosphorylated by Pbs2p and translocated to the nucleus, where it promotes the activation of several genes involved in the production of glycerol, which accumulates inside the cell (O'Rourke and Herskowitz, 2004).

The *in silico* analysis of PbAESTs describes the components of the HOG pathway in *P. brasiliensis* (Table 1). The comparison of the Pb HOG proteins with their homologues in *C. albicans* and *S. cerevisiae*, as well as the multiple sequence alignment in Figure 2, which shows the high conservation of the MAPK domain and the kinase-associated domain 1 of Hog protein, all suggest that *P. brasiliensis* is able to use this same strategy to avoid cell dehydration.

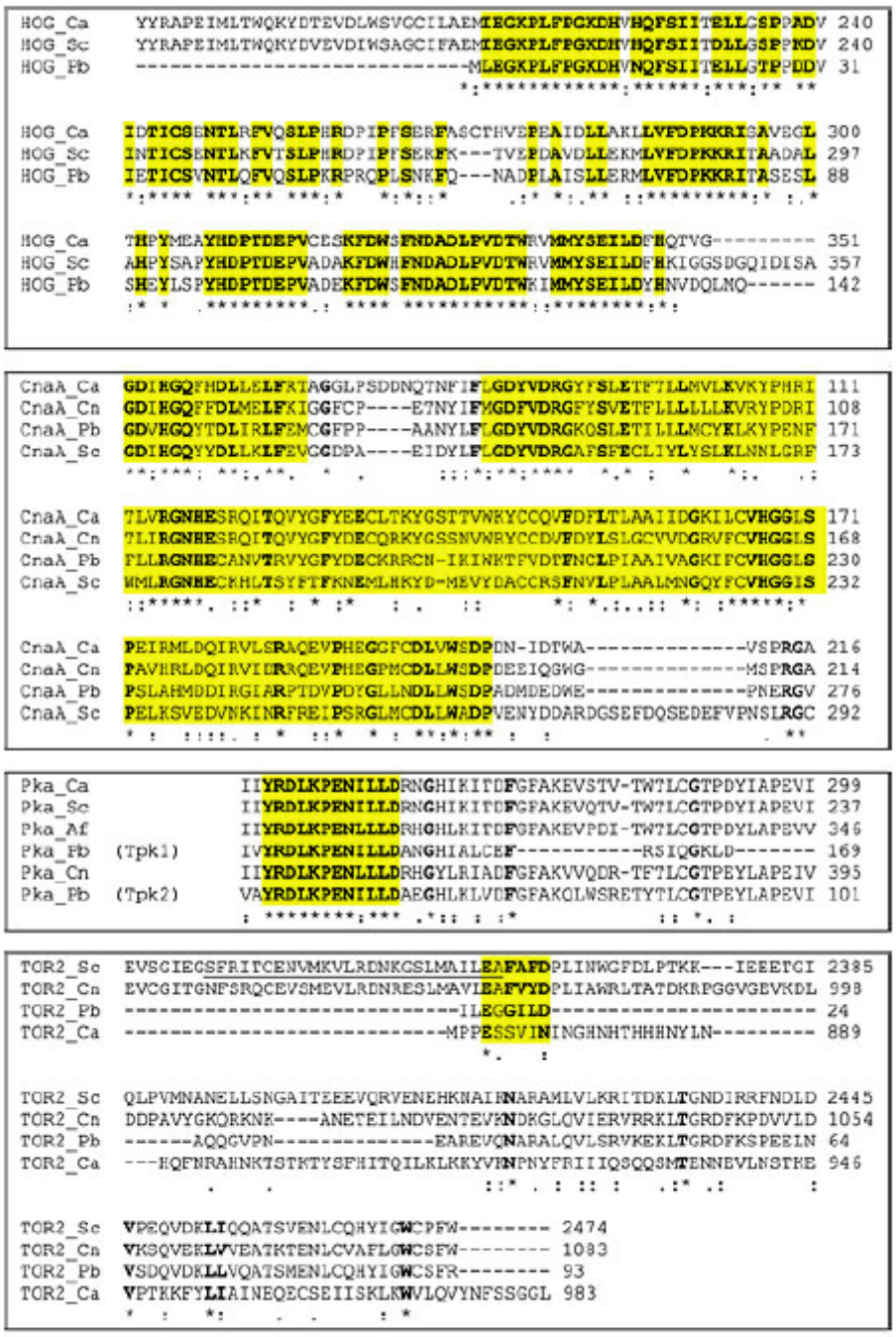

Figure 2. Multiple sequence alignment of the common core of HOG, calcineurin (CnaA), Pka and TOR from *Candida albicans* (Ca), *Saccharomyces cerevisiae* (Sc), *Aspergillus fumigatus* (Af), *Cryptococcus neoformans* (Cn), and *Paracoccidioides brasiliensis* (Pb), as performed in the CLUSTAL W server (Aiyar, 2000). The common domains identified by MIPS (http://mips.gsf.de/proj/yeast/CYGD/db/) are in yellow. The amino acids that are identical within and outside the MIPS domain are in bold.

cAMP/PKA pathway

Cyclic AMP (cAMP) is the regulatory component of a well-characterized signaling pathway implicated in a variety of cellular process among fungal species. Various components of the cAMP signaling pathway were identified through transcriptome analysis of *P. brasiliensis*, including adenylate cyclase Cyr1p, two cAMP-dependent protein kinase catalytic subunits, Tpk1p and Tpk2p, cAMP-dependent protein kinase regulatory subunit Bcy1p, and a low affinity phosphodiesterase, Pde1p (Table 1).

cAMP/protein kinase A (PKA) pathway elements have been dissected in various fungi. In *C. neoformans*, the cAMP signaling cascade is necessary for production of the capsule, mating filaments and melanin, all of them considered virulence factors for this organism. Mutants of the *CAC1* gene for adenylate cyclase in *C. neoformans* are defective in capsule production and melanin synthesis; they fail to produce mating filaments and were found to be avirulent in a mouse model of cryptococcosis (Pukkila-Worley and Alspaugh, 2004). In *C. albicans*, adenylate cyclase - Ca*CDC35*, PKA catalytic subunits (Tpk1p and Tpk2p) and Efg1p (hypha-specific transcriptional activator), all of them elements of the cAMP cascade, are involved in the regulation of vegetative growth and are essential for the serum-induced switch of yeast to hyphae, which has to do with the virulence of this fungus (Rocha et al. 2001; Cloutier et al., 2003). Mutants of Ca*CDC35* are unable to switch from yeast to hyphae, and they are unable to establish vaginal infection in a mouse model of candidiasis; they are also avirulent in systemic disease (Rocha et al., 2001). In the human pathogen *A. fumigatus*, elements of the cAMP pathway, such as ACYA (adenylate cyclase), PKAC and PKAR (PKA catalytic and regulatory subunits, respectively) have recently been shown to regulate the virulence gene pksP (Liebmann et al., 2003). The Δ*acyA* mutants of *A. fumigatus* have severely impaired sporulation and growth (Liebmann et al., 2003) and are more susceptible to being killing by human monocyte-derived macrophages.

The yeast prototype, *S. cerevisiae*, in which the cAMP signaling pathway elements are best known. This cascade is activated by nitrogen starvation, and it transduces the signals that control the phenotypic switch in haploid cells that promote invasive growth. In diploid cells, these signals transform budding yeast into elongated forms that constitute a chain known as a pseudohypha. In addition, cAMP controls cell growth, metabolism, stress resistance, and agar adherence (Lengeler et al., 2000). The regulation of adenylate cyclase activity differs among the species. In *S. cerevisiae*, Ras proteins and the Gα protein *GPA2* activate adenylyl cyclase in response to nutrient conditions and intracellular acidification (Colombo et al., 1998); however, in *Schizosaccharomyces pombe*, adenylate cyclase is regulated by Gpa2p, and Ras1p has no effect (Fukui et al., 1986). In *C. neoformans* and *A. fumigatus*, *GPA1* and *GPAB* were identified; both of their Gα protein homologues are involved in the activation of this pathway (Madhani et al., 1999; Liebmann et al., 2003). The G-protein-coupled-receptor (*Gpcr*), the upstream component responsible for sensing the nitrogen limitations in *S. cerevisiae* (Pan et al., 2000), was also identified in *P. brasiliensis*, although it is still not described in *C. neoformans*, *C. albicans* and *A. fumigatus*. In *S. cerevisiae*, *Cyr1p* (adenylate cyclase) is an essential gene responsible for the progression of cells through the G1 phase (Mosch et al., 1999), while in *C. albicans*, *A. fumigatus* and *C. neoformans*, it is not an essential gene.

Despite the high degree of conservation of the signaling cascade elements among fungi, their specific functions differ greatly. The multiple sequence alignment in Figure 2 shows that *P.*

brasiliensis has two different catalytic PKA subunits, defined as Tpk1p or *TPK1* (PBAEST1562) and Tpk2p or *TPK2* (PBAEST3488), with a very conserved serine/threonine protein kinase catalytic domain. The enzymatic activity of these protein kinases is controlled by the phosphorylation of specific residues in the activation segment of the catalytic domain.

The differences in the roles of adenylate cyclase can be explained by the fact that the cAMP pathway in *S. cerevisiae* is primarily involved in mediating nutritional signals to the cell cycle machinery, whereas in pathogenic or opportunistic fungi, the main function of these components is to sense and transduce stress signals from the host environment to the morphogenetic machinery that controls virulence and pathogenicity factors, thus establishing infection and allowing survival of the pathogen.

In the dimorphic human pathogen *Histoplasma capsulatum*, it has been shown that during the transition from budding yeast to mycelium, which is dependent on a temperature shift from 37° to 25°C, there is an increase in intracellular levels of cAMP associated with this dramatic morphological switch. In addition, exogenous cAMP and phosphodiesterase inhibitors allow the Y-M transition, even at 37°C (Sacco et al., 1981).

In our biological model, *P. brasiliensis*, evidence that cAMP has a role in the dimorphic transition, which is closely related to virulence, was first reported in 1985 by Paris and Duran, who observed that exogenous cAMP inhibits rather than induces the process of filamentation in this organism. The identification of cAMP/PKA signaling pathway components suggests a possible mechanism for cAMP involvement in the temperature-dependent dimorphic transition from mycelium to yeast. However, the complexity of the pathogenesis regulation in *P. brasiliensis* requires further studies of each signaling element.

The roles of Ras

RAS proteins belong to the *Rho* family - a superfamily of GTPases. It is a small guanine nucleotide-binding protein bound to the plasma membrane and involved in the regulation of several cell functions. It hydrolyses GTP; the GTP-bound form is active and GDP is inactive (Waugh et al., 2002). In human cells, this class of proteins plays a role in transmitting growth regulation signals from membrane-localized tyrosine-kinase receptors to different pathways, and ultimately to the nucleus. Some mutations in the RAS1 gene have been associated with unchecked cell growth, resulting in cancer (Barbacid, 1987). In *S. cerevisiae*, the Ras proteins regulate pseudohyphal growth and act upstream of the MAPK and cAMP/PKA pathways (Mösch et al., 1999). In *C. neoformans*, Ras1 protein is involved in filamentation, mating and high-temperature growth. The *ras1* mutants are avirulent in meningitis animal models; they have a growth defect at 37°C that arrests them as large unbudded cells with depolarized actin and loss of cytoskeletal asymmetry. They are unable to produce filaments (Waugh et al., 2002). A homologue of RAS was also identified in *P. brasiliensis* (Table 1) and in *C. albicans*, where it is not an essential gene, but it is involved in hyphal growth, since *ras1/ras1* mutants have a severe defect in filamentation in response to serum (Leberer et al., 2001). In *C. albicans*, Ras1p is also implicated in the cross talk between the MAPK and cAMP/PKA systems for the activation of the hypha-specific transcriptional factor Efg1, which is involved in the dimorphic transition from yeast to hyphae. The *RAS* gene should be further investigated in *P. brasiliensis* to elucidate its function in the Y-M transition and its possible involvement in pathogenicity.

Calcium-calmodulin-calcineurin dealing with stress

Among the diverse activities performed by cells, adaptation to changes in the environment is crucial for their viability. Calcium signaling is important in eukaryotes in numerous cellular processes, including the alteration of gene expression in response to external stimuli. Two important mediators of calcium signals in eukaryotic cells are the Ca^{2+}-binding protein calmodulin (Camp) and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin.

Calmodulin is present in all eukaryotic cells, including *P. brasiliensis* (Table 1). In the cytoplasm, it has been implicated in the regulation of protein kinases, protein phosphatases, transcription factors, motor proteins, and cytoskeletal components. Calmodulin is usually genetically represented by a single copy gene that varies from 1 to 15 kb (de Carvalho et al., 2003; Kraus and Heitman, 2003). Inhibitors of the calmodulin pathway are able to impair the transition from mycelium to yeast cells in *P. brasiliensis* (de Carvalho et al., 2003).

In *S. cerevisiae* studies using temperature-sensitivity mutants of Camp, it has been found that this is an essential protein for cytoskeletal actin organization, endocytosis, and nuclear division (Desrivieres et al., 2002). In *C. albicans*, calcium signaling via calmodulin is important for the transition from the yeast to the filamentous form. Camp, which regulates cell proliferation and mediates the secondary messenger functions of Ca^{2+}, was also identified in several fungi, including *Phycomyces blakesleeanus*, *Neurospora crassa* and *A. nidulans*. Recent studies reported that Camp plays a vital role in chitin synthase activation by mediating phosphorylation of specific microsomal protein kinases during chitin formation in *N. crassa* (Suresh and Subramanyam, 1997).

Calcineurin is a serine-threonine-specific phosphatase that is conserved among eukaryotes. It consists of a catalytic subunit (CnaAp) and a Ca^{2+}-binding regulatory subunit (CnaBp), and the association of the two subunits is essential for activity (Watanabe et al., 1996). Activation of calcineurin occurs when Ca^{2+}/calmodulin binds to the C-terminal region of CnaAp, resulting in a conformational change that releases the active site from an auto-inhibitory domain. In *C. neoformans*, it is required for mating and haploid fruiting, for growth at elevated temperature and for virulence. Calcineurin participates in the morphogenesis of *S. pombe* by altering septal positioning and aberrant spindle body organization (Yoshida et al., 1994; Odom et al., 1997). Calcineurin was identified in *P. brasiliensis* transcriptome analysis and aligns well with its known homologues (Figure 2), which is also true for the catalytic serine/threonine domain specific to 2A phosphatases.

In *S. cerevisiae*, the expression of the truncated CnaAp resulted in cell elongation with a unipolar budding pattern (Mendoza et al., 1996). In *C. neoformans*, calcineurin plays a role in hyphal elongation, observed in mating and in the survival of the heterokaryon produced by cell fusion. It is also required for hyphal elongation in diploid strains and during asexual haploid budding of MATα cells in response to nitrogen limitation (Cruz et al., 2001).

Activated calcineurin dephosphorylates and activates the transcriptional factor Crz1p/Tcn1p, which enters the nucleus and, in turn, activates a set of responsive genes by binding to calcineurin-dependent responsive elements. It has been well documented that Crz1p/Tcn1p mediates most of the transcriptional responses driven by the activation of calcineurin under stress conditions (Viladevall et al., 2004). The genes that are controlled by Cna1p/Crz1p encode products that promote cell survival under stress. Crz1p is the best-characterized substrate of calcineurin in yeast, although recent studies on the identification of new substrates promise to

provide novel insights into additional functions of calcineurin *in vivo* (Cyert, 2003). Most Tcn1p-dependent genes can be differentially induced based on mechanisms of sensitivity to Ca^{2+} signals and other regulatory inputs (Matheos et al., 1997).

Calcium-mediated signaling mechanisms are used by virtually every eukaryotic cell to regulate a wide variety of cellular processes, including the maintenance of cell integrity under various types of stress. *Paracoccidioides brasiliensis* may use this machinery to protect itself against the harsh environment of the host.

TOR signaling pathway

TOR (target of rapamycin) is a phosphatidylinositol kinase-related protein kinase that controls cellular functions necessary for cell growth and proliferation in response to nutrients (Helliwell et al., 1998). In a recent study, it was reported that TOR acts within a growth regulatory network mediated by nutrient availability. This network affects all aspects of gene expression, including transcription, translation, and protein stability (Powers et al., 2004). In addition, the TOR pathway regulates the developmental program of pseudohyphal differentiation in concert with highly conserved MAPK and PKA signaling cascades (Rohde and Cardenas, 2004).

In *S. cerevisiae* and *S. pombe*, there are two TOR proteins, Tor1p and Tor2p; however, other organisms, such as *C. albicans* and *C. neoformans*, have only one TOR homologue (Cruz et al., 1999). The function of Tor1p is conserved throughout evolution; it is involved in translation initiation, transcription, ribosome biogenesis and tRNA synthesis (Schmelzle et al., 2004). In addition, Tor2p has two functions: one that is redundant to that of Tor1p and another that is unique, controlling the organization of the actin cytoskeleton during the cell cycle. Tor2p is not sensitive to rapamycin, being inhibited only by nitrogen starvation (Rohde and Cardenas, 2004).

Tor2p activates Rho1p GTPase, exchanging the GDP of the inactive form for GTP. This exchange is achieved by Rom2p protein, a guanidine nucleotide exchange factor, whereas Sac7p is a GTPase-activating protein. Rho1p phosphorylates *Pkc1p*, which activates the MAPK cascade Bck1p, Mkk1/2p and Mpk1p (Hay and Sonenberg, 2004).

In the course of *P. brasiliensis* transcriptome analysis, *TOR2*, *ROM2*, *RHO1*, *SAC7*, *PKC1*, *BCK1*, *MKK1*, *MPK1*, and *FPR1* homologues were identified (Table 1). Although Tor1p was not identified, some components of its signaling pathway, such as Cdc55p, Tpd3p and Sit4p are present in *P. brasiliensis*. Therefore, Tor1p may be present in the *P. brasiliensis* genome, unless Tor2p fulfils both functions in *P. brasiliensis*. Multiple sequence analysis of Tor2p revealed a kinase domain (Figure 2). Tor protein has a C-terminal region homology with the catalytic domain of phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase. This domain is characteristic of the TOR family and is essential to its function (Crespo and Hall, 2002).

CONCLUSIONS

Transcriptome analyses are a powerful tool to investigate organisms such as *P. brasiliensis*, in which the direct genetic analysis has many drawbacks and for which there is no efficient method of direct genetic analysis. By this comparative approach, it was possible to identify several components of signaling pathways in *P. brasiliensis* known to regulate cell events, such as morphogenesis and virulence. These components were found to be similar to

those of other fungi. We suggest that *P. brasiliensis* has co-opted a conserved signaling pathway to regulate phenotypes required for virulence, as have the other pathogenic microorganisms. However, there are still regulatory connections that need to be understood, in particular the broad transcriptional effects caused by the loss of the aforementioned cascades. The identification of pathogen-specific signaling events will help us understand *P. brasiliensis* pathogenesis, which could lead to the development of new antifungals.

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Transcriptional profile of *ras1* and *ras2* and the potential role of farnesylation in the dimorphism of the human pathogen *Paracoccidioides brasiliensis,*

Title: Transcriptional profile of *ras1* and *ras2* and the potential role of farnesylation in the dimorphism of the human pathogen *Paracoccidioides brasiliensis*

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Running title: *ras* expression and farnesylation role in *P. brasiliensis*

Abstract:

Paracoccidioides brasiliensis is a thermo-dimorphic fungus that causes a human systemic mycosis with high incidence in Latin America. Due to their participation in the control of pathogen morphogenesis, differentiation and virulence we decided to characterize *ras* genes in *P. brasiliensis*. We identified *ras1* and *ras2* coding for two different proteins with high identity. The *ras* transcriptional pattern was investigated by Reverse Transcription PCR (RT-PCR) during mycelium-to-yeast (M→Y) transition, heat shock at 42 ºC and after internalization of yeast cells by murine macrophages. Both genes were down regulated inside macrophages and *ras1*, at 42 ºC. In contrast, *ras* genes did not show any transcriptional variation during the M→Y transition. The fact that Ras proteins are attached to the membrane via farnesylation prompted us to use a farnesyltransferase inhibitor to investigate the importance of that process to vegetative growth and dimorphic transition. Farnesylation blockage interfered with vegetative growth of yeast cells and stimulated germinative tube production even at 37 ºC. During Y→M transition the inhibitor increased filamentation in a dose-dependent manner, indicating that impaired farnesylation favours the mycelium form of *P. brasiliensis*. The results suggest that *ras* genes might have a role in dimorphism, heat shock response and in host-pathogen interaction.

Introduction

Paracoccidioides brasiliensis (Pb) is the aetiological agent of the paracoccidioidomycoses (PCM), one of the human systemic mycosis with high prevalence in Latin America (Brummer *et al.,* 1993). About 10 million people are estimated to be infected with this pathogen (Restrepo *et al*., 2001). Despite the high incidence, little is known about how the infective form enters its host. The main hypothesis is that the disease is acquired through inhalation of propagules, since the ecological niche of this fungus is probably the soil (De Albornoz, 1971; Ferreira *et al*., 1990; Silva-Vergara *et al*., 1998).

P. brasiliensis is a thermo-dimorphic fungus, appearing as mycelium at 22 ºC and as yeast at 37 ºC. Both forms are multinucleated (San-Blas, 1993). No teleomorphic (sexual) state has been determined as of yet; the same is also true for the exact ploidy; all of which makes classical genetic analysis unfeasible. Genome size ranges from 25.8 to 75.6 Mb, while the estimated ploidy and number of nuclei per cell varies with the isolate (Feitosa *et al*., 2003). Some strains that are unable to switch from mycelium to yeast are avirulent, which implies that the pathogenicity of *P. brasiliensis* appears to be related to the ability to differentiate (San-Blas & Nino-Vega, 2001). The components of the signalling pathways of *P. brasiliensis* such as: cAMP/PKA, Ca^{+2}/calmodulin-calcineurin (de Carvalho *et al*., 2003) and MAP-Kinases were found in *P. brasiliensis* through transcriptome analysis and reverse annotation. (Felipe *et al*., 2005; Fernandes *et al*., 2005).

The *ras* genes are conserved from fungi to humans (Barbacid, 1987). Some mutations that cause dominant activation of RAS are highly associated with tumours in mammalian cells (Barbacid, 1987). The Ras proteins belong to a small GTP-binding proteins family with GTPase activity. The conversion of Ras from an inactive GDP-bound form to an active GTP-bound one occurs in response to a variety of extracellular stimuli (Wiesmuller & Wittinghofer, 1994). For Ras proteins to work properly they must be attached to the membrane by the addition of a farnesyl group, a lipid intermediate from ergosterol biosynthesis in fungi, to their carboxy terminal region, a step catalyzed by the farnesyltransferase enzymes. Ras genes have been identified as key components in signalling cascades in diverse fungi species such as *Saccharomyces cerevisiae* (DeFeo-Jones *et al*., 1983), *Cryptococcus neoformans* (Alspaugh *et al*., 2000; Waugh *et al*., 2002), *Candida albicans* (Feng *et al*., 1999; Leberer *et al*., 2001), *Aspergillus fumigatus* (Fortwendel *et al*., 2004; Fortwendel *et al*., 2005), *Penicillium marneffei* (Boyce *et al*., 2005), and others. In these organisms Ras proteins act as sensors of environmental conditions to control diverse cellular processes including cell cycle progression, cAMP synthesis, cell differentiation and morphogenesis, cytoskeleton organization and expression of virulence genes in pathogens. In the budding yeast *S. cerevisiae* Ras proteins are involved in life span, response to stress including heat

The *ras2* of *P. brasiliensis* (Pb01 isolate) was first identified in the Pb01 transcriptome project analysis (Felipe *et al.*, 2003; Felipe *et al.*, 2005). The *C. neoformans RAS2* gene sequence was used to blast search on the PbAEST (*P. brasiliensis* Assembled EST) Bank, thus identifying PbAEST983, which shows 76.5 % identity to the probe and had an alignment e-value of 1e-09. The corresponding clone was sequenced and found to be the *ras2* gene of *P. brasiliensis* isolate Pb01. Based on *ras1* and *ras2* sequences, PCR amplification was carried out using genomic DNA as template to reconstitute the complete gene sequences and to evaluate the presence of introns.

Total RNA extraction of *P. brasiliensis*

P. brasiliensis total RNA was extracted using Trizol reagent (Invitrogen), following suppliers' recommendations, as previously described (Venancio *et al.*, 2002). To remove any genomic DNA contamination, RNA was treated with RNase-free-DNase I (Promega) followed by enzyme inactivation (EDTA 2,5 mM; 65 °C / 10 min) and ethanol precipitation.

Infection of J774 macrophage cell line with *P. brasiliensis* yeast cells and total RNA extraction

The experiments were performed according to Tavares *et al.*, 2007. Briefly, the macrophage-like cell line J774.1 (ATCC TIB-67) was cultured as an adherent monolayer in RPMI-1640; then infected with 2×10^6 opsonized yeast cells, with a yeast-to-macrophage ratio of 1:5, and incubated for nine hours at 37 °C in an atmosphere containing 5 % CO_2. Yeast cells that were not internalized in the period were removed by washing with warmed RPMI-1640 and macrophages were lysed with guanidine thiocyanate based solution (Monahan *et al.*, 2002). Intact fungi were harvested by a rapid centrifugation followed by Trizol RNA extraction and amplification using the MessageAmp aRNA kit (Ambion) was performed. A single macrophage infection experiment produces about 3 μg of *P. brasiliensis* RNA; because of this limitation, the RNA yields from 3-4 independent macrophage infection experiments were pooled. Total RNA from *P. brasiliensis* yeast cells cultured in Fava-Netto's medium for seven days was extracted and amplified exactly as described above, and used as control.

Southern blot

Total DNA was extracted following Raeder & Broda (1985). A hundred micrograms of DNA were digested with the restriction endonucleases *Bam*HI (QBiogene), *Eco*RI, *Hind*III, *Pst*I and *Xba*I (all from Promega) in the proportion of 5 $U \bullet \mu g^{-1}$ DNA as recommended by Sambrook & Russel (2001). All digestions were carried out at 37 °C and incubated over night. Ten micrograms of DNA from each digestion were electrophoresed in a 0.8 % agarose gel. The gel was blotted

onto a charged nylon membrane (GE Healthcare) by upward capillary transfer. The *ras1* and *ras2* cDNA probes were amplified by PCR using specific primers (for *ras1*: ras1/1-5'-CTTGCTGGTCTACTCCATCACTTC, ras1/3-3'-CAGATGAATACGAGGACATCTC; for *ras2*: ras2/1-5'-CAACGGGACTCAGCTACC, ras2/2-3'-CCACAAGCATTACTGGAAC). A total of ten nanograms of purified product were chemically labelled using the AlkPhos Direct labelling and detection system (GE Healtchare). All procedures were based on suppliers' recommendations.

Semiquantitative RT-PCR

The transcriptional analysis of *P. brasiliensis ras1* and *ras2* was carried out employing sqRT-PCR methodology. Briefly, the cDNA first strand was synthesized from two micrograms of DNAse-treated total RNA using the SuperScript II Reverse Transcriptase (Invitrogen) following supplier's recommendations. Five microlitres of reaction were amplified in a final reaction volume of 25 μL containing *Taq* DNA Polymerase buffer 1X, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM specific primers (*ras1:* ras1/1-5'-CTTGCTGGTCTACTCCATCACTTC, ras1/3-3'-CAGATGAATACG AGGACATCTC or *ras2:* ras2/1-5'-CAACGGGACTCAGCTACC, ras2/4-3'-GGAGAGCTCCATCATTTCCC), two units of *Taq* DNA Polymerase (Cenbiot-RS/Brazil) and 0.2 μM *l34* ribosomal protein or clathrin light chain primers (*clat*) as internal controls (*l34*: l34-5'-GTCCGCATCATCAAGACTCC, l34-3'- CTTGACACAGCCAGCGCAG; *clat*: clat-5'-CCTGGGTGAAGATGCGGATC, clat-3'-GGATGTGCCTGTGATGGTTC). The PCR programme consisted of a first step of denaturation (94 ºC for 2 min), 24 cycles of 94 ºC for 1 min, 60 ºC for 1 min and 72 ºC for 1 min; and a final extension at 72 ºC for 10 min. The appropriated number of cycles, in which the amplification is on the exponential range, was defined testing a range from 24 to 35. The optimal number of cycles should be in the same range for the specific gene of interest and for the housekeeping gene used as an internal control (Marone *et al*., 2001). Since the intensity of specific amplification products reached a plateau at around 30 cycles (data not shown), all semiquantitative analyses employed 24 cycles. Amplicons were analyzed by 1.5 % agarose gel electrophoresis. Each set of reactions always included a negative control containing RNA instead of cDNA, to rule out genomic DNA contamination. The quantification of gene expression levels was performed by densitometry analysis (Scion Image software) available on-line (http://www.scioncorp.com). Amplified product intensity was expressed as relative absorbance units (AU). The ratio between the relative AU determined for the amplified gene of interest and the internal control was calculated to normalize variations for sample concentration and as a control for reaction efficiency. Mean and standard error of all performed experiments were calculated relative to the amplification of the control gene. Statistical analyses were

performed using the software "Mynova", version 1.3 (S. Brooks, Copyright 1993). The statistical test applied was Student´s *t* test. A *P* value ≤ 0.05 was considered significant.

Drug susceptibility testing

Drug susceptibility tests were adapted from the international standard M27-A2 (National Committee for Clinical Laboratory Standards, 2002). *P brasiliensis* yeast cells were grown on Fava-Netto semisolid medium for 5 – 7 days at 37 °C, harvested and diluted in RPMI-1640 medium to a final concentration of 10^5 cells·mL^{-1}. The experiment was carried out in U-shaped 96-well plates with 100 µL of final volume per well. Amphotericin B was also used as control and the concentrations assayed varied from 4 µg·mL^{-1} to 0.39 ng·mL^{-1}. The FPT Inhibitor III ((E,E)-[2-Oxo-2-[[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]amino]ethyl]phosphonic Acid, (2,2-Dimethyl-1-oxopropoxy)methyl Ester, Na) (Calbiochem) was ressuspended and diluted in the culture media at concentrations varying from 500 to 0.43 µM. The microplate was incubated 7 – 10 days at 37 ºC. Yeast growth inhibition was quantified via the OD_{595nm} and each drug concentration was tested in triplicate.

To assess the effect of FPT Inhibitor III on the vegetative growth of yeast and mycelium of *P. brasiliensis*, the same procedure was performed, except that the culture medium employed was the defined McVeigh Morton (MVM) medium (Restrepo & Jiménez, 1980) supplemented with 2 % glucose as previously described by Hahn & Hamdan (2000). For the mycelium, hyphal fragments were inoculated into Brain Heart Infusion (BHI) and growth was allowed for ten days at 22 ºC under agitation of 150 rpm. The supernatant was then removed and hyphae were washed three times with 0.9 % saline solution and dispersed by passing through a hypodermic syringe with a 30 gauge needle to produce small mycelium fragments, which were counted in a haematocytometer. Density was adjusted to about 10^5 fragments•mL^{-1}. The microplate was incubated for ten days at 22 ºC.

FPT Inhibitor III testing during the *P. brasiliensis* differentiation.

The effect of the farnesylation inhibitor on the *P. brasiliensis* temperature-driven dimorphic transition was determined on the same concentrations previously assayed for the MIC testing. Inocula for yeast and mycelium were also prepared as described above. After preparation, yeast and mycelium plates were kept for 48 hours at 37 ºC and 22 ºC, respectively. Each plate was then shifted to the other temperature to allow the dimorphic transition and incubated for ten days. After

the incubation period, cell morphology was observed under a Nikon microscope. Pictures were taken using a Sony DSC-W5 digital camera and three-fold optical zoom.

Results and discussion

Identification and molecular characterization of *ras1* and *ras2* genes in *P. brasiliensis*

The *ras1* gene is composed of two exons separated by a single intron of 99 bp. The *ras1* 636-bp open reading frame (ORF) encodes a putative protein sequence of 212 amino acids with four conserved GTP-binding domains, an effector domain and the farnesylation site (CVIM). The complete *ras1* sequence was deposited at GenBank under accession number **DQ157363**. The *ras2* gene contains two exons separated by one intron of 89 bp, an ORF of 714 bp that codifies a putative protein of 238 amino acids also with the same five domains and a farnesylation site (CLIL). All these consensus motifs are characteristic of Ras proteins (Bourne *et al.*, 1991). The complete *ras2* sequence was deposited at GenBank under accession number **AY910576** (**Fig. 1a**).

The *in silico* analysis of the *P. brasiliensis* Ras1 and Ras2 deduced protein sequences revealed through a multi-alignment programme that all Ras-GTPases are closely related. Blast search showed that both *P. brasiliensis* Ras are highly conserved relative to other fungi, with similarity varying from 45.7 to 79.3 % for Ras1 and 39 to 82 % for Ras2; best matches were to *A. fumigatus* RasA and RasB, respectively. Similarity and identity of 41.3 and 51.4%, respectively, were observed between *P. brasiliensis* Ras1 and Ras2. Molecular masses were estimated at 24.19 kDa (Ras1) and 26.51 kDa (Ras2), well in the range of small GTPases.

The comparison of Ras sequences shows them to be quite conserved on the four GTP-binding sites and the effector domain, but the prenylation site is variable. It is important to note that the prenylation site (CAAX) of Ras2, which is CLIL, has leucine for X, which favours the addition of a geranyl group instead of a farnesyl group as expected for the Ras-GTPase family. The presence of a leucine as X in the carboxy region is also observed in *C. neoformans* Ras1 (CVVL) and *A. fumigatus* RasB (CVIL). Special attention must be given to the Ras2 region of about 20-30 amino acids that is absent in Ras1 (**Fig. 1a**). This region (22 amino acid residues in *P. brasiliensis*) is found in almost all Ras2 sequences analyzed, including *A. fumigatus* RasB, *C. neoformans* Ras2 and *Neurospora crassa* Ras2. A phylogenetic tree based on sequence homology has been generated in which it is clear that Ras1 and Ras2 groups are clustered apart. Both *P. brasiliensis* Ras are grouped alongside Ras proteins from *A. fumigatus* and *N. crassa*, suggesting that these fungi are in close proximity (**Fig. 1b**).

Figure 1

To confirm the presence and the copy number of the two *ras* genes in *P. brasiliensis* isolate Pb01, we chose *EcoR*I, *Hind*III, *Pst*I, which do not have cut within either sequence, to digest genomic DNA. *BamHI* has one site in *ras2* but not in *ras1*. It is important to mention that the probe for *ras2* hybridization was the 75 bp corresponding to the 22-residue region that is not present in the *ras1* sequence, thus enabling specific hybridization of *ras2* and not *ras1*. For the *ras1* probe we select the 300-bp of the 3' region because of its low similarity to *ras2*. Hybridization detection revealed a single band in all lanes probed for each gene (**Figure 2**). These results demonstrate that *P. brasiliensis* has two different *ras* genes, *ras1* and *ras2* on its genome as observed for most other fungi, except for *C. albicans* and *Schizosaccharomyces pombe*, in whose genome just one *RAS* gene was identified (Leberer *et al*., 2001, Fukui & Kaziro, 1985).

Figure 2

Transcriptional profiles of *P. brasiliensis ras1* and *ras2* under different conditions

To investigate the *ras* transcriptional pattern we first performed a northern-blot analysis using RNA extracted from mycelium and yeast of *P. brasiliensis* but we were unable to detect any transcripts of either *ras* genes in any conditions, even with 15µg of total RNA per lane (data not shown). Such an impossibility using this same approach was previously reported for the *ras* genes of *C. neoformans* (Waugh *et al*., 2002), *A. fumigatus* (Fortwendel *et al.,* 2004) and *P. marneffei* (Boyce *et al.,* 2005). We decided to evaluate the *ras* transcriptional pattern by semiquantitative RT-PCR. As we had not any information about the *ras1* and *ras2* in the pathobiology of *P. brasiliensis*, we started by analysing the transcriptional pattern of *ras* genes on the temperature-dependent dimorphic transition from mycelium to yeast, under heat shock at 42 ºC, and during internalization of yeast in murine macrophage cells, which mimics several aspects of host infection.

***In vitro* expression of *ras1* and *ras2* of *P. brasiliensis* during dimorphism and under heat shock at 42 ºC**

As Ras proteins are key components of cascades controlling cellular events such as serum-induced pseudo-filament formation in *C. albicans* (Feng *et al*., 1999; Leberer *et al*., 2001) and haploid fruiting in *C. neoformans* (Alspaugh *et al*., 2000; Waugh *et al*., 2002), we speculated that they would suffer some regulation pointing the course of the dimorphic transition in *P. brasiliensis*. We evaluated expression levels of *ras1* and *ras2* on cells undergoing the first 24

hours of the mycelium-to-yeast transition. It is important to mention that we chose as the internal control the gene coding for the chlathrin light chain (*clat*) since its corresponding PbAESTs were equally distributed in the mycelium and yeast partial transcriptomes analysed by our laboratory (Felipe *et al*., 2003 and Felipe *et al*., 2005). The sqRT-PCR confirmed its usefulness as constitutive expression control (data not shown). Total RNA from each point of the differentiation was used in sqRT-PCR experiments with specific primers directed to *ras1* and *ras2* genes and to the internal control *clat*. Amplicons corresponding to *ras1*, *ras2* and *clat* transcripts were quantified by densitometry. **Fig. 3a** shows the quantification levels of the *ras1* or *ras2* product during the temperature-dependent dimorphic transition. After appropriate statistical analysis, we did not observe significant difference on expression levels of *ras1* or *ras2* at any point of the differentiation. The cellular differentiation dependent upon temperature change in dimorphic fungi is an event in which many genes must be tightly regulated. Nunes *et al.* (2005) identified by microarray 2583 genes with modulated expression at some point of the differentiation of *P. brasiliensis* isolate Pb18. Among them figured as positively regulated in the M to Y direction: the calcineurin regulatory subunit, the Ca^{2+}/calmodulin-dependent protein phosphatase, protein kinase A and G proteins α and β. These proteins are common components of known signalling cascades that are involved in morphogenesis, differentiation, cell integrity, growth at high temperature and virulence in several pathogens. Our results indicate that *P. brasiliensis ras* genes do not have the expression modulated by temperature shifts and appear as constitutive genes during the dimorphic transition.

The involvement of Ras protein-controlled pathways in the heat shock response was reported previously for *S. cerevisiae* (Engelberg *et al.*, 1994). Independently of heat shock factor (HSF), the authors showed induction of Heat Shock Proteins (HSPs) when Ras/cAMP/ PKA signalling was blocked in yeast cells exposed to heat shock conditions, resulting in a thermo-tolerant phenotype. To analyze whether *P. brasiliensis ras* genes suffer any transcriptional modulation under heat shock, yeast cells were subjected to temperature shifts from 37 ºC to 42 ºC for 0, 15, 30, 60 and 120 min. The result showed that transcription of *P. brasiliensis ras1* is modulated by heat shock at 42 ºC. After 30 to 120 min *ras1* expression decreases sharply, falling to the lowest level at the 60-minute point (**Fig. 3b**). Conversely, *ras2* is not induced or repressed during heat-shock. As Ras proteins are important signalling molecules needed for diverse responses to different stimuli, it may be hypothesized that *P. brasiliensis* yeast cells keep *ras2* as a constitutive expressed gene to respond immediately to stimuli other than temperature shift and *ras1* be modulated specifically by heat shock at 42 ºC.

Figure 3

310

311 ***In vivo* expression of *ras1* and *ras2* genes following *P. brasiliensis* yeast cells**
312 **internalization by murine macrophages**

313 When *P. brasiliensis* infects the host, the first defence line against the pathogen is lung
314 macrophages. Inside non-activated macrophages fungal propagules have to deal with the harsh
315 phagosome environment before switching to the yeast pathogenic form. These cells may develop a
316 latency state enabling survival until environmental conditions favours fungal replication and
317 dissemination. The ability to survive is crucial for the establishment and progression of the
318 infection in susceptible hosts and, according to Tavares *et al.* (2007); it appears to be a
319 consequence of genetic re-programming of the pathogen. Those authors provided evidences that
320 the fungus responds to the microenvironment in order to adapt mainly to the nutritional and
321 oxidative stress generated by the phagosome.

322 In order to study the transcriptional profile of *ras* genes in the setting of the host-pathogen
323 interaction, we used RNA obtained from *P. brasiliensis* yeast cells after nine hours of
324 internalization by murine J774 macrophages. Our semiquantitative analysis revealed that *ras1* and
325 *ras2* are highly repressed – six-fold and three times, respectively, when compared to cells grown
326 *in vitro* in Fava-Netto medium as control (**Fig. 4**). It is the first time that fungal *ras* expression
327 modulation is detected in the macrophage microenvironment.

328 Our results lead to the hypothesis that the modulation of *ras* expression inside macrophage
329 cells is a response to the nutritional starvation which can be noted by the repression of the
330 glycolytic pathway (Tavares *et al.*, 2007), accompanied by the induction of the glyoxylate cycle
331 (Derengowski, L.S., personal communication) and the overall gluconeogenetic status of the yeast
332 cell, as reported before. In good accordance to our data, Breviario *et al.* (1988) demonstrated that
333 *RAS1* and *RAS2* transcripts are down regulated under glucose starvation in *S. cerevisiae*. In
334 addition, Wang *et al.* (2004), by analyzing the transcriptional framework in response to glucose
335 when Ras2 is induced, showed a repression of gluconeogenesis in *S. cerevisiae*. In fact, the
336 phagosome is a nutritionally hostile environment with an extremely low pH and a surfeit of
337 oxidative reactive species produced to counter the presence of the pathogen. Yeast cells respond to
338 this aggression by modulating the transcriptional apparatus to survive even in the presence of the
339 macrophage defences. The pathogen latency state seems to be the most reasonable strategy of the
340 yeast cells to evade the host immune system. Based on this fact, down regulation displayed by
341 both *ras* genes in yeast engulfed by host cells completely makes sense, since the situation is
342 unfavourable to proliferation or differentiation.

343

344 **Figure 4**

345

346 **The potential role of farnesylation in the dimorphism of *P. brasiliensis***

347 The high frequency of Ras mutations in human cancer and the elucidation of the role of
348 farnesylation in proper membrane addressing of Ras motivated an increased search for
349 farnesyltransferase inhibitors against malignant cells (Basso *et al.*, 2006). For our studies we chose
350 the commercially available FPT Inhibitor III (Calbiochem) due to its cell permeability and the
351 previous results with the fungal pathogens *C. albicans* (McGeady *et al.*, 2002) and *C. neoformans*
352 (Vallim *et al.*, 2004). This compound blocks farnesyltransferase by competing with FPP for the
353 active site of the enzyme. It did not produce any effect on the vegetative growth of *C. albicans*,
354 but caused a decreased rate in serum-induced conversion from yeast to pseudohyphae by this
355 pathogen (McGeady *et al.*, 2002). Subsequently, Vallim *et al.* (2004) also detected no interference
356 by FPT Inhibitor III on the vegetative growth of *C. neoformans*, but it was able to impair two
357 cellular differentiation events in this pathogen, the haploid fruiting and mating. Since Ras proteins
358 must be farnesylated to proper membrane signalling, we decided to use FPT Inhibitor III in *P.*
359 *brasiliensis* to investigate a role for Ras and other possible farnesylated proteins in this pathogen.
360 We tested FPT Inhibitor III during vegetative growth of mycelium at 22 ºC and yeast at 37 ºC
361 and during dimorphic transition from mycelium to yeast and back again. The MIC for Pb01 yeast
362 cells was higher than 500 µM. The MIC data corroborates with Vallim *et al.* (2004) and McGeady
363 *et al.* (2002), who reported that high concentrations of the compound act on the vegetative growth
364 of *C. neoformans* and *C. albicans*, respectively. No morphological difference to the control was
365 detected on the vegetative growth of mycelium cells at 22 ºC in the presence of the inhibitor even
366 at 10 days of incubation at 500 µM (**Fig. 5a**). Similarly, no alteration was detected during
367 differentiation from mycelium to yeast at any of the concentrations tested (**Fig. 5b**).
368 However, when we evaluated the vegetative growth of yeast cells at different concentrations of
369 the inhibitor at 37 ºC, the cells presented germinative tubes typical of mycelium that were not
370 observed in the control (**Fig. 5c**). It is important to note that this phenomenon in *P. brasiliensis* is
371 strictly dependent on the temperature shift from 37 ºC to 22 ºC and that the blockage of
372 farnesylation mimicked the process, thus favouring the filamentous form of the fungus.
373 In the yeast-to-mycelium transition experiment yeast cells were incubated at 22 ºC in the
374 presence of 0, 125, 250 and 500 µM of the inhibitor. As can be seen, yeast cells also presented
375 germinative tubes developing to produce true hyphal filaments in the presence of the inhibitor
376 (**Fig. 5d**). However, we detected an increased amount of yeast cells with germinative tubes or true
377 filaments. The data show that the percentage of yeast cells displaying germinative tubes increased
378 up to nearly twice normal transition values for the highest concentration (**Fig. 5e**). It is concluded

379 that the inhibitor favours transition to mycelium in a dose-dependent fashion and perturbs the
380 yeast phase by making the yeast-to-mycelium transition a temperature-independent event.
381
382 **Figure 5**
383
384 As the farnesylation inhibitor is not specific for Ras protein incorporation to the membrane,
385 the defects observed might result from inhibition of other Ras-related proteins that are also
386 farnesylated. It is improbable that FPT Inhibitor III has any direct interference on the Rho-GTPase
387 superfamily that includes RhoA, Rac and Cdc42, as they are geranylated instead of farnesylated
388 (Arellano *et al*., 1999). From the 30 proteins known to be farnesylated in the human cells (Appels
389 & Schellens, 2005), two of them, Rheb and Ras, are reported to be involved in growth and
390 differentiation in response to different stimuli in fungi (Aspuria & Tamanoi, 2004). The Rheb
391 protein has been proposed to activate the Tor signalling pathway, which in turn controls cell
392 differentiation in *S. pombe* (Alvarez & Moreno, 2006; Uritani *et al.,* 2006), *C. neoformans* and *C.*
393 *albicans* (Rohde & Cardenas, 2004). As for Ras, in a wide range of fungi it is an upstream
394 component of several signalling cascades. Ras proteins activate Cdc42 to promote cell polarity
395 and mating process in *S. pombe* (Marcus *et al.,* 1995), filamentous growth in *S. cerevisiae* (Mosh
396 *et al.,* 1996), vegetative growth and serum-induced dimorphic transition in *C. albicans* (Leberer *et*
397 *al.,* 2001), spore germination and polarized growth in *P. marneffei* (Boyce *et al.,* 2005) and high-
398 temperature growth and cellular differentiation in *C. neoformans* (Vallim *et al.,* 2005).
399 In this work we have shown that impaired farnesylation promoted alterations on vegetative
400 growth of *P. brasiliensis* yeast cells and induced dimorphic transition from yeast to mycelium, but
401 we have been unable to determine how and which farnesylated protein, if Ras, Rheb or both, is
402 controlling these events. We know that *P. brasiliensis* possesses all signalling components
403 involved in cellular differentiation and morphogenesis described for other fungi (Fernandes *et al.,*
404 2005), including Tor- and Ras-controlled pathways, but the way they work and are connected and
405 which signals activate them are yet to be elucidated.
406 Our studies show that *P. brasiliensis* has two *ras* genes that codify the Ras1 and Ras2 proteins.
407 These genes are modulated negatively when yeast cells of this pathogen are in contact with host
408 cells and this probably stems from the nutrient limitation and the harsh environment met by the
409 fungus. However, only *ras1* displayed heat-shock triggered mRNA suppression, although neither
410 gene responds to dimorphic transition. These data suggest a modulation of *ras* based upon the
411 signal that is conveyed to the cell. Still, the cascades that Ras controls and their connections must
412 be further studied; but the importance of Ras proteins in the pathobiology of *P. brasiliensis* is
413 already notable. To address the many questions that have arisen from this work we are developing

an RNA interference strategy for functional gene analysis in this pathogen. Gene silencing is preferable to classical disruption due to the unfeasibility of knock-out approaches on *P. brasiliensis* multinucleated and multi-budding yeast cells. This first analysis of *ras* genes opens an avenue of possibilities for characterization of *P. brasiliensis* signalling cascades and their components, which will certain lead to new insights into adaptive responses, morphogenesis control, virulence and pathogenicity in this fungus.

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553 **Figure legends**

554 **Fig. 1.** *P. brasiliensis* Ras1 and Ras2 sequences analysis. **(a)** ClustalW alignment of Ras1 and
555 Ras2 from *P. brasiliensis*. Gray boxes indicate identical residues between the two sequences, and
556 the conserved Ras domains are indicated above the sequences: G1-G4 are the GTP-binding
557 domains, ED is the GTPase effector domain, PS is the prenylation site. **(b)** Phylogenetic tree
558 analysis based on a clustering algorithm to evaluate the relatedness of *P brasiliensis* Ras proteins
559 to those of different fungi. Bootstrap values are indicated on each branch of the tree. Af_RasA: *A.*
560 *fumigatus* RasA (**AAB07703**), Af_RasB: *A. fumigatus* RasB (**AAP94030**), Ca_Ras1: *C. albicans*
561 Ras1(**AF177670**), Cn_Ras1: *C. neoformans* Ras1 (**AF294647**), Cn_Ras2: *C. neoformans* Ras2
562 (**AF294349**), Human H-Ras (**AF493916**), Nc_Ras1: *N. crassa* Ras1 (**P22126**), Nc_Ras2: *N.*
563 *crassa* Ras2 (**BAA03708**), Pb_Ras1: *P. brasiliensis* Ras1 (**DQ157363**), Pb_Ras2: *P. brasiliensis*
564 Ras2 (**AY910576**), Sc_Ras1: *S. cerevisiae* Ras1 (**CAA99298**), Sc_Ras2: *S. cerevisiae* Ras2
565 (**AAA34959**), Sp_Ras: *S. pombe* Ras (**CAA27399**).

566

567 **Fig. 2.** *P. brasiliensis ras1* and *ras2* copy numbers by Southern blot analysis. Ten-microgram total
568 DNA samples digested with the restriction endonucleases *Bam*HI (B), *Eco*RI (E), *Hind*III (H) and
569 *Pst*I (P) were loaded and separated by 0.8 % agarose gel electrophoresis, blotted onto charged
570 nylon membrane and hybridized against chemically labelled *ras1* (panel **a**) and *ras2* (panel **b**).
571 The sizes in kb of hybridized bands are indicated at the bottom of each panel.

572

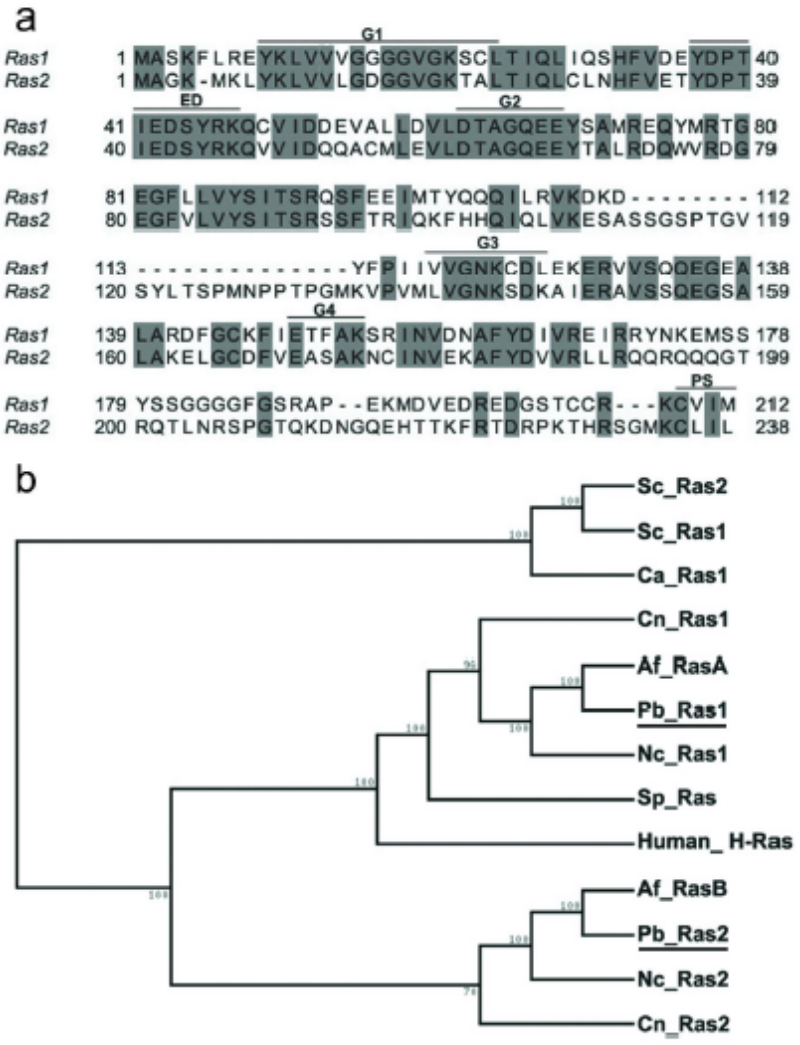
573 **Fig. 3.** *ras1* and *ras2* expression analysis during the dimorphic transition from mycelium to yeast
574 (**a**) and in response to heat shock at 42 ºC (**b**). **Panel a** represents the relative quantification of
575 *ras1* and *ras2* transcripts measured in triple RT-PCR experiments at several time points (0.5h, 1h,
576 2h, 6h and 24h) of the dimorphic transition as well as during the vegetative growth of mycelium
577 (M) at 22 ºC and yeast cells (Y) at 37 ºC. **Panel b** depicts similar quantifications of *ras* mRNA
578 transcripts from yeast cells heat-shocked for 0, 15, 30, 60, 90 or 120 minutes at 42 ºC. The
579 semiquantitative analysis of *P. brasiliensis ras* gene expression was performed by densitometry
580 employing the Scion Image software (http://www.scioncorp.com). The *ras1* and *ras2* transcript
581 levels were calculated relative to the internal control, *l34* for heat shock and *clat* for dimorphic
582 transition. Gray columns correspond to *ras1* transcripts and white columns correspond to *ras2*
583 transcripts. Bars represent standard errors and asterisks indicate $P < 0.05$.

584

585 **Fig. 4.** *ras1 and ras2* transcriptional responses after yeast internalization by murine macrophages.
586 *P. brasiliensis* yeast cells were exposed to murine macrophage J774 cells for nine hours. The total

RNA from internalized yeast cells was extracted and used in RT-PCR experiments, as described in Material and Methods. The control was the total RNA from *P. brasiliensis* yeast cells cultivated *in vitro* in Fava Netto's medium for seven days. **Panel a** shows the agarose gel electrophoresis analysis of RT-PCR products obtained for each condition tested, i.e. inside macrophage and control. **Panel b** represents the semiquantitative analysis of *P. brasiliensis ras1* and *ras2* mRNA expression at each condition. The *ras1* and *ras2*, transcript levels were calculated relative to the internal control *l34*. The gray columns correspond to *ras1* transcripts and white columns correspond to *ras2* transcripts. The semiquantitative analyses were performed exactly as described in figure 3. Bars represent standard errors of RT-PCR quadruple experiments, and asterisks indicate $P < 0.05$.

Fig. 5. Farnesylation inhibition effects on mycelium and yeast cells and during the differentiation of *P. brasiliensis*. **Panel a**, farnesylation inhibition on the vegetative growth of mycelium, where fragments of mycelium were incubated in the presence of 500 µM FPT Inhibitor III in MVM medium at 22 ºC for ten days. The control was mycelium fragments grown in MVM medium, bar is 10µm. **Panel b**, the dimorphic transition from mycelium to yeast, in which fragments of mycelium were incubated for 48h at 22 ºC and shifted to 37 ºC for 10 days to induce yeast differentiation in the presence or absence of 500 µM FPT Inhibitor III, magnification bar, 10µm. **Panel c**, perturbation of yeast vegetative growth of *P. brasiliensis* in the presence of farnesylation inhibitor. Yeast cells were incubated in the presence of 500 µM FPT Inhibitor III in MVM medium at 37 ºC for 7-10 days (upper panel). The control was yeast cells grown in MVM medium in the same conditions described above (bottom panel). Black arrowheads indicate germinative tubes emerging from yeast cells when farnesylation is blocked, magnification bar, 10µm. **Panel d,** dimorphic transition from yeast to mycelium in the presence of farnesylation inhibitor. The yeast cells were inoculated into MVM medium and incubated for 48h at 37 ºC and then shifted to 22 ºC for 10 days to induce differentiation to the mycelium form in the presence of 500, 250 and 125 µM of FPT Inhibitor III and in the absence of the compound (control). In all concentrations the yeast cells produce germinative tubes, the magnification bar in the upper panel stands for 1 µm and in the bottom panel, for 10 µm. To quantify differences in the germination rate for each inhibitor concentration we performed a random counting of yeast cells with germinative tubes and calculated their proportion relative to the total cell number in four randomly chosen fields of three different lamina in three independent experiments. Results were plotted in a graphic as percentage of yeast cells with germinative tubes versus the inhibitor concentration assayed (**panel e**). Bars represent standard errors of triple experiments, with *a*=$P < 0.001$ and *b*=$P < 0.01$.



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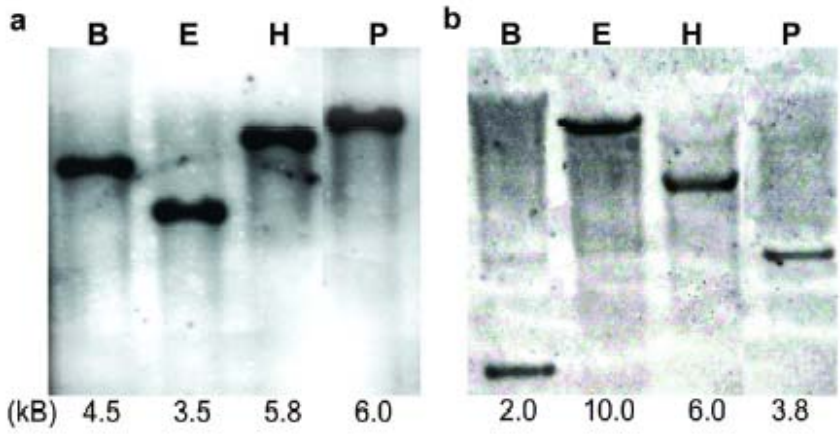

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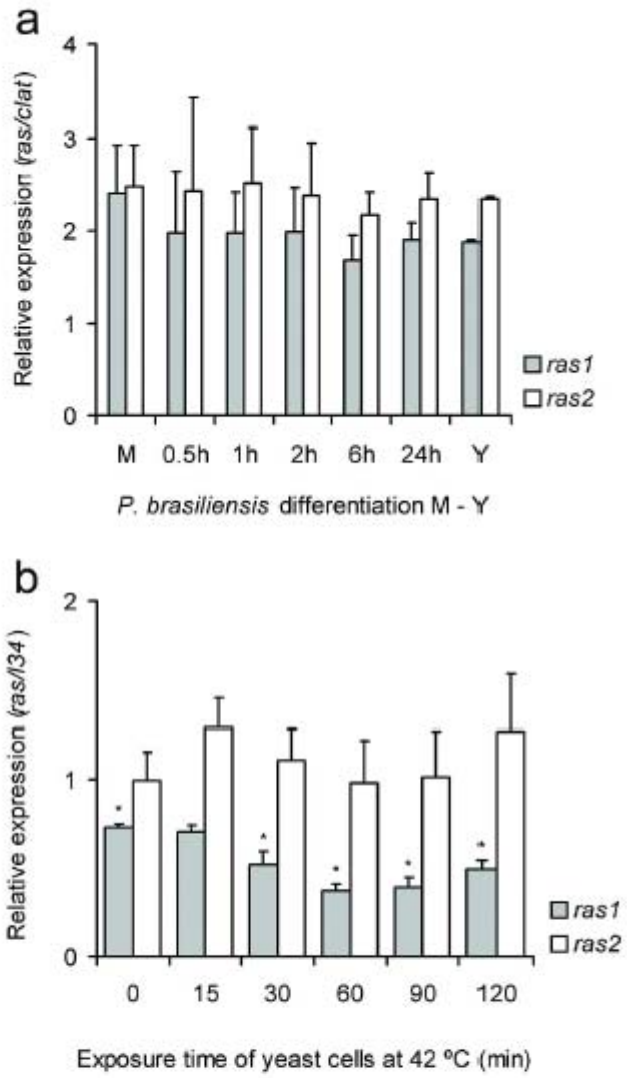

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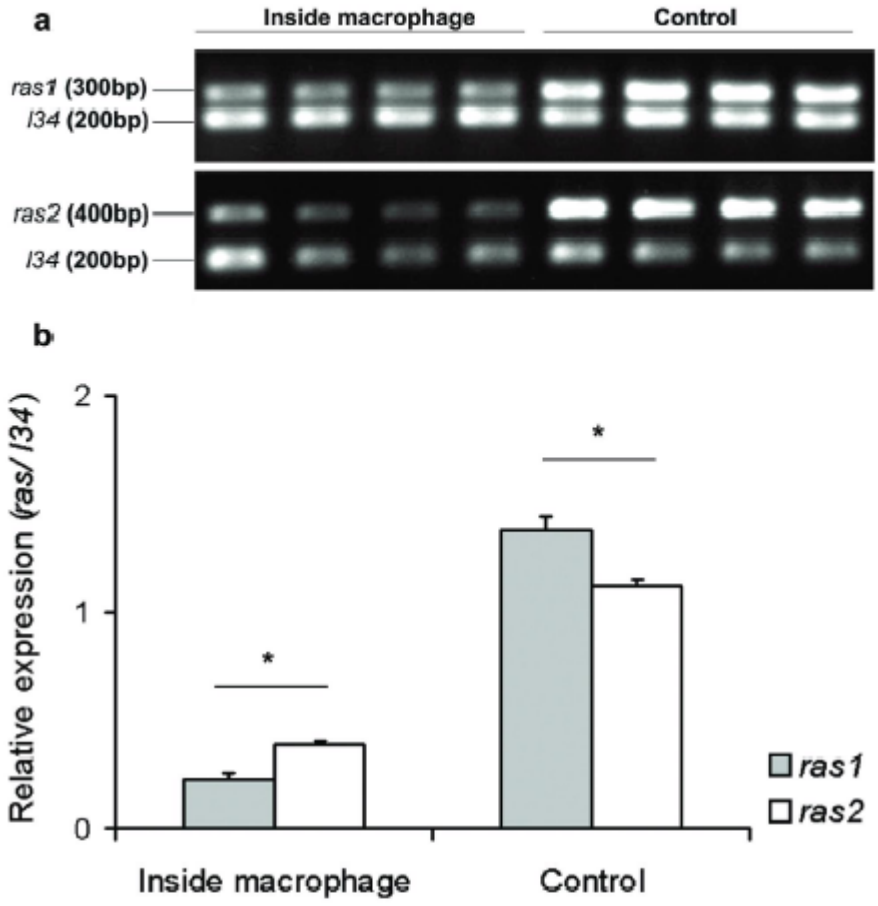

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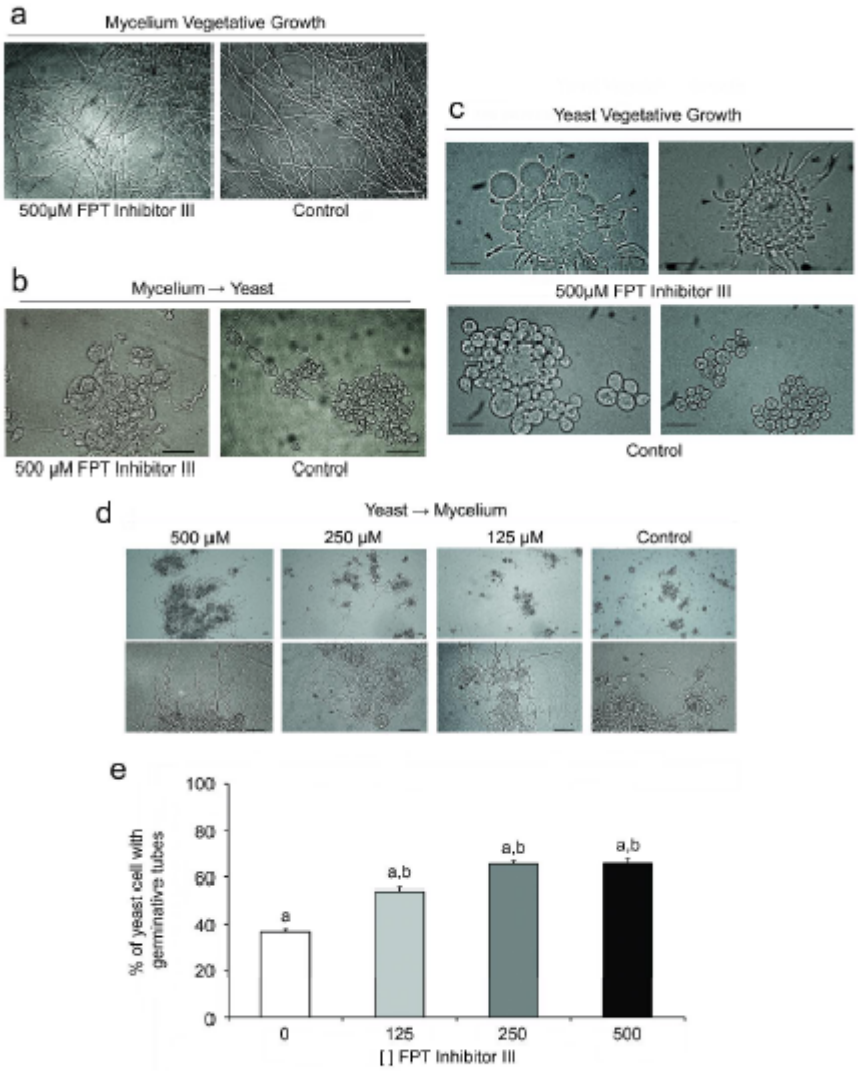

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Regulatory networks in the host-fungal pathogen interaction

Regulatory networks in the host-fungal pathogen interaction

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Running title – **Signaling in host-fungal pathogen interaction**

Abstract

The host innate immune response is critical for protection against infection, responsible for recognizing and countering microbial invasion. Macrophages and neutrophils mainly participate in this response, producing and releasing cytokines and chemokines. In addition, they present microbial antigens to lymphocytes, leading to the development of a highly specific immune response. Receptors are required for detection of microbial components triggering signaling pathways to activate the host immune response. On the other hand, sensing and responding to the environment is required for fungal survival. Fungi have sophisticated and conserved signaling cascades to sense and respond to different types of stress including osmotic shock, temperature, oxidative or nitrosative damage. This review focuses in the main pathways that host cells uses to recognize, interact and respond to the infecting fungal pathogens and highlights the cascades that regulate environmental response by the most studied human fungal pathogens.

Introduction

Signal transduction is a very important mechanism by which the cell exercises its regulatory response depending on the stimulus. The signal transduction communicates extracellular signals to the cell interior through the alternative phosphorylation of numerous proteins, including transcription factors, cytoskeleton proteins, kinases and other enzymes, influencing gene expression, metabolism, cell division, cell morphology, cell survival and homeostasis (Cobb and Goldsmith, 1995).

Fungi are models on the environmental response as the signal transduction pathways were evolutionarily conserved and are often equivalent to multicellular eukaryotic organisms. Fungi may be divided according to the presence or absence of a differentiation pattern that marks the switch from a mycelium to a yeast form. This division overlaps poorly with phylogenetic relationships; for example, in the group of dimorphic fungi we find both *Candida albicans* and the distantly related, thermo-dimorphic ascomycetes – *Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum, Paracoccidiodes brasiliensis, Penicillium marneffei and Sporothryx schenkii*; whereas in the non-dimorphic group we find both the filamentous ascomycete *Aspergillus fumigatus* and the basidiomycete *Cryptococcus neoformans*. There is no obvious connection to the life cycle of the fungi, since *C. albicans* and *C. neoformans* are to be found in commensalism with men and birds or *Eucalyptus* trees, respectively, while the others are known or supposed to exist as free-living moulds.

Here we review the current state of the knowledge on environmental adaptation focusing in the host-pathogen interaction cascades in fungi. In this review we discuss the main pathway that host cells recognize and interact to respond to the infecting fungal pathogens. Additionally, we also compare and highlight cascades that regulate environmental response by those human fungal pathogens in relation to morphogenesis, thermal differentiation, oxidative stress adaptation, virulence and pathogenicity.

1. Signaling on pathogen recognition by the host cells

The anti-pathogen innate immune response is critical for protection against infection. It is the first line of host defense, responsible for recognizing and countering microbial invasion. This response is comprised mainly of phagocytic cells, such as macrophages and neutrophils, which can ingest and kill the invading pathogens. These cells produce and release cytokines and chemokines. In addition, they present microbial

antigens to lymphocytes, leading to the development of a highly specific immune response (Janeway, 1992).

The first interaction occurs between Pattern-Recognition Receptors (PRRs) and conserved molecular structures of microbes know as Pathogen-Associated Molecular Patterns (PAMPs), which are generally essential for the survival of the microbes. The ability of the innate immune system to distinguish between pathogens has been of considerable interest, although much is still not understood. The discovery of the Toll-Like Receptor (TLRs) and the non-TLR has given an insight into the mechanisms of intracellular signaling following microbial sensing **(Table 1).**

Insert table 1

PRRs are localized in serum or tissue fluid, membrane or cytoplasm, and are subdivided into related families according to their structure and/or function. Generally, each receptor recognizes a range of microorganisms based on its individual ligand specificity. Leukocytes use these receptors to recognize pathogen either directly (non-opsonic recognition) or indirectly (opsonic recognition). During infection, microbial recognition occurs through multiple interactions at multiple sites and involves many receptors (Janeway and Medzhitov, 2002; Romani, 2004). Following recognition, some PRRs promote microbial uptake. Shortly thereafter, microbial killing occurs in the phagosome through various mechanisms such as drop in pH and attack by hydrolytic enzymes and toxic metabolites. The fungal cell wall consists mainly of carbohydrates, including mannose-based structures, β-glucan (more than 50%) and chitin. The recognition of β-glucan could contribute to an effective immune response.

1.1. Recognition of fungal structures by Toll-Like Receptor (TLR)

TLRs are a family of ten innate immune recognition receptors that are required for detection of a broad range of microbial products including LPS, flagellin, and bacterial lipoproteins. They are distinct from each other in ligand specificities, expression patterns, and signaling pathways, but all act in the initiation and activation of immunity.

TLR2 and TLR4 have been implicated in anti-fungal immunity and recent studies have demonstrated a crucial involvement of these in the recognition of fungal pathogens such as *C. albicans*, *A. fumigatus* and *C. neoformans.* (Netea *et al.*, 2004a; Netea *et al.*, 2006). Fungal infection in knock-out mice deficient in either TLRs or TLR-associated

adaptor molecules showed that specific TLRs such as TLR2 and TLR4 play differential roles in the activation of the various arms of the innate immune response. Recent data also suggest that TLRs offer escape mechanisms to certain pathogenic microorganisms, especially through TLR2-driven induction of antiinflamatory cytokines (Netea *et al.*, 2004b). TLR2 is the most powerful receptor and recognizes a wide variety of PAMPs from bacteria, yeast, fungi, parasites and viruses. The natural ligands of the TLRs are evolutionary conserved microbial components. Those recognizing bacterial and fungal PAMPs are generally displayed on the surface of cells, and those recognizing viral components are found inside the cells **(Figure 1).**

Insert Figure 1

TLRs are believed to function as dimers. Though most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, each dimer having different ligand specificity. TLRs may also depend on other co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of LPS. When activated, TLRs recruit adapter molecules within the cytoplasm of cells in order to propagate a signal. Four adapter molecules are known to be involved in signaling, known as MyD88, TIRAP, TRIF, and TRAM. The adapters activate other molecules within the cell, including certain protein kinases (TAK1, IKK complex, IRAK1, IRAK4, TBK1) that amplify the signal, and ultimately lead to the induction or suppression of genes that orchestrate the inflammatory response. In all, various genes are activated by TLR signaling, and it constitutes one of the most powerful and important gateways for gene modulation. Following activation by ligands of microbial origin, several reactions are viable. Immune cells produce signaling factors as cytokines which trigger inflammation.

Study of TLRs has significantly enhanced the understanding of innate and adaptive immune responses and provides novel therapeutic approaches against infectious and inflammatory diseases. Recent developments provide crucial information for clarifying the mechanisms of fungal recognition by cells of the immune system, and provide hope for designing new therapeutical approaches to fungal infections**.**

1.2. Recognition of fungal structures by non-Toll Like Receptor (non-TLR)

1.2.1. Receptors that recognize mannose

C-type lectins are classical molecules characterized by their ability to bind carbohydrates in a Ca^{2+}-dependent manner. They have a common domain, named carbohydrate recognition domain (CRD), which contains 18 highly conserved amino acid residues including two disulfide bonds. Phagocytes express many kinds of C-type lectin receptors on their surface for antigen capture. Some of these domains are known by the acronym CLEC (C-type lectin domain family) (Kanazawa, 2007).

The mannose receptor (MR) is one of such receptors, consisting of an N-terminal cystein-rich domain, a fibronectin type II domain and 8 or 10 CRDs. MR preferentially recognizes α-linked oligomannose with branched rather than linear structures (Kanazawa, 2007) and is capable of recognizing a variety of antigens, ranging from self-molecules to pathogens (Geijtenbeek *et al*., 2004). Recognition of *C. albicans* by human/murine macrophages is mediated by a mannose receptor that can bind to N-linked mannosyl residues and by TLR-4 bound to O-linked mannosyl residues (Netea *et al.*, 2006). It was demonstrated that the protective T-cell response to the pathogenic yeast *C. neoformans* is dependent on the recognition of mannoproteins by multiple MR present on the surface of dendritic cells. Mansour *et al.* (2006) suggest that these cells provide the crucial link between innate and adaptive immune responses to *C. neoformans* via a process that is dependent upon the efficient uptake of mannoprotein by MR. Following the interaction between *Pneumocystis carinii* and human alveolar macrophages, IL-8 is released. This event is partially dependent on the transcription factors NF-kappaB, ERK1/2 and JNK MAPKs. It also requires the interaction between the fungus and both receptors MR and TLR2 (Tachado *et al*., 2007) **(Figure 1)**. As for *P. brasiliensis,* the higher mannose receptor expression is important for macrophage phagocytic capacity (Jiménez *et al*., 2006). Another receptor able to recognize mannose residues present on the surface of a wide spectrum of microorganisms is the CLEC-1b (C-type lectin like receptor 1). The gene encoding this receptor is induced during macrophage infection with *P. brasiliensis* (S.S. Silva, unpublished).

1.2.2. Receptors that recognizes β-glucan

The recognition of β-glucan occurs by a family of non-classic C-type lectin receptors called Dectin-1. Dectin-1 receptors lack a cysteine residue in its stalk region and contain an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail, which is involved in cellular activation. After phosphorylation of the second tyrosine occur

various effects such as induction of phagocytosis, production of reactive oxygen species (ROS) and cytokine production mediated by nuclear factor (NF)-kB activation (Brown, 2006).

The synergism of Dectin-1 and TLR2 signaling enhances NF-kappaB activation and regulates cytokine production **(Figure 1).** Dectin-1 signaling via CARD9, that is an adaptor molecule containing an N-terminal caspase-recruitment domain (Dostert & Tschopp, 2007), and also directly modulates gene expression via activation of NFAT (Goodridge *et al*., 2007). Dectin-1 was originally thought to be a dendritic cell-specific receptor, from which its name "dendritic-cell-associated C-type lectin-1" was derived (Ariizumi *et al*., 2000). This receptor recognizes fungal β-glucan without opsonic molecules. It therefore recognizes several fungal species, including *C. albicans* (Taylor *et al.,* 2007), *P. carinii* (Saijo *et al*., 2007), *Coccicioides posodaii* (Viriyakosoi *et al*., 2005) and *A. fumigatus* (Gersuk *et al*., 2006). Their expression on macrophage is up-regulated by IL-4 and IL-13 and down-regulated by LPS, IL-10 and dexamethasone. The loss of this receptor might explain why some fungi escape the host immune surveillance (Brown, 2006).

The protection role of dectin-1 against all fungal infections is not a consensus. Taylor *et al.* (2007) described that dectin-1-knockout mice were more susceptible than wild-type mice to *C. albicans* infection. In contrast, Saijo *et al*. (2007) showed that wild-type and dectin-1-knockout mice were equally susceptible to *Candida* infection. The recognition of *C. albicans* by macrophages is a complex process involving multiple receptor systems that recognize sequentially the various layers of the outer portion of the fungal cell wall. The components of the cell wall of a pathogenic fungus (*N*-linked mannans, *O*-linked mannans, and β-glucans) are involved in the recognition by monocytes/macrophages by MR, TLR4 and dectin-1/TLR2 respectively and in the subsequent induction of pro- and anti-inflammatory cytokine release (Netea *et al*., 2006).

The absence of interaction between dectin-1 and β-glucan contributes to fungal evasion of immune system. The yeast form of *C. albicans* induces a host-protective response while hyphae do not, since the last lacks the surface exposed β-glucan (Heinsbroek *et al*., 2005). *C. neoformans* encapsulation also mask the cell-wall β-glucan (Cross and Bancroft, 1995) and the changes in proportion of cell wall α/β glucan of *P. brasiliensis* (San-Blas *et al*., 1984; Silva *et al*., 1994) contributes to the evasion of specific response. The subversion of host immune response is effective by dectin-1 receptor blockage by cell wall α-glucan, as demonstrated for *H. capsulatum* (Rappleye *et al*., 2007).

The macrophage response to resting spores and invasive form of the *A. fumigatus* is dependent on the dectin-1 interaction with β-glucan present in the cell wall. β-glucan is absent on the surface of dormant conidia, but is present after cellular swelling and loss of the hydrophobic proteinaceous cell wall. Dectin-1 binding to germ tubes increases, but is not required, for TLR2-mediated inflammatory cytokine secretion. In addition, it stimulates TNF-α production in the absence of both TLR2 and MyD88 signaling (Gersuk *et al*., 2006).

Cells that do not express dectin-1 receptor and still have an important role in anti-fungal immunity express other receptors, such as the complement receptor 3 (CR3), lactosylceramide and scavenger receptors. All these receptor also recognize β-glucan. CR3 (also called CD11b-CD18 or MAC1), the complement fragment inactivated C3b (C3bi), β-glucan and endogenous ligands are involved in cell adhesion and migration. CR3 consists of several domains, including a lectin domain that mediates carbohydrates recognition and regulates adhesion (Taylor *et al*., 2002; Xia *et al*., 1999). The importance of CR3 in fungus uptake is demonstrated for *P. brasiliensis* when the opsonic conidia are extensively phagocyted by macrophages. The heat-inactivation of serum used for opsonization or the treatment of macrophages with anti-CR3 decreased phagocytosis by macrophages (Jiménez *et al*., 2006).

Lactosylceramide, also called CDw17 and Galβ4Glcβ1Cer, is a glucosphingolipid PRR found in plasma membrane micro-domains of many cells types. Lactosylceramide-mediated recognition of β-glucan induces various cellular responses *in vitro*, including cytokine production, the respiratory burst and NFkB activation (Wakshull *et al*., 1999.). The scavenger receptors are a family of cell-surface glycoprotein PRRs that differ greatly in their structure but are all capable of recognizing modified low-density lipoproteins. These receptors have also been implicated in β-glucan recognition (Rice *et al*., 2002).

Phagocytic cells are crucial for host defence against invading pathogens such as bacterial and fungus. The macrophage phagosome is believed to be a poor source of glucose and amino acids (Fan *et al*., 2005; Lorenz *et al*., 2004). Such nutrient deprivation inside the macrophage induces a similar adaptative response of intracellular bacterial and fungal pathogens (Chatterjee *et al*., 2006; Fan *et al*., 2005; Lorenz *et al*., 2004). Further, we will focus in the signaling pathways underwent by fungal pathogens in order to activate the genetic re-programming necessary to the survival in the host cells.

2. Signaling response in fungal pathogens

2.1. Morphogenesis and differentiation

In this topic we focused on signaling components that have been demonstrated by knock-out experiments to be involved in fungal pathogenesis using animal models. The differentiation process observed in fungal pathogens is that of thermo-dimorphic fungi. The species that cause human disease probably follow a similar genetic programme that causes them to change from mycelium to yeast upon exposure to the higher temperature of the host. Recently, Nemecek *et al*., 2006 identified a histidine kinase (HK) receptor as the dimorphism switch - denominated Drk1, for Dimorphism-related kinase - in *B. dermatitidis*. They also provided evidence that the mechanism is conserved in the other species when they reproduced the effects of silencing the corresponding drk1 gene in *H. capsulatum*. These effects were to lock the fungi in the mycelial phase, which was correlated with a severe impairment of their ability to cause infection. HKs have only been proposed as membrane receptors in *B. dermatitidis* and *H. capsulatum*. Exactly how these receptors sense environmental changes and which factors function downstream in morphogenetic process remains to be elucidated.

MAPKinase cascades are responsible for fungal differentiation and virulence. The key regulator molecules in this case are the small GTPase Ras and its immediate downstream effectors best known is the Rho-GTPase Cdc42. This pathway was first characterized in *C. albicans* (Feng *et al.*, 1999) and *C. neoformans* (Alspaugh *et al*., 2000), and more recently in *A. fumigatus* (Fortwendel *et al.*, 2004; Fortwendel *et al*., 2005) and *P. marneffei* (Boyce *et al*., 2005). The Ras-controlled processes seem to be both more global and more conserved among species. Cdc42 regulates the actin component of the cytoskeleton and thus the vegetative growth of all pathogens studied so far. The pathway is also indispensable for growth at 37°C and therefore knock-out strains are avirulent in animal models. Still, some Ras-triggered events are known to be species-specific: in *C. albicans*, the pathway is necessary for serum-induced filamentation (Feng *et al*., 1999; Leberer *et al*., 2001); in *P. marneffei*, no influence in dimorphism has been identified for Ras and its effectors to date, but the maintenance of cell polarity by this cascade has been well characterized for that fungus (Boyce *et al*., 2005); and in *A. fumigatus*, spore germination and virulence are determined by Ras-dependent mechanisms (Fortwendel *et al*., 2005).

There is ample evidence that Ras acts not only as a relay protein in signaling processes, but also that it functions in the cell as a bifurcation, exerting modulation over

several pathways. Recent data indicate that more than one MAPKinase cascade is modulated by Ras through downstream Rho-GTPases other than Cdc42 (Nichols *et al*., 2007). Furthermore, a completely different branch cascade is also modulated by Ras, namely the cAMP/PKA pathway **(Figure 2)**. In that case the immediate effector of Ras is adenylate cyclase, and there seems to be functional redundancy with the MAPKinase pathway, for both seem to influence the same morphogenetic events and pathogenicity. Apart from *P. marneffei*, the only thermo-dimorphic pathogen where members of the Ras-mediated pathways have been studied *in silico* is *P.brasiliensis* (Fernandes *et al*., 2005; Felipe *et al*., 2005).

Insert Figure 2

2.2. Signaling in response to oxidative stress

Phagocytosing macrophages is responsible to release oxygen and nitrogen compounds as part of the antimicrobial burst (Densen and Mandell, 1995). In this way, the study of the oxidative stress in fungi, which suffers phagocytosis, is expected to elucidate how this pathogen survives the oxidative killing mechanism by the host macrophage system.

Reactive oxygen species (ROS) such as hydrogen peroxide, produced externally or during normal metabolism can damage different cell components and usually trigger a counteracting antioxidant response (Aguirre *et al*., 2005). Fungi have several mechanisms to handle ROS, such as the presence of a large number of antioxidant enzymes and the ability to produce secondary metabolites with antioxidant function (Kawasaki and Aguirre, 2001; Lee *et al*., 2005).

A number of studies indicate that reactive nitrogen species (RNS) are important antimicrobial effectors produced by macrophages (Missall *et al*., 2004). In contrast to the redundancy in the ROS elimination, there are few mechanisms for the disposal of NO (de Jesus-Berrios *et al*., 2003). To survive the oxidative and nitrosative attack initiated by phagocytic cells of the host, pathogens must respond appropriately. This antimicrobial attack is established by two main systems including the nitric oxide synthase, which generates reactive nitrogen species and the NADPH oxidase pathway, generating reactive oxygen species (Fang, 2004). To cause infection, pathogens must evade the immune system by initiating a response to the stress encountered.

2.2.1. Signal transduction mechanisms in fungal responses to ROS and RNS

An important example of an environmental challenge to which fungi must rise is the high levels of ROS produced by neutrophil cells during the oxidative burst. The oxidative killing of fungal cells by this host defense mechanism represent an important line of elimination of pathogens, as demonstrated by the correlations between the function of oxidative stress response in pathogenic fungi and their ability to proliferate in host cells (Wysong *et al*., 1998). Studies have demonstrated that eukaryotes evolved novel mechanism to perceive and eliminate ROS. Those studies had identified three major modules that govern the response to oxidative stress, the stress responsive MAPKinase cascade (also denominated SAPK for Stress Activated Protein Kinases), the most well-characterized signal transduction cascades regulating fungal adaptation to the environmental stress, a histidine kinase (HK) system (a prokaryotic-type multistep phosphorelay system) and the AP-1-like transcription factors (Posas *et al*., 1996; Grefen and Harter, 2004).

2.2.2. Fungal MAPK and HK components systems

In eukaryotic cells, phosphorelay systems (HKs) have been found in slime molds, fungi and plants where they connect environmental stress signals to the MAPKinase cascades (reviewed in Kruppa and Calderone, 2006). In a prototypical two-component system, a sensor HK phosphorylates a histidine residue within its kinase domain, referred as an H-box; subsequently the HK phosphorylates a phosphotransfer protein (HPt), which in turn transfers the phosphate group to an aspartate residue in a receiver domain of a response regulator protein (RR). Most characterized HKs, both bacterial and eukaryotic, require the additional phosphorelay step through the HPt domain protein and a second RR protein. This additional phosphorelay step may allow the organism to integrate multiple input signals into a single output. In fungi, a classical target of the two-component system is the Hog1 MAPKinase pathway and the phosphotransfer from the HK is mediated by Ssk proteins **(Figure 3)**, which possess aspartate residues that are phosphorylated by the kinase (Kruppa and Calderone, 2006).

Insert Figure 3

The ascomycete *C. albicans* is the most common fungal pathogen in immunocompromised patients (Fridkin and Jarvis, 1996). In order to defend against

colonization of this pathogen, the host immune system utilizes the ROS production. Studies have suggested that macrophages ingest and kill the fungus through an oxygen-dependent mechanism (Vazquez-Torres and Balish, 1997). One of the major signal transduction pathways in *C. albicans* is the HOG pathway, which senses oxidative stress and is also involved in sensing osmotic stress (Bahn *et al*., 2007). Three HK genes, Sln1, Chk1 and Nik1, which are distinct and redundant, as well as the homologue of the HPt protein, Ypd1, have been reported in *C. albicans* (Nagahashi *et al*., 1998; Calera *et al*., 1998; Calera *et al*., 2000). Additionally, *C. albicans* presents two RR, Ssk1 and Skn7; the last is supposed to regulate its action as a transcriptional factor (Lee *et al*., 1999). Ssk1 mediates gene activity by interacting with the MAPKKK Ssk2 of the MAPK pathway (Chauhan *et al*., 2003). The MAPKinases module in *C. albicans* is composed of Ssk2 (MAPKKK), Pbs2 (MAPKK) and Hog1 (MAPK). When phosphorylated, Ssk1 (RR) is not able of inducing activation of the MAPKKK, Ssk2. Under oxidative/osmotic stress, phosphotransfer through Sln1-Ypd1-Ssk1 activates Ssk2, which activates Pbs2 and finally Hog1 (reviewed in Chauhan *et al*., 2006). Observations are consistent with the idea that *C. albicans* MAPKinase (Hog1) pathway is required for the fungal resistance to oxidative stress. Hog1 mutant strains are sensitive to the oxidants menadione and H_2O_2 (Alonso-Monge *et al*., 2003). Also, the Pbs2 MAPKK component is crucial for the fungal anti-oxidant adaptation (Arana *et al*., 2005). Transcriptional analysis of ssk1 mutants has shown that a considerable portion of the altered genes encode for proteins related to the stress adaptation (Chauhan *et al*., 2003). The role of Hog1 in the regulation of global transcriptional responses to stress has been studied in *C. albicans*. DNA microarrays were used to characterize the global transcriptional responses of HOG1 and hog1 cells to osmotic, oxidative and heavy metal stress. Inactivation of HOG1 attenuated transcriptional responses to osmotic and heavy metal stresses, but not to oxidative stress. It has been suggested that Hog1 plays a central role in the regulation of osmotic and heavy metal stress-induced gene expression, but a less central role in the regulation of oxidative stress genes in *C. albicans* (Enjalbert *et al*., 2006).

The basidiomycete *C. neoformans* is encapsulated yeast that predominantly infects the central nervous system in immunocompromised patients causing life threatening fungal meningitis (Mitchell and Perfect, 1995). In *C. neoformans* a two-component phosphorelay system has been investigated (Bahn *et al*., 2006). Unlike the ascomycete fungi, the HOG pathway of *C. neoformans* is constitutively phosphorylated under normal conditions, via the Pbs2 MAPKK and an unknown MAPKKK. In response to diverse stress conditions a

variety of sensor kinases, including the characterized Tco1 and Tco2, activates Ssk1, which in turn activates a Hog1-specific phosphatase resulting in rapid dephosphorylation of Hog1 and the consequent induction of appropriated cellular response to the environmental stimuli **(Figure 3)**. Tco2 is partially responsible for response to oxidative damage and osmotic shock, as described (Bahn *et al.,* 2006).

By comparison to known sequences of the MAPK module in other fungi, all the components of the MAPK module had been identified in *A. fumigatus* (Aguirre *et al*., 2006) and homologues of the HOG-MAPK pathway proteins had been studied in this fungal pathogen. The sakA gene (also called hogA), has a role in protection against oxidative stress, since SakA mutants of *A. fumigatus* are oxidant sensitive (Xue *et al*., 2004). Additionally, *A. fumigatus* contains a second SAPK, called MpkC; its role in the oxidative stress response and pathogenicity has not yet been evaluated (May *et al*., 2005).

P. brasiliensis is an important dimorphic fungal pathogen located predominantly in Central and South America. *In silico* search of *P.brasiliensis* database has revealed a number of two-component signal transduction proteins, as well as MAPK pathways including HOG1 (Felipe *et al*., 2005; Fernandes *et al*., 2005) Accordingly the fungal database predicts a sln1 sensor kinase orthologue, one ypd1 orthologue, as well as ssk1 (RR), ssk2 (MAPKKK), pbs2 (MAPKK), hog1 (MAPK), suggesting that this fungal pathogen can utilize the modules that govern the response to oxidative stress, the stress responsive MAPKinase cascade and the multistep phosphorelay system for dealing with oxidative stress, which constitutes another hint at the phylogenetic conservation of this mechanism.

2.2.3. Fungal AP-1-like transcription factors

Fungal cells have targeted nuclear localization of several components of their oxidative-stress response machinery for modulation. AP-1 (Activating Protein 1) describes a group of members of the Jun, Fos and ATF family proteins that form homo/heterodimers to regulate cell proliferation and differentiation, apoptosis and stress response (Karin *et al*., 1997). Studies had demonstrated the role of the AP-1 homologues in the redox homeostasis in fungi, putatively as a positive regulator of gene expression (Harshman *et al*., 1988).

Fungal AP-1-like factors had been first identified in *Saccharomyces cerevisiae* (Yap1, for yeast AP-1) and had been characterized as a transcription factor, which regulates antioxidant genes, such as thioredoxin and gluthatione peroxidase (Kuge and

Jones, 1994; Tsuzi *et al*., 2004). *C. albicans* presents a homologue of the *S. cerevisiae* Yap1, denominated Cap1. The role of Cap1 in the fungal adaptation to oxidant stress has been investigated. The protein is induced in the presence of oxidative stress and localizes to the nucleus, in an oxidant–responsive fashion, inducing a set of genes necessary to the response to this stress (Zhang *et al*., 2000). Fungal cells lacking *CAP1* are hypersensitive to diamide and H_2O_2 (Zhang *et al*., 2000). Although the detailed mechanisms of *CAP1* regulation needs to await further studies, the oxidative stress sensing by Cap1 seems to involve a cysteine residue in the C-terminal domain, as described for its homologues (Zhang *et al*., 2000). In *C. albicans*, Cap1 has been shown to activate transcription via the Yap response element (YRE: TKACTAA) (Fernandes *et al*., 1997) and *cap1* mutant cells display increased sensitivity to reactive oxygen species (Alonso-Monge *et al*., 2003). Cap1 seems to act independently from the HOG pathway in *C. albicans*, since deletion of *HOG1* has no effect on the Cap1 translocation to the nucleus and *CAP1* deletion does not inhibit Hog1 phosphorylation (reviewed in Chauhan *et al*., 2006). Other pathways, such as the Cap1 pathway, could play key roles in the regulation of oxidative stress genes, as suggested (Enjalbert *et al*., 2006). Homologues of Yap1 have been described in other fungi. *A. fumigatus* contains Yap1 homologues, but its function is still unknown (Aguirre *et al*., 2006).

In contrast to the redundancy in the systems for H_2O_2 elimination in response to ROS, there are few mechanisms for the disposal of NO. Resistance to nitric oxide has been shown to be important to the stress defense and virulence of *C. neoformans*. It has been shown that macrophages produce nitric oxide in response to cryptococcal cells and that the macrophage antifungal activity of macrophages is mostly dependent on RNS (Gross *et al*., 1999; Tohyama *et al*., 1996). Proteomic and transcriptional response of *C. neoformans* to nitric oxide stress have been investigated. One stress-related protein up-regulated in the presence of nitric oxide stress was glutathione reductase (GLr1); the corresponding mutant was sensitive to nitrosative stress and macrophage killing. Additionally the mutant was avirulent in mice. Interestingly, mutants deficient in Glr1 are not sensitive to peroxide stress, suggesting the specificity of this antioxidant enzyme to the nitrosative stress response in this fungal pathogen (Missall *et al*., 2006). The fungus depicts a single gene for NO oxigenase and also a single gene for 5-nitroglutathione reductase. It has been described that NO generated in mammal hosts exerts a fungistatic effect against *C. neoformans* and that NO oxigenase is able of protecting fungal cells during infection and promotes fungal virulence (de Jesus-Berrios *et al*., 2003).

Two candidate factors, an alternative oxidase, presumably providing a means of fungal cells to continue respiratory growth in the face of RNS, and a nitric oxide reductase has been suggested in *H. capsulatum* mechanism for combating nitrosative stress. Also a P450 nitric oxide reductase, Nor1, is induced in *H. capsulatum* exposed to nitrosative stress. In support of its role in combating host defenses, overexpression of NOR1 in *H. capsulatum* shows slightly increased growth rates in the presence of a nitrosative agent compared with wild-type cells (Nittler *et al*., 2005).

2.2.4. Antioxidant enzymes as effectors of oxidative stress of the pathogens

Fungi have enlisted several classes of antioxidant enzymes defenses to cope with the variety of ROS that is available in the phagocytes. O_2^- is eliminated by superoxide dismutases (SOD), present at cytosol, mitochondria and also secreted forms. A battery of enzymes decomposes H_2O_2, such as catalases, thioredoxin, glutathione reductase and gluthatione peroxidase. Those enzymes are considered for the majority fungal pathogens virulence factors due to their ability to handle with the oxidative stress implied by the host environment. Some of the genes that encode those antioxidant enzymes when impaired produce mutants with virulence attenuated or completely avirulent (Chauhan *et al*., 2006). The virulence factors from the fungal pathogens are going to be discussed below.

3. Signaling in response to temperature stress

Thermotolerance in fungi is the ability to grow at 37°C, which is a *sine qua non* condition for human infection and dissemination. The ability to exist in different forms and to reversibly switch from one to the other during infection provides the metabolic flexibility reflecting as an evolutionary adaptation to different environments **(**Tekaia and Latge, 2005)**.** The evolutionary mechanisms may involve the expression of temperature-induced genes or even the stress resistance ones that might confer pathogenicity and/or virulence. Since the thermotolerance is a universal virulence trait across pathogenic fungi, the understanding of how pathogenic fungi grow at high temperatures may provide important clues to pathogenesis in humans.

3.1. MAPKinases

MAPKinases are key elements in the environmental stress in fungi. Their essential role in sensing different signals enables the pathogenic fungi to integrate, amplify and

modulate the adaptive response and virulence (Roman *et al*, 2007). The genetic manipulation in several pathogenic fungi revealed the important relationship between signal-transduction pathways, virulence and stress response.

In *C. albicans*, the HOG pathway is able to respond to a myriad of environmental stimuli, including sexual development, oxidative stress response, morphological differentiation and growth at low temperature (for revision, see Bahn *et al*, 2007). *MKC1* gene encodes a mitogen-activated protein (MAP) kinase, which is phosphorylated in response to low-temperature shock **(Figure 4).** Recently it has been found that the oxidative-stress-mediated phosphorylation of Mkc1 is partially dependent on an intact HOG pathway (Arana *et al.*, 2005), which might indicate a crosstalk between the temperature and oxidative stress responses.

Insert Figure 4

In *C. neoformans*, MAPKinases together with calcineurin pathway are reported to be involved in cell wall integrity and remodeling in response to elevated growth temperature (Kraus *et al.*, 2003). The *C. neoformans* Mpk1 is required for growth at 37ºC *in vitro*. The *mpk1* mutants were unable to grow at 37ºC and also the virulence was attenuated in the mouse model of cryptococcosis. Mpk1 is activated by phosphorylation in response to drugs that are able to alter the cell wall integrity, such as calcofluor white, nikkomycin Z and caspofungin (Kraus *et al*, 2003). The cross-talk between both pathways was recently reinforced by the isolation of a hypervirulent *eca1* mutant that is unable to grow at mammalian body temperature. Eca1 is likely involved in the calcium maintenance at the ER. The mutant behavior for virulence was tested in different host models where the ability of infection was attenuated at 37ºC (Fan *et al.*, 2007).

In *P. brasiliensis*, Bastos *et al*. (2007) described the differentially expressed sequences during transition from mycelium to yeast cells, which is essentially temperature-dependent event. MAPKinase ESTs were identified in the transition transcriptome. A MAPKinase and PKC proteins were induced in the transition library suggesting their involvement in the fungal cell wall biosynthesis as a response of adaptation to the environment and also consequently to survive and proliferate within the host.

3.2. Ca $^{2+}$-Calmodulin-calcineurin

Calcineurin is a major player in calcium-dependent signal transduction pathways in eukaryotes. Calcineurin is a conserved serine-threonine-specific Ca^{2+}-calmodulin activated protein phosphatase that mediates cell-stress responses. Calcineurin B binds to a α-helical extension of calcineurin A distinct from the active site. In response to calcium influx, calmodulin binds to calcineurin A and blocks the action of the auto- inhibitory C-terminal domain of calcineurin A, resulting in the formation of the active calcineurin complex.

Calcineurin acts on transcription factors and governs the expression of genes in a species-dependent fashion. It is critical for virulence in pathogenic fungi that infect humans as *C. neoformans* and *C. albicans* (Kraus *et al.*, 2005) and *A. fumigatus* (Steinbach *et al.*, 2007). It was recently reviewed by Steinbach and coworkers (2007) the role of calcineurin as a novel drug target against invasive fungal infections caused by *C. neoformans, C. albicans* and *A. fumigatus* which most causes infection in immunocompromised patients. De Carvalho *et al*. (2003) have shown the inhibitory effect of several drugs in the calcium-calmodulin signalling pathway in *P. brasiliensis* during the mycelium-to-yeast transition. Additionally, an *in silico* search of the *P. brasiliensis* database has also revealed a calcium-calmodulin signalling pathway **(Figure 4)** that might be controlling the temperature shift (Felipe *et al.*, 2005; Fernandes *et al.*, 2005). The potential role of this pathway in the dimorphism control and possible in pathogenesis of *P. brasiliensis* was re-inforced by the up regulation of calcineurin B during the transition from mycelium to yeast (Bastos *et al*., 2007).

Calcineurin is critical for virulence and pathogenicity of *C. neoformans*, since calcineurin A mutant strains are viable but fail to grow in high temperature and are avirulent in animal model of cryptococcal meningitis (Odom *et al*., 1997). Down-regulation of calmodulin was associated with impaired growth at 37°C **(Figure 4)** and, in the absence of calcineurin function the *C. neoformans cam1* mutant displayed impaired bud formation, which implicates the involvement of Ca^{+2}-calmodulin-calcineurin in morphogenesis and high-temperature growth (Kraus *et al*., 2005).

Calcineurin is not required for the serum induced transition of *C. albicans*, however the involvement of calcineurin in the virulence and consequently in the growth on the high temperature is dependent on the host niche. In mouse models of systemic disseminated candidiasis the calcineurin is essential to the infection while in vaginal or pulmonary infection the absence of calcineurin function does not affect the virulence of the pathogen (Bader *et al.,* 2006). In addition, in *C. albicans* the deletion of either the catalytic subunit

or regulatory subunit abrogates calcineurin activity resulting in cells that are sensitive to multiple stress conditions (Bader *et al.*, 2006).

Recent studies in *A. fumigatus* suggest that calcineurin controls key steps in polarized hyphal growth, and is therefore critically important for tissue invasion. The *Aspergillus* calcineurin pathway was not directly involved with heat stress, but the over-expression of calcineurin was observed under several stresses. The link between calcineurin and *A. fumigatus* pathogenicity was supported by animal models in which the blockage of calcineurin leads to a decrease in fungal growth, halt tissue invasion and decrease host mortality (Steinbach *et al.*, 2006).

3.3. RAS

Ras proteins, small guanine-nucleotide binding proteins, are highly conserved and they control the activation of diverse signaling pathways. Among microorganisms, Ras proteins regulate fundamental and cellular processes such as morphological transitions, mating and microbial pathogenesis.

Ras proteins control several distinct cellular processes in *C. neoformans* including the maintenance of the cytoskeletal integrity, allowing this organism to respond to changing environments and to external stress. *C. neoformans* Ras1 has a role in the high temperature growth since *ras1* mutants arrest as large, unbudded cells with depolarized actin, also are unable to grow at 37ºC and are avirulent in animal models of meningitis. The *RAS2* gene when over-expressed in the *ras1* mutant rescues the ability to grow at high temperature, indicating a redundant effect in the signaling pathways that responds to temperature (Alspaugh *et al*., 2000; Waugh *et al*., 2002). Over-expression of either *RAC1* or *CDC42* also suppresses the high-temperature growth defect of the *ras1* mutant strain **(Figure 4)**. Cdc24 was recently identified as the downstream effector of Ras1 that activates Cdc42 to regulate thermo-tolerance and ability to cause infection in *C. neoformans* (Nichols *et al*., 2007). Over-expression of *CDC42* but not *RAC1* suppresses the *cdc24* temperature-sensitive growth defect. Therefore, Rac1 is similar to the Cdc42 protein and may play some overlapping functions downstream of Ras1. However, the primary role of Rac1 appears to be as a downstream effector of Ras1 to control hyphal formation during sexual differentiation working in coordination to function with other similar protein as an alternative pathway (Vallim *et al*., 2005). Differently, Cdc24 and Cdc42 proteins likely mediate Ras function in establishing and maintaining polarized growth in the budding yeast cell in the face of cell stresses, such as elevated temperature.

Ras signaling during morphogenesis of *C. albicans* has been studied extensively and it regulates both mitogen-activated protein kinase-dependent and cAMP–PKA-dependent responses (Phillips *et al.*, 2006). The morphogenetic switching relies on the activation of Ras-GTPase by extracellular signals as environmental sensing and induces two downstream cascades: a MAPkinase and the cAMP pathway, resulting in hypha-specific gene expression (Martin *et al*., 2005). The deletion of RAS gene in *C. albicans* is not essential; however the mutants presented defects in morphology, impaired ability to switch from yeast to filaments and reduced pathogenicity (Leberer *et al*., 2001). The defects observed in the *ras* mutant suggest the involvement of Ras signaling in control the high temperature growth and morphogenesis in the fungal pathogen *C. albicans*. Also, two *ras* genes were identified in *A. fumigatus* and despite the morphogenetic defects and the virulence attenuated observed in the *ras* mutant, there is just a suggestion of the indirect involvement of Ras on the high temperature growth control of this pathogen (Fortwendel *et al*., 2005).

In the dimorphic human pathogens as *P. marneffei* and *P. brasiliensis*, two *ras* genes were identified. In the first case, *ras* genes have been implicated in a signaling cascade to regulate morphogenesis (Boyce *et al*., 2005); however there is no evidence of the direct role of those genes in the dimorphic process of *P. marneffei*. In the case of *P. brasiliensis ras* genes were not regulated during the transition temperature dependent from mycelium to yeast, however *ras1* and not *ras2* showed a negative regulation after heat shock at 42ºC. Also both genes, *ras1* and *ras2*, were repressed inside the macrophage cells indicating the potential role of Ras in the response to temperature and host environment stimuli in *P. brasiliensis* (L. Fernandes, unpublished).

3.4. cAMP - PKA

Known as a nutrient sensing pathway the cAMP-PKA cascades involves a G protein couples receptor, Gpr1, the G proteins Gpa2 and Ras2, adenylyl cyclase, cyclic AMP (cAMP) and cAMP-dependent protein kinases. The role of cAMP is to activate protein kinase A (PKA), a heterotetramer consisting of two subunits, cAMP binds to two sites on each regulatory subunit and causes the release of free and active catalytic subunits, which may phosphorylate serine and threonine residues on target proteins which, in turn, stimulates enzymes involved in a myriad of cellular events. For example in *C. albicans* this pathway controls the utilization of storage carbohydrates, represses stress-activated genes,

regulates entry into stationary phase and promotes pseudohyphal morphogenesis (Jung and Stateva, 2003).

The cAMP-dependent pathway, which regulates yeast-to-hypha morphogenesis in *C. albicans*, is controlled by changes in cAMP levels. Both low-and high-affinity cAMP phosphodiesterases are encoded in the *C. albicans* genome. Deletion of *PDE2* causes elevated cAMP levels and responsiveness to exogenous cAMP, higher sensitivity to heat shock, severe growth defects at 42^0C and highly reduced levels of *EFG1* transcription (Jung and Stateva, 2003).

In order to understand the cyclic adenosine 5′-monophosphate (cAMP)/protein kinase A (PKA) signaling pathway, Hu *et al*., 2007 studied the transcriptional regulation of *PKA* in *C. neoformans* by the evaluation of the transcriptional changes in the *pka* mutants. They reported alterations of gene response involved with virulence, ribosome biogenesis, the response to stress, vesicle (protein) trafficking, membrane transport, and cell wall biogenesis. Among the genes related to high temperature growth that suffered any modulation by PKA were the *TPS1* that codes for trehalose-6-phosphate synthase, required in trehalose synthesis, and is involved in the ability of the fungus to grow at 37°C; *ILV2* encoding acetolactate synthase and *SPE3/LYS9* chimeric gene (spermidine synthase/saccharopine dehydrogenase) which are involved in amino acid metabolism and also necessary for growth at elevated temperature and virulence, and finally the cyclophilin A which is related to growth at host temperature.

3.5. Other genes

Up to now two genes have been directly related to thermophyly in *Aspergillus*: the *thtA* gene that allows the fungus to grow at 48°C (Chang *et al*., 2004) and the *cgrA* gene (Bhabhra and Askew, 2005). The *thtA* encodes a putative protein of 141 kDa with unknown function and the HA-tagged ThtA protein accumulated to similar levels in cultures grown at either 37°C or 48°C. ThtA was shown to be essential for growth of *A. fumigatus* at high temperatures but does not contribute to the pathogenicity of the species. In contrast, the *cgrA* gene seems to be implicated in the virulence of *A. fumigatus*. CgrA is a highly conserved nucleolar protein involved in ribosome biogenesis. The disruption of *cgrA* decreases the growth rate at 37°C *in vitro* and *in vivo* (Bhabhra *et al*., 2004).

The use of reverse-genetic allowed the access to the contribution of stress response genes implicated in high-temperature growth of *C. neoformans*. Giles and coworkers (2005) showed that Sod2, as a critical component for cell viability, is also implicated in the

adaptation to growth at elevated temperatures. The authors showed that Sod2 acts as a regulator of the steady-state concentration of reactive oxygen species at higher temperatures.

4. Signal transduction pathways as regulators of fungal virulence.

Virulence factors and events as dimorphism, growth at elevated temperatures, adherence to host cells, cell wall components, enzyme production are important for the ability of human pathogenic fungi to create a microenvironment that is favorable for its penetration and dissemination into the host, by digesting cell membranes, absorbing the nutrients derived from this digestive process, suppressing the host immune system, and finally growing through the host tissues. By the definition, a gene to be considered involved in virulence of one pathogen, its null mutant has to cause an attenuated infection compared to the wild type and reconstituted strains, based on "Molecular Koch's postulates" (Falkow, 1988; Falkow, 2004). Fungal pathogens have adapted the conserved signaling components involved in diverse cellular functions to activate their virulence factors in order to survive and establish the pathogenesis. Despite many virulence genes have been reported previously for diverse fungi, it is still unknown all signaling cascades events and components that elicits the activation of the pathogen virulence. Then we decide to explore the signaling pathways involved with virulence and also the virulence factors that still do not have an activation mechanism elucidated.

4.1. Signaling cascades

There are several genes in pathogens the contribution of which to oxidant adaptation in vitro can be correlated with their survival and virulence *in vivo*. In *C. albicans* the HOG pathway is activated in response to oxidative stress and *hog1* mutants are more sensitive to oxidants *in vitro* (Alonso-Monge *et al*., 2003). A strain of C. *albicans* lacking *SLN1* has reduced virulence in an animal model (Yamada-Okabe *et al*., 1999) and mutants in the two component response regulator Ssk1 are more easily killed by human neutrophils (Du *et al*., 2005), further confirming the role of both genes in the transmission of the phosphorylation signal to Hog1 MAPKinase.

The Skn7 response regulator has been described in *C. neoformans* (Wormley *et al*., 2005), in which it works independently of the Hog1 MAPK pathway (Bahn *et al.,* 2006). Animal model experiment revealed that *SKN7* disruption strongly attenuates cryptococcal

virulence *in vivo*, promoted increased susceptibility to oxidant stress and decreased intracellular survival in endothelium (Coenjaerts *et al*., 2006).

Only recently have histidine kinases (HK) – with receptor function or not – been identified as virulence determinants in fungi **(Figure 2)**. They trigger "two-component" systems that relay the received stimulus to other signaling pathways that target both nucleus and cytoplasm. Two such kinases, Tco1 and Tco2, have been implicated in genetic reprogramming and adaptation to the host by *C. neoformans* (Bahn *et al.,* 2006). Similarly, the kinase Chk1 from *C. albicans* is known to control cell wall biosynthesis, biofilm formation and adaptation to heat and oxidative stress, all of which are determinants of virulence (Chauhan *et al*., 2003; Kumamoto and Vinces, 2005a). In *A. fumigatus*, work with the HK (Fos1) has begun to unravel its function in the ability of the fungus to grow invasively (Clemons *et al*., 2002). In all these fungi, a classical target of the two-component system is the Hog1 MAPkinase pathway and the phosphotransfer from the HK is mediated by Ssk proteins, which possess aspartate residues that are phosphorilated by the kinase (Kruppa and Calderone, 2006). Members of both the two-component system and of the Hog1 pathway have been identified *in silico* for *P. brasiliensis* (Fernandes *et al*., 2005), which constitutes another hint at the phylogenetic conservation of this mechanism.

The cyclic AMP-dependent protein kinase A (PKA) is also a regulator of the morphogenesis, differentiation, stress response and virulence in eukaryotic microorganisms. In *A. fumigatus* deletion of one or more catalytic subunits of PKA has been shown to decrease growth, to reduce tolerance to oxidative stress and also virulence of this pathogen (Liebmann *et al*., 2003). A *pkaR* mutant, deleted for the regulatory subunits of PKA, presents reduced growth and germination rates, increased susceptibility to oxidative stress and attenuated virulence (Zhao *et al*., 2006). The *C. neoformans* Gα subunit, Gpa1, plays a key role in cAMP signaling and virulence (Alspaugh *et al*., 1997). A mutant in the Gpa1 protein showed reduced virulence and inability to synthesize melanin, which could be partially restored by the addition of extracellular cAMP, suggesting that Gpa1 regulates cAMP production (Alspaugh *et al*., 1998).

The calmodulin/calcineurin pathway is another determinant of virulence. Its action in *C. neoformans* seems to be preserving cell integrity at host temperature and thus protect the fungus against the new environment (Kraus and Heitman, 2003). As expected, mutants are avirulent. Both calmodulin and calcineurin seem to be essential for fungal pathogenicity and a similar role has been defined for the pathway in *C. albicans* and *A. fumigatus* (Bader *et al*., 2006; Steinbach *et al*., 2006).

4.2. Melanin as virulence factor

Many pathogenic fungi produce melanin, which are hydrophobic compounds though to confer protection from harsh environmental conditions. Melanin formation might protect fungal cells by quenching released free radicals, acting as a buffer against external ROS. Different types of melanin have been described as important virulence factors for numerous fungi. Mutants of *C. neoformans* lacking melanin pigment showed a reduced virulence in a murine animal model (Wang *et al*., 1995). DOPA-melanin protects the cells from damage by hypochlorite or hydrogen peroxide, which is part of the immune response (Wang *et al*., 1995). *C. neoformans* melanin has been postulated to inhibit TNF-α production and proliferation of lymphocytes, thus reducing the immune response to fungal infection (Huffnagle *et al*., 1995). *C. neoformans* encodes two laccases, with are both regulated by oxidative and nitrosative stress and deletion of both encoding genes reduces survival of *C. neoformans* in primary macrophages (Missall *et al*., 2005).

Both the MAPkinase and the cAMP signaling cascades regulate two important and very well described virulence factors - the melanin and capsule - in *C. neoformans*. Melanin and capsule genes are co-regulated at the level of transcription by cAMP pathway. In *C. neoformans*, Pka1 is required for both melanin and capsule production (D´Souza *et al*., 2001). Unlike *gpa1* mutant strains, exogenous cAMP does not restore melanin or capsule production in *pka1* mutant strains, suggesting that Pka1 is the target of cAMP activation. Strains with defects in the cAMP signaling cascade are unable to induce melanin and capsule formation and these strains are highly attenuated for virulence (Pukkila-Worley *et al*., 2005). A striking feature of the *C. neoformans* Hog1 MAPK pathway is the cross talk with the cAMP signaling pathway in controlling biosynthesis of capsule and melanin (Bahn *et al.,* 2005). In addition, Tco1 was found to be a key sensor histidine kinase negatively regulating melanin synthesis; disruption of the *TCO1* gene dramatically enhanced melanin production via the Pbs2-Hog1-pathway and the *tco1* mutant was less virulent in a mice model of infection, as cited above (Bahn *et al*., 2006).

In *A. fumigatus* DHN-melanin is though to protect the conidia from the host immune system by quenching reactive oxygen species. A mutant producing pigment- less conidia showed a reduced virulence (Tsai *et al*., 1998). The *pksP* mutant is more sensitive to hydrogen peroxide and sodium hypochlorite and more susceptible to damage by murine macrophages *in vitro* than the wild strain (Jahn *et al*., 1997; Jahn *et al*., 2000). Despite those descriptions, melanin seems not to be a major factor for *A. fumigatus* virulence, since

deletion of additional genes in the DHN-melanin pathway do not result on fungal virulence decrease (Tsai *et al*., 1999).

P. brasiliensis melanin-like pigments were detected in cells growing *in vitro* and during infection (Goméz *et al*., 2001) and also melanization of *P. brasiliensis* yeast cells reduces their paghocytosis by macrophages in culture (Silva *et al*., 2005). Transcripts encoding tyrosinase and aromatic-L-amino acid decarboxylase are up regulated in *P. brasiliensis* yeast cells recovered from murine infected tissues suggesting an active synthesis of melanin during experimental infection (Bailão *et al*., 2006). The other dimorphic fungus, *P. marneffei*, produces melanin *in vitro* as well as *in vivo*. Skin tissue of infected patients with this fungus was demonstrated to contain yeast cells that were labeled by melanin-binding antibodies (Youngchim *et al*., 2005). Melanization can also be detected in conidia and yeast cells of *H. capsulatum in vitro* and during infection (Nosanchuk *et al*., 2002). Despite of the presence of melanin like pigments in those dimorphic human pathogens the pathways that activate the melanin synthesis is still not elucidated. However, as the components of the cellular cascades are much conserved among the species probably that involved in melanization in those fungi may be the same as described for others as for *C. neoformans*.

4.3. Cell wall components – chitin, glucans and lipids

Cell wall, for almost fungi, is a complex structure, made by the ordered arrangement of different components. This composite provides protection to the cell against physical, chemical and biological aggression, and is responsible for the morphology and as an important factor for the virulence.

The crystalline arrangement of the polysaccharide chitin and its linkage to β-1,3-glucan constitute the basic cell-wall scaffold to which mannoprotein are covalently associated. The chitin amount in cell wall contributes to the virulence of *P. brasiliensis*, since the yeast contains three-fold higher chitin than mycelium (San-Blas and San-Blas, 1977). Proteins, related with fungal virulence, binds to chitin such as Yps3 from virulent strains of *H. capsulatum* (Bohse and Woods, 2005) and Bad1 from *B. dermatitidis* (Brandhorst and Klein, 2000). These yeast-phase-specific proteins are homolog and are involved in adhesion of fungi and can suppress cytokine production (Rappley and Goldman, 2006). Chitin synthases have been proposed to be a target for the control of mycosis considering the importance of chitin in the structure of the fungal cell wall and its absence in the host.

Glucans are the most abundant cell wall component. The β-(1,3)-glucan and α-(1,3)-glucan are essential components of the cell wall. The α-(1,3)-glucan, a virulence determinant, is a cell wall polysaccharide common to most medically important fungi, including all the dimorphic pathogenic species. Spontaneous loss of α -(1,3)-glucan correlated with decreased virulence, indicating this may be a conserved mechanism of fungal pathogenicity as described to *B. dermatitidis*, *C. neoformas, A. fumigatus, C. immitis* and *P. brasiliensis* (San-Blas and San-Blas, 1977; Klimpel and Goldman, 1988; Hogan and Klein, 1994, Bernard and Latge, 2001; Cole and Hung, 2001). The role of α-(1,3)-glucan in virulence was demonstrated by RNA interference in *H. capsulatum* (Rappleye *et al*., 2004) and also in *C.neoformans* (Reese and Doering, 2003). The silencing of *AGS1* expression in *H. capsulatum* cells impaired the ability of the fungus to proliferate, to kill macrophage in culture and showed decreased TNF-α production (Rappleye *et al*., 2004; Rappleye *et al*., 2007). The activity of the β-1, 3-glucan synthase is regulated by Rho-GTPase, and protein kinase C (PKC) signaling molecules. The Rho –GTPase was already identified in pathogenic fungi such as *Candida, Aspergillus, Cryptococcus, Pneumocystis* and *Paracoccidioides*, constituting a promising drug target against fungal diseases (Liu and Balasubramanian, 2001).

Lipids, in general, constitute the minor components of the fungal cell wall. In *C. albicans* the phospholipomannan are involved in organization of the glucan chains and have relevance in adhesion, protection and signaling (Ruiz-Herrera *et al*., 2006). The phospholipomannan mediated the *C. albicans* escape from macrophages and induction of cell apoptosis (Mille *et al*., 2004).

4.4. Antioxidant enzymes as virulence factors in response to oxidative stress

Copper-and zinc-containing superoxide dismutase (Cu/ZnSOD) is suspected to be one of the anti-oxidant enzymes and virulence determinants active in some pathogenic microorganisms. The *C. albicans SOD1* encoding a Cu/ZnSOD was disrupted; the resulting *sod1/sod1* mutant showed increased sensitivity to menadione compared to the isogenic wild-type strain. Furthermore the *C. albicans* lacking the Cu/ZnSOD showed increased susceptibility to macrophage attack and had attenuated virulence in mice, which suggests that the Cu/ZnSOD is required for the protection of *C. albicans* against oxidative stresses and for the full virulence of the organism (Hwang *et al*., 2002). Furthermore, *C. albicans* virulence is attenuated by the inactivation of catalase (*CAT1*) and *SOD5* (Wysong *et al*., 1998; Martchenko *et al*., 2004). Nevertheless, the significance of oxidative stress

responses in the virulence of *C. albicans* has been emphasized by the observations that mutations that inactivated such responses attenuate the virulence of this fungal pathogen. In *C. neoformans* the expression of the *SOD1* gene increases at the host temperature and the mutant lacking this Cu/ZnSOD can be killed by ROS in a cell free system and is less virulent than the wild-type strain in a murine model of infection (Cox *et al*., 2003). Strains of *C. neoformans* variety *gattii* lacking *SOD1* and *SOD2* are unable to produce experimental disease (Narasipura *et al*., 2005). Although little is known about the role of Sod in *A. fumigatus*, a Cu/Zn Sod is known to be immunoreactive with sera of patients with aspergillosis (Holdom *et al*., 2000). Four genes encoding for two Sod isoenzymes, a Mn containing and a Cu/Zn containing protein, had been described in the *P. brasiliensis* transcriptome (Campos *et al*., 2005). The *sod3* gene was induced upon internalization of *P. brasiliensis* yeast cells by *in vitro* cultured macrophages (Tavares *et al*., 2007). *H. capsulatum* has three catalase genes encoding *CATA*, *CATB* and *CATP*, presenting differential expression. Among them, *CATB* and *CATP* are constitutively expressed, whereas *CATA* expression is restricted to the mycelial phase on when yeast cells are exposed to H_2O_2 (Johnson *et al*., 2002). *P. brasiliensis* presents three catalases, one of which *catP* is up-regulated in the parasitic phase and when yeast cells are exposed to H_2O_2 (Moreira *et al*., 2004; Campos *et al*., 2005). The transcript encoding a homologue of *catA* is induced upon incubation of yeast cells in human plasma (Bailão *et al*., 2007).

The gene encoding for a thiol peroxidase (*TSA1*) of *C. neoformans* was shown to be necessary to hydrogen peroxide fungal resistance. In cryptococcosis inhalation mouse model the gene *TSA1* was proven to be necessary for virulence (Missall *et al*., 2004). Also the ability of the fungal pathogen *C. neoformans* to evade the mammalian innate immune response and cause disease is partially due to its capacity to respond and survive under nitrosative stress. A fungal deletion mutant for the glutathione reductase gene (*GLR1*) is sensitive to nitric oxide stress, but not peroxide stress, revealing specificity between oxidative and nitrosative stress response in *C. neoformans*. Additionally the *glr1* mutant is sensitive to macrophage killing being avirulent in mice (Missall *et al*., 2006). Peroxiredoxin homologues had been identified in the *P. brasiliensis* transcriptome (Campos *et al*., 2005), although the role in the antioxidant mechanisms remains to be elucidated. Also, a member of the thioredoxin family was up-regulated in the *P. brasiliensis* yeast cells recovered from liver of infected mice (Soares, CMA, unpublished).

4.5. Secreted virulence factors: phospholipases, proteases and other metabolites

Pathogenic fungi use extracellular enzymes to degrade the cell membrane, which is the main structural barrier of the host. The main enzymes are phospholipases and proteases. Since phospholipases target membrane phospholipids and digest these components, direct host cell damage and lyses have been proposed as a major mechanism contributing to virulence. Such host cell injury would be expected to facilitate the penetration of the infecting agent (Ghannoum, 2000; Ganendren *et al*., 2006).

Phospholipases constitute a heterogeneous group of enzymes which can hydrolyze one or more specific ester bonds in phospholipids. These enzymes are classified as: phospholipaseA (PLA_1, PLA_2), B, C and D. Besides the lysophospholipase activity, PLB also bears a transacylase activity (LPTA). The phospholipases observed in C. *neoformans* are similar to those found in *C. albicans* (Chen *et al*., 1997). *A. fumigatus* secretes PLA, PLB and PLC (Birch *et al*, 1996). In *P. brasiliensis* transcriptome a *plB* orthologue to *C. albicans* was found (Tavares *et al*., 2005). In this case, it is well known that *P brasiliensis* invades endothelial cells (Mendes-Giannini *et al*., 2004) and the action of phospholipases would therefore contribute to tissue invasion (Tavares *et al*., 2005). In *C. albicans* the amount of phospholipase varies according to the isolate and also is related with the infection location. For instance, blood isolates produce higher levels of phospholipases when compared with wound or urine isolates (Price *et al*, 1982). In *C. albicans*, PLB-deficient mutants when compared to parental strains revealed that the disruption of *PLB* does not affect growth and germination, but reduces the ability to secrete this enzyme (Leidich *et al*, 1998). In *C. neofarmans* a disruption gene (*PLB1*) also does not affect significantly fungi growth, but the inhibition of enzyme secretion reduces invasion levels at the lungs (Noverr *et al*., 2003; Santangelo *et al*., 2004). Besides contributing to virulence by causing cell damage in the host, phospholipases are also involved in survival inside macrophage, destruction of lung and in the production of eicosanoids with modulate phagocyty activity (Cox *et al*., 2001; Noverr *et al*., 2003).

In animal tissues the structural barriers are composed by phospholipids and proteins. Thus the fungus requires proteases to invade them, secreting large amounts of these enzymes during infection. Fungal extracellular proteases constitute a large and heterogeneous group of proteins. SAP protease (aspartyl peptidase) was broadly studied in *C. albicans* and was demonstrated to be related with the degradation of several human proteins such as albumin, hemoglobin, keratin and secreted immunoglobulin A (Hube *et al*., 1998). *C. albicans* presents nine different *SAP* (*SAP* 1 to 9) genes (Yang , 2003). *In vitro* studies demonstrated that *SAP* 1, 2 and 3 are expressed by yeast cells only, but *SAP* 4,

5 and 6 are expressed in the transition from yeast to hyphae at neutral pH (Hube *et al*, 1998, Schaller *et al*., 1999). The *SAP* gene in *C. albicans* is regulated at the transcriptional level and processed by a signal peptidase and Kex2-like protease (Newport and Agabian, 1997). Thus, production of Sap is a highly regulated and tightly controlled process, which appears to be an important virulence factor and is indicative of the multiple functions of this gene family, including digesting molecules for nutrient acquisition, destruction of host cell membrane aiding host tissue invasion, degradation of host surface molecules to enhance adhesion and degradation of cells and molecules of the host immune system (Naglik *et al*., 2003, dos Santos *et al*., 2006). *C. neoformans* protease activities degrade host proteins such a collagen, elastin, fibrinogen, immunoglobulins and complement factors (Chen *et al*., 1996). Degradation of host cells may protect *C. neoformans* from the host immune response and possibly aids in the escape of the fungal cells from phagosomal compartments (Chen *et al*., 1996, Steenbergen and Casadevall, 2003). Fungal proteases may induce local airway inflammation by recruiting inflammatory cells via activation of epithelial cells (Tomee *et al*., 1997). Serine proteases have been described in human pathogenic fungi such as *C. albicans* (Santos *et al*., 2006), *P. brasiliensis* (Carmona *et al*., 1995), *C. neofarmans* (Rodrigues *et al*., 2003) and *A. fumigatus* (Larcher *et al*., 1992). The serine protease activity in *A. fumigatus* causes cell desquamation and induces production of IL-8 , IL-6 and monocyte chemotactic protein 1 (MCP-1) in human pulmonary epithelial cell lines (Tomee *et al*., 1997). The production of these pro-inflammatory cytokines may trigger an inflammatory cascade involving macrophages, monocytes, lymphocytes and neutrophils causing local inflammation which may create a microenvironment for fungal attachment, penetration and mobilization of nutrients for fungal growth. The fungal attachment and penetration may further enhance inflammation and create a self-perpetuated cycle of inflammation (Tomee and Kauffman, 2000).

Besides phospholipases and proteases, another secreted enzyme which has to be highlighted is the urease. This enzyme catalyses the hydrolysis of urea yielding carbon dioxide (CO2) and ammonia (NH3) and further ammonium hydroxide, therefore creating an alkaline microenvironment. This enzyme is found in several fungi species such as *C. immitis, C. neoformans* and *P. brasiliensis* (Yu *et al*, 1997; Cox *et al*, 2000, Tavares *et al*., 2005). In *C. neoformans,* when the urease gene (*URE1*) was disrupted, the mutants did not show differences regarding capsule size or colony formation unit counts in the central nervous system of rabbit model (Cox *et al*., 2000). In contrast, in murine intravenous and inhalation models, mutants were attenuated when compared to the parental strain, which

imply the urease in the pathogenesis of *Cryptococcus*, probably in a species and /or infection site manner (Tavares *et al*., 2005).

Other important virulence factors are the metabolites such as gliotoxin, fumagillin and restictocin which facilitate fungal growth by causing generalized immunosuppression. One of the best studied fungal toxins is gliotoxin, first described as an antibiotic. Gliotoxin may be lethal at relative low concentrations and is produced by many fungi including *A. fumigatus*. Gliotoxin immunossupressive activity includes inhibition of phagocytosis, T cell proliferation, cytolytic T lymphocytes activation and adherence of macrophage, blood monocytes, fibroblasts ((Tomee and Kauffman, 2000). This inhibitory activity of gliotoxin may be exerted by inducing DNA damage through production of reactive oxygen species **(**(Tomee and Kauffman, 2000). Other special classes of toxins are the ribotoxins produced by *Aspergillus*. These toxins are small basic ribosome-binding proteins that inactivate the large ribosomal subunit impairing protein synthesis.

4.6. Biofilm as a virulence factor in fungal pathogens

The ability to modify the surrounding environment by secretion of an extracellular matrix is the landmark of biofilm formation. *C. albicans* is capable to structure biofilms on several kinds of surface, both organic and inorganic, and this accounts for the resilience of systemic candidiasis and its resistance to treatment in several cases (Kumamoto and Vinces, 2005b). The secretion of matrix components seems to be regulated by the Histidine kinase Chk1, but in this case more can be said about the triggering mechanism. Morphogenetic and virulence processes in this pathogen are influenced by the recently described mechanism of eukaryotic quorum-sensing. Tyrosol (Chen *et al*., 2004) and farnesol (Hornby *et al*., 2001) are the quorum-sensing molecules (QSM) of *C. albicans*. The former induces biofilm synthesis and the latter represses it (Alem *et al*, 2006). Recent data indicate that farnesol exerts its inhibitory action through the Chk1 two-component system (Kruppa *et al*., 2004). The mechanism of action of tyrosol remains unknown, but its actions go beyond matrix production. In an apparent positive feedback system, tyrosol is known to induce tube germination and biomass expansion in *Candida* biofilms (Alem *et al*, 2006). As for farnesol, there seems to be at least one kind of cell response to this molecule that could contribute to fungal virulence: farnesol-conditioned media have conferred *in vitro* resistance of *C. albicans* to oxidative stress (Westwater *et al*., 2005).

Concluding remarks

Understanding the host-pathogen interaction may provide insights to host defenses and the tactics used by pathogens to circumvent these defenses. The discovery of the TLR and the non-TLR implicated in anti-fungal immunity has given an insight into the mechanisms of intracellular host signaling following microbial sensing. In counterpart, signal transduction pathways provide mechanisms for fungal adaptation to stress conditions. Transduction pathways, including the MAPkinases, calcineurin, Ras and cAMP/PKA, are responsible for activating transcription factors to control gene expression in cell survival, osmotic shock, morphogenesis, thermal and oxidative stresses and virulence in fungi. Molecular understanding of those signaling networks increases the knowledge in cell communication and provides new landscapes for drug targets. Besides, understanding the mechanisms of signal transduction pathways may facilitate the development of new drugs and therapies for fungal infections disease.

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Figure Legends

Figure 1 – Membrane receptors recognizing yeast components – The fungi molecules bind to the toll-like (TLR), Dectin and/or mannose receptors inducing intracellular signaling cascades that result in cell-specific responses such as phagocytosis, burst respiratory and cytokines production. **TAK1 -** TGF-β-Activated Kinase 1**; NIK** - NF-kβ-Inducing Kinase**; NF-κB -** Nuclear factor κB; **IKK -** I-kB kinase; **SYK -** Spleen Tyrosine Kinase; **CARD9 -** adaptor molecule containing an N-terminal caspase-recruitment domain.

Figure 2 – Proposed mechanism of cAMP/PKA and MAPkinases pathways cross-talking by Ras-GTPases in pathogenic fungi that regulates morphogenesis, high temperature growth and virulence. In *P. brasiliensis* the dotted arrowhead indicates that the mechanism was not still demonstrated, however all the components listed on the pathways were found by *in silico* annotation of the pathogen transcriptome.

Figure 3 – Mechanism of stress-response in pathogenic fungi. The diagram depicts the multi-step phosphorelay pathways in *C. albicans*, *C. neoformans* and *P. brasiliensis*. Arrowheads depict known routes; dotted arrowheads indicate unconfirmed signaling pathways.

Figure 4- Comparative cascades signaling that control temperature stress response pathways in *C. neoformans*, *C. albicans* and *P. brasiliensis.* The Cam1/Cna1 corresponds to the calcium-calmodulin/calcineurin and PKC1/Mkk1/Mkc1 to the MAPkinase pathways, respectively. Arrowheads depict known routes; dotted arrowheads indicate unconfirmed signaling pathways.

Table 1 – Patter-Recognition Receptors (PRR) and Pathogen-Associated Molecular Patterns (PAMPs) involved in the host-fungal pathogen interaction

PRR	Fungal PAMPs	Microorganisms
Non-TLR		
CD14	glucuronoxylomannan	*Cryptococcus neoformans*
CR3	mannose, β-glucan, N-acetylglucosamine, methylmannoside, methylglucoside, complement opsonized pathogen	*C. albicans, C. neoformans, P. brasiliensis*
Classical C-type lectin receptor	mannose	*C. albicans, P. carinii*,
Non-classical C-type lectin receptor	β -glucan	*C. albicans, P. carinii, H. capsulatum, Coccidioides posadasii, Aspergilus fumigatus*.
Lactosylceramide	β -glucan	*P. carinii, C. neoformans, C. albicans*
Mannose-binding lectin	mannose, fucose, glucose	*C. neoformans, A. fumigatus, C. albicans*
TLR		
TLR2	phospholipomannan, zymosan, lipoproteins, lipopeptides, glycolipids	*C. neoformans, C. albicans, A. fumigatus, H. capsulatum.*
TLR4	mannan, glucoronoxylomannan	*C. neoformans, C. albicans, A. fumigatus*

Adapted and modified from Brown, 2006. CD14 – cluster of differentiation 14; CR3 - complement receptor 3; PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptor; TLR, Toll-like receptor.

Figure1.

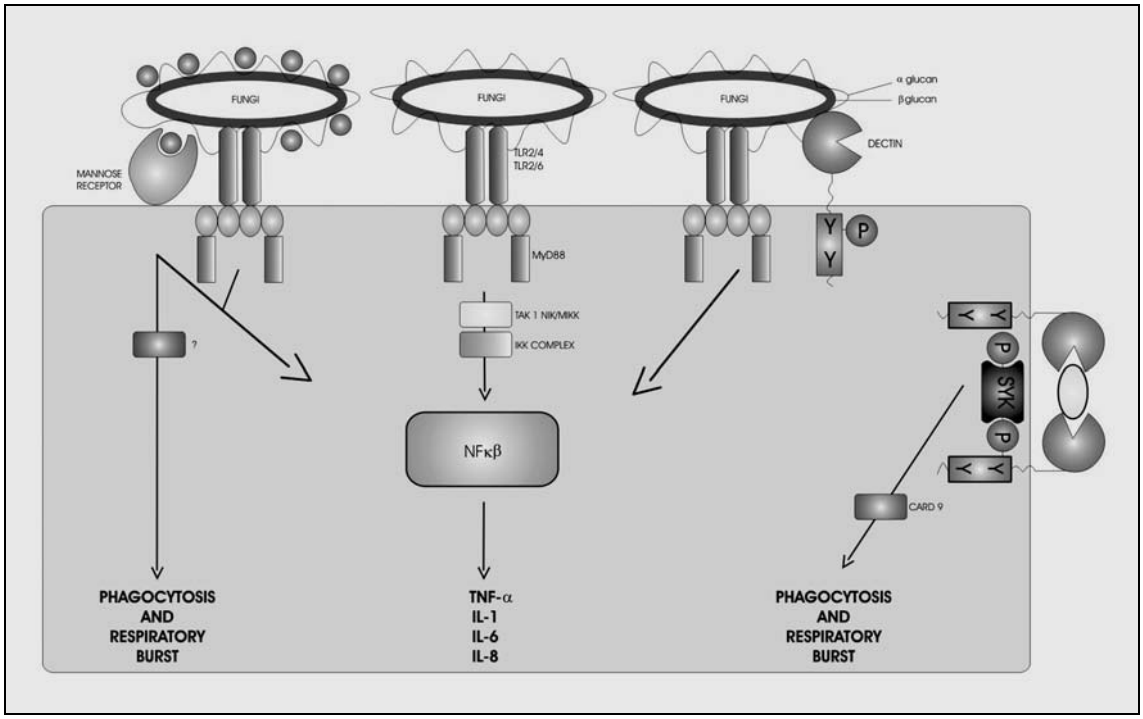

FUNGI
FUNGI
FUNGI
α glucan
βglucan
DECTIN
MANNOSE RECEPTOR
TLR2/4
TLR2/6
MyD88
TAK 1 NIK/MKK
IKK COMPLEX
?
NFκβ
SYK
CARD 9
PHAGOCYTOSIS AND RESPIRATORY BURST
TNF-α IL-1 IL-6 IL-8
PHAGOCYTOSIS AND RESPIRATORY BURST

Figure2.

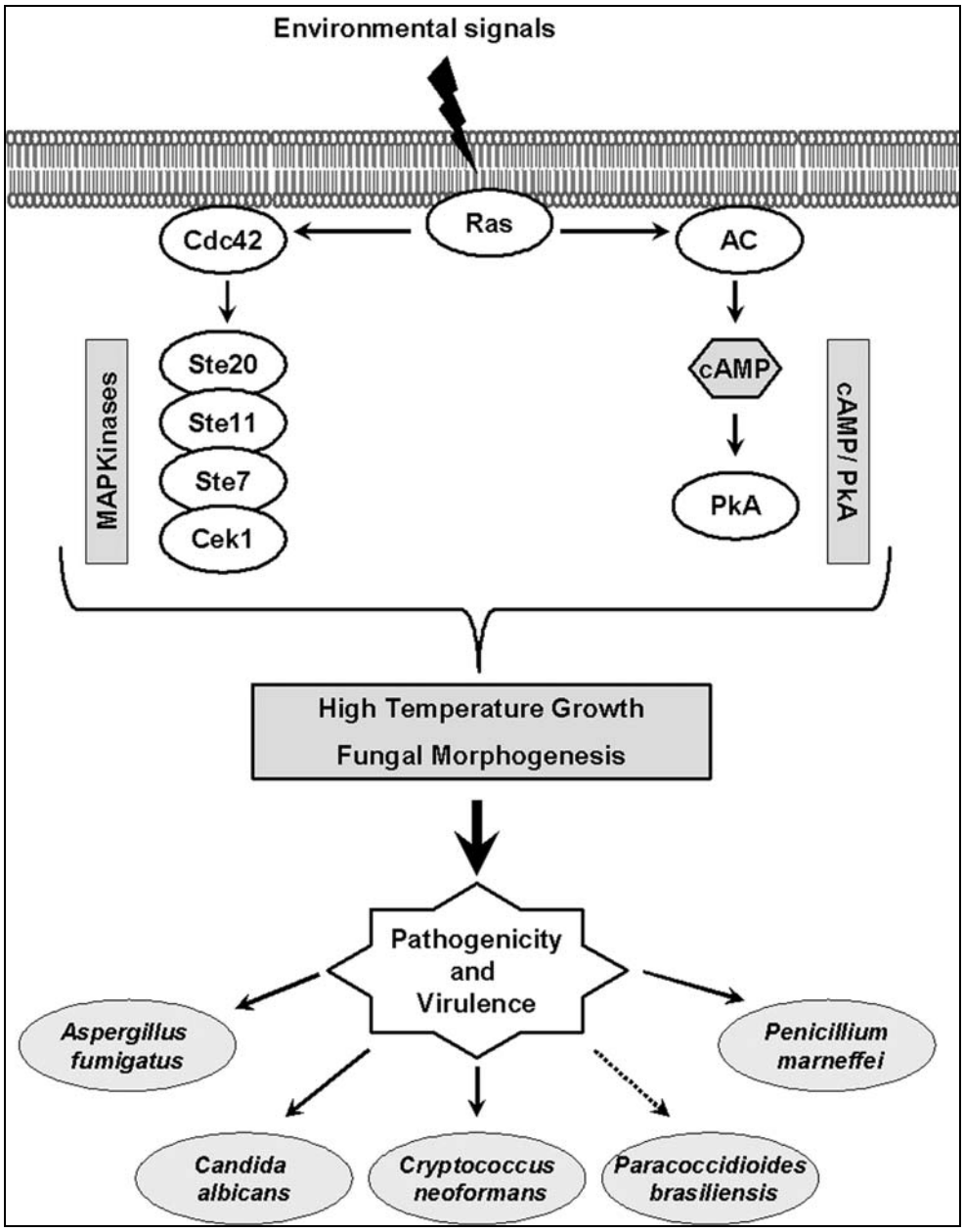

Environmental signals
Ras
Cdc42
AC
MAPKinases
Ste20
Ste11
Ste7
Cek1
cAMP
PkA
cAMP/ PkA
High Temperature Growth
Fungal Morphogenesis
Pathogenicity
and
Virulence
Aspergillus fumigatus
Penicillium marneffei
Candida albicans
Cryptococcus neoformans
Paracoccidioides brasiliensis

Figure 3

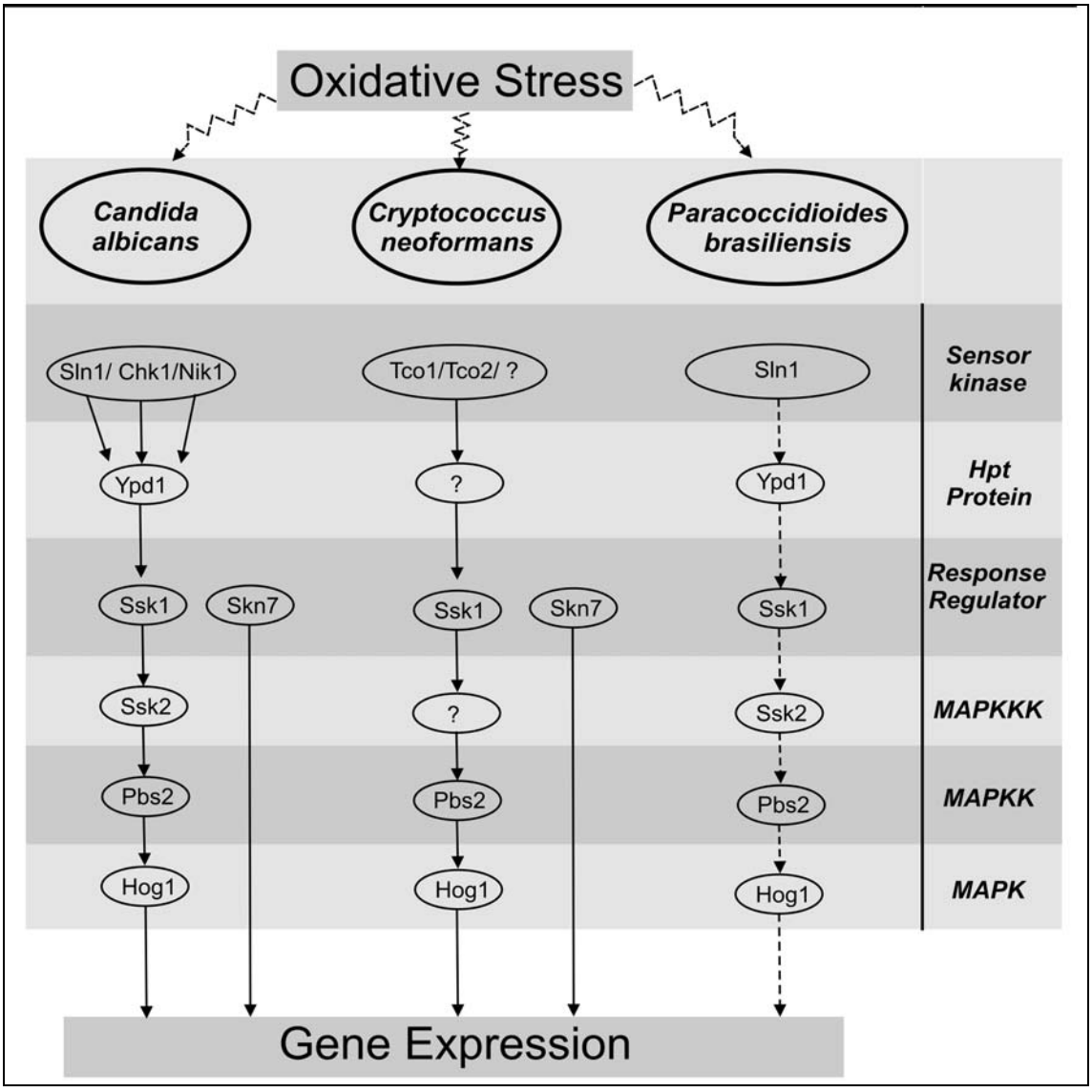
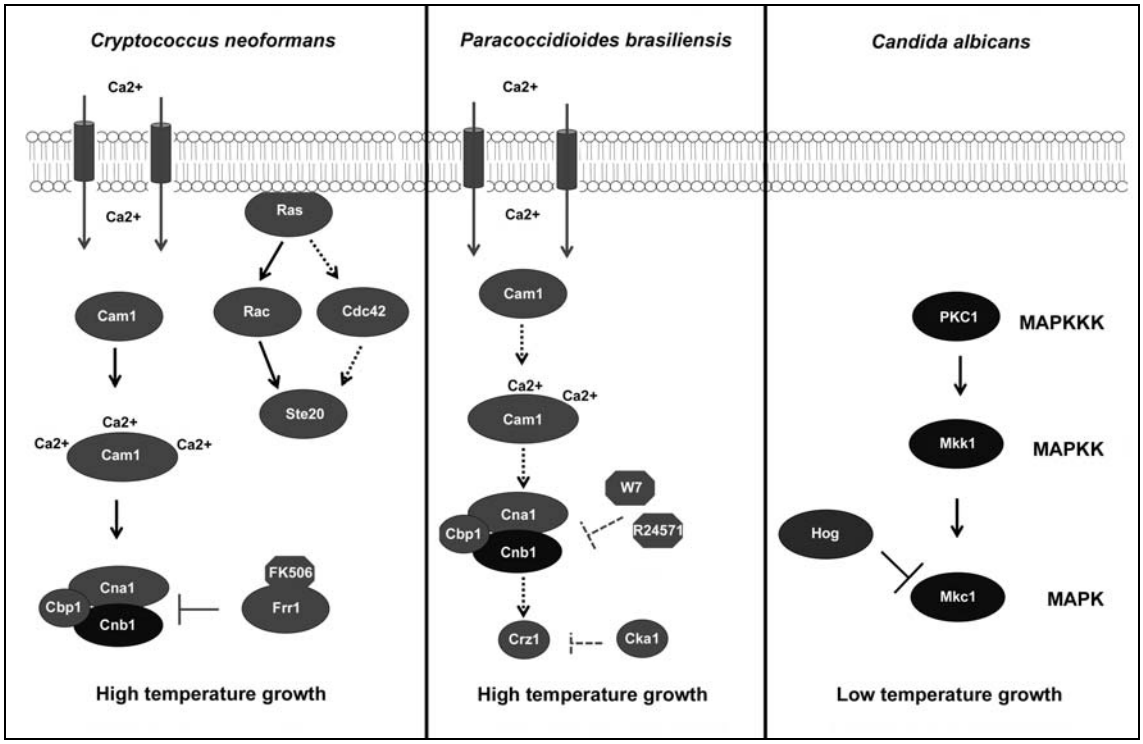

Oxidative Stress
Candida albicans
Cryptococcus neoformans
Paracoccidioides brasiliensis
Sln1/ Chk1/Nik1
Tco1/Tco2/ ?
Sln1
Sensor kinase
Ypd1
?
Ypd1
Hpt Protein
Ssk1
Skn7
Ssk1
Skn7
Ssk1
Response Regulator
Ssk2
?
Ssk2
MAPKKK
Pbs2
Pbs2
Pbs2
MAPKK
Hog1
Hog1
Hog1
MAPK
Gene Expression

Figure 4


Cryptococcus neoformans
Ca2+
Ca2+
Ras
Cam1
Rac
Cdc42
Ste20
Ca2+
Ca2+
Cam1
Ca2+
Cna1
Cbp1
Cnb1
FK506
Frr1
High temperature growth
Paracoccidioides brasiliensis
Ca2+
Ca2+
Cam1
Ca2+
Ca2+
Cam1
W7
Cna1
Cbp1
Cnb1
R24571
Crz1
Cka1
High temperature growth
Candida albicans
PKC1
MAPKKK
Mkk1
MAPKK
Hog
Mkc1
MAPK
Low temperature growth

Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells.

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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.

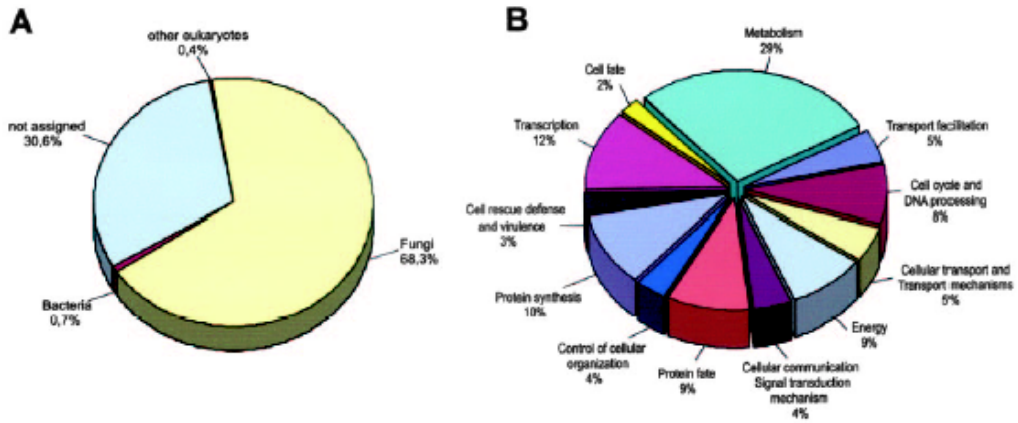

FIG. 1. ***P. brasiliensis* transcriptome characterization.** *A*, distribution of blast best hit among organisms. Each PbAEST was tested against the GenBank™ nr data base, and the best hit organism was computed. A PbAEST was considered as not assigned when the best hit exceed an *E* value of 10^{-10}.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

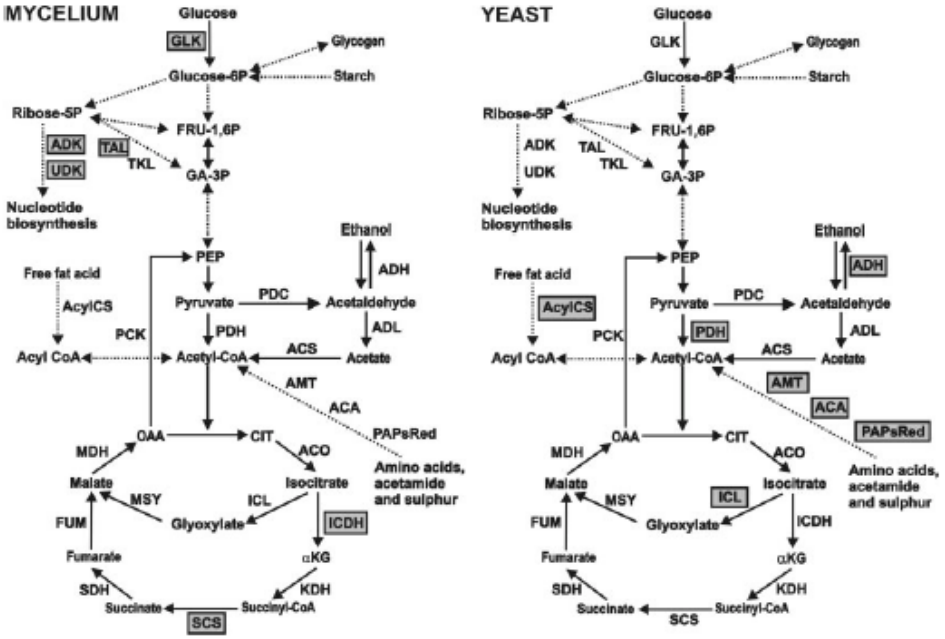

FIG. 2. **Comparison of the expression pattern of genes encoding for enzymes in mycelium-to-yeast cell differentiation of *P. brasiliensis*.** For the detailed metabolic comparison between mycelium and yeast metabolism, see Supplemental Table IV, since we have presented in this figure only the central 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.

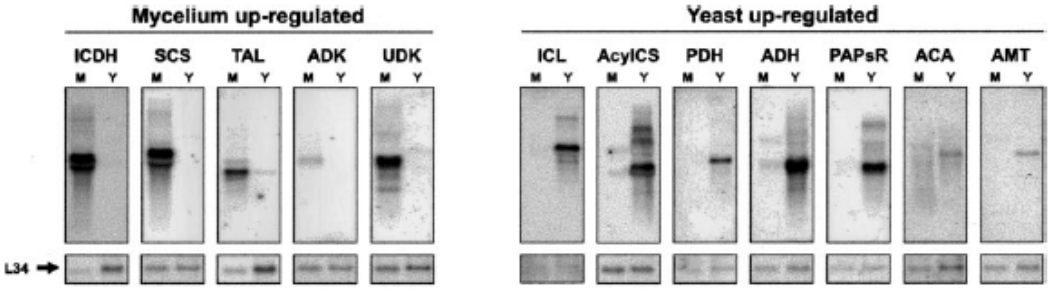

FIG. 3. **Northern blot analysis of mycelium and yeast up-regulated genes of *P. brasiliensis*.** Total RNA samples from both mycelium (*M*) and yeast (*Y*) were blotted onto nylon membranes and hybridized against gene-specific radiolabeled probes. *ICDH*, isocitrate dehydrogenase; *SCS*, β-succinyl-CoA-synthetase, *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

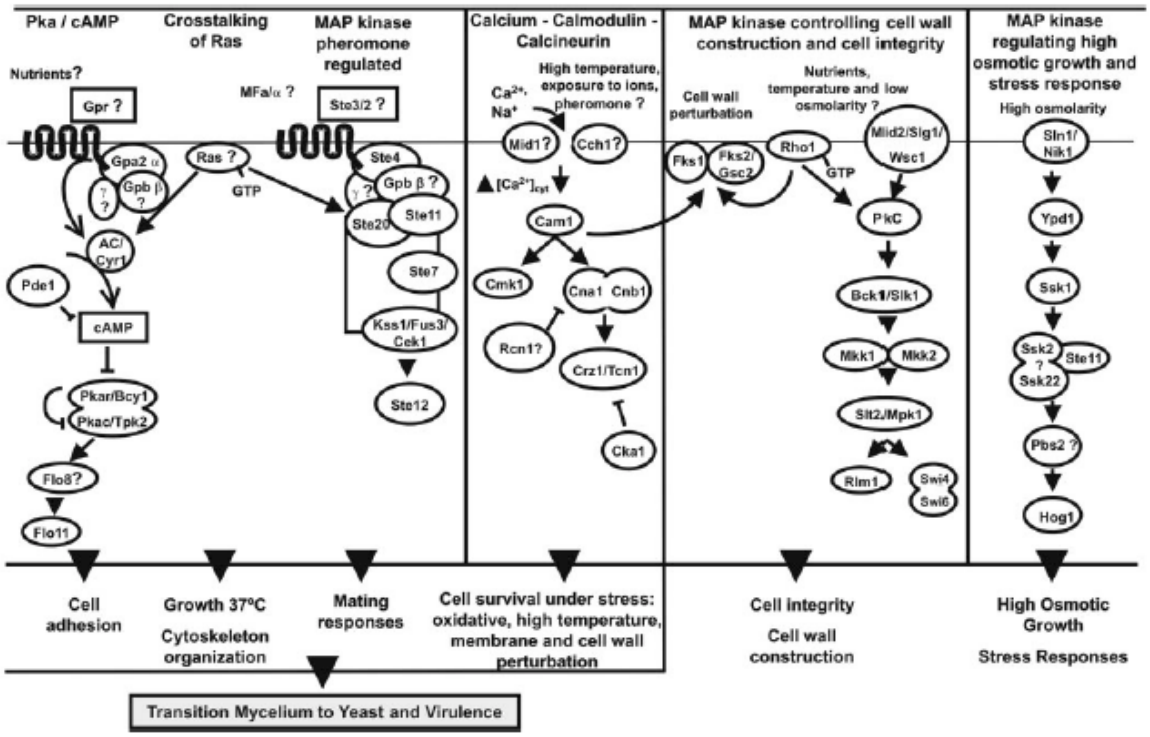

FIG. 4. **Signaling pathways in *P. brasiliensis*.** Shown are cAMP/protein kinase A regulating fungal development and virulence; MAPK signaling for cell integrity, cell wall construction, pheromone/mating, and osmoregulation; calcium-calmodulin-calcineurin controlling cell survival under stress conditions; 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	*gnt1*[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	AAL18064	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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Therapeutic targets in *Paracoccidioides brasiliensis*: post-transcriptome perspectives

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Therapeutic targets in *Paracoccidioides brasiliensis*: post-transcriptome perspectives

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ABSTRACT. The rise in antifungal resistance, observed as a result of the increasing numbers of immunocompromised patients, has made the discovery of new targets for drug therapy imperative. The description of the *Paracoccidioides brasiliensis* transcriptome has allowed us to find alternatives to refine current therapy against paracoccidioidomycosis. We used comparative analysis of expressed sequence tags to find promising drug targets that have been addressed in other pathogens. We divided the analysis into six different categories, based on the involvement of the targeted mechanisms in the cell: i) cell wall construction, ii) plasma membrane composition, iii) cellular machinery, iv) cellular metabolism, v) signaling pathways, and vi) other essential processes. Through this approach, it has been possible to infer strategies to develop alternative drugs against this pathogen.

Key words: Drug targets, Fungi, *Paracoccidioides brasiliensis*

INTRODUCTION

The incidence and severity of mycoses have grown to alarming levels worldwide. Patients with immune deficiency have contributed to this scenario since they are more susceptible to unconventional, more aggressive forms of classical mycoses, and they also tend to harbor resistant varieties of the pathogens. Antifungal agents currently available for treatment of systemic mycoses include four groups of drugs, namely Polyenes (amphotericin B), Fluoropyrimidines (flucytosines), Azoles (ketoconazole, fluconazole, itraconazole), and Echinocandins (caspofungin and mycafungin). More recently, compounds from the Allylamine group (terbinefine) have shown high synergy with drugs from the Azole group, but even this drug association is unable to treat some fungal infections adequately. In addition, antifungals cause serious side effects, such as nephrotoxicity. In this context, the search for alternative therapies and the development of more specific antifungal drugs are urgently needed (Georgopapadakou and Walsh, 1996; Wills et al., 2000; Kontoyiannis and Lewis, 2002).

Some strategies have been employed to develop antifungal drugs. One of them is the classical method, in which screening is done *in vitro* with a great variety of compounds, either extracted from plants and animals or synthesized *in vitro* (Wills et al., 2000). The refinement of classical drugs or of administration methods is a promising alternative to optimize pharmacological action at the site of infection or to increase specificity. More recently, nanotechnology has been envisaged to administer safe and effective low dosages of drugs, diminishing side effects and complications for the patients, such as amphotericin B (Fukui et al., 2003; Sobue and Sekiguchi, 2004).

In this era of genomic science, genome sequencing and bioinformatics drive the discovery of new antifungal targets. We have made efforts to identify such targets, or at least candidates, in *Paracoccidioides brasiliensis*, based on transcriptome analysis and on sites of action of antimycotic drugs already described in the literature, either for *P. brasiliensis* or for other fungal pathogens.

CELL WALL SYNTHESIS

The cell wall of human pathogenic fungi is in close contact with the host and serves as a barrier against host defense mechanisms. Human cells are ineffective in degrading cell wall polysaccharides of *P. brasiliensis*, as shown by ultrastructural studies of yeast cells ingested by activated macrophages, which revealed empty cells with intact walls (Brummer et al., 1990). The cell wall has an essential role in the pathobiology of *P. brasiliensis*, since it is directly linked to the morphogenetic changes associated with the fungal life cycle. The cell wall remains to be explored in detail as a target for antifungals (Selitrennikoff and Nakata, 2003). Genes involved in cell wall metabolism in *P. brasiliensis* have been isolated, and many others were identified in the transcriptome (Table 1). Some are differentially expressed, such as chitin synthases (Niño-Vega et al., 2000), hydrophobins (Albuquerque et al., 2004), mannosyltransferase-*Pbymnt* (Costa et al., 2002), and chitin deacetylase - *cda* (Felipe et al., 2005) and are candidates for drug targets.

Table 1. Potential drug targets related to cell wall construction.

PbAEST	Orthologue name	Product	References	Remarks	Known inhibitor(s)
Enzymes and synthases					
5198	*Pb*FKS1	1,3-β-glucan synthase	Pereira et al. (2000)	Synthesize glucan polymer	Candins
4988	*Pb*AGS	1,3-α-glucan synthase		Present in the yeast phase	
944	*RHO*	RHO		Regulate 1,3-β-glucan synthase	
1011, 742, 4968	PGI, GFA and GNA	Phosphoglucose isomerase, ketol-isomerase and glucosamine-6-phosphate acetyltransferase		Precursors of the chitin	
1147, 1927, 3456, 5473, 954	*Pb*CHS1, *Pb*CHS2, *Pb*CHS3, *Pb*CHS4, and *Pb*CHS5	Chitin synthase	Niño-Vega et al. (1998, 2000)	Expression changed during dimorphic transition	Polyoxins and nikkomycins
3958	*Pb*CHS6	Chitin synthase		Present in the mycelium phase	
Remodeling enzymes and others					
	*Pb*YMNT	Mannosyltransferase	Costa et al. (2002)	Expressed preferentially in the yeast phase	
2980	PMT1	Mannosyltransferase		Required for dimorphism (Ernst and Prill, 2001). Present in yeast phase	
3607	*KTR1*	Mannosyltransferase			
1063	KTR3	Mannosyltransferase		Present in the mycelium phase	
191	MNN2	Mannosyltransferase			
3220	MNN9	Mannosyltransferase		Present in the yeast phase	
202	BGL2	Glucosyltransferase		Transglucosylase	
1370	GAS1	Endotransglycosylase		May be involved in extending and rearranging 1,3-β-glucan chains (Popolo and Vai, 1999)	
5441	CRH1			Localized at the cell surface, near chitin-rich areas (Rodriguez-Peña et al., 2000)	
1527, 2375, 1370	GEL1, GEL 2 and GEL 3	β-1,3-glucanosyltransferase	AY380566, AY340235 and AY324033	Transglucosylase	
	*Pb*NAG1	N-acetyl-β-D-glucosaminidase	Santos et al. (2004)		
1058	EGL	β-1,3-endoglucanase			
1195	CTS1	Chitinase		Daughter cell specific expression	Allosamidin and demethylallosamidin
1281, 1511	SCW1, DSE4	Glucanase		Daughter cell specific expression	
0737	CDA	Chitin deacetylase		Expressed preferentially in the yeast phase	

PbAEST = *Paracoccidioides brasiliensis* assembled expressed sequence tag.

Synthases

1,3-β-glucan synthase

In *P. brasiliensis*, 1,3-β-glucan synthase requires uridine diphosphate (UDP)-glucose as the preferred nucleotide precursor to the *in vitro* synthesis of β-glucan (San-Blas, 1979). Only one homolog to 1,3-β-glucan synthase, *PbFKS1*, has been cloned from this fungus (Pereira et al., 2000). The presence of putative regulatory signals suggests flexible and possibly complex control mechanisms for the expression of *PbFKS1*. Although the UDP-glucose-binding motif was not found, domain analogs to cellulose synthase, proposed by Kelly et al. (1996), are present in *PbFKS1*. Analyses suggest that PbFks1p is localized within the cytoplasmic membrane and possesses a catalytic cytoplasmic domain between two transmembrane regions. PbFks1p seems to assemble the phosphorylated glucan polymer and simultaneously extrude it out of the membrane, since a phosphotransferase system-phosphoryl carrier protein component phosphorylation site motif has been found.

1,3-β-glucan synthase is regulated by the RHO GTPases, which is a multifunctional regulator related to numerous proteins (Douglas, 2001). The role for Rho1p in the regulation of 1,3-β-glucan synthase has been described in pathogenic fungi, such as *Candida albicans* (Kondoh et al., 1997), *Aspergillus fumigatus* (Beauvais et al., 2001) and *Cryptococcus neoformans* (Tanaka et al., 1999). Two *RHO* genes were identified in the transcriptome of *P. brasiliensis*. *RHO1* is a key regulator of 1,3-β-glucan synthase in budding and fission yeasts and in *C. albicans* (Kondoh et al., 1997). In *Schizosaccharomyces pombe*, Rho2 GTPase functions as a regulatory factor of 1,3-α-glucan synthase (Calonge et al., 2000).

Echinocandins disturb the membrane and cause a loss of the enzymatic activity of 1,3-β-glucan synthase without any direct binding to the catalytic site, thus compromising the structure and osmolarity of the cell wall (Klepser, 2003). Three echinocandins are currently in clinical development: caspofungin (Merck & Co., Inc., Rahway, NJ, USA), micafungin (Fujisawa Inc., Deerfield, IL, USA), and anidulafungin (formerly LY303366; Versicor Inc., Freemont, CA, USA). The 1,3-β-glucan synthase inhibitors papulacandin B, micafungin and cilofungin are more active against the mycelial form of *P. brasiliensis* (Dávila et al., 1986; Hanson and Stevens, 1989; Nakai et al., 2003). A new generation of semi-synthetic candins is emerging (Tkacz and DiDomenico, 2001) and natural-product screening by a series of new methods has also identified other 1,3-β-glucan synthase inhibitors (Onishi et al., 2000; Barrett, 2002).

1,3-α-glucan synthase

1,3-α-glucan synthase is proposed as a virulence factor in *P. brasiliensis* (San-Blas et al., 1977), as well as in *Blastomyces dermatitidis* (Hogan and Klein, 1994) and *Histoplasma capsulatum* (Klimpel and Goldman, 1988). We identified the 1,3-α-glucan synthase gene, Ags2, in the yeast phase of the *P. brasiliensis* transcriptome. Few 1,3-α-glucan synthase genes have been isolated to date. *Aspergillus fumigatus* mutants show a slight reduction in the growth rate and in the concentration of cell wall 1,3-α-glucan synthase (Bernard and Latgé, 2001). To our knowledge, no inhibitors of 1,3-α-glucan synthase have been identified. The search for these inhibitors is very important since the yeast pathogenic phase presents 1,3-α-glucan as an almost exclusive (95% of the cell wall) glucan polymer (Kanetsuna et al., 1972).

Chitin synthase

Membrane-bound chitin synthase catalyses the polymerization of GlcNAc (N-acetyl-β-D-glucosaminidase) from cytosolic UDP-GlcNAc into polysaccharide chains that are extruded to the cell wall (Ruiz-Herrera, 1992; Gooday, 1995). Chitin synthesis in fungi is a complex process (Horiuchi and Takagi, 1999), regulated by multigene families and involved in distinct physiological processes (Cabib, 1991; Gaughran et al., 1994). Chitin synthases are inhibited by nucleoside tri- and dipeptide molecules, polyoxins and nikkomycins, respectively. These inhibitors, structurally analogous to UDP-GlcNAc, are transported by peptide permeases into the cell, where they probably act by binding to the catalytic site of the chitin synthase. Transport failure and low permeability of the inhibitors result in resistance (Ruiz-Herrera and San Blas, 2003; Gozalbo et al., 2004).

Although it is possible to express *CHS* genes in a heterologous host (Silverman et al., 1988; Bulawa et al., 1986), these transmembrane proteins are not soluble recombinant molecules amenable to direct high-throughput screening. Studies of the molecular mechanism of chitin polymerization (Ruiz-Herrera and San Blas, 2003) and crystallization (Ruiz-Herrera, 1992) may reveal new drug targets in the future.

Five chitin synthases were identified by PCR amplification of conserved *CHS* gene domains in *P. brasiliensis*. Despite the fact that yeast cells contain more chitin than do hyphae, the levels of mRNAs for *PbCHS1*, *PbCHS2*, *PbCHS4*, and *PbCHS5* are higher in the mycelium (Niño-Vega et al., 2000), suggesting that post-transcriptional regulation of *CHS* gene expression is important for morphogenesis. We have identified, by means of transcriptome analysis, a new chitin synthase, *CHS6*, differentially expressed in the mycelium phase of *P. brasiliensis*.

Phosphoglucose isomerase, glutamine fructose-6-phosphate amidotransferase and glucosamine-6-phosphate acetyltransferase are critical enzymes in the synthesis of cell wall precursors. Since low levels of these precursors result in cell wall abnormality, leading to fungal cell death, these enzymes, also present in the *P. brasiliensis* transcriptome, are potential targets for antifungal drugs (Selitrennikoff and Nakata, 2003).

Remodeling enzymes

Mannosyltransferase

Some proteins present in the cell wall are glycosylated on their serine or threonine amino acids by the addition of mannose residues. Mannosyltransferases, Pmt1p and Mnt1p, are important for cell wall structure, adhesion and virulence (Gozalbo et al., 2004). In addition PMT1 is required to dimorphism (Ernst and Prill, 2001). Another protein involved in the synthesis of *N*-linked outer chain mannans is codified by the MNN9 gene. Disruption mutants for this gene exhibit phenotypes with characteristic defects in cell wall biosynthesis and/or assembly (Wills et al., 2000). Benanomicins and pradimicins bind selectively to the terminal D-mannosides of mannans in the cell wall and cell membrane of fungal cells in a calcium-dependent manner, resulting in insoluble complexes (Gozalbo et al., 2004). The PMT1, MNN2, KTR1, KTR3, MNN9, and *Pb*YMNT1 genes identified in *P. brasiliensis* transcriptome are promising targets for future antifungal therapy.

Cross-linking among cell wall components

Cell wall polymers are linked differentially to form the final architecture responsible for different morphologies in fungi (Klis et al., 2001). Enzymes involved in the integration of 1,3-β-glucans have been described. They are Gas1p, an endotransglycosylase that may be involved in extending and rearranging 1,3-β-glucan chains (Popolo and Vai, 1999), Bgl2p, an endotransglycosylase that introduces intrachain 1,6-β-linkages (Goldman et al., 1995), Crh1p (Rodrigues-Peña et al., 2000) and the homolog to GPI-anchored 1,3-β-glucanosyltransferases Gel1p (Beauvais and Latgé, 2001). Genes that encode these enzymes were identified in the *P. brasiliensis* transcriptome. Transglycosidases play an active role in cell wall synthesis and in fungal morphogenesis (Beauvais and Latgé, 2001). Since enzymes responsible for the branching of 1,3-β-glucan or the linkage of chitin to 1,3-β-glucan are active in the periplasmic space and involve essential biosynthetic steps, they present good candidates for drug development. To our knowledge, no inhibitors of these enzymes have been identified.

Hydrolases

Hydrolytic enzymes have different roles in the morphogenetic events (Wessels, 1988). In contrast to the other fungal β-1,3-endoglucanases reported in the literature, which are exocellular, the first cell wall-associated fungal β-1,3-endoglucanase was identified in *A. fumigatus* (Mouyna et al., 2002). In addition, several cell wall-associated and secreted chitinases have been identified (Mellor et al., 1994; Hearn et al., 1998). Inhibition of chitinases by antibiotics, such as allosamidin or demethylallosamidin, leads to the formation of clumps, as daughter cells are unable to separate from mother cells during budding (Gozalbo et al., 2004). The chitinase and β-1,3-endoglucanase genes were identified in the transcriptome of *P. brasiliensis.* Our group has cloned and characterized *Pb*NAG1, encoding an NAG, a glycosyl hydrolase family 20 protein (Santos et al., 2004). Another predicted NAG gene, *Pb*NAG2, is currently under study.

PLASMA MEMBRANE COMPOSITION

The fungal plasma membrane is the most important target for antifungal drugs in current therapy. It is based on the fact that the plasma membrane acts as the main interface between the cell and the environment of all organisms. Disturbances in the environment are perceived by the cell across transmembrane proteins, and a cascade of signals is started to allow for adaptation. In this context, the transduction of signals will culminate in membrane composition alterations to adapt to different situations (Georgopapadakou and Walsh, 1996; Wills et al., 2000).

The cell has the ability to adapt to different levels of pressure, pH variation and mechanical stress. Ergosterol and sphingolipids are responsible for the shape and rigidity of the plasma membrane. If some steps of their biosynthetic pathways are blocked, the intermediates may accumulate inside the cell and promote an excessive stiffness in the membrane (Wills et al., 2000; Theis and Stahl, 2004).

Pump-mediated efflux confers the cell with a way to exchange ions and nutrients, granting optimal pH and energy reserves. Depletion in the mechanisms of pump efflux may impair synthesis of important cellular components, including cell wall precursors (Luo et al.,

1999; Theis and Stahl, 2004). Since some drugs can affect the integrity of the cell by forming pores in the plasma membrane and promoting the leakage of crucial components, they constitute natural candidate antimycotics.

Ergosterol

The ergosterol biosynthetic pathway is the best-explored target of current therapy. The final product is ergosterol itself, which is the main exclusive fungal sterol, and it localizes to the cytoplasmic membrane. Some enzymes participating in ergosterol biosynthesis are not present in human cells.

Different classes of antifungal agents target components of ergosterol biosynthesis (Georgopapadakou and Walsh, 1996; Wills et al., 2000; Odds et al., 2003; Onyewu et al., 2003). The polyene antimycotic, amphotericin B, is an important antifungal agent that acts only against ergosterol and not against mammalian cholesterol synthesis (Odds et al., 2003). Despite its fungicidal effect, it has serious side effects, such as nephrotoxicity. With the advent of nanotechnology, some formulations of this drug have been developed to overcome these undesired reactions. This drug has a large spectrum of action and is indicated for treatment against *Candida* spp and other pathogenic fungi, including *P. brasiliensis* (Wills et al., 2000; Odds et al., 2003; Onyewu et al., 2003). *Erg6* encodes an S-adenosylmethionine:Δ24-methyltransferase and its disruption in *C. albicans* and *S. cerevisiae* results in increased resistance to polyenes and decreased ergosterol content (Young et al., 2003).

The azoles act in several steps of ergosterol biosynthesis by inhibiting enzymes in this pathway, which varies among the different fungal species. The main target for this class of drug is cytochrome P450 lanosterol 14-α-demethylase, which is encoded by *Erg11* and also has an iron protoporphyrin moiety at the active site, where the drug binds. Ketoconazole, itraconazole and fluconazole have been used in antifungal therapy. They basically prevent the demethylation of lanosterol, resulting in defective synthesis or in the depletion of ergosterol (Wills et al., 2000; Smith and Edlind, 2002; Odds et al., 2003; Theis and Stahl, 2004). *Paracoccidioides brasiliensis* presents ERG6 and ERG11 in its transcriptome (Table 2). Although some enzymes of the ergosterol pathway are not exclusively fungal, they can be exploited as potential targets for drug development.

Sphingolipids

The sphingolipid pathway is considered a potential target for the development of new antimycotics. Sphingolipids are involved in several intracellular responses, including heat shock-induced stress, endocytosis, apoptosis, and signal transduction. They have important roles in the maintenance of the plasma membrane of eukaryotic cells, and they are thus required for cellular growth and viability (Luberto et al., 2001; Obeid et al., 2002; Thevissen et al., 2004). Although sphingolipids are present in both mammalian and yeast membranes, some differences are found between the respective synthetic pathways (Obeid et al., 2002).

The most abundant sphingolipid in the plasma membrane is inositol phosphoryl ceramide (IPC), synthesized by IPC synthase; the corresponding gene IPC1 (also named AUR1) has also been identified in the *P. brasiliensis* transcriptome. This enzyme transfers phosphorylinositol from phosphatidylinositol to the 1-hydroxy group of phytoceramide to form IPC (Zhong et al.,

Table 2. Drug targets in plasma membrane composition.

PbAEST	Target	Ortholog accession number	e-value	Remarks	Drug/inhibitor
1959	Δ(24) Sterol C-Methyltransferase (ERG6)	T50969	4e-44	Ergosterol biosynthesis	Polyene: Amphoterycin B
1797	Cytochrome P450 lanosterol 14-α-demethylase (ERG11)	EAK97836	6e-75	Ergosterol biosynthesis	Azoles: Fluconazole, Itraconazole, Ketoconazole
200	Inositol phosphoryl ceramide (IPC1)/ Aureobasidin resistance (AUR1)	AAD22750	1e-43	Sphingolipid biosynthesis	Aureobasidin A
120	Pump membrane ATPase (PMA1)	P49380	1e-111	Plasma membrane ATPase	Bafilomycin Concanamycin

PbAEST = *Paracoccidioides brasilienses* assembled expressed sequence tag.

2000; Wills et al., 2000). IPC1/AUR1 is absent from mammalian cells, although both mammals and yeast use the same substrate to produce sphingomyelin and inositol phosphoryl ceramide, respectively (Wills et al., 2000; Heung et al., 2004). In *C. neoformans*, *Ipc1* is involved in the production of melanin, a virulence factor and a component used to protect the fungus against the immunological host response (Heung et al., 2004). Down-regulation of this gene causes a deficient response at low pH, whereby, as can be inferred in an *in vivo* model, virulence is diminished (Luberto et al., 2001).

Some studies have demonstrated that IPC is essential for survival of some fungi. Sensibility tests are being carried out with a variety of compounds to find inhibitors of IPC synthase. One prominent compound is the Aureobasidin A, a cyclic nonadepsipeptide, extracted from *Aureobasidium pullulans* (Zhong et al., 2000; Wills et al., 2000), which is active against *C. albicans*, *S. cerevisiae* and *C. neoformans* at lower concentrations when compared to other antifungals (Zhong et al., 2000). As IPC synthases are not present in human cells, the use of IPC inhibitors as antifungal agents is a good possibility for the therapy of systemic mycoses in humans. The *Ipc1* gene has been found in the *P. brasiliensis* transcriptome (Table 2). Previous studies in our laboratory have demonstrated that IPC1 is a promising target for drug development against *P. brasiliensis*.

Proton ATPases

Plasma membrane ATPase plays important roles in the maintenance of cell homeostasis by regulation of pH through ionic exchange. This mechanism creates a membrane potential and an electrochemical gradient that allows the uptake of ions and nutrients required for cellular physiology. The proton pump mechanism enables the cell to tolerate different pH gradients (Luo et al., 1999; Wills et al., 2000; Burghoorn et al., 2002; Lee et al., 2002; Wang and Chang, 2002; Pizzirusso and Chang, 2004). Recently, studies have demonstrated that bafilomycin inhibits ATPase

activity with high specificity and potency. Bafilomycin and concanamycin, antibiotics with similar structures, have been used as potent anti-tumor agents. Experiments with mutations in different sites of ATPase protein in strains of *Neurospora crassa* caused this fungus to overcome the toxic effects of concanamycin (Bowman and Bowman, 2002).

We identified the gene PMA1 in the *P. brasiliensis* transcriptome (Table 2); it is known to encode the plasma membrane ATPase in other fungi, including *S. cerevisiae*. Although PMA1 is also present in the human transcriptome, several domains are exclusive to fungi, and gene deletion has been demonstrated to be lethal for some of those microorganisms. ATPases are promising targets for the development of antimycotics, and the differences between fungal and mammalian proteins need to be further investigated (Georgopapadakou and Walsh, 1996; Wills et al., 2000).

CELLULAR MACHINERY

Several components of cellular machinery were demonstrated in other fungi as potential drug targets, and promising inhibitors to them are being tested (Table 3). Topoisomerase 1 (Top1) and elongation factors belong to the nuclear machinery, where they are key components in DNA-RNA-protein synthesis. Moreover, the heat shock protein Hsp90, which is required for proper protein refolding upon thermal stress, and three different proteins involved in post-translational modifications, N-myristoyltransferase (NMT), farnesyltransferase (FTase) and geranylgeranyltransferase (GGtase) I, were examined by our project.

Topoisomerase

Topoisomerases are enzymes that act on chromosome replication, transcription, recombination, and segregation processes by controlling the topological state of DNA. Their classification is related to how they cleave the DNA strand: TOP1 cleaves and rearranges one DNA strand, while TOP2 can cleave both DNA strands (Stewart et al., 1998). Topoisomerase-specific inhibitors, for example camptothecin or its analog topothecan, stabilizes the complex topoisomerase-DNA, leading to DNA damage and cell death (D'Arpa and Liu, 1989). TOP1 has been demonstrated to be essential and a virulence factor for some fungi, and its deletion in *C. albicans* induces slow cellular growth and aberrant cell morphology (Fostel et al., 1992; Jiang et al., 1997). The fungal TOP 1 gene has a considerable number of regions that are not present in the human transcriptome (Stewart et al., 1996). Selective inhibition of fungal (*C. albicans*) Top1p catalytic activity when compared to the human enzyme has already been observed using the aminocatechol A-3253 (Fostel and Montgomery, 1995). We found the *Top1* in the *P. brasiliensis* transcriptome, with identities of 90, 80 and 58% with *A. nidulans* (EAA66126.1), *N. crassa* (XP_331510.1) and human (CAA18536.1), respectively, indicating that the differences between human and fungal Top1p can be explored by drug design.

Elongation factors

Elongation factors required for protein synthesis are being focused on as potential drug targets in fungi. Some sordarin derivatives, such as GM 222712 and GM 237354, have shown *in vitro* activity against a wide range of pathogenic fungi, including *Aspergillus* spp, *Candida* spp,

Table 3. Cellular machinery components as promising drug targets in *Paracoccidioides brasiliensis*.

Target	PbAEST	Ortholog accession number/ organism	Percentage identity/ e-value	Known inhibitor
Topoisomerase I (Top1)	4267	NP_014637.1 *Saccharomyces cerevisiae*	70.548% / 3.4e-43	Camptothecin
Elongation factor 3 (EF-3)	845	NP_013350.1 *Saccharomyces cerevisiae*	79.672% / 3.7e-91	Sordarins
Heat shock protein 90 (Hsp90)	1656	NP_013911.1 *Saccharomyces cerevisiae*	74.330% / 1.7e-123	Geldanamycin
N-myristoyltransferase (Nmt1)	668	NP_013296.1 *Saccharomyces cerevisiae*	50.388% / 8e-30	Myristic acid analogs
FTase β-subunit (Ram1)	3641	NP_594251.1 *Schizosaccharomyces pombe*	40.288% / 6.7e-16	FPT inhibitors, FTI-275, FTase inhibitors, α-hydroxyfarnesyl-phosphonic acid, L-744,382, manumycin (Calbiochem®)
GGTase I β-subunit (Cdc43)	5030	NP_594142.1 *Schizosaccharomyces pombe*	51.471% / 8.6e-11	GGTI-287, GGTI-297, GGTI-2133, GGTI-2147 (Calbiochem®)
Commom α-subunit of FTase and GGTaseI (Ram2)	1926	NP_593518.1 *Schizosaccharomyces pombe*	40.722% / 5.7e-18	

PbAEST = *Paracoccidioides brasilienses* assembled expressed sequence tag.

C. neoformans, and other filamentous fungi (Andriole, 2000; Wills et al., 2000). Sordarins act by blocking the elongation cycle at the initial steps of translocation, prior to GTP hydrolysis, thus preventing the formation of peptidyl-[(3)H] puromycin on polysomes in *C. albicans*. Elongation factor 3 is a soluble form required for fungal translation machinery that is absent from other organisms, including humans, and hence a very attractive target for antifungal therapy (Wills et al., 2000). It was also identified in the *P. brasiliensis* transcriptome.

Hsp90

Hsps are highly conserved among different organisms. Hsp90 is a heat shock protein of approximately 90 kDa that is required for the refolding or degradation of cellular proteins upon heat shock, and it appears to be linked to the immune response against fungal pathogens (Matthews et al., 1998). The *P. brasiliensis* Hsp90 is differentially expressed by the pathogenic yeast phase (Felipe et al., 2005).

Geldanamycin is a benzoquinone ansamycin antibiotic produced by *Streptomycetes* that binds with high specificity within the ADP/ATP binding pocket of Hsp90, thereby inhibiting its function. Cells lacking hsp90 functions are severely compromised and cannot progress through the cell cycle (Cardenas et al., 1998; Piper, 2001). Despite the high conservation of Hsp90

between yeast and humans, and the evidence that geldanamycin is not toxic to wild-type yeast strains (Cardenas et al., 1998), development of specific antifungal inhibitors of Hsp90 deserves attention due to its role in *P. brasiliensis* dimorphism and pathogenesis.

N-myristoyltransferase

NMT catalyses the transfer of myristate from Co-enzyme A to the amino-terminal Gly residue of a number of cellular proteins involved in signal transduction pathways (Nagarajan et al., 1997). This process is observed only in eukaryotic cells. Genetic studies have shown that NMT is essential for the viability of the several fungal pathogens that cause systemic infection in immunosuppressed individuals (Lodge et al., 1994). Comparative analysis of the peptide substrate specificities of human and yeast NMT revealed distinct differences in peptide recognition, despite the high degree of conservation in the acyl-Co-enzyme A substrate specificities (Rocque et al., 1993). These differences in peptide recognition provide an opportunity to develop species-specific enzyme inhibitors that act as antifungals. Moreover, myristic acid analogs proposed as inhibitors of NMT were tested *in vitro* for activity against the budding yeasts *S. cerevisiae*, *C. albicans*, *C. neoformans*, and the filamentous fungus *Aspergillus niger* (Parang et al., 1996). In addition, in 1997, Nagarajan et al. described the development of a potent peptidomimetic class-specific inhibitor for the *C. albicans* NMT. In the *P. brasiliensis* transcriptome analysis we identified the gene Nmt1, which codifies glycylpeptide N-tetradecanoyltransferase, commonly known as NMT.

Prenyltransferases: farnesyltransferase and geranylgeranyltransferase

Prenylation is a post-translational protein modification process, in which there is an addition of a prenyl group - farnesyl diphosphate or geranylgeranyl diphosphate, derived from ergosterol biosynthesis, to the C-terminus of the protein, resulting in its correct localization to cell membranes. This process is mediated by prenyltransferases (PTFs), such as FTase, and GGTases I and II.

Prenylated proteins participate in a variety of cellular functions, such as control of cell growth, differentiation, cytokinesis, membrane trafficking, and signal transduction. Proteins that are prenylated include small GTP-binding proteins, lipopeptide pheromones, nuclear lamins, and trimeric G-proteins (Schafer and Rine, 1992). Many of these GTPases also participate in cell signaling pathways, which are likely to be involved in the pathogenesis of *C. albicans* and *C. neoformans* (McGeady et al., 2002; Vallim et al., 2004).

All PTFs in yeasts and mammals consist of α- and β-subunits. The α-subunit is encoded by the RAM2 gene and is shared between FTase and GGTase I; the β-subunits of FTase and GGTase I are encoded by RAM1 and CDC43, respectively. In the *P. brasiliensis* transcriptome analysis, we identified all of these subunits, suggesting the ability of this pathogen to use both PTFs in its morphogenesis, in contrast with *C. neoformans*, for which just the FTase has been identified to date (Vallim et al., 2004).

Compounds that inhibit protein prenylation have been developed and studied, as potential agents to treat human malignancies, mainly due to the observation that activated Ras mutations are associated with a significant number of human cancers. Initial trials indicated that inhibiting prenylation could result in a reduction in the growth rate of some tumor lines (Kohl et

al., 1995). More recently, these compounds were assayed *in vitro* in the opportunistic pathogens, *C. albicans* and *C. neoformans*, to assess the effect of blocking the prenylation process as a way to avoid differentiation and pathogenesis (McGeady et al., 2002; Vallim et al., 2004). The results are very promising, in that prenylation inhibitors blocked the serum-induced conversion from the yeast to the filamentous form (this latter being closely related to pathogenesis) in *C. albicans* and impaired hyphal differentiation in *C. neoformans*. Moreover, the poor similarity between fungal and human PTFs makes them very attractive drug targets to be evaluated in *P. brasiliensis*.

SIGNALING PATHWAYS

Pathogenic organisms have co-opted conserved signaling pathways to sense and respond to the host harsh conditions and to activate the genes required for virulence and pathogenesis. For this reason, many studies are focusing on signaling cascade components as possible targets for antifungal therapy. In our biological model, *P. brasiliensis*, several signaling pathways have been described; nonetheless, only calcineurin and TOR will be discussed below.

Calcineurin

Calcineurin, a serine-threonine specific phosphatase, is conserved among eukaryotes, and consists of a catalytic and a regulatory subunit, which play a crucial role to maintain the perfect homeostasis of the cell under stress conditions. This protein is activated by calmodulin in response to increases in intracellular calcium and is required to control cell survival under oxidative stress, high temperature and membrane/cell wall perturbation. Fernandes et al., 2005, this issue, pages 216-231, have identified all components of the calcineurin-signaling pathway in the *P. brasiliensis* transcriptome. According to recent studies, its requirement for growth and virulence-increasing properties in fungal pathogens, such as *A. fumigatus*, *C. neoformans* and *C. albicans* (Odom et al., 1997), make it a potential drug target. Moreover, Odom et al., 1997 proposed calcineurin as the conserved target of two immunosuppressive drugs: cyclosporin A (CsA) and tacrolimus (FK-506), which have been isolated from bacteria and fungi, respectively. In spite of the difference in chemical structures - FK-506 is a macrolide, whereas CsA is a cyclic peptide, they are both hydrophobic and thought to diffuse across the plasma membrane. FK-506 and CsA form complexes with the immunophilins FKBP12 and cyclophilin A (CyPA), respectively. Immunophilins are small, ubiquitous, cytosolic proteins that catalyze *cis-trans* prolyl isomerization, which is required for protein folding. When FK-506 binds to FKBP12 and CsA binds to CyPA, the inhibition of prolyl-isomerase activity is observed, as is the most exacerbated effect: inhibition of calcineurin signaling through the binding of the drug-immunophilin complexes to the regulatory subunit (Figure 1) (Cardenas et al., 1998). FK-506 and CsA analogs have been described that are specific for fungal calcineurins and also do not cause immunosuppression (Odom et al., 1997).

TOR

TORs are phosphatidylinositol kinase-related proteins known as key regulators of cell growth in response to nutritional and mitogenic signals and as targets for the immunosuppres-

sive and anti-cancerous drug rapamycin. Rapamycin or sirolimus bind to the immunophilin receptor, FKPB, and the complex rapamycin-*Fkpb* binds to the *Tor* protein, blocking signal transduction and arresting cells in G1 (Figure 1) (Brown and Schreiber, 1996). Rapamycin also blocks filamentation in a number of important human and plant pathogens, and its mechanism of action is conserved among eukaryotes. A recent study performed by Steinbach et al. (2004) reports *in vitro* and *in vivo* rapamycin activity against *A. fumigatus*, *C. albicans*, *C. neoformans*, and *S. cerevisiae* (Singh and Heitman, 2004). The rapamycin target TOR protein was found in the *P. brasiliensis* transcriptome, as well as the downstream components of this pathway (Fernandes et al., 2005, this issue, pages 216-231). The antimicrobial properties of less immunosuppressive analogs of rapamycin hold promise for the development of an effective antifungal therapy (Dickman et al., 2000).

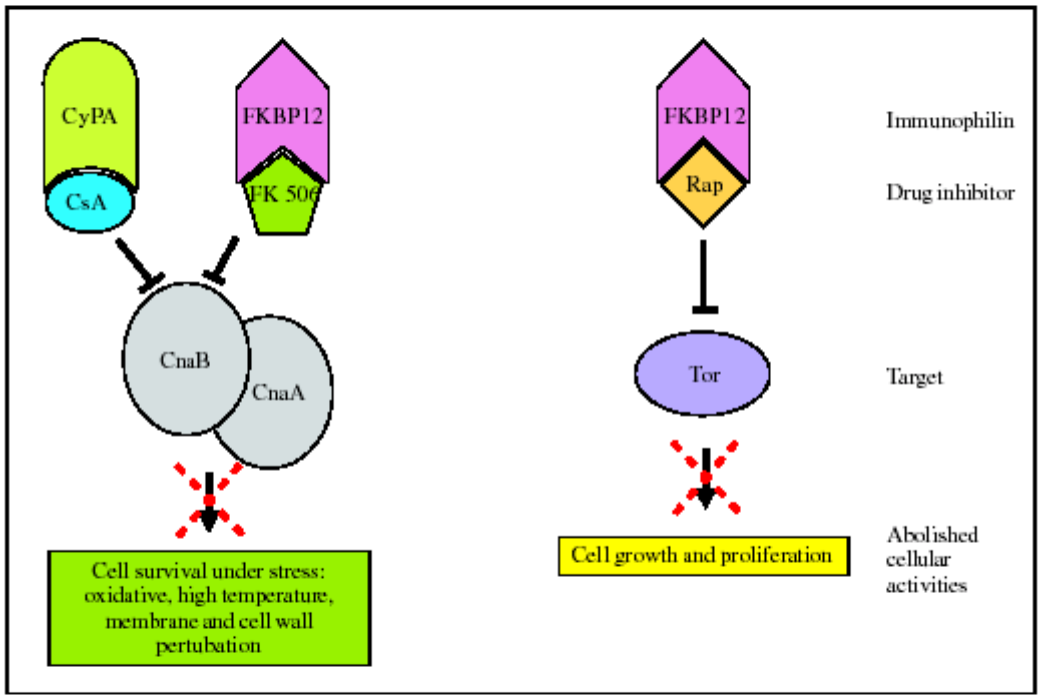

Figure 1. Mechanisms of action of tacrolimus (FK-506), cyclosporin A (CyPA) and rapamycin (Rap). FK-506 and CyPA bind to the immunophilins FKBP12 and cyclophilin A (CsA), respectively. The formed complexes bind to the calcineurin regulatory subunit (CnaB), thus inhibiting the transduction of signals in response to stress conditions. Likewise, Rap also binds to the immunophilin FKBP12, and the complex FKBP12-Rap binds to Tor protein through the FRB domain (FKBP12-rapamycin-binding domain), resulting in the blockage of cell cycle progression and consequent impairment of cell growth and proliferation (Adapted from Cardenas et al., 1998).

CELL METABOLISM

Glyoxylate cycle

The glyoxylate cycle is an alternative pathway that helps fungi to obtain energy. Two key enzymes participate in it: isocitrate lyase (Icl1) and malate synthase (Mls1). They are

activated when fungus cells are phagocytosed by the macrophage, which appears to be a glucose-limiting environment. The resultant fungal response is an up-regulation of enzymes that use poor carbon sources, including lipids, to synthesize cell wall precursors (Selitrennikoff and Nakata, 2003). These lipids are metabolized into two-carbon units that are brought into the tricarboxylic acid cycle via acetyl-Co-enzyme A. ICL1 cleaves isocitrate, producing glyoxylate and succinate, while MLS1 catalyses the reaction of glyoxylate with acetyl-Co-enzyme A to produce malate. These two reactions in succession bypass the two oxidative decarboxylase steps of the citric cycle (Clemons and Stevens, 2001; Selitrennikoff and Nakata, 2003). Several reports show the importance of the glyoxylate cycle for fungal pathogenicity (Honer et al., 1999; Lorenz and Fink, 2002). Both genes were found in the *P. brasiliensis* transcriptome (Table 4). These enzymes are not present in human cells and should be assessed for drug design (Lorenz and Fink, 2002; Selitrennikoff and Nakata, 2003).

Table 4. Potential drug targets from cellular metabolism.

Target	PbAEST	Ortholog accession number/ organism	Percentage identity	e-value	Remarks
Isocitrate lyase (ICL1)	1688	NP_010987.1 *Saccharomyces cerevisiae*	58.929%	8.1e-73	Required by glyoxylate cycle, up-regulated in *P. brasiliensis* yeast cells
Malate synthase (MLS1)	829	NP_014282.1 *Saccharomyces cerevisiae*	67.292%	4.3e-93	Required by glyoxylate cycle
Urease	2456	NP_594813.1 *Schizosaccharomyces pombe*	81.407%	1.5e-61	Involved in sporulation and pathogenesis
Urate oxidase	4129	NP_588354.1 *Schizosaccharomyces pombe*	41.315%	1.8e-27	Involved in sporulation and pathogenesis

PbAEST = *Paracoccidioides brasilienses* assembled expressed sequence tag.

Urease

Urease is a metalloenzyme that catalyses the hydrolysis of urea to ammonia and carbamate. Under physiological conditions, this reaction results in an increase of pH. Many pathogenic fungi have urease activity, among which are *C. nerformans*, *Coccidioides immitis*, *H. capsulatum*, *P. brasiliensis* (Table 4), *Sporothrix sckenchii*, and some species of *Trichosporon* and *Aspergillus*. The first urease gene cloned from a human pathogenic fungus was that of *C. immitis* (Yu et al., 1997). In this fungus, the urease gene plays a role in sporulation, pathogenesis and virulence (Cole, 1997). Urease is hypothesized to contribute to alkalinity of the microenvironment in which the fungus grows, mainly due to the release of ammonia and ammonium ions. However, it is evident that *C. immitis* urease activity is not responsible for the total amount of ammonia secreted during *in vitro* growth of the pathogen (Mirbod et al., 2002). Being a virulence factor in pathogens, including fungi and bacteria, and absent in humans, ure-

ase is an important enzyme involved in the colonization of the host, and it may serve as a potential drug target.

Urate oxidase (uricase)

Urate oxidase or uricase is an enzyme of the purine degradation pathway that was found in the *P. brasiliensis* transcriptome (Table 4). It is responsible for the conversion of uric acid into allantoin and hydrogen peroxide (Nahm and Marzluf, 1987). Functional uricase is absent in higher primates, which excrete uric acid as the end product of purine degradation (Elion et al., 1968; Friedman et al., 1985). Since uricase is a powerful scavenger of free radicals, it plays an important role in protecting pathogens during macrophage ingestion (Ames et al., 1981; Whiteman and Halliwell, 1996), and therefore its blockage may lead to their impaired growth.

ESSENTIAL GENES

Roemer et al. (2003) developed a very efficient strategy to identify essential genes in the human pathogen *C. albicans*. The technique called GRACE™ (gene replacement and conditional expression) is based on deletion of the first allele (*C. albicans* is a diploid organism) by PCR-generated cassettes containing a selectable marker, and a tetracycline-repressed promoter linked to a second selectable marker replaces the second allele. By this approach, Roemer et al., 2003 studied 1152 genes, but only 61% (567) were confirmed experimentally to be essential in *C. albicans*. Most of the genes are required for cellular processes such as cell growth and division, as well as DNA synthesis. The main goal of the present study is that by this technique it is possible to identify and validate the target *in vitro* and *in vivo*, in *C. albicans*, an opportunist diploid pathogen with no obvious sexual cycle, which impairs classic genetic analysis. Based on the similarities with our biological model of interest, *P. brasiliensis*, the GRACE™ strategy is a very promising way to screen for essential genes and further develop novel therapies against paracoccidioidomycosis. In our analysis, we have decided to search for the *C. albicans* essential genes in the *P. brasiliensis* transcriptome; we identified 15 genes, such as *alg7* (UDP-N-acetylglucosamine-1-phosphate transferase) and *sec14* (phosphatidylinositol/phosphatidylcholine transferase). Though they are present in *P. brasiliensis*, further studies such as knockout or GRACE experiments, may confirm their true importance in this organism.

CONCLUDING REMARKS

We have explored different possible targets for the development of alternative drug therapies against *P. brasiliensis*. Some of the potential targets discussed here, which are fungus-specific and have been dissected as promising targets for the majority of fungal pathogens, were identified in our *P. brasiliensis* transcriptome analyses. Targets present in both host and pathogen should not be discarded due to the possibility to explore their differences, as is the case for Top1. Since current therapy is aimed at targets related to plasma membrane biosynthesis of both fungi and mammals, the respective drugs appear to be specific for the fungal enzymes. Bearing this in mind, the refinement of current antifungals is a great option to develop new specific therapies. One source of potential targets that was recently proposed by Roemer et al.

(2003) is the identification of essential genes.

Our comparative study was made possible by the *P. brasiliensis* transcriptome-sequencing project, which has enabled us to compare and validate targets described in other pathogenic organisms with the expressed sequence tags found in our database. Together with the great advances in understanding fungal pathogenesis, the development of methods for screening in combinatorial chemistry libraries and improved molecular modeling computational programs, the molecular revolution holds new promises and perspectives in the newly born field of antifungal design.

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Cell organisation, sulphur metabolism and ion transport-related genes are differentially expressed in *Paracoccidioides brasiliensis* mycelium and yeast cells

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Cell organisation, sulphur metabolism and ion transport-related genes are differentially expressed in *Paracoccidioides brasiliensis* mycelium and yeast cells

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Abstract

Background: Mycelium-to-yeast transition in the human host is essential for pathogenicity by the fungus *Paracoccidioides brasiliensis* and both cell types are therefore critical to the establishment of paracoccidioidomycosis (PCM), a systemic mycosis endemic to Latin America. The infected population is of about 10 million individuals, 2% of whom will eventually develop the disease. Previously, transcriptome analysis of mycelium and yeast cells resulted in the assembly of 6,022 sequence groups. Gene expression analysis, using both *in silico* EST subtraction and cDNA microarray, revealed genes that were differential to yeast or mycelium, and we discussed those involved in sugar metabolism. To advance our understanding of molecular mechanisms of dimorphic transition, we performed an extended analysis of gene expression profiles using the methods mentioned above.

Results: In this work, continuous data mining revealed 66 new differentially expressed sequences that were MIPS(Munich Information Center for Protein Sequences)-categorised according to the cellular process in which they are presumably involved. Two well represented classes were chosen for further analysis: (i) control of cell organisation – cell wall, membrane and cytoskeleton, whose representatives were *hex* (encoding for a hexagonal peroxisome protein), *bgl* (encoding for a 1,3-β-glucosidase) in mycelium cells; and *ags* (an α-1,3-glucan synthase), *cda* (a chitin deacetylase) and *vrp* (a verprolin) in yeast cells; (ii) ion metabolism and transport – two genes putatively implicated in ion transport were confirmed to be highly expressed in mycelium cells – *isc* and *ktp*, respectively an iron-sulphur cluster-like protein and a cation transporter; and a putative P-type cation pump (*pct*) in yeast. Also, several enzymes from the cysteine *de novo* biosynthesis pathway were shown to be up regulated in the yeast form, including ATP sulphurylase, APS kinase and also PAPS reductase.

Conclusion: Taken together, these data show that several genes involved in cell organisation and ion metabolism/transport are expressed differentially along dimorphic transition. Hyper expression in yeast of the enzymes of sulphur metabolism reinforced that this metabolic pathway could be important for this process. Understanding these changes by functional analysis of such genes may lead to a better understanding of the infective process, thus providing new targets and strategies to control PCM.

Background

The availability of great amounts of raw genomic and transcriptome data collected from several organisms has prompted the development of large-scale gene expression analysis which will ultimately help to unravel the function of many genes in diverse biological contexts. Different approaches such as cDNA microarrays [1-3], *in silico* ESTs subtraction [4,5] and serial analysis of gene expression – SAGE [6,7] are widely employed to assess differential gene expression patterns leading to the discovery of a great number of genes that are over or under expressed in each physiological context. The successful use of the cDNA microarray approach in fungal pathogens such as *Candida albicans* [8-13], *Histoplasma capsulatum* [14] and *Cryptococcus neoformans* [15] has resulted in the identification of genes involved in cell viability and opened new experimental perspectives to understand host-parasite interactions and thus develop new therapeutic approaches to systemic mycoses [8,11].

Paracoccidioidomycosis (PCM) is a human illness endemic to Latin America [16]; its area of incidence spreads non-uniformly from Mexico to Argentina [17], being higher in Brazil, Venezuela, Colombia and Argentina [18,19,16]. An estimation for Brazil points to an incidence rate between 1 and 3 and a mortality rate of 1.4 per million [20]. McEwen *et al.* [21] reported an overall infected population of 10 million individuals in Latin America, 2% of whom will eventually develop the disease. In nature, another important mammalian host is the armadillo *Dasypus novemcinctus* [22]. PCM affects the skin, lymph nodes and various internal organs, including the lungs – where it causes granulomatous processes – and the central nervous system [19,23]. Its clinical presentations range from a localised and benign disease to a progressive and potentially lethal systemic infection [24]. The disease is more frequent in adult males, who account for up to 90% of all cases. Healthy rural workers are the main targets, but PCM affects immunosuppressed individuals as well [25,26], including as much as 30% of AIDS patients [27]. All patients from whom the fungus is isolated must be treated and, in spite of new antifungal drugs, pulmonary fibrosis is still the most frequent sequel. The outcome of infection depends on several factors, including host responses and the virulence of the infecting isolate.

The causative agent of PCM, the thermo-regulated dimorphic fungus *P. brasiliensis*, is believed to be a free-living mycelium saprobe that undergoes transition to the yeast pathogenic form upon temperature change from the environmental 24–26 °C to the mammalian body temperature of 37 °C. This switch is necessary and sufficient to trigger morphotype interconversion *in vitro*, which makes this fungus an interesting model to study fungal cell differentiation at the molecular level. The biochemical events regulating dimorphic transition in *P. brasiliensis* are yet poorly defined, although relevant molecular-level information on this process has been partially described in the transcriptome analyses of two different *P. brasiliensis* isolates [28-30].

The exact ecological niche of this pathogen is still unknown [17], but *P. brasiliensis* can be retrieved from the soil. The fungus *Penicillium marneffei* is greatly similar in that it is a human opportunistic pathogen that also undergoes thermally-controlled dimorphic transition upon infection, can also infect a wild mammal (the bamboo rat) and has an yet unknown natural reservoir. Genomic data provided evidence that, in the case of *P. marneffei*, the fungus may have a sexual stage as a free-living organism [31].

Phylogenetic analysis of members of the order Onygenales demonstrated a close relationship of *P. brasiliensis* with the pathogenic fungi *Blastomyces dermatitidis, Emmonsia parva* and *Histoplasma capsulatum* [32]. *P. brasiliensis* can be fitted with *B. dermatitidis* and *E. parva* in the family Onygenacea [33]. Recently it was reported that *P. brasiliensis* is in fact a complex of at least three closely correlated phylogenetic species [34]. So far, the sexual phase of the ascomycete *P. brasiliensis* was not reported limiting our knowledge about the mechanisms that contribute to its dimorphism, pathogenicity, and virulence. *P. brasiliensis* isolates shows chromosomal polymorphism; it contains 4–5 chromosomal DNA molecules with molecular sizes ranging from 2–10 Mb [35,36]. The genome size was estimated to be around 30 Mb [37] and DNA sequencing of ~ 50 Kb revealed a density of one gene per 3.5–4.5 Kb, suggesting a total of 7,500–9,000 genes [38].

Recently, our group analysed the transcriptome of the Pb01 isolate, represented by a set of 6,022 clusters. The 16 genes that were then found to be differentially expressed

by both methods used – *in silico* EST subtraction and cDNA microarray – were categorised by function. We chose to discuss in that work those that were involved in core metabolic pathways such as sugar metabolism [28]. Now, continued overlap analysis from raw data revealed 66 new genes that are differentially expressed in one or other morphotype. Upon categorisation by known databases we have selected two MIPS [39] classes, which were chosen to be confirmed by northern blotting. Here we present the result of this extended analysis, and discuss the putative roles the differential genes – related to cell organisation and ion metabolism and transport – play in the corresponding morphotype of this pathogen. One of the discussed pathways – *de novo* cysteine synthesis from inorganic sulphate, a branch of sulphur metabolism – was almost entirely up-regulated in the yeast form. The importance of sulphur metabolism to the life cycle of pathogenic fungi has been extensively reviewed elsewhere [40,41] and recently new data from microarray experiments have arisen from work in *H. capsulatum* that support a role of organic sulphate in the maintenance of the yeast phase [14]. In a previous report [42], the importance of organic sulphates to the growth and differentiation of *P. brasiliensis* was assessed. This phenomenon demanded further investigation and prompted us to assess up- and downregulation of sulphur metabolism genes in mycelium and yeast cells and also dimorphic transition in both directions without inorganic sulphate as a sulphur source. We have thus found that this compound is unnecessary for the process.

Results and discussion

Differentially expressed genes identified by in silico *EST subtraction and cDNA microarray*

Comparative gene expression profiling in dimorphic fungi can reveal key proteins involved in commitment to differentiation and gene regulation. From the 66 new PbAESTs (*P. brasiliensis* assembled expressed sequence tags) identified in this work, thirteen of which correspond to up-regulated genes in mycelium and fifty four which are differential for yeast cells (Tables 1 and 2). This set complements the one generated previously [28], which included 16 genes that were differential by the same overlap analysis and also 30 genes that were differential

Table 1: Mycelium up-regulated genes identified by *in silico* ESTs subtraction and cDNA microarray.

PbAEST	Acession Numbers (GenBank)	Annotated function	Number of reads		P-value[a]	Fold change	Accession Number/Best-hit organism/E-value	Functional categories
			M	Y				
202	CA582032	1,3-beta-glucosidase*	7	2	0.036942	12.3	AAL09828.1/*C. immitis*/ 1.0E-132	Control of cell organization: Cell wall and membrane
2155	CA582352	Peroxisomal membrane protein PEX16 (peroxin-16)	7	0	0.004174	1.4	EAL88469.1/*A. fumigatus*/ 3.0E-64	
186	CA583085	HEX*	13	8	0.049272	3.4	EAL91716.1/*A. fumigatus*/ 3.0E-66	
2496	CA583518	Iron-sulphur cluster nifU-like protein*	5	1	0.048854	1.7	EAL90111.1/*A. fumigatus*/ 8.0E-58	Ion transport
4179	CN245816	Potassium transporter protein*	0	1	-[b]	5.2	CAA08814.1/*N. crassa*/ 4.0E-22	
1420	CN247275	U1 small nuclear ribonucleoprotein	9	1	0.00526	1.6	EAL91268.1/*A. fumigatus*/ 1.0E-60	Transcription
1029	CA582332	Methyltransferase	32	1	0.000000	2.1	EAL84975.1/*A. fumigatus*/ 1.0E-56	Others
2096	CA581148	Unkown	20	1	0.000006	5.6	-	
514	CA583322	Unkown	15	1	0.000138	23.4	-	
1045	CA581951	Unkown	13	2	0.001769	24	-	
1178	CN247241	Unkown	10	0	0.000535	8.5	-	
1664	CN247289	Unkown	10	3	0.018648	2.5	-	

[a] FDR = 4,8% and Q-value < 5%.
[b] Not significant by Audic-Claverie's method.
* Up-regulated genes confirmed by northern blotting.
** Not assayed by cDNA microarray but confirmed as up-regulated in mycelium by northern blotting.

Table 2: Yeast up-regulated genes identified by *in silico* ESTs subtraction and cDNA microarray.

PbAEST	Accession Numbers (GenBank)	Annotated function	Number of reads		P-value[a]	Fold change	Accession Number/Best-hit organism/E-value	Functional categories
			M	Y				
1422	CA581980	Alpha-1,2-mannosyltransferase (Alg11)	4	11	0.019803	2.0	EAL88400.1/A. *fumigatus*/1.0E-130	Control of cellular organization: Cell wall and membrane
4988	CN253911	Alpha 1,3-glucan synthase[#]	-	1	-	5.7	AAV52833.1/P. *brasiliensis*/4.0E-93	
2162	CN238153	Putative WW domain protein (probable membrane protein)	4	12	0.013092	3.6	EAL85876.1/A. *fumigatus*/6.0E-17	
136	CA582283	Involved in cytoskeletal organization and cellular growth (verprolin)[#]	4	10	0.029289	4.0	NP_013441.1/S. *cerevisiae*/2.3	
667	CA583397	Adenylylsulphate kinase	3	8	0.038949	2.1	EAL90409.1/A. *fumigatus*/1.0E-88	Ion transport and metabolism
48	CA582091	ATP-sulphurylase	10	18	0.023038	4.8	EAL92915.1/A. *fumigatus*/0.0	
2031	CA581274	Outer mitochondrial membrane protein porin	1	14	0.000207	1.3	XP_323644.1/N. *crassa*/1.0E-108	
2724	CA581633	P-type Cu(2+) transporting ATPase[#]	0	1	-[b]	3.8	NP_009854.1/S. *cerevisiae*/1.7E-20	
635	CN247312	ATP citrate lyase	1	7	0.014984	1.9	EAL88915.1/A. *fumigatus*/0.0	Energy
2016	CN242578	ATPase inhibitor; Inh1	2	14	0.000835	2.7	NP_010100.1/S. *cerevisiae*/4.0E-08	
563	CA583982	Cytochrome c oxidase subunit VII	11	43	0.000002	2.1	AAT77147.1/P. *brasiliensis*/3.0E-26	
2398	CN240705	Disulfide isomerase	3	8	0.038949	2.1	EAL91387.1/A. *fumigatus*/3.0E-61	
540	CN240558	Cytochrome C oxidase biogenesis protein	0	5	0.015111	1.8	XP_214182.2/R. *norvegicus*/1.0E-06	
578	CA582837	Pyruvate dehydrogenase e1 component beta subunit	2	7	0.033994	1.6	EAL86696.1/A. *fumigatus*/2.0E-99	
407	CA583387	Succinyl-CoA synthetase alpha subunit	7	19	0.004468	2.6	EAL91981.1/A. *fumigatus*/1.0E-155	
284	CN239025	Ubiquinol-cytochrome C reductase complex ubiquinone-binding protein QP-C	0	4	0.030475	1.5	EAL90680.1/A. *fumigatus*/7.0E-29	
378	CA580847	Argininosuccinate synthase	0	6	0.007492	1.7	NP_229577.1/T. *maritima*/4.0E-77	Amino acid metabolism and transport
1618	CA583639	Aromatic-L-amino-acid decarboxylase	1	33	0.00000	17.2	EAL86509.1/A. *fumigatus*/0.0	
125	CA583825	Glycine cleavage system h protein	4	9	0.042192	1.4	EAL90537.1/A. *fumigatus*/6.0E-36	
1674	CA583874	Aldolase	5	14	0.010368	37.9	AAL34519.2/P. *brasiliensis*/0.0	C-compound and carbohydrate metabolism
42	CA581692	Phosphoglycerate kinase	1	10	0.002512	2.6	EAL90363.1/A. *fumigatus*/0.0	
9	CA581893	Beta-ketoacyl synthase (Cem1)	1	5	0.045709	2.2	EAL87667.1/A. *fumigatus*/1.0E-88	Lipid, fatty-acid and isoprenoid metabolism
780	CA581145	GPR/FUN34 family protein	0	11	0.000225	14.9	EAL87502.1/A. *fumigatus*/6.0E-67	
1989	CA581550	Acetyl-coenzyme A synthetase (AcetatE– CoA ligase) (Acyl-activating enzyme)	1	9	0.004605	2.0	EAL89682.1/A. *fumigatus*/0.0	
1550	CA582818	NADH-cytochrome b5 reductase	0	6	0.007492	5.4	EAL88164.1/A. *fumigatus*/1.0E-86	

Table 2: Yeast up-regulated genes identified by *in silico* ESTs subtraction and cDNA microarray. *(Continued)*

300	CAS81927	Nucleoside diphosphate kinase	6	58	0.00000	1.6	AAP85295.1/A. *fumigatus*/2.0E-67	Nucleotide metabolism	
547	CAS83473	6,7-dimethyl-8-ribityllumazine synthase	0	6	0.007492	1.4	AAD53721/A. *fumigatus*/9.0E-56	Metabolism of vitamins, cofactors, and prosthetic groups	
924	CN240624	Coproporphyrinogen III oxidase	2	7	0.033994	2.7	EAL88456.1/A. *fumigatus*/0.0		
867	CAS80742	NADH pyrophosphatase	1	5	0.045709	5.7	EAL85969.1/A. *fumigatus*/1.0E-159		
1490	CAS83063	Pyridoxamine 5'-phosphate oxidase	0	10	0.000453	3.5	AAC28862.1/S. *commune*/2.0E-32		
447	CAS80589	NADH:ubiquinone oxidoreductase B18 subunit	1	10	0.002512	1.4	EAL92195.1/A. *fumigatus*/9.0E-33		
488	CAS82788	Exonuclease II	1	5	0.045709	1.9	EAL85993.1/A. *fumigatus*/1.0E-138	Transcription, translation and ribosome structure	
165	CN241393	RNP domain protein	3	13	0.003962	1.8	EAL89070.1/A. *fumigatus*/5.0E-81		
2436	CAS80512	Splicing factor u2af 35 kd subunit	2	7	0.033994	2.5	EAL86523.1/A. *fumigatus*/1.0E-103		
253	CN240426	Zinc finger, C3HC4 type	0	5	0.015111	1.4	NP_593329.1/S. *cerevisiae*/3.0E-10		
551	CN239696	Ribosomal protein L35[***]	5	10	0.044755	-	AAL08563.1/P. *brasiliensis*/1.0E-63		
979	CAS82572	60S ribosomal protein L7/L12 precursor	1	8	0.008358	1.3	EAL89813.1/A. *fumigatus*/4.0E-49		
175	CAS81863	Complex I intermediatE-associated protein CIA30 precursor	4	15	0.003399	5.6	EAL92946.1/A. *fumigatus*/1.0E-114	Protein fate and Secretion	
832	CN242383	Glutathione S transferase	1	7	0.014984	2.0	NP_588171.1/S. *pombe*/7.0E-42		
2387	CAS84103	Non-classical export protein (Nce1)	1	7	0.014984	55.6	EAL87256.1/A. *fumigatus*/1.0E-29		
1823	CAS83903	Profilin	1	5	0.045709	1.3	NP_014765.1/S. *cerevisiae*/8.0E-14		
4188	CN245872	Mating type protein (MAT1-2)[*]	1	0	-	8.0	EAL89707.1/A. *fumigatus*/2.0E-36	Mating Type	
50	CAS81392	Cu-Zn superoxide dismutasE-related[*]	0	8	0.001842	2.1	CAB97297.1/N. *crassa*/3.0E-30	Virulence and oxidative stress	
2059	CN241260	Ribosome associated protein (Stm1)	6	31	0.000007	1.7	EAL92489.1/A. *fumigatus*/2.0E-32	Others	
2005	CAS80764	Signal peptide protein	1	6	0.026442	2.3	EAL93249.1/A. *fumigatus*/7.0E-68		
39	CAS81046	Unknown	0	6	0.007492	2.2	-		
33	CAS82496	Unknown	0	8	0.001842	3.1	-		
1442	CAS81846	Unknown	3	16	0.000836	4.5	-		
2399	CAS81839	Unknown	1	5	0.045709	2.5	-		
512	CAS83749	Unknown	0	6	0.007492	4.3	-		
639	CAS81506	Unknown	0	7	0.003715	1.7	-		
718	CN247621	Unknown	0	6	0.007492	1.8	-		
765	CAS81478	Unknown	0	10	0.000453	3.9	-		
529	CAS80398	Unknown	1	5	0.045709	18.8	-		

[a]FDR = 4.8% and Q-value < 5%.

[b] Not significant by Audic-Claverie's method.

[*] Up-regulated genes confirmed by northern blotting.

[***] Not assayed by cDNA microarray but confirmed as up-regulated in yeast by northern blotting.

according to *in silico* EST subtraction alone. MIPS functional categories [43] were used to classify the 66 PbAESTs into 14 major groups (data not shown). Gene categorisation revealed some that are involved in energy production (11%) – this was expected considering the adaptation process that is required for the mycelium-to-yeast transition; control of cell wall organisation (10%); ion metabolism and transport (8%); transcription, translation and ribosome structure (8%); virulence and oxidative stress (4%). Manual annotation under stringent criteria of sequence alignment with other dimorphic fungi gene sets allowed us to ascribe a putative biological function to many of those genes. The genes that belonged in two categories – cell wall organisation and ion metabolism and transport – were selected for confirmation by northern blotting.

Mycelium and yeast up-regulated genes involved in cell organisation

The *hex* and *bgl* genes, which code for the hexagonal peroxisome protein and 1,3 β-glucosidase, respectively, were up-regulated in mycelium cells and are categorised as involved in cell wall, membrane and cytoskeleton organisation (Figure 1a). The hexagonal peroxisome protein has been identified in different filamentous ascomycetes such as the plant pathogen *Magnaporthe grisea* [44] and in *Neurospora crassa* [45], being the major protein of the Woronin body, a septal pore-associated organelle [46,47]. HEX1p has been shown to seal septal pores in response to cellular damage and is strongly implicated in cell integrity maintenance [45]. In *M. grisea*, *hex1* mutants present delayed host penetration and subsequent disruption of invasive hyphal growth in plants. Inability of these mutants to survive under nitrogen starvation *in vitro* has also been observed [44]. Deletion of *hex1* in *N. crassa* eliminates Woronin bodies from the cytoplasm and results in hyphae that exhibit a cytoplasmic-bleeding morphotype in response to cell lysis [45]. It was thus proposed that the Woronin body represents a new category of peroxisome acting in the maintenance of cellular integrity and virulence in filamentous fungi [45]. We hypothesise that these highly specialised vesicles are involved in the protection of *P. brasiliensis* against cellular damage as well as its survival during invasive growth and host colonisation in the process of infection. Future investigations are required to elucidate the role of Woronin bodies/HEX1 protein in *P. brasiliensis*.

Another mycelium up-regulated gene codes for β-1,3-glucosidase, an enzyme that hydrolyses the O-glycosidic linkages of β-glucan. This polysaccharide is an important cell wall constituent in *P. brasiliensis* mycelium cells in contrast with α-glucans, which predominate in the yeast cell wall [48]. A hypothesis formulated by Kanetsuna *et al.* [49] and modified by San Blas and San Blas [50] explains

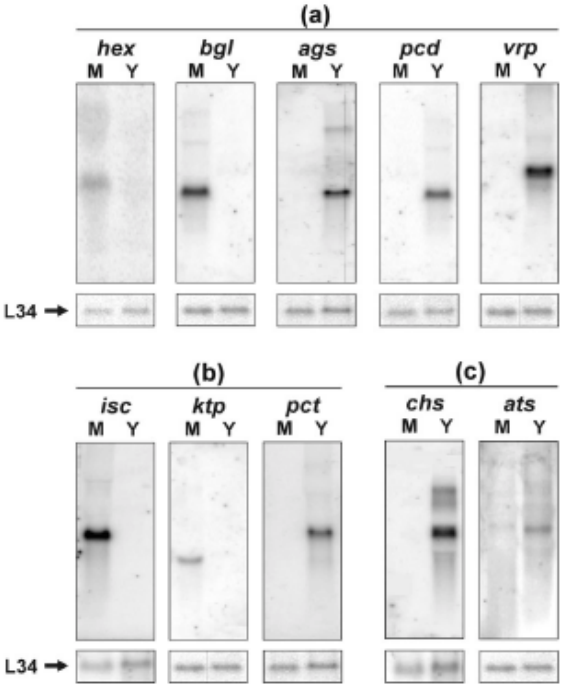

Figure 1
Northern blot analysis of mycelium and yeastup-regulated genes of *P. brasiliensis*. Total RNA samples from both mycelium (M) and yeast (Y) were blotted onto nylon membranes and hybridised against gene-specific radiolabelled probes: **(a)** Control of cell organisation: *hex* – Hexagonal peroxisome protein, *bgl* –1,3 beta-glucosidase, *ags* – alpha 1,3-glucan synthase, *cda* – Chitin deacetylase, *vrp* – Verprolin; **(b)** Ion transporters: *isc* –Iron-sulphur cluster-like protein, *ktp* – Potassium transporter, *pct* – Putative P-type Cu(2+) transporting ATPase; **(c)** Sulphur metabolism: *chs* – Choline sulphatase, *ats* – ATP sulphurylase. The constitutive 60S ribosomal protein L34 was used as a loading control.

the differentiation from mycelium to yeast and vice-versa based on a change on cell wall composition. At 37°C, there is an increased synthesis of chitin and α-glucan, and low levels of β-glucan, which results in the yeast form. In contrast, at 22°C, α-glucan synthesis occurs at low rates and long β-glucan fibrils are formed in the budding spots. In keeping with these morphological and biochemical events, 1,3-β-glucosidase increased levels are correlated to the shift to the mycelium phase.

Other three genes coding for proteins from the same category were confirmed to be up-regulated in yeast cells: *ags* (α-1,3-glucan synthase), *cda* (chitin deacetylase) and *vrp*-verprolin (Fig. 1a). The *P. brasiliensis* 1,3-α-glucan synthase gene was first described by Pereira *et al.* [51].

Recently, it was demonstrated that it is strongly up-regulated in yeast cells [28,52], which was confirmed in this work by northern blotting analysis. Rappleye *et al*. [53] silenced the 1,3-α-glucan synthase gene in *H. capsulatum* and demonstrated that α-(1,3)-glucan is an important virulence factor and affects the ability of *H. capsulatum* to kill macrophages and colonise murine lungs. In *C. neoformans*, mutants for 1,3-α-glucan synthase failed to assemble the capsule, which is an important virulence factor of this pathogen [54]. Morphogenetic transition is the essence of *P. brasiliensis* life cycle: for instance, low levels of α-1,3-glucan in the cell wall of the yeast form have been correlated with low virulence [55]. Virulent cultures of *P. brasiliensis* isolates grown i*n vitro* for long periods have thinner cell walls, low α-1,3-glucan levels and are consequently less virulent [56]. Our results suggest that α-glucan synthase is involved in the dimorphic transition of *P. brasiliensis* and possibly in its virulence. The cell wall is an essential and dynamic fungal structure that has been implicated in several pathogenic processes. Being absent in mammalian cells, it may be a relevant target to drug therapies. In this context, the gene that encodes α-1,3-glucan synthase was demonstrated to be a virulence factor using RNAi approaches in *Cryptococcus neoformans* [54] and *H. capsulatum* [53], and seems to be an ideal target for new antifungal drugs. In *P. brasiliensis* glucan polymers constitute 95% of yeast cell wall [49] and thus any interference in cell wall synthesis through glucan synthases is likely to affect virulence directly.

Chitin deacetylase enzyme (CDA) catalyses the conversion of chitin to chitosan by deacetylation of N-acetyl-D-glucosamine residues. Chitosan is a flexible, soluble polymer that integrates the cell wall of some fungi, such as *S. cerevisiae* [57] and *C. neoformans* [58]. In *S. cerevisiae*, chitosan is only found during sporulation [59]. The molecular characterisation of two sporulation-specific chitin deacetylase genes, *CDA1* and *CDA2*, both of which contribute to spore wall rigidity, was described previously [59]. In *S. cerevisiae*, *cda1* mutants present a more diffuse chitosan layer, while their surface layer remains intact. In *cda2* mutant cells, by comparison, the chitosan layer is not detected at all. In the spore walls of *cda1* and *cda2* mutants both outer layers are missing due to defects on wall maturation. However, in *C. neoformans*, a study reported that chitin is present in the yeast cell wall and most of it is continually deacetylated to chitosan. Mutants for chitin deacetylase show suppression of growth due to the lack of chitosan and therefore have a reduced infection capability [58]. The same study hypothesized that this constant remodelling of the cell wall contributed to cellular integrity in this fungus. In *P. brasiliensis*, we identified a highly expressed *cda* gene in yeast cells that presents similarity to the *C. neoformans*. If the *C. neoformans* model is closer to what is found in *P. brasiliensis*, then chitin synthase and chitin deacetylase may be potential targets to antifungal therapy.

Verprolin is required for a fully polarised distribution of cortical actin patches and viability at high temperature. This is the first time that verprolin is described in *P. brasiliensis*, a pathogen that has as an intrinsic characteristic the ability to grow at the human body temperature, 37°C. The inability of *vrp-1* mutants to grow at 37°C was reported by Naqvi *et al*. [60] in the non-pathogenic yeast *S. cerevisiae*. Likewise, we hypothesise that verprolin is involved in the ability of *P. brasiliensis* to grow at 37°C and in cell cytoskeleton organisation since this gene is over expressed in yeast cells. Considering that the actin cytoskeleton plays a crucial role on fundamental processes such as cell growth, differentiation and migration, localised membrane growth, endocytosis, and cell division [61], this protein is likely to play a key role in cell maintenance and viability of *P. brasiliensis* inside the host cell.

Mycelium and yeast up-regulated genes involved in ion metabolism and transport

Two genes putatively implicated in ion transport were confirmed to be highly expressed in mycelium cells: *isc* and *ktp*, an iron-sulphur cluster protein and a cation transporter, respectively. In contrast, a putative P-type cation pump (*pct*) was up-regulated in the yeast form (Figure 1b).

It has been reported that the ISC protein is responsible for mitochondrial uptake of iron and seems to monitor the cytoplasmic levels of this ion. In *S. cerevisiae*, the double knock-out of the homologues *ISU1* and *ISU2* is lethal. Defective mutants are distinguished by iron accumulation in the mitochondrial matrix and its respective decrease in the cytosol [62]. In *C. neoformans*, complementation, cloning and sequencing of such genes has recently been accomplished [63]. It has long been hypothesised that iron is a limiting factor for infectivity during cryptococcosis as well as in other systemic mycoses, in that the host normally provides only limited amounts of this compound. Arango and Restrepo [64]demonstrated iron availability to be essential for growth of mycelium and yeast of *P. brasiliensis*; but especially for mycelium, whose growth was totally prevented by the addition of the iron chelator phenanthroline to the medium, an effect observed only to a lesser extent in yeast. The effect of phenanthroline was reversed partially in mycelium and totally in yeast by addition of excess iron. This is in good agreement with the overexpression of the ISC protein in the mycelial phase. In *P. brasiliensis* it could be involved in monitoring the amount of iron in the environment and in providing a means of storage of this metal.

The *ktp* sequence from *P. brasiliensis* aligned best with potassium transporter proteins of the HAK family, which are mainly implicated in the resistance to potassium starvation. In *N. crassa*, the closest homolog of *P. brasiliensis*, KTP coexists with another potassium transporter of the TRK family [65]. It has been hypothesised that soil organisms are universally equipped with a powerful K^+-concentrating apparatus, as these organisms are faced with a very diluted and variable environment, thus being forced to pump potassium in against a steep gradient [65]. This is likely to be the case of *P. brasiliensis*, whose ecological niche for the mycelium form is thought to be the soil.

Another yeast up-regulated gene is *pct*, a putative member of the E1-E2 (P-type) family of ATPases. These are ATP-dependent proteins which regulate transmembrane flow of all relevant cations, including Na^+, H^+, Mg^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+} and K^+ [66]. In *C. albicans*, the E1-E2 ATPase gene, *CDR1*, confers resistance to both copper and silver, the latter being used as an antimicrobial agent [67]. A similar function could be attributed to the *P. brasiliensis pct* gene, although alignment data are insufficient to identify which cation this protein transports.

Sulphur metabolism

Several enzymes from the cysteine *de novo* biosynthesis pathway (Figure 2) were shown to be up-regulated in the yeast form of *P. brasiliensis*. Our previous analysis [28] had already confirmed over expression of paps reductase (the third in the pathway). *In silico* EST subtraction and cDNA microarray showed yeast up-regulation for atp sulphurylase and aps kinase; the former was confirmed by northern blotting (Figure 1c). Thus, we can strongly suggest that the yeast form synthesises cysteine actively from inorganic sulphate.

In order to reinforce these data, we have evaluated the importance of inorganic sulphate to growth and differentiation. Auxotrophy of *P. brasiliensis* yeast for several sources of organic sulphate – including cysteine itself and sulphydrylic compounds – has been reported before [42]. It was concluded then that organic sulphate deprivation suppressed growth in the yeast phase and prevented mycelium-to-yeast differentiation, whereas the mycelial phase is able to grow on either inorganic or organic sulphur [68]. Also, the saprophytic, mycelial form of *H. capsulatum* is prototrophic while the pathogenic yeast form requires cysteine [69]. It has been reported that exogenous cysteine is required for both yeast phase growth and morphological transition from mycelium-to-yeast of *H. capsulatum* [41,70]. In this work, both mycelium and yeast cells of *P. brasiliensis* were incubated in modified MVM medium without inorganic sulphate, apart from the negligible amounts present in the trace elements solution. Dimor-

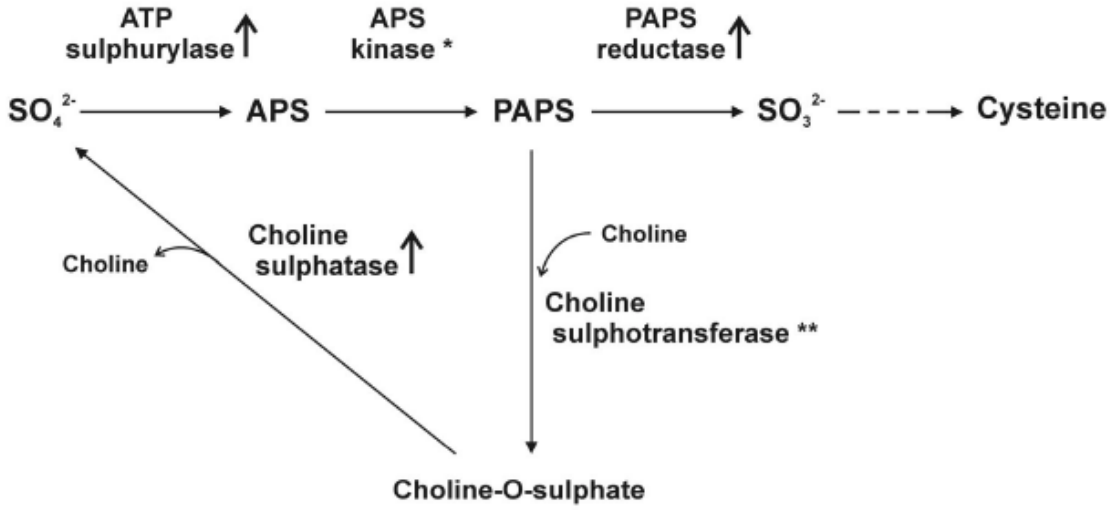

Figure 2
Up-regulated genes encoding enzymes from the cysteine *de novo* biosynthesis pathway. Arrows indicate enzymes identified as up-regulated both by *in silico* subtraction, cDNA microarray and confirmed by northern blotting experiments. (*) enzyme identified as up-regulated by both *in silico* subtraction and cDNAs microarray but not assayed by northern blotting. (**) indicates an enzyme not found in the transcriptome of *P. brasiliensis*.

phic transition was assessed in the mycelium to yeast direction and in the opposite way.Sustained growth was observed for both morphotypes (data not shown) and, upon the corresponding temperature shifts, differentiation was successfully triggered in both directions (Figure 3). Thus, inorganic sulphate seems to be unnecessary for the transition, quite contrarily to organic sulphate. In this context, it is interesting to consider a branch of the cysteine biosynthetic pathway (Fig. 2). In fungi and plants a fraction of PAPS, which is toxic to fungi if it reaches high cytosolic levels, is used by choline sulphotransferase to produce choline-O-sulphate [40], which serves as an osmoprotectant and cytosolic sulphur store in these organisms. We have not found a homologue of choline sulphotranferase in *P. brasiliensis* to date, but the enzyme choline sulphatase, which degrades its product to choline and sulphate, is also over expressed in the yeast morphotype, as confirmed here (Figure 1c) and previously reported [52]. The *C. neoformans met3* mutant, which lacks ATP sulphurylase activity, had a substantial defect in melanin formation, significantly reducedgrowth rate, and greatly increased thermotolerance. In the murine inhalation infection model, the *met3* mutant was avirulent and was deficient in its ability to survive in mice [71]. In this context, disrupting the genes encoding choline sulphatase or ATP sulphurylase in *P. brasiliensis* should reveal its role in the growth, maintenance of yeast cells and pathogenicity of this fungus. It is interesting that another intracellular pathogen of humans, the bacterium *Mycobacterium tuberculosis*, depends on sulphur compounds for expression of its full virulence, drug resistance and overall survival inside the macrophage. It has developed a very efficient sulphate activation pathway (SAC) that ensures constant synthesis of PAPS at high rates, from which sulphate may be distributed to other synthetic pathways [72]. The SAC includes the bacterial counterparts of ATP sulphurylase and APS kinase, the latter of which performs PAPS synthesis by coupling it with GTP hydrolysis by a GTPase that is also present in SAC. Whether similar mechanisms are present in pathogenic fungi such as *P. brasiliensis* remains to be investigated.

Conclusion

Taken together, these data show that several genes involved in cell organisation and ion metabolism/transport are differential in their expression along dimorphic transition, which is in accordance with the proposed model for this process in Figure 4. While α-glucan is synthesised during yeast phase due to high expression of 1,3 α-glucan synthase, β-glucan is degraded by the action of 1,3 β-glucosidase during hyphal growth. The *cda* gene is probably involved in the cell wall synthesis of yeast cells,

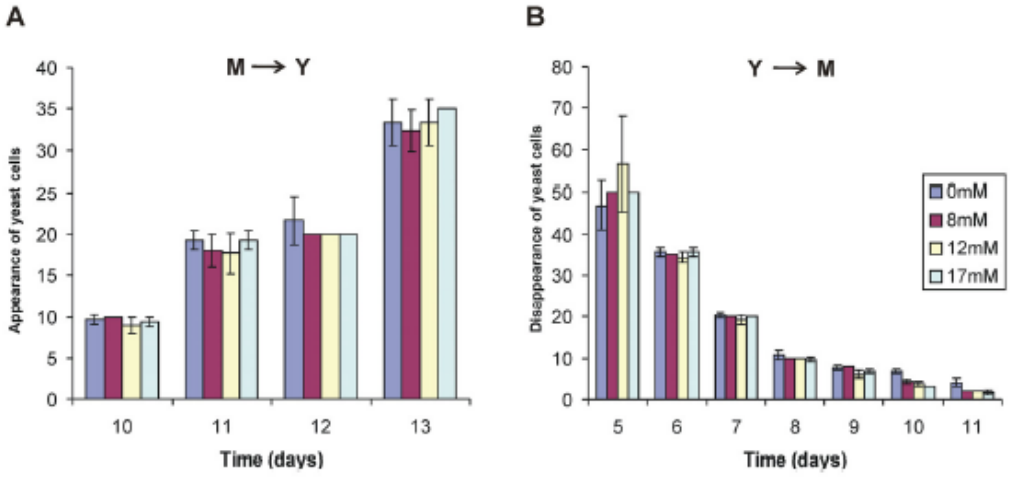

Figure 3
Cell differentiation of *P. brasiliensis* in modified MVM medium without inorganic sulphate. The fungus was grown in four different concentrations of sulphate salts (0, 8, 12 and 17 mM; the latter is the original concentration of MVM medium). (A) The appearance of yeast cells was verified daily in the transition from mycelium to yeast after temperature shift to 37°C. (B) The disappearance of yeast cells was verified daily in the transition from yeast to mycelium after temperature shift to 22°C. Triple samples were counted for each time point. The coloured boxes indicate the average of the three samples and bars represent the standard deviation of the mean. As observed, the presence or absence of inorganic sulphate did not affect transition in either direction.

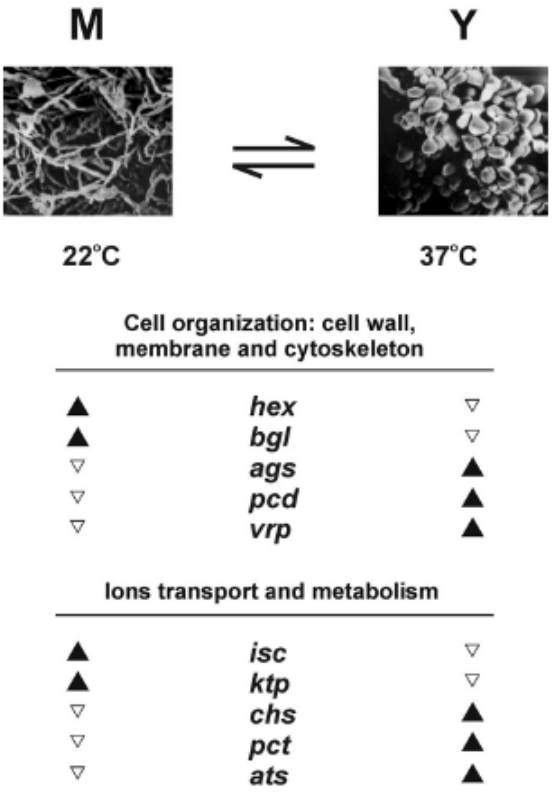

Figure 4
Genes involved in cell organisation (cell wall, membrane and cytoskeleton), sulphur metabolism and ion transport. Genes that were identified as up-regulated in mycelium (22°C) or yeast (36°C) cells of *P. brasiliensis* are represented by black arrowheads. Electron microscopy was performed by Silva et al. [78].

since it is over expressed in this phase. In addition, genes related to septal sealing and cytoskeleton organisation (*hex* and *vpr*) are also probably implicated in the stabilisation and maintenance of mycelium and yeast cells in the environment and at 37°C in the human host. Also, the differential expression pattern of genes that control metabolism and transport of potassium, iron, copper and sulphur ions suggests that they may influence directly the pathogen adaptation to the host environment. Specifically, in spite of the undisturbed growth and differentiation on depletion of inorganic sulphate, the over expression of enzymes from *de novo* cysteine synthesis lends support to previous findings about the importance of this pathway to yeast metabolism. Understanding these changes by functional analysis of such genes may lead to a better understanding of the infective process, thus providing new targets and strategies to control PCM.

Methods

Strains and cultures

P. brasiliensis clinical isolate Pb01 (ATCC-MYA-826) was used in this study. Cells from both mycelium and yeast forms were grown on semi-solid Fava Neto's medium [73] for 7 to 10 days at 22°C or 37°C, respectively.

Overlap analysis – in silico EST subtraction and cDNA microarrays

This work was based on the output of previous large-scale expression analysis experiments, as outlined in reference 28. Briefly, we have constructed a λZAP II® (Invitrogen) cDNA library from mycelium and yeast mRNA fractions and 5'-sequenced the mass-excised cloned fragments with the T7 vector primer. Raw sequence data were quality-assessed by PHRED and assembled by CAP3, thus generating a set of 6,022 PbAESTs (singlets and contigs). For functional annotation of sequences we used the nr (NCBI) database. *In silico* electronic subtraction was performed according to the Audic and Claverie [74] statistical approach, with a 95% confidence rate. For cDNA microarray 1,152 clones were selected and PCR-amplified for spotting onto nylon-membranes in triple experiments. Hybridisation against [α-^{33}P] dCTP-labeled total RNA from mycelium or yeast and phosphor imager signal capture were performed as in [28]. After signal quantification and background subtraction [75], statistical analysis was carried out with the SAM (Significance Analysis of Microarrays) method [76]. Data from both experiments were overlapped to identify differential genes, thus generating the set of 66 sequences we used in this work.

Northern blot analysis

Total RNA (15μg) from mycelium and yeast cells of *P. brasiliensis* was separated on denaturing 1,5 % agarose gel and blotted onto a Hybond-N membrane (GE Healthcare). Probes were radiolabeled using [α-P^{32}]dATP by random priming according to supplier's instructions (Invitrogen), purified and used in overnight hybridisation (50% formamide, 4X SSPE, 5X Denhardt's solution, 0,1% SDS, 100μg/ml herring sperm DNA) at 42°C. The membranes were then washed under stringency conditions of 2X SSPE-0.1% SDS at 65°C for 1h. Signal bands were visualised using the Typhoon 9210 Phosphor Imager (GE HealthCare).

Dimorphic transition without inorganic sulphate

We incubated both mycelium and yeast cells on modified versions of McVeigh and Morton's medium – MVM [77] where ammonium and magnesium sulphate salts were gradually replaced by their chloride counterparts, in the following chloride concentration set points: 0, 8, 12 and

17 mM, where the first corresponds to the original recipe and the last, to virtual absence of inorganic sulphate, apart from negligible amounts in the trace elements solution (~ 8 μM). Molar concentrations of both magnesium and ammonium were thus conserved. We have also evaluated whether dimorphic transition occurred normally in the medium without inorganic sulphur. To achieve this, five flasks containing 100 ml of modified MVM were inoculated with comparable amounts of mycelium (100 mg wet mass) and yeast (2.5×10^7 cells) previously grown on standard MVM. Samples were incubated in rotating shakers (120 rpm) at 36 and 22°C, respectively, thus triggering dimorphic transition. Fungal viability and progress of transition were assessed by serial 100 μl sampling every 24 hours (three independent samples). Each sample was coloured with Janus Green and the number of yeast cells was counted in a light microscope with the aid of a Neubauer counting chamber.

Accession numbers

The accession numbers of the EST sequences analysed in this work are shown in the Tables 1 and 2.

Abbreviations

ags alpha 1,3-glucan synthase

aps adenosine 5'-phosphosulphate

ats ATP sulphurylase

bgl 1,3 beta-glucosidase,

BLAST basic local alignment search tool

cda chitin deacetylase

cDNA complementary DNA

chs choline sulphatase

COG clusters of orthologous groups

e-value extreme value distributionESTs

ESTs expressed sequence tags

GO gene ontology

hex hexagonal peroxisome protein

isc iron-sulphur cluster-like protein

ktp potassium transporter

MIPS Munich information center for proteins sequences

PAPS phosphoadenylyl-sulfate reductase

PbAETs *P. brasiliensis* assembled EST sequences

PCM paracoccidioidomycosis

pct putative P-type Cu(2+) transporting ATPase

SAGE serial analysis of gene expression

SAM significance analysis of microarrays

vrp verprolin

Authors' contributions

RA and MF planned and designed the study, developed the experiments and the data analysis, wrote the main draft of the paper and support the preparation of the figures and tables. HP supports the discussion of the results and revised the manuscript. AN participated in the *in silico* ESTs subtraction analysis of the raw data generated by the transcriptome project. MC analysed the results of the microarray experiments, helped in the manuscript edition, and prepared the figures. AL executed the microarray experiments. MC, RC and MB participated in the normalization process of the microarray raw data and helped to make the statistical analyses. SS participated of the differentiation experiment involved of the inorganic sulphur, and of the preparation of the RNA of *P. brasiliensis* to make the microarray experiments. LF participated of the analysis of the cell wall organization. SP helped in the ESTs amplification and on the analysis of the sulphur metabolism. GP, ES, ED designed the microarray experiments. CS participated on the Pb ESTs annotation. All authors read and approved the final manuscript.

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Insights into the pathobiology of *Paracoccidioides brasiliensis* from transcriptome analysis – advances and perspectives.

Title: Insights into the pathobiology of *Paracoccidioides brasiliensis* from transcriptome analysis – advances and perspectives

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Running title: Insights from *Paracoccidioides brasiliensis* transcriptome

Abstract

Paracoccidioiddes brasiliensis is a thermo-dimorphic fungus endemic to Latin America, where it causes the most prevalent systemic mycosis, paracoccidioidomycosis (PCM). DNA microarray technology has been used to identify patterns of gene expression when a microbe is confronted with conditions of interest, such as *in vitro* and/or *ex vivo* interaction with specific cells. *P. brasiliensis* is one organism that has benefited from this approach. Even though its genome has not been sequenced yet, much has been discovered from its transcriptome and DNA array analyses. In this review, we will outline the current knowledge in *P. brasiliensis* transcriptome, with focus on differential expression analysis *in vitro* and on the discussion of the genes that are controlled during the host-pathogen interaction *ex vivo* in order to give insights into the pathobiology of this fungus. *In vitro*

experiments enabled the delineation of whole metabolic pathways; the description of differential metabolism between mycelium and yeast cells and of the mainly signaling pathways controlling dimorphism, high temperature growth, thermal and oxidative stress and virulence/pathogenicity. Recent *ex vivo* experiments provided advances on the comprehension of the plasticity of response and indicate that *P. brasiliensis* is not only able to undergo fast and dramatic expression profile changes but can also discern subtle differences, such as whether it is being attacked by a macrophage or submitted to the conditions of the bloodstream route conditions.

Introduction

The fungus *P. brasiliensis* is a thermally-controlled dimorphic pathogen endemic to Latin America. It causes the most prevalent systemic mycosis, paracoccidioidomycosis (PCM) with around ten million people infected, of which about 2% will develop the illness [1]. PCM ranges in clinical onset from an acute infection to a chronic, disseminated form that may compromise several organs besides its initial focus (usually the lungs). Infection is thought to be contracted by inhalation of fungal propagules – conidia or mycelial fragments – and is triggered by the dimorphic shift that characterizes this fungus and which consists of its change upon exposure to the body temperature to the yeast form. It is a primary pathogen, infecting immunocompetent hosts, and has a strong bias towards males. The disease is fatal if left untreated and late treatment may result in disabling sequelae.

The phylogenetic classification of *P. brasiliensis* is based primarily on sequence analysis. It is placed alongside other pathogenic, free-living fungi such as *Aspergillus fumigatus* and *Penicillium marneffei* inside Phylum Ascomycota, Subphylum Pezizomycotina, and Class Eurotiomycetes. Narrower classification puts it with thermodimorphic *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Coccidioides*

immitis inside Order Onygenales, family Onygenaceae. This classification of dimorphic fungi has been proposed in spite of the fact that no teleomorphic stage has been yet identified for some of them (this stage is known for *H. capsulatum* only). Recent progress on phylogeny of *P. brasiliensis* may be reviewed in detail elsewhere [2]. One of the tasks for the post-genomic era of *P. brasiliensis* will be to establish the cladistic relationships of the fungus with other ascomycetes and trace the history of the interaction of the fungus with animal and human hosts. This will perhaps help us to explain how it evolved to cause paracoccidioidomycosis and the precise determinants of its virulence.

P. brasiliensis has been shown to be refractory to classical genetic analysis. Recently, however, careful analyses have established it has a genome of 26-35Mb distributed in four or five chromosomes [3-5]. Ploidy is not consensual; some groups have proposed several isolates to be diploid [3, 4], whereas other have used different techniques to propose that most isolates be either haploid or aneuploid [5]. It presents some degree of phenotypic variability; some of them retaining the yeast form even at lower temperatures [6]. Several isolates also differ in their ability to sporulate [7] and cause disease [8]. These presumably reflect a corresponding degree of genetic diversity, which has been supported by recent data [8]. Transcriptome data suggest that the fungus possess sex-related genes [9]. Also, Matute *et al*. [10] presented compelling evidence of intra-specific recombination, although no sexual reproduction has been detected to date.

Work on genetic variability of *P. brasiliensis* has been carried out for more than a decade. The early works with random amplified polymorphic DNA analysis [11] had already proved to be able to separate isolates into discrete groups. More recent work combining sequencing and phylogenetic analyses has led researchers to postulate the existence of at least three cryptic species [10]; the same group of researchers has also

developed a microsatellite analysis protocol that enables the correct classification of isolates according to each phylogenetic species [12].

The genome constitutes the informational core of all biological processes and the study of living organisms depends heavily on our ability to access its contents. For many decades the experimental approach consisted of isolating single genes or regulatory elements and characterizing each one at a time by means of loss-of-function and/or gain-of-function experiments; or by identifying effectors (proteins, cofactors or metabolites) and studying their roles and interactions.

In keeping with the informational flow in the cell, genome has been closely followed by transcriptome and proteome. The focus of research has shifted from looking into single genes to understanding global processes and refined controls, including the identification of non-coding RNAs and large-scale phenotypic screening of random mutants [13, 14]. Non-coding sequences have attracted much interest in recent years with the discovery and elucidation of RNA interference (RNAi) mechanisms [15]. RNAi has already been used as an experimental tool to characterize genes by means of loss-of-function experiments in organisms that are refractory to common gene disruption mechanisms [16]. None of these phenomena have been explored in *P. brasiliensis* and thus a large avenue of investigation remains to be opened.

The study of pathogens and their interaction with hosts is of special interest. Computer-aided data mining has enabled unambiguous identification of open reading frames, and transcriptional profiling has yielded relevant information concerning differential gene expression [9, 17]. Microarray technology, in conjunction with statistical and experimental validation, has been used to identify patterns of gene expression – selected according to previous genome or transcriptome information – when the microbe is

confronted with conditions of interest, such as *ex vivo* interaction with specific cells or exposure to therapeutic agents, signalling molecules or stressors [18, 19].

The fungus *P. brasiliensis* is one organism that has benefited from these approaches. Even though its genome has not been sequenced yet, much has been discovered from the transcriptome and DNA array analyses of this fungus. In this review, we will outline the current knowledge from *P. brasiliensis* transcriptome, with a focus on differential expression analysis *in vitro* and how it has helped to advance research in recent years. We have also focused on the discussion of the genes that are controlled during the host-pathogen interaction *ex vivo* in order to give insights into the pathobiology of this fungus.

The transcriptome projects and array studies of *P. brasiliensis*

The main global gene analysis in *P. brasiliensis* were performed using the EST (Expressed Sequence Tag) approach, which worked efficiently and seemed to be a useful method to provide the most valuable genetic information of this fungal pathogen. Felipe *et al*. [9, 20] and Goldman *et al*. [21] reported the analysis of 6,022 (from Pb01 isolate) and 4,692 (from Pb18 isolate) assembled groups, respectively. Those first results allowed the delineation of whole metabolic pathways, the differential metabolism between mycelium and yeast cells and the mainly signaling pathways controlling dimorphism, high temperature growth, thermal and oxidative stress and virulence/pathogenicity of the fungus. Also, the transcriptome projects highlighted the importance of differential expression genes in both phases – mycelium and yeast, the potentially related virulence factors and possible drug targets.

Among the metabolic features generated by the transcriptome, one of the most interesting found, is the differential metabolism between mycelium and yeast cells of *P. brasiliensis*. The mycelium cells appears to have an aerobic metabolism which is suggested

by the up-regulation of isocitrate dehydrogenase and succinyl-CoA synthetase enzymes, involved in citrate cycle and also glucokinase, adenylate kinase, uridine kinase and transaldolase. On the other hand, the yeast cells presented induction of genes coding alcohol dehydrogenase I and pyruvate dehydrogenase which evidenced an anaerobic metabolic characteristic favoring fermentation of the pathogenic phase of this fungus [9]. The differential metabolism between mycelium and yeast was reinforced by Nunes *et al*. [22] studies on the transcriptional response of *P. brasiliensis* during the dimorphism, in which they observed an increased expression of alcohol dehydrogenase I and pyruvate decarboxylase genes in the differentiation process from mycelium to yeast, where almost 90% of the cells are already in the yeast form.

Marques *et al.* [23] and Andrade *et al*. [24] using the array methodology evaluated the differentially expressed genes in mycelium and yeast cells, which in many cases are important keys to understand the pathobiology of *P. brasiliensis*. Andrade *et al.* [24] reported 66 transcriptional modulated genes in mycelium or yeast categorized into two classes, the first group, cell organization includes genes involved in maintenance of cell wall, membrane and cytoskeleton, as the mycelium up-regulated gene hex which encodes a hexagonal peroxisome protein controlling cell integrity, and *bgl* coding 1,3-β-glucosidase involved with cell wall modification during the dimorphism. Among the modulated genes from the yeast phase are *vrp* (verprolin), *cda* (chitin deacetylase), *ags* (α-1, 3-glucan synthase). The α-1,3-glucan synthase also reported in [23] to be positively regulated in the pathogenic form of *P. brasiliensis* can be easily correlated with the biology of this fungus. The α-1,3-glucan is the main component of the yeast cell wall, and it is closed related to the virulence due to its ability to mask the host recognition mechanism of the pathogen, as also reported in *H. capsulatum* [25] promoting the fungal escape from the host defenses which contribute to the fungal pathogenesis. The second group of genes consist those

involved in metabolism and transport of ions. Genes that regulate the ion metabolism and transport as *isc* (iron-sulphur cluster) and *kpt* which is related to potassium availability were positively regulated in mycelium cells. Those genes appear to be involved in availability of iron and potassium, respectively important for the saprophytic life of *P. brasiliensis* on its ecological niche, the soil. The *pct* gene coding a P-type cation pump is reported to be up-regulated in yeast cells.

The observations of Paris *et al.* [26] that yeast cells of *P. brasiliensis* were unable to grow in the presence of inorganic sulphur, were confirmed by the array experiments in which the genes coding sulphur metabolism enzymes such as atp sulphurylase, aps kinase, paps redutase and choline sulphatase showed to be up- regulated in yeast cells of this pathogen, indicating the auxotrophic status for cysteine of the pathogenic phase of this fungus [23, 24, 27].

The *in silico* analysis of the transcriptome allowed the identification of almost all components of the conserved signaling pathways already characterized in other eukaryotes as MAP kinases (Mitogen activated protein kinases), PKA/cAMP (Protein kinase A/ cyclic adenosine monophosphate), Ras- GTPases, calcineurin-calmodulin, two component system (histidine kinase), and G proteins coupled receptors [9]. In addition, Nunes *et al.* [22] using microarray reported activation of genes coding for proteins from conserved signaling pathways during the dimorphic transition from mycelium to yeast of *P. brasiliensis*. Among them were G-proteins, Ser/Thr protein kinases, protein kinase A, calmodulin-like protein and calcineurin regulatory subunit. Recently, Bastos *et al.* [28] evaluated the genes regulated on the beginning of cellular transition from mycelium to yeast of *P. brasiliensis*. In accordance, induction of MAP kinases, calcineurin regulatory subunit, serine/threonine kinases and also the histidine kinase and two component sensor kinases was observed [22]. The histidine kinase gene (*drk*) was reported to control the global dimorphism in fungal

pathogens [14]. These studies suggest the involvement of those signaling pathways in the cellular differentiation process of *P. brasiliensis.*

Fungal pathogens use the conserved mechanism of signaling to promote cell survival under different conditions. The activation of virulence factors is also dependent of the signaling triggered by those cascades, and the main consequence of this activation is the ability of the pathogen to infect and disseminate on the harsh host environment. By a comparison of the *P. brasiliensis* ESTs with *Candida albicans* genes, Felipe *el al*. [9] identified some genes potentially related to fungal virulence. In a more detailed search scan, Tavares *et al*. [29] categorized 30 putative virulence genes into the following classes: metabolism, cell wall, detoxification-related, secreted factors, and others. Genes as *icl1* and *mls1* coding respectively, isocitrate lyase and malate synthase of the glyoxylate cycle were potentially correlated with virulence due to their activation on poor carbon growth conditions. As those enzymes are not present in humans, they are also possible drug targets candidates. Other genes related to virulence are identified such as: *ade2* (phosphoribosylaminoimidazole carboxylase), *nmt1* (N-myristoyltransferase) and *fas2* (fatty acid synthase α-subunit), *tps1* (trehalose-6-phosphate synthase), respectively involved in nucleotide, lipids and glucose metabolism. Among the genes from the second group are those necessary to the cell wall integrity during the vegetative growth and differentiation of *P. brasiliensis*, and includes: *ags1* (α-glucan-synthase gene), *chs3 (*chitin synthase 3), *gna1 (*glucosamine-6-phosphate acetyltransferase), *pmt1* (mannosyl transferase) and *mnt1 (*α-1,2-mannosyltransferase), *phr1* and *phr2* (1,3-β-glucanosyltransferases). The third group comprises the pathogen virulence genes that function on the detoxification of oxidative radicals: as Cu/Zn superoxide dismutase (*sod1*), thiol peroxidase (*tsa1*) alternative oxidase (*aox1*) and catalase (*cat1*). As *P. brasiliensis* is a pathogen that survives intracellularly, the yeast cells have to minimize the toxic substances

present in the phagossomes of the macrophage cells, and by this reason the fungus exhibits an antioxidant arsenal of enzymes that are necessary to the survival and consequently virulence of the pathogen. The secreted virulence factors, also reported by Tavares *et al*. [29] include genes encoding proteinases, phospholipases and urease.

The *in vitro* transcriptome studies opened a new window on the understanding of *P. brasiliensis* biology. Recently, the genes modulated in the host-pathogen interaction were evaluated trough the transcriptional response of *P. brasiliensis* when yeast cells were internalized into macrophage cells [30]. In addition, Bailão *et al*. [31] also analyzed genes with differential expression when yeast cells were in contact with human blood and rescued from infected mice, to identify the genes required to the *P. brasiliensis* adaptation on the host interaction.

Global patterns of gene expression in the host-pathogen interaction

Understanding host-pathogen interactions may provide insights into host defences and the tactics used by pathogens to overcome them. In recent years, several approaches such as cDNA micro arrays, cDNA representational difference analysis (RDA) and serial analysis of gene expression (SAGE) have been developed to identify general profiles of gene expression [32, 33]. Specifically, the cDNA micro array technology has been applied to the analysis of bacterial and fungal interactions with phagocytes, thus identifying key effectors of pathogen virulence and host defence [32, 34-36].

Transcriptional profile of *P. brasiliensis* upon infection

The first defence line encountered by *P. brasiliensis* upon infection is pulmonary resident macrophages. Despite being phagocytized, *P. brasiliensis* conidia germinate into the parasitic yeast form, which is equipped to resist the harsh intraphagosomal environment, thus surviving and replicating in non-activated murine and human macrophages. It has been proposed, for PCM and for other systemic mycoses such as

histoplasmosis and coccidioidomycosis, that fungal intracellular parasitism is a landmark event for establishment and progression of disease in susceptible hosts, since it enables fungal latency and/or dissemination from the lungs to other organs and tissues [37]. The understanding of this process should reveal key aspects of how these pathogens manage to survive and replicate intracellularly and eventually lead to the development of new antimicrobial drugs. The macrophage phagosome is believed to be a poor source of glucose and amino acids [18, 38]. Nutritional deprivation inside the macrophage induces a similar adaptative response by intracellular bacterial and fungal pathogens [18, 38, 39]. Micro array analysis showed that following phagocytosis, *C. albicans* and *Listeria monocytogenes* present a strong reduction in the expression of genes involved in glycolysis an amino acid metabolism [38, 39]. In order to focus the research on the interaction of *P. brasiliensis* with the human host, 1,152 cDNA clones of interest were selected from the transcriptional database, based on previous findings of *P. brasiliensis* transcriptome [9], including putative virulence factors, general metabolism enzymes, heat shock proteins, cell-wall synthetic enzymes and also some of unknown function. In addition, the protocol of RNA extraction from *P. brasiliensis* yeast cells internalized by murine macrophages, without any additional fungal *in vitro* growth was standardized [30]. They observed that early phagocytized *P. brasiliensis* also sense and respond to the phagosomal environment. Genes implicated in glucose and amino acid depletion (*pfkA* - phosphofructokinase, *gapdh* - glyceraldehyde-3-phosphate dehydrogenase, *pgk* - phosphoglycerate kinase, *gpma* - phosphoglycerate mutase, *eno* - enolase, *metG*- cystathionine β-lyase), cell wall metabolism (*fks* - â-glucan synthase) and oxidative stress (*sod3* - Cu,Zn superoxide dismutase and *hsp60* - 60 kDa heat shock protein) were differentially expressed by *P. brasiliensis* upon macrophage infection. The data showed a considerable degree of transcriptional plasticity by *P. brasiliensis* in response to the hostile environment of

macrophages, which is expected to underlie its adaptability and consequent survival inside that cell [30].

From the primary site in the lungs, paracoccidioidomycosis may evolve with fungal dissemination via the bloodstream and/or lymphatic system to many organs [40]. Recently, using cDNA – RDA technology, Bailão *et al.* [31] reported the differential expression profile of *P. brasiliensis* in conditions that mimic the haematological route of fungal propagation. Under such conditions, several genes, including a transport facilitator (*ctr3*), stress response proteins (30, 70 and 90 kDa heat shock proteins - *hsp30, hsp70* and *hsp90*) and cell-wall remodelling (*sho1* - transmembrane osmosensor, *pas-li*ke - protein with PYP-like sensor domain - PAS domain, *septin-1*), were induced upon blood contact.

Nutrient deprivation inside the phagosome induces a similar adaptive response from intracellular bacterial and fungal pathogens and early phagocytized *P. brasiliensis* also sense and respond to the glucose-depleted environment, repressing genes related to glycolysis and amino acid synthesis. In contrast, when *P. brasiliensis* are exposed to blood, genes related to glucose and amino acid metabolism are induced, probably because the milieu is quite rich. Similar results were described upon incubation of *C. albicans* in human blood [41].

After internalization, macrophages challenge the pathogens releasing a group of toxic antimicrobial molecules, including reactive oxygen and nitrogen intermediates (ROI and RNI, respectively). In response to the oxidative stress generated by the macrophage, the *P. brasiliensis* counter attacks inducing antioxidant gene such as *sod3* [30]. *In silico* analysis showed that the deduced amino acid sequence of the *P. brasiliensis sod3* homologue codes a putative membrane GPI-anchored Cu,Zn SOD [42], which would make it more directly accessible to host-derived superoxide anions and thus be more efficient at ROI detoxification. This idea is reinforced by *C. albicans* GPI-anchored Cu,Zn SOD gene,

which is induced *in vitro* by oxidative species generators and upon internalization by neutrophils. [41, 43]. Also, genes encoding molecules involved with thermal stress-response as *hsp60* were induced when *P. brasiliensis* was ingested by macrophages [30] as well as in exposure to blood as *hsp30*, *hsp70 and hsp90* [31]. These proteins may contribute to the protection of *P. brasiliensis* yeast cells from damage following stress, which occur during infection.

Of particular interest for PCM pathogenesis are the genes involved in cell-wall remodelling. The gene for β-glucan synthase (*fks*) is down-regulated in response to the macrophage environment. It has been shown for *P. brasiliensis* and other fungi that the degree of virulence from different isolates in an experimental model of murine infection is related to the relative α-to-β-glucan levels present in their cell wall – avirulent yeast isolates contains less α- and more β-glucan [44, 45]. The mechanism of β-glucan host protection may be related to its capacity to stimulate inflammatory mediators such as tumor necrosis factor-alpha (TNF-α). Since TNF-α has been associated with resistance to *P. brasiliensis* [46, 47], *fks* down-regulation may be an important adaptive mechanism of *P. brasiliensis* to reduce the inflammatory response elicited by the host [30]. In addition, transcripts related to remodelling cell wall and osmotic stress (*gln1* - glutamine synthetase, *septin-1*, *sho1*, *pas-like*) were also induced in *P. brasiliensis* upon blood contact [31]. The induction of *gln1* and *septin-1* has been speculated to be important in the mediation of chitin deposition. Chitin synthesis has been shown to be essential in the compensatory response to cell-wall stress in fungi, preventing cell death [48]. The *sho1* and *pas-like* genes belong to different classes of osmotic stress sensors. In *C. albicans*, *sho1* is related to fungal morphogenesis by interconnecting two pathways involved in cell wall biogenesis and oxidative stress [49]. Up-regulation of *sho1* and *pas-like* implies their involvement in osmolarity sensing during fungal dissemination through the blood. Fig. 1 shows the

proposed model for adaptative changes of *P. brasiliensis* to the host environment, considering the environment of macrophage cells and exposure to blood.

Figure 1

The transcriptional programme of macrophage-ingested cells displays little resemblance to that obtained from *P. brasiliensis* cells exposed to blood. These pioneering works provided advances on the comprehension of the plasticity of response and indicate that *P. brasiliensis* is not only able to undergo fast and dramatic expression profile changes but can also discern even subtle differences, such as whether it is being attacked by a macrophage or submitted to the conditions of the bloodstream route conditions.

Transcriptional response of macrophage cells upon infection with *P. brasiliensis*

The host cell response to pathogens is among the best studied examples of cellular reactions to external stimuli. Pathogen-induced phenotypic changes in host cells are often accompanied by marked changes in gene expression. DNA microarrays technology has greatly expanded our ability to monitor changes in the abundance of transcripts in a host upon infection with a microorganism. Macrophages, neutrophils and peripheral blood mononuclear cells (PBMCs) respond to a broad range of microbial stimuli with common transcriptional activation programmes [32, 36, 50]. In this regard, microarray analyses have shown an increase of genes related to innate immunity in macrophages infected with *Mycobacterium tuberculosis*, *C. albicans* and *A. fumigatus* [51, 35, 36]. Recently, for the first time, Silva *et al.* (in revision) [52] used cDNA microarray analysis to identify differential genes of macrophages infected with *P. brasiliensis* that are related to several immune processes such as inflammation, cell membrane regulation, transcriptional regulation, signal transduction and apoptosis. This last work and the results obtained by Tavares *et al*. [30] studies provided the first data of differential gene expression from host-*P. brasiliensis* interaction, at 6 hours of infection, the same time and experimental

conditions. In this sense, we have reported by cDNA microarray analysis that, in response to the harsh macrophage microenvironment, *P. brasiliensis* expressed genes primarily associated with glucose and amino acid limitation, cell wall construction, and oxidative stress [30]. In counterpart, macrophages at the same time point up-regulate genes related to inflammation (chemokines and cytokines) and phagocytosis, probably as an effort to avoid host fungal dissemination into different organs and tissues [52].

Concluding remarks

For many decades genetic information of *P. brasiliensis* was not easily and widely accessed at the molecular in order to gain information about the biology, virulence, pathogenicity, interaction with the host and onset of disease. In recent years, the new approach of global gene expression analysis gave us the opportunity to understand the general and differential metabolism in both phases of dimorphic fungi. In addition, it has made possible the identification of potential candidate molecules that contribute to virulence, pathogenicity and others that may be used for drug targeting. Furthermore, it has opened the possibility to access the whole transcriptional response during host-pathogen interaction. Finally, the identification of genes that are differentially expressed under *in vitro* dimorphic transition experiments, *ex vivo* macrophage infection or exposure of *P. brasiliensis* to blood has advanced current knowledge to a better understanding of PCM.

As a main consequence of post-transcriptome analysis, the scientific community around *P. brasiliensis* is allocating efforts in order to develop an efficient genetic toolbox to prove and validate the gene function of many of those candidate genes highlighted by the transcriptome data. For example, the generation of random, T-DNA insertional mutants of fungi by means of co-cultivation with *Agrobacterium tumefaciens* has proved to be a powerful technique that has solved at least one major question in the biology of dimorphic fungi [14]. The molecular toolbox to implement this approach in *P. brasiliensis* is still to

be developed. Furthermore, these data contribute to a global picture of this systemic illness that may help us to devise comprehensive therapeutic approaches in the near future.

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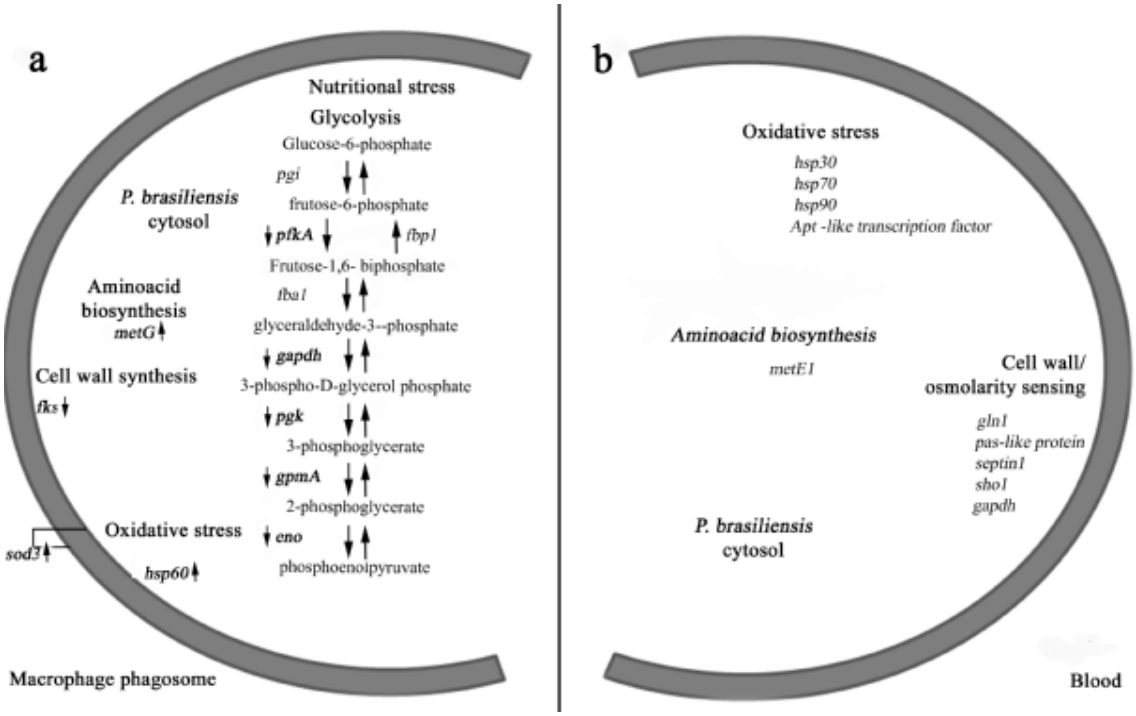
Figure Legend

Figure 1. Model for adaptative changes of *P. brasiliensis* to the host environment. The small arrows ↑ and ↓ indicated induced and repressed genes, respectively. (**a**) ***P. brasiliensis* survival in macrophage phagosome:** oxidative and thermal stress – *sod3*, and *hsp60;* nutritional stress (aminoacid byosynthesis) – *metG*; glycolysis – *pfkA*, *gapdh, pgk, gpma* and *eno*; cell wall synthesis – *fks* [30]. (**b**) **Induction of *P. brasiliensis* genes during blood exposure:** thermal stress – *hsp30, hsp70* and *hsp90, Ap1-like transcription factor*; nutritional response – *metE1* (s-adenosylmethionine synthetase)*;* cell wall/osmolarity sensing – *gln1, pas-like protein., septin, sho1* and *gapdh* [31].

Figure 1


a
Nutritional stress
Glycolysis
Glucose-6-phosphate
pgi
P. brasiliensis
cytosol
frutose-6-phosphate
pfkA
fbp1
Frutose-1,6- biphosphate
Aminoacid
biosynthesis
metG
fba1
glyceraldehyde-3--phosphate
gapdh
Cell wall synthesis
fks
3-phospho-D-glycerol phosphate
pgk
3-phosphoglycerate
gpmA
2-phosphoglycerate
Oxidative stress
eno
sod3
hsp60
phosphoenoipyruvate
Macrophage phagosome
b
Oxidative stress
hsp30
hsp70
hsp90
Apt -like transcription factor
Aminoacid biosynthesis
metE1
Cell wall/
osmolarity sensing
gln1
pas-like protein
septin1
sho1
gapdh
P. brasiliensis
cytosol
Blood