Universidade de São Paulo
Faculdade de Ciências Farmacêuticas de Ribeirão Preto

**Caracterização molecular de genes preferencialmente expressos
na fase leveduriforme patogênica de *Paracoccidioides brasiliensis*
através das técnicas de “Macroarray” e de SSH (“Suppression
Subtractive Hybridization”)**

Everaldo dos Reis Marques

Ribeirão Preto

2005

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas de Ribeirão Preto
Programa de Pós Graduação em Biociências Aplicadas à
Farmácia

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Tese de Doutorado
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Orientado: Everaldo dos Reis Marques
Orientador: Prof. Dr. Gustavo Henrique Goldman

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AUTORIZO A DIVULGAÇÃO TOTAL OU PARCIAL DESTE TRABALHO, POR QUALQUER MEIO CONVENCIONAL OU ELETRÔNICO, PARA FINS DE ESTUDO E PESQUISA, DESDE QUE CITADA A FONTE.

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RESUMO

Paracoccidioides brasiliensis, um fungo termodimórfico, é o agente causador da paracoccidioidomicose (PCM), a micose sistêmica prevalente da América Latina. A patogenicidade aparenta estar intimamente relacionada com a transição dimórfica da forma de micélio para a de levedura, que é induzida pela mudança da temperatura do ambiente pela temperatura do hospedeiro mamífero. Há poucas informações disponíveis sobre genes de *P. brasiliensis* que são necessários durante a fase patogênica. Nós, então, realizamos as técnicas de SSH (“Suppression Subtraction Hybridization”) e de “Macroarray” com o objetivo de identificar genes que sejam preferencialmente expressos na fase leveduriforme do isolado Pb18. Genes identificados em ambos os procedimentos estão mais expressos na fase leveduriforme e estão envolvidos em metabolismo básico, transdução de sinal, crescimento e morfogênese e metabolismo do enxofre. Para testar se as mudanças observadas na expressão gênica refletem as diferenças entre as condições de crescimento usadas para obter as duas formas morfológicas preferivelmente às diferenças intrínsecas dos tipos celulares, nós realizamos experimentos com RT-PCR em tempo real utilizando preparações de RNA derivadas de ambas as fases, micélio e levedura, crescidas a 26°C e 37°C nos meios de cultura completos (YPD e Sabouraud) e meio mínimo. Vinte genes, incluindo *AGS1* (α -1,3-glucan synthase) e *TSA1* (thiol-specific antioxidant), foram mostrados como mais expressos na levedura patogênica em relação ao micélio. Embora a expressão de RNA mensageiro foi bastante diferente em relação aos meios completos e meio mínimo, mostramos uma tendência geral para que esses genes serem mais expressos nas células leveduriformes patogênicas de *P.*

brasiliensis. Além disso, mostramos a complementação dos genes *METR* e *SCONC* de *P. brasiliensis* e uma cepa com estes genes deletados de *Aspergillus nidulans*, sugerindo uma possível homologia entre eles. Mostramos também a análise de genes da via do metabolismo do enxofre foram mais expressos na levedura patogênica de *P. brasiliensis* em relação ao micélio saprofítico.

ABSTRACT.

Paracoccidioides brasiliensis, a thermodimorphic fungus, is the causative agent of paracoccidioidomycosis (PCM), a prevalent systemic mycosis in Latin America. Pathogenicity appears to be intimately related to the dimorphic transition from the hyphal to the yeast form, which is induced by a shift from environmental temperature to the temperature of the mammalian host. Little information is available on the *P. brasiliensis* genes necessary during the pathogenic phase. We have therefore undertaken Suppression Subtraction Hybridization (SSH) and macroarray analyses with the aim of identifying genes that are preferentially expressed in the yeast phase. Genes identified by both procedures as being more highly expressed in the yeast phase are involved in basic metabolism, signal transduction, growth and morphogenesis, and sulfur metabolism. In order to test whether the observed changes in gene expression reflect the differences between the growth conditions used to obtain the two morphological forms rather than differences intrinsic to the cell types, we performed real-time RT-PCR experiments using RNA derived from both yeast cells and mycelia that had been cultured at 37 and 26°C in either complete medium (YPD or Sabouraud) or minimal medium. Twenty genes, including *AGS1* (1,3-glucan synthase) and *TSA1* (thiol-specific antioxidant), were shown to be more highly expressed in the yeast cells than in the hyphae. Although their levels of expression could be different in rich and minimal media, there was a general tendency for these genes to be more highly expressed in the yeast cells. Moreover, complementation of *P. brasiliensis* *METR* and *SCONC* genes in strains of *Aspergillus nidulans* with these genes deleted suggested a possible homology between them. We show the analyses of genes involved in the

sulphur metabolism pathway and these genes were more expressed in the pathogenic yeast than saprophytic mycelia of *P. brasiliensis*.

I-Introdução

1.1. O FUNGO DIMÓRFICO *Paracoccidioides brasiliensis*.

O fungo *Paracoccidioides brasiliensis* é o agente etiológico da paracoccidioidomicose (PCM), a qual é uma doença granulomatosa. É um fungo termodimórfico que se apresenta sob a forma miceliana à temperatura ambiente (23 a 28°C) e de levedura à temperatura de 35 a 37°C (Lacaz, 1994 a,b). A fase leveduriforme caracteriza-se pela presença de células esféricas ou ovais, com paredes bem definidas, refringentes, aparentemente duplas, que podem apresentar brotamento multipolar característico (forma de roda de leme) (San-Blas & Niño Vega, 2001). A fase miceliana apresenta hifas delgadas, longas, dependendo das condições de cultivo. Benoliel et al (2005) relataram a presença de genes ortólogos aos das enzimas hidrolíticas identificados no transcriptoma de *P. brasiliensis* e atividade de enzimas hidrolíticas no sobrenadante de culturas de micélio e levedura de *P. brasiliensis*. Os ensaios enzimáticos mostraram atividade de celulase e xilanase mais altas no micélio que em levedura e a atividade das enzimas amilase e quitinase foram detectados somente na fase miceliana, sugerindo que essa fase é realmente saprófita. Splendore (citado por Del Negro et al 1982) foi um dos primeiros cientistas a estudar alguns casos de PCM com caracterização das culturas dos fungos isolados no intervalo dos anos 1909 a 1912. Atualmente, apenas a forma imperfeita do fungo (anamórfica) é conhecida, o que dificulta a sua classificação taxonômica e inferências de ordem filogenéticas. Um estágio teleomórfico (sexual) não foi ainda determinado, causando um grande atraso no conhecimento da biologia do fungo através da análise genética clássica. Entretanto, estudos moleculares baseados no seqüenciamento e comparação dos genes das

subunidades menor (18S) e maior (28S) do RNAr (RNA ribossômico) permitiram conhecer as relações filogenéticas de fungos cujas formas perfeitas (teleomórficas) não são conhecidas, (incluindo *P. brasiliensis*), demonstrando que *P. brasiliensis* poderia ser enquadrado no Filo ou Divisão *Ascomycota* (San-Blas 2002). Bialek et al (2000) sugeriram através de análise das seqüências das unidades ribossomais, que o fungo está próximo geneticamente do fungo *Blastomyces dermatitidis*, *Emmonsia parva* e *Histoplasma capsulatum*.

Feitosa (2000) investigou mais de 12 isolados de *P. brasiliensis* vindos de diferentes regiões e hospedeiros, estimando através de “Pulsed field gel electrophoresis” (PFGE) o genoma haplóide de *P. brasiliensis* entre 23,3 e 36,8 Mpb (mega pares de bases) e em isolados potencialmente diplóides entre 38,3 e 76,7 Mpb, com possibilidade de ter um número haplóide de 4 ou 5 cromossomos. Feitosa et al (2003) complementando este estudo, observaram diferenças genéticas relacionadas às diferentes isolados estudados, com possibilidades de estado haplóide, diplóide ou aneuplóide em relação ao *P. brasiliensis* sendo ainda hoje não muito clara a ploidia do fungo.

1.2. A EPIDEMIOLOGIA DE *Paracoccidioides brasiliensis*

Dados epidemiológicos indicam uma larga distribuição geográfica do fungo *P. brasiliensis* nas Américas Central e do Sul, estendendo-se do México até a Argentina, e ocorrendo principalmente na Colômbia, Venezuela e Brasil (para uma revisão, ver San-Blas e Niño-Veja, 2001), porém o seu habitat ainda é uma questão a ser elucidada (Restrepo et al 2001). Além do homem, o tatu (*Dasypus novemcinctus*), parece ser o hospedeiro ou o reservatório natural do fungo (Bagagli

et al 1988), mas também há relatos de infecções em animais domésticos, como cães (Ricci et al 2004) e a descrição do isolamento de *P. brasiliensis* de fezes de pingüim (Garcia et al 1993).

É estimado que 10 milhões de pessoas estejam infectados pelo *P. brasiliensis*. Acredita-se que o ser humano é geralmente infectado pela inalação de microconídeos propagados no ar a partir da fase miceliana, os quais se localizam no epitélio alveolar onde sofrem modificação para a fase leveduriforme patogênica (Restrepo et al 2001). A doença paracoccidioidomicose afeta principalmente homens, na proporção de 13 homens para 1 mulher (Aristizabal et al 1998), já que estrogênios, tais como -estradiol, parecem bloquear a transição morfológica (Aristizabal et al 1998).

1.3. A DOENÇA PARACOCCIDIOIDOMICOSE (PCM).

A doença apresenta-se em duas formas: aguda (tipo juvenil) e crônica (tipo adulto). A forma aguda (tipo juvenil) é responsável por 3 a 4% dos casos, com disseminação hematogênica ou linfática, comprometimento sistêmico e hipertrofia do baço, fígado e linfonodos. A forma crônica (tipo adulto) representa a maioria dos casos da doença e é encontrada predominantemente em homens na faixa etária dos 30 aos 50 anos (para uma revisão, ver Brummer et al 1993), daí a sua importância já que atinge o homem na sua fase mais produtiva.

O desenvolvimento da PCM depende da interação entre o *P. brasiliensis* e componentes do hospedeiro (imunocomprometimento, alimentação, maior exposição ao fungo) (Hanna et al 2000). Há casos em que o hospedeiro consegue desenvolver um quadro benigno da doença, com infecção localizada, resposta

celular preservada, baixos níveis de anticorpos, granulomas compactos e fungos pouco numerosos ou ausentes. Porém, existem casos de micose sistêmica em que o hospedeiro fica extremamente imunodeprimido, havendo uma disseminação e formação de granulomas frouxos e numerosas células fúngicas, comprometimento sistêmico, e hipertrofia do baço, fígado e linfonodos (Feitosa, 2000). Em ambas as formas, os doentes graves apresentam depressão da resposta imune celular específica e inespecífica.

Calcagno et al (1998) mostraram em seu trabalho a análise de 33 isolados de *P. brasiliensis* originados de 5 países da América Latina, utilizando a técnica de RAPD ("Randomly Amplified Polymorphic DNA") onde mostraram que foi possível separar os isolados de acordo com sua origem geográfica, porém não sugeriram a possibilidade de relacionar essa separação em relação a patogenicidade, virulência ou forma da doença. Curiosamente, trabalhando com 15 isolados de *P. brasiliensis*, Molinari-Madlum et al (1999), utilizando também RAPD, conseguiram mostrar a separação de dois grupos com somente 17% de identidade genômica de acordo com as lesões observadas (número e tamanho dos granulomas, disseminação do fungo nos diferentes órgãos) em camundongos, sugerindo a possibilidade da utilização da técnica para determinar padrões de virulência.

Motta et al (2002) realizaram um trabalho com 35 isolados de *P. brasiliensis* através da técnica de RAPD, na tentativa de definir padrões para as formas das doenças e manifestações clínicas. Neste trabalho utilizaram isolados de forma aguda, crônica, virulência atenuada, avirulentas entre outras e não foi possível estabelecer uma relação quanto ao grau de virulência e o tipo de manifestação clínica relativa à infecção clínica e o padrão genético.

Recentemente, Moraes et al (2000) seqüenciaram o gene da gp43 de dezessete isolados de *P. brasiliensis* e mostraram regiões altamente conservadas do gene, notadamente o epitopo P10, que possuem grande potencial para o desenvolvimento de uma vacina universal (Iwai et al 2003).

Nascimento et al (2004) demonstraram através de análises de microsatélites em seqüências de DNA genômico de *P. brasiliensis* que não foi possível relacionar as formas da doença (aguda e crônica) com o background genético de 23 isolados (4 ambientais, 18 clínicos e o isolado Pb18) de *P. brasiliensis*.

1.3.A. O tratamento da PCM

O tratamento da paracoccidioidomicose é motivo de inúmeras controvérsias, não havendo até hoje um estudo clínico controlado, com metodologia adequada, comparando as múltiplas opções terapêuticas já disponíveis. A maioria dos grupos opta por um tratamento inicial (denominado tratamento de ataque) com o objetivo de obter a remissão imediata dos sinais clínicos. Após esta fase, realiza-se um tratamento de consolidação (denominado tratamento de manutenção) visando diminuir as chances de ocorrer recidiva (Camargo, 2002).

1.3.B. Derivados Sulfamídicos.

A PCM era considerada uma doença incurável até o ano de 1940, quando foi utilizado pela primeira vez um derivado sulfamídico (sulfamidopirimidina). Desde de então, outros sulfamídicos foram empregados com sucesso. A sulfadiazina, uma sulfa de excreção rápida, foi largamente utilizada, e ainda hoje é o tratamento de escolha de alguns grupos. A combinação mais utilizada é sulfametaxazol-

trimetropin, por apresentar alta eficácia, baixa toxicidade e baixo custo (Camargo, 2002). O tratamento consta de um período de ataque (960mg/12hs) por 6 meses; após este período, as doses são reduzidas pela metade e mantidas com acompanhamento por um período adicional de até 3 anos de tratamento. Este longo período dificulta a eficácia do tratamento.

1.3.C. Anfotericina B

Anfotericina B é a segunda droga mais utilizada para o tratamento da PCM, ligando-se ao ergosterol, que é a parte constituinte da membrana do fungo, levando à deformação estrutural da membrana plasmática e a consequente morte do fungo. A anfotericina B tem eficácia comprovada, porém constatou-se que é tóxica aos rins, sendo utilizada somente em casos graves da doença ou quando é verificada resistência clínica às outras drogas utilizadas (Camargo, 2002).

1.3.D. Derivados azólicos

Os azóis vêm sendo bastante utilizados como drogas antifúngicas (para uma revisão, ver Georgopapadakou & Walsh, 1996). Eles interferem com a síntese do ergosterol. O fungo *P. brasiliensis* é extremamente sensível, *in vitro*, aos azóis (San-Blas & Niño Vega, 2001), com valores de Concentração Mínima Inibitória (MIC) e Concentração Mínima Fungicida (MFC) para a fase leveduriforme patogênica do *P. brasiliensis* que alcançam entre 10^{-7} e 10^{-10} M (equivalente à $6,7 \cdot 10^{-2}$ — $6,7 \cdot 10^{-5}$ g/ml) para diferentes superconazoles (San-Blas e Calcagno, 1993). O primeiro derivado azólico a ser empregado no tratamento da PCM foi o cetoconazol, com doses de 200 a 400 mg/dia, com eficácia de 90% em estudos com acompanhamento de até um ano. Porém, o cetoconazol apresenta alguns

efeitos colaterais, tais como, problemas gastrintestinais e alterações hepáticas. Uma droga mais recente no tratamento da PCM é o itraconazol, que tem mostrado menores índices de recidiva e poucos efeitos colaterais, além de ser apontado como terapêutica de escolha por vários autores. A droga fluconazol tem sido utilizada com sucesso, porém a experiência clínica com fluconazol é ainda restrita (Camargo, 2002).

1.3.E. Diagnóstico da PCM.

A principal forma de diagnóstico de ocorrência do *P. brasiliensis* é a observação microscópica no material clínico de leveduras com parede dupla e múltiplos brotamentos, com posterior isolamento em cultura. Atualmente, o imunodiagnóstico tem adquirido cada vez mais credibilidade na PCM, sendo valioso no acompanhamento da doença. A especificidade dos testes sorológicos depende do antígeno utilizado. Botteon et al (2002) realizaram em doadores de sangue o primeiro estudo epidemiológico utilizando o teste de Elisa. Os autores demonstraram que 21% do grupo de 700 pessoas da zona rural *versus* 0,9% do grupo de 350 pessoas da zona urbana foram positivos para exo-antígenos e gp43 (discutida e apresentada abaixo), confirmando que as pessoas da zona rural estão mais expostas ao *P. brasiliensis*. O principal componente antigênico descrito em *P. brasiliensis* é a gp43, uma glicoproteína exocelular que contém uma única cadeia de oligossacarídeos (Puccia et al 1986; Cisalpino et al 1996). Foi demonstrada uma forte resposta humoral que pode ser detectada no soro de pacientes com PCM (Mendes Gianini et al 2000). A gp43 é um potencial fator de virulência associado ao *P. brasiliensis* o qual se liga a laminina de murinos, resultando no aumento da patogenicidade das células leveduriformes (Vicentini et al 1994). Embora a

seqüência de proteína codificada pela gp43 seja similar àquelas das exo-1,3- -D-glucanases de *Saccharomyces cerevisiae* e *Candida albicans*, a sua atividade hidrolítica está ausente. A gp43 é reconhecida no soro da maioria dos pacientes com PCM, cujos anticorpos são dirigidos contra epitopos peptídicos conformacionais. Pacientes com PCM severa têm altos títulos de anticorpos contra gp43, os quais tendem a decrescer com o tratamento bem sucedido (Travassos et al 1995).

1.4. A VIRULÊNCIA E A PATOGENICIDADE DE *Paracoccidioides brasiliensis*.

As vias de sinalização que controlam as mudanças morfológicas em *P. brasiliensis* ainda não estão bem esclarecidas, mas o envolvimento de vias de transdução de sinais do AMPc (mono-fosfato de adenosina cíclico) e MAPK (“mitogen- activated protein kinase”) têm sido relatadas em outros fungos dimórficos (Lengeler et al 2000). Tem sido descrito que o AMPc exógeno inibe a transição de levedura para micélio em *P. brasiliensis*, mantendo a patogenicidade da forma leveduriforme (Paris e Duran, 1985; Borges-Walmsley e Walmsley, 2000). Contudo, recentemente foi observado em nosso laboratório que o AMPc inibe a transição de micélio para levedura (Walmsley et al manuscrito em preparação). Em *Candida albicans* a transição de levedura para micélio é controlada pelo AMPc e o AMPc exógeno estimula pseudohifas que são potencialmente capazes de invadir células de mamíferos (Rocha et al 2001).

Aparentemente, a patogenicidade está ligada à transição dimórfica, pois isolados de *P. brasiliensis* (e também de *H. capsulatum* e *B. dermatitidis*) que são incapazes de se transformar em leveduras não são virulentos (Medoff et al 1987; San-Blas e Niño Vega, 2001; Borges-Walmsley et al 2002 e Rooney e Klein, 2002). A levedura de *P. brasiliensis* também pode ligar-se a componentes da matriz extracelular, tais como fibronectina e colágeno (Mendes-Gianini et al 2000). A adesão às células do hospedeiro e à superfície da mucosa é um passo fundamental no estabelecimento da infecção. Esta correlação entre aderência e virulência tem sido sugerida a partir da quantificação da adesão fúngica em culturas de células epiteliais de mamíferos, onde isolados virulentos têm maior capacidade de adesão (Mendes-Gianini et al 2000).

Carvalho et al (2004) trabalharam com isolados de *Paracoccidioides brasiliensis* com diferentes características de virulência. Curiosamente o isolado mais virulento (Pb12) foi a que produziu menores quantidades de anticorpos anti-gp43, os autores sugeriram que a expressão da gp43 é regulada transcricionalmente, mas também que a expressão do gene pode ser regulada pela proteína e/ou por níveis de secreção.

A gp43 nativa gera uma resposta imune protetora em camundongos contra a virulência do *P. brasiliensis* (Pinto et al 2000). Investigações feitas sobre a resposta imune e proteção pelo plasmídeo expressando o gene da GP43 sob o controle do promotor de um gene da CMV (citomegalovírus), mostram que a vacinação de DNA provoca uma resposta imune específica com alta proteção contra a fase leveduriforme infectiva de *P. brasiliensis* (Pinto et al 2000). San-Blas et al (2005) foram capazes de desenhar primers originados de uma sequência

consenso de isolados de *P. brasiliensis* que detectam um fragmento específico em torno de 300 pares de bases com uma alta sensibilidade para amostras clínicas, mostrando que o diagnóstico molecular pode ser uma opção valiosa para um diagnóstico preciso.

Uma protease extracelular serina-tiol foi caracterizada em *P. brasiliensis*, sendo um provável fator de virulência que pode hidrolizar componentes da membrana basal, como fibronectina, laminina, colágeno IV e proteoglicanas (Carmona et al 1995; Puccia et al 1998 e Puccia et al 1999). Esta atividade enzimática, em colaboração com a gp43, poderia aumentar a capacidade de disseminação de *P. brasiliensis*. Isto possibilitaria a exposição de sialoglicoconjugados à superfície celular de *P. brasiliensis*, que podem também modular a interação entre o fungo e o tecido do hospedeiro (Soares et al, 1998).

Quando ocorre a mudança para a fase leveduriforme, um aumento no conteúdo de quitina é observado na parede celular, seguido da mudança da estrutura anomérica das glucanas de α -1,3 para β -1,3-glucana (San-Blas e Niño-Veja, 2001). A superfície de β -1,3-glucana pode se refletir em uma função protetora da camada da levedura contra mecanismos de defesa do hospedeiro uma vez que fagócitos são incapazes de digerir β -1,3-glucana (San-Blas, 1982). Rappleye et al (2004) demonstraram através da técnica de interferência de RNA (RNAi) que o gene *AGS1* (β -1,3-glucan synthase) silenciado em *H. capsulatum* promoveu a diminuição da virulência do fungo, pois verificou-se a atenuação da habilidade de eliminar macrófagos e colonizar pulmões de murinos.

1.5. O DIMORFISMO EM FUNGOS PATOGÊNICOS.

Em diferentes fungos dimórficos, estudos recentes têm demonstrado que a mudança morfológica está associada com a patogenicidade (Braun e Johnson 1997; Leberer et al 1997; Lo et al 1997; Stoldt et al 1997; Yaar et al 1997; Csank et al 1998; Borges-Walmsley et al 2002; Romani et al 2003). Diante dessa premissa, a identificação de genes preferencialmente expressos na fase leveduriforme pode evidenciar informações sobre a patogenicidade de *P. brasiliensis*.

A variação da temperatura seria o único fator responsável pela transição dimórfica em *P. brasiliensis*, *H. capsulatum* e *Blastomyces dermatitidis* (Gilardi & Lafer, 1962; San-Blas & Centeno, 1977; San-Blas & San-Blas, 1982; San-Blas & San-Blas, 1984). Paris et al (1985) verificaram, em *P. brasiliensis* que a variação da temperatura é essencial para a conversão da forma miceliana para a leveduriforme, porém, fatores nutricionais também estão envolvidos. Estes autores mostraram que a forma miceliana cresce em meio mínimo utilizando enxofre inorgânico, enquanto a forma leveduriforme cresce somente quando enxofre orgânico é adicionado ao meio. Rippon (1968) verificaram que o início da conversão de micélio para levedura em *H. capsulatum* requer mudança na temperatura e também redução do potencial óxido-redução do meio de cultura. Entretanto, a manutenção da fase leveduriforme requer compostos orgânicos contendo enxofre, especialmente cisteína (Scherr, 1957; McVeigh & Houston, 1972). A possibilidade de um requerimento nutricional para grupos -SH foi sugerida (Garrison et al 1970), mas não explorada. Boguslawski et al (1976) verificaram que as células leveduriformes de *H. capsulatum* não possuem a enzima sulfito redutase, de modo que estas células não

podem reduzir sulfito inorgânico para sulfeto, e deste modo, não podem sintetizar cisteína. Em contraste, as células micelianas que contêm, com esta redutase ativa, podem crescer sem suplemento com cisteína. Estes autores verificaram, ainda, que a ausência da atividade da sulfito redutase na forma leveduriforme não pode ser explicada pela instabilidade térmica desta enzima nas condições de crescimento da levedura (37°C), pois a enzima extraída do micélio funciona melhor a 37°C que a 23°C (Boguslawski et al 1976). Foi verificado, em *H. capsulatum*, que o ácido *p*-cloromercufenilsulfônico (PCMS), uma droga inibidora de grupos sulfidrilas, bloqueia a transição de micélio para levedura a 37°C (Maresca et al 1977) e bloqueia a presença, somente na fase de levedura, da enzima cistina redutase (Maresca et al 1978). Posteriormente, Maresca et al (1981), distinguiram três estágios na transição de micélio para levedura em *H. capsulatum* e sugeriram que o metabolismo da cisteína possui função fundamental no processo de diferenciação celular deste fungo. No primeiro estágio, ocorre progressivo decréscimo na frequência respiratória e na concentração intracelular de cisteína e outros aminoácidos; no estágio 2, a respiração cessa por completo e a concentração de cisteína livre cai para baixos níveis e no estágio 3, ocorre a ativação da respiração mitocondrial e conclusão da transição morfológica. Cisteína exógena é necessária durante o segundo estágio para que ocorra ativação da respiração mitocondrial e conclusão da transição morfológica (Maresca et al 1981). Medoff et al (1987b) verificaram que a transição de micélio para levedura dos fungos dimórficos *B. dermatitidis* e *P. brasiliensis* é muito semelhante àquela observada no *H. capsulatum* e concluíram que estes três patógenos dimórficos possuem mecanismos paralelos na indução da transição dimórfica.

1.6. GENES DIFERENCIALMENTE EXPRESSOS EM *Paracoccidioides brasiliensis*.

Existem poucos relatos na literatura acerca do isolamento de determinantes genéticos especificamente expressos na fase leveduriforme patogênica de *P. brasiliensis* (Cunha et al 1999 e da Silva et al 1999). Na procura por proteínas que são diferencialmente expressas na fase leveduriforme patogênica do *P. brasiliensis*, da Fonseca et al (2001) identificaram e caracterizaram novos antígenos homólogos à catalase, frutose-1,6-bifosfato desidrogenase, malate desidrogenase e triosefosfato isomerase, que são preferencialmente sintetizados por células leveduriformes. Silva et al (1999) evidenciaram a expressão de um membro da família das proteínas de choque térmico, a Hsp70 foi caracterizada e a sua expressão diferencial foi demonstrada na fase de levedura. Uma forte indução de Hsp70 em células leveduriformes do *P. brasiliensis* Pb01 pode estar relacionada à interação parasita-hospedeiro durante o processo infeccioso e a resposta imune no hospedeiro mamífero.

Venancio et al (2002) usaram a técnica de “differential display” para isolar e identificar genes diferencialmente expressos de *P. brasiliensis*, nas duas fases celulares, assim como em diferentes intervalos de tempo durante a transição induzida pela temperatura de micélio para levedura (M-Y). Três fragmentos de cDNA foram específicos para a forma miceliana enquanto dois fragmentos de cDNA foram super-expressos durante a transição de micélio para levedura.

Cunha et al (1999) utilizando géis bidimensionais, caracterizaram duas proteínas diferencialmente expressas em *P. brasiliensis*: uma chamada de PbM46 é

expressa nas duas fases, porém com alta concentração na fase miceliana do *P. brasiliensis* e a outra chamada de PbY20, exclusiva e altamente expressa na fase leveduriforme de *P. brasiliensis*. A PbM46 é similar à enolases de outras espécies, porém com alta similaridade com a proteína expressa pela levedura *S. cerevisiae* (80%), enquanto a PbY20 possui 34 resíduos de aminoácidos com 90% de identidade com uma pequena proteína alergênica de *Alternaria alternata* e 82% idêntica a uma proteína alergênica de *Cladosporium herbarium* (Cunha et al 1999). A PbY20 foi imunologicamente detectada somente em extratos de células de levedura, sugerindo um importante papel desta proteína na diferenciação ou na manutenção da forma leveduriforme patogênica. Além disso, a proteína PbY20 foi recentemente reconhecida como um membro da família da flavina mononucleotídeos fladodoxina-like (WrbA) a qual está envolvida em sistemas biológicos de estresse por choque de temperatura e oxidativo (Daher et al 2005).

No ano de 2003, mostramos um levantamento de genes expressos na fase leveduriforme patogênica de *P. brasiliensis* utilizando o isolado Pb18 (Goldman et al 2003). Até então haviam sido depositadas no banco do NCBI (www.ncbi.nlm.nih.gov) pouquíssimas seqüências de *P. brasiliensis*, aumentando assim consideravelmente as informações de transcritos preferencialmente expressos. Foram obtidas 13490 ESTs (“Expressed Sequences Tags”) de ambas as extremidades (5 e 3’), que foram agrupadas e funcionalmente classificadas somando 4692 genes expressos (1397 “contigs” que são seqüências repetidas pelo menos 2 vezes e 3295 “singlets” que são seqüências únicas). Foram identificados “*in silico*” vários genes homólogos de *P. brasiliensis* envolvidos em virulência e patogenicidade em *Candida albicans*. Foi verificada a expressão aumentada de

genes que codificam proteínas de “heat shock” (*HSP 70-82* e *-104*), de poliubiquitina (*UBI1*) e uma desaturase (*OLE1*) durante a transição de micélio para levedura. Estes resultados sugerem que a estabilização de proteínas e mudanças na organização da membrana são fatores importantes para a transição morfológica em altas temperaturas.

Felipe et al (2003, 2005) mostraram em seu trabalho uma análise *in silico* de 6022 ESTs de micélio e levedura do isolado Pb01 de *P. brasiliensis* e uma análise de “macroarray” de 1152 clones selecionados. Nunes et al (manuscrito aceito para publicação) mostraram a análise estatística das hibridações feitas do RNA total extraído de células em transição micélio-levedura com o biochip de 4692 ESTs de *P. brasiliensis*. Este trabalho mostrou 2583 genes que apresentam modulação da expressão em pelo menos um ponto amostral durante a transição de micélio para a levedura. Estes trabalhos acrescentam importantes informações para genes diferencialmente expressos e ressaltam a necessidade de estudos posteriores para vias específicas e comprovação experimental dos dados mostrados.

1.7- O METABOLISMO DO ENXOFRE.

O enxofre participa de vias metabólicas que são requeridas para uma variedade de funções metabólicas críticas: biosíntese de aminoácidos, produção de poliaminas, todas as reações de transferência do grupo metil e biosíntese de nucleotídeos. Somando-se a isto, várias enzimas envolvidas no metabolismo do enxofre usam vitaminas como cofatores como as B6, B12, riboflavinas e ácido fólico, conectando o metabolismo do enxofre ao das vitaminas (Christopher et al 2002).

A assimilação e o metabolismo do enxofre estão sujeitos a um sofisticado controle metabólico e o controle da expressão de genes estruturais envolve fatores regulatórios atuando tanto positiva quanto negativamente (Marzluf, 1997; Thomas and Surdin-Kerjan, 1997; Paszewski et al 2000). As proteínas regulatórias que atuam positivamente no metabolismo de enxofre foram identificadas em *Neurospora crassa* e leveduras (Marzluf, 1997). O fator de transcrição CYS3 de *N. crassa* é o principal regulador positivo para o metabolismo do enxofre (Marzluf & Metzenberg, 1968). O CYS3 é um membro da família bZIP de proteínas que se ligam ao DNA e funcionam como um homodímero (Kanaan et al 1992). A proteína reconhece a seqüência palindrômica 5'-ATGRYRYCAT-3' presente nos promotores de genes de assimilação de enxofre (Natorff et al 2003). Natorff et al (2003) identificaram e caracterizaram um gene que regula positivamente o metabolismo do enxofre (*METR*) em *A. nidulans*. Mutações no gene *METR* afetam a expressão de vários genes incluindo *SCONB* (gene regulatório negativo), *SB* (sulfato permease), *CYSD* (homocisteína sintase) e *CYSB* (cisteína sintase) (Paszewski et al 2000; Natorff et al., 2003). O gene *METR* não é essencial, pois mutantes com deleção neste gene são viáveis e têm o mesmo fenótipo de isolados com mutações pontuais. A proteína MetR é similar às proteínas cys3 de *N. crassa* e Met4 de *S. cerevisiae*, bem como à proteína CAA94632 de *Schizosaccharomyces pombe*, de função desconhecida (Natorff et al 2003).

Na presença de fontes orgânicas de enxofre, como a metionina, o sistema de repressão do metabolismo do enxofre reduz a expressão de genes que codificam algumas enzimas que participam da via de assimilação do enxofre, como sulfohidrolases e homocisteína sintase (Paszewski & Grabski, 1974; 1975). Quatro

genes que atuam regulando negativamente o metabolismo do enxofre foram identificados em *A. nidulans* (*sconA*, *B*, *C* e *D*) (Natorff et al 1993). Mutações nos genes *scon* resultam na perda da repressão de metabólitos do enxofre, levando à síntese constitutiva de várias enzimas do metabolismo do enxofre, incluindo sulfato permease. Como consequência, mutantes *scon*, ao contrário do tipo selvagem, são sensíveis ao selenato, um análogo tóxico do sulfato, na presença de metionina (Arst, 1968; Natorff et al 1998). Fenotipicamente, estes mutantes assemelham-se às mutações *scon-1* e *scon-2* de *Neurospora* e MET30 em *S cerevisiae* (Burton & Metzenberg, 1972; Natorff et al 1993; Thomas et al 1995) e apresentam um domínio F-box altamente conservado, também encontrado em muitas outras proteínas (Bai et al 1996; Patton et al 1998b). Em *S. cerevisiae*, as proteínas F-box ligam-se à Skp1, Cullin ou Cdc53 em um complexo conhecido como SCF. Esse complexo é uma “E3-like” ubiquitina ligase o qual interage com ambas as enzimas, E2 e Cdc34, e com seus substratos. O complexo SCF regula vários processos celulares, incluindo associação dos cinetocoros (Connelly & Hieter, 1996), progressão do ciclo celular (Bai et al 1996; Feldman et al 1997) e sensoriamento da glicose (Li & Johnson, 1997). A especificidade do complexo SCF por uma proteína é determinada pela proteína F-box enquanto a Skp1 é responsável por tornar o substrato alvo para a ubiquitinação (Bai et al 1996; Lyapina et al 1998; Patton et al 1998a; 1998b). A interação entre as proteínas Met30 e Skp1 demonstrada por Patton et al (1998a), fortemente sugere que o complexo SCF está envolvido na regulação do metabolismo do enxofre na levedura (Piotrowska et al 2000). A proteína SconC contém seqüências PEST que são comuns em proteínas que estão sujeitas a uma rápida reciclagem. As seqüências PEST são ricas em prolina (P),

ácido glutâmico (E), serina (S) e treonina (T) que são alvos para rápida destruição (Rechsteiner e Rogers, 1996).

II-Objetivos

O principal objetivo deste trabalho foi mostrar através das técnicas de “macroarray” e SSH (“Suppression Subtractive Hybridization”) genes preferencialmente expressos na levedura patogênica de *P. brasiliensis*. Para alcançar estes objetivos iniciais estabelecemos:

(i) Utilizar o banco de dados de ESTs de *P. brasiliensis Pb18* para realizar um “screening” diferencial de 1397 “contigs” utilizando a tecnologia dos “Macroarrays”, visando assim evidenciar genes mais expressos na fase leveduriforme patogênica de *P. brasiliensis*;

(ii) Isolar cDNAs que correspondem a genes preferencialmente expressos na fase leveduriforme patogênica de *P. brasiliensis Pb18* utilizando a técnica de SSH;

(iii) Analisar a expressão de genes selecionados a partir destas duas técnicas por RT-PCR em tempo real, a fim de confirmar ou não a expressão aumentada na fase específica leveduriforme patogênica de *P. brasiliensis*.

Um outro objetivo de nosso trabalho foi a investigação do metabolismo do enxofre, já que a levedura patogênica apresenta auxotrofia para aminoácidos contendo enxofre orgânico. Assim, estabelecemos as seguintes prioridades:

(i) o isolamento e caracterização dos genes *METR* e *SCONC* de *P. brasiliensis*;

(ii) a verificação da funcionalidade de *METR* e *SCONC* através de ensaios de complementação do mutante *metR* de *A. nidulans*;

(iii) a análise da expressão dos genes envolvidos na assimilação de enxofre durante a transição micélio-levedura.

III-Materiais e Métodos

3.1. Soluções e meios de cultura gerais.

GET:

23 ml de glicose 20%.

10 ml de EDTA 0,5 M, pH 8.0.

13 ml de Tris HCl 1M, pH 7.4.

Água qsp (quantidade suficiente para) 500ml.

Acetato de potássio 3M.

147,2 g de KOAc

7,5ml de Ácido Acético Glacial.

Água qsp 500 ml.

Estoque de RNase A (Amershan Biosciences) a 10mg/ml.

Preparar na concentração adequada, ferver por 15 minutos, resfriar a temperatura ambiente e estocar em freezer.

Tampão TAE 50X concentrado pH8.0.

Tris-base	242 gr.
Ácido acético glacial	57,1 ml.
EDTA 0,5M	100 ml.
Água qsp	1000ml

Tampão MOPS 10X {3-(N-morpholino) propanesulfonic acid.} pH final entre 6.6 e 7.8.

MOPS	0,2 M
Acetato de Sódio	0,08M
EDTA	0,01M

Solução de Denhardts 50x concentrado.

Ficol	5 gr.
Polivinilpirrolidona	5 gr.
soro albumina bovina	5 gr.
Água qsp	500ml.

SSC 20x concentrado pH 7.0 ajustado com NaOH.

Cloreto de Sódio	175,3gr.
Citrato de Sódio	88,2gr.
água qsp	1000ml.

Meio de cultura 2xYT.

Bacto Triptona	16 gr.
Bacto Extrato de Levedura	10 gr.
Cloreto de Sódio	5 gr.
Água destilada (qsp)	1000 ml.

Brometo de Etídio (Solução estoque)

Brometo de Etídio	1% em água destilada.
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TAE 50x pH 8.0

Tris Base	242 g
CH ₃ COOH (Ácido Acético Glacial)	57,1 mL
EDTA	0,5M pH 8,0100 mL
Água destilada q.s.p.	1 L

Dissolver em água, acertar para o pH 8.0 e acertar o volume final

Tampão de amostra para eletroforese de DNA.

Azul de Bromofenol (Sigma)	0,25%
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Xileno Cianol FF (Sigma)	0,25%
Ficoll Tipo 400 (Sigma)	30%

Dissolver em água destilada e acertar o volume final.

Tampão de amostra para eletroforese de RNA.

Azul de Bromofenol (Sigma)	0,1 mg/mL
Glicerol (Sigma)	50%
Tampão MOPS	1x

Dissolver em água destilada e acertar o volume final.

Tampão de extração de DNA

Tris-HCl pH 8,5	200 mM
NaCl	250 mM
EDTA	25 mM
SDS	0,5%

Dissolver em água destilada.

3.2. Meio de cultura para *P. brasiliensis*:

Meio Mínimo (Restrepo & Jiménez, 1980):

Glicose	10,0g
KH ₂ PO ₄	1,5g
MgSO ₄ .7H ₂ O	0,5g
CaCl ₂ .2H ₂ O	0,15g
(NH ₄) ₂ SO ₄	2,0g
L-Asparagina	2,0g

L-Cistina	0,2g
Suplemento vitamínico	10,0ml
Elementos traços	1,0ml
Água Destilada q.s.p.	1L

Ajustar o pH para 7.0 com NaOH 1N e autoclavar.

Observações: Dissolver a cistina em NaOH 1 N, antes da adição ao meio. Todos os componentes, com exceção do suplemento vitamínico, devem ser misturados e o pH ajustado para 7.0 com NaOH. Esterilizar a solução de vitaminas por filtração e adicioná-la ao meio de cultura, após autoclavação. Quando foi utilizado apenas o Meio Mínimo (sem adição de cistina, cisteína ou metionina), este meio foi preparado sem cistina.

Solução de vitaminas (Restrepo & Jiménez, 1980):

Cloreto de tiamina	6,0mg
Niacina	6,0mg
Pantotenato de cálcio	6,0mg
Inositol	1,0mg
Biotina	0,1mg
Riboflavina	1,0mg
Ácido fólico	10,0mg
Cloreto de colina	10,0mg
Cloreto de piridoxina	10,0mg
Água destilada q.s.p.	100ml

Esta solução deve ser esterilizada por filtração e adicionada ao meio de cultura, após autoclavação.

Solução de elementos traços (Restrepo & Jiménez, 1980):

H ₃ BO ₃	5,7mg
CuSO ₄ .5H ₂ O	15,7mg
Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O	140,4mg
MnSO ₄ .14H ₂ O	8,1mg
(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	3,6mg
ZnSO ₄ .7H ₂ O	79,2mg
Água destilada q.s.p.	100ml.

3.3. Meios de cultura e soluções para ensaios com *Aspergillus nidulans*.

Meio de cultura mínimo

Solução de Sais	1X
Elementos traços	0,1%
Glicose	1%
Ágar (Difco)	2%

Ajustar para o pH 6,5, completar o volume com água destilada e autoclavar.

Meio completo (YAG, YUU, YG e YG+UU)

Extrato de levedura	0,5%
Glicose	2%
Ágar	2%
Elementos Traços	0,1%

Completar o volume com água destilada e autoclavar.

*Obs: 1- **YUU**: meio de cultura completo YAG suplementado com uridina 4mM e uracila 10mM; 2- **Meio de cultura + KCl (“Meio de regeneração”)**: meio completo mais 0,6M de KCl; 3- Os meios **YG** e **YG+UU** são iguais, aos meios YAG e YUU sem Ágar 2%, respectivamente; e 4- Top Agar: 1% de ágar.

Solução de elementos traços 1000x

ZnSO ₄ .7H ₂ O	75 mM
H ₃ BO ₃	180 mM
MnCl ₂ .4H ₂ O	25 mM
FeSO ₄ .7H ₂ O	18 mM
CoCl ₂ .5H ₂ O	6 mM
Cu(SO ₄).5H ₂ O	6 mM
(NH ₄) ₆ MO ₇ O ₂₄ .4.H ₂ O	1 mM
EDTA	140 mM

Adicionar os sólidos na ordem listada em 1/8 do volume final de água destilada. Dissolver cada um completamente antes de adicionar o próximo. Aquecer a solução até 100°C. Resfriar para 60°C e ajustar o pH entre 6,5 e 6,8 com NaOH. Resfriar até atingir a temperatura ambiente e completar para o volume final.

Solução de protoplastização

Solução 1 de Protoplastização	20 mL
Solução 2 de Protoplastização	20 mL
MgSO ₄ .7H ₂ O 1M	6,5 mL

BSA	400 mg
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Glucanex	300 mg
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Dissolver a BSA e a Glucanex nas soluções 1 e 2 de protoplastização e $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Filtrar com filtro 0,045µm da Millipore.

Solução 1 de protoplastização

$(\text{NH}_4)_2\text{SO}_4$	0,8M
------------------------------	------

$\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ (Ácido Cítrico) pH6,0	0,1M
---	------

Dissolver em água destilada, completar o volume e autoclavar.

Solução 2 de protoplastização

Extrato de Levedura	1%
---------------------	----

Sacarose	2%
----------	----

Dissolver em água destilada, completar o volume e autoclavar.

Solução 3 de protoplastização

$(\text{NH}_4)_2\text{SO}_4$	0,4 M
------------------------------	-------

Sacarose	1%
----------	----

$\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ (Ácido Cítrico) pH 6,0	50 mM
--	-------

Dissolver em água destilada, completar o volume e autoclavar.

Solução 4 de protoplastização

PEG6000	25%
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$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	100 mM
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KCl	0,6 M
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Tris HCl pH7,5	10 mM
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Dissolver em água destilada, completar o volume e autoclavar.

Solução 5 de protoplastização

KCl	0,6 M
-----	-------

CaCl ₂	50 mM
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MES pH 6	10 mM
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Dissolver em água destilada, completar o volume e autoclavar.

Solução de sais 20x

NaNO ₃	3,2 M
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KCl	0,14 M
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KH ₂ PO ₄	0,2 M
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MgSO ₄ .7H ₂ O	0,04 M
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Dissolver em água destilada e completar o volume.

3.4. Protocolos utilizados.

3.4.A. Extração de DNA plasmidial. (miniprep).

Foi realizada extração plasmidial utilizando o seguinte protocolo:

1) Separados os clones de interesse, descongelar se for o caso, e inocular em 1,5 ml de meio de cultura 2xYT (ver item meio de cultura de Materiais e Métodos) para cada poço da placa de cultura, mais a presença do antibiótico ampicilina (0,1mg/ml de meio) para a seleção do plasmídeo. A inoculação pode ser feita com um replicador de placas, com palitos ou com ponteiros (tudo esterilizado).

2) Selar a placa com um adesivo e perfurar com uma agulha cada poço para que haja aeração, do contrário o crescimento bacteriano não será satisfatório.

3) Colocar a placa em um agitador a 37°C a 300 RPM por 22 a 24hs. (anotar qualquer anormalidade no crescimento).

4) Centrifugar a 4000 RPM por 6 min para precipitar as células.

5) Remover o adesivo e descartar o sobrenadante adequadamente, deixar a placa secando virada para baixo em papel absorvente por uns 10 minutos.

(OBS: SE, NESTE PONTO, FOR NECESSÁRIO INTERROMPER A EXTRAÇÃO, PODE-SE GUARDAR AS PLACAS NO FREEZER ATÉ POR ALGUMAS SEMANAS)

Para as próximas etapas ligue um forno de aquecimento a 90°C para que ele estabilize nesta temperatura.

6) Adicionar 240 μ l de GET em cada poço, selar a placa e agitá-la em “vortex” até as células estarem totalmente suspensas.

7) Centrifugar por 6 min a 4000 RPM para precipitar as células, remover o adesivo e descartar o sobrenadante, deixar as placas secando viradas para baixo em um papel absorvente por 5 min.

8) Acrescentar em cada poço 80 μ l de GET+5 μ l de RNase A. Preparar um “mix” GET/RNase).

9) Ressuspender as células em “vortex”, centrifugar rapidamente até 3000 RPM e ressuspendê-las novamente em “vortex”.

10) Transferir 60 μ l das células ressuspensas para uma placa receptora (TOWER TEAR , essa placa tem que suportar os 90°C do forno). Adicionar 60 μ l de

NaOH/SDS em cada poço (para 220 amostras usar 1300 l de SDS 10% + 650 l de NaOH 4M+ 13 ml de água destilada esterilizada). Selar a placa com um adesivo e misturar por inversão 10 vezes moderadamente.

11) Deixar na bancada por 10 minutos. Centrifugar por alguns segundos até 3000 RPM.

12) Adicionar 60 l de Acetato de Potássio 3M (armazenado em geladeira 4°C) a cada poço da placa receptora e misturar por inversão 10 vezes.

13) Deixar na bancada por 10 minutos. Centrifugar por alguns segundos.

14) Remover o adesivo e colocar a placa em uma estufa ou forno a 90°C por EXATAMENTE 30 minutos.

15) Resfriar a placa em gelo por 10 minutos e centrifugar por 5 minutos a 4000 RPM a 20°C.

Enquanto a placa estiver centrifugando, ou até antes, montar o sistema de Placa filtro MILLIPORE (MAGV N22) fixada com fita adesiva em cima de uma placa de 96 poços fundo em “U”, que receberá suas amostras filtradas.

16) Transferir todo o sobrenadante com uma pipeta multicanal para a placa filtro (tentar não mexer no precipitado), centrifugar por 4 minutos a 4000 RPM a 20°C.

17) Remover e descartar a placa filtro. Adicionar, em cada poço da placa que recebeu o filtrado, 110 l de isopropanol absoluto, selar a placa com um novo adesivo e misturar por inversão 20 vezes rapidamente.

18) Centrifugar por 45 minutos a 4000 RPM a 20°C.

19) Descartar o sobrenadante e adicionar em cada poço 200 μ l de etanol 70% gelado (4°C), selar e centrifugar por 5 minutos a 4000 RPM a 20°C e descartar o sobrenadante novamente.

20) Se preferir, centrifugar a placa invertida em papel absorvente por 2 minutos, a 800 RPM, isso ajuda a secar mais rapidamente.

21) Deixar a placa secando por 1 hora à temperatura ambiente.

22) Ressuspender o DNA por uma noite (“overnight”) em 40 μ l/poço de água milliQ autoclavada.

Checar em gel de agarose e usar a quantidade de plasmídeo necessária para a sua reação de seqüenciamento.

Após a extração plasmidial, cada amostra é checada em gel de agarose 2% em tampão TAE 1X e coradas com brometo de etídeo (10 μ g/ μ l). Se for observada degradação do DNA extraído, repete-se a extração.

3.4.B. Amplificação do clone representativo do “contig”.

Para realizar o “screening” diferencial, um representante da região 5’ de cada um dos 1397 “contigs”, foram reorganizados em 15 placas de 96 poços fundo em “U”. Após a escolha do clone representativo, os mesmos foram inoculados em meio de cultura comercial “Circle Grow” para uma posterior PCR de colônias utilizando o seguinte protocolo:

A biblioteca de cDNA está em placas em fundo em “U” de 96 poços. A biblioteca foi descongelada e replicada em outra placa contendo 150 μ l de meio de cultura Circle Grow mais o antibiótico ampicilina (0,1mg/ml). As placas foram embaladas em filme plástico e incubadas a 37°C por 22 horas.

Após a incubação, foi feita a verificação de homogeneidade do crescimento bacteriano e possível contaminação da cultura. Foi adicionado, dentro de um fluxo laminar, 75 l de Glicerol 70% esterilizado em cada poço da placa, seguido de homogenização.

Preparamos, então uma diluição 10X de cada amostra da placa de cultura em água deionizada, destilada e esterilizada. Utilizamos 1 l dessa diluição para reação de PCR.

Mistura para PCR de culturas.

PCR Buffer 10X	1,5 l.
dNTPs 1,25mM de cada	1,5 l.
MgCl ₂ 50mM	0,45 l.
“Primer” “anti-sense” 5pmoles/ l (“primer” poli dTV Gibco BRL)	0,63 l
“Primer” “sense” 5pmoles/ l M13R Gibco BRL)	0,63 l
Taq DNA polimerase 5U/ l	0,2 l.
Água milliQ esterilizada	9,19 l
Cultura diluída 10X	1,0 l
Volume final	15 l/poço.

Condições de ciclo.

Pré-desnaturação de 95°C/4minutos, seguida de 35 ciclos de 95°C/45segundos; 55°C/45 segundos; 72°C por 1minuto. Finalmente foi feita uma incubação adicional de 5 minutos a 72°C.

Após a reação, o produto de PCR de cada poço das placas foi submetido a eletroforese em gel de agarose 2% em tampão TAE 1X.

Para realizarmos a hibridização para o “screening” diferencial com sondas da 1ª fita de cDNAs do micélio e levedura precisamos, primeiramente, obter RNA total íntegro, isto é, não degradado e para isso utilizamos os seguintes protocolos:

3.4.C. Extração de RNA total de *P. brasiliensis*.

(Observações: Para qualquer extração de RNA deve-se lavar toda a bancada com SDS (dodecil sulfato de sódio, Sigma) 10% ou detergente comum, deixar de molho as partes plásticas das pipetas com solução de HCl 0,1N por 30 minutos e enxaguar muito bem. Enxaguar com etanol absoluto a parte metálica da pipeta e também a bancada e, se possível, usar ponteiros com barreiras para todo o procedimento de extração de RNA total, RNA mensageiro ou tratamento com DNase.)

3.4.C1 Extração de RNA total da levedura:

1. Colocar em relação às leveduras do *P. brasiliensis* uma proporção de 3:1 (v/v) de Trizol e 1:1 (v/v) de “Glass beads” (425 a 600 micron Sigma) (tratadas com água contendo DEPC (dietil pirocarbonato Sigma) 0,1% em um tubo tipo “Falcon” novo e esterilizado de 50 ml e agitar vigorosamente em “vortex” por 10 minutos).
2. Aliquotar 1ml em tubos tipo “eppendorf”.
3. Adicionar para cada 1ml aliquotado 200 μ l de Clorofórmio e homogeneizar cuidadosamente por 15 segundos manualmente.
4. Centrifugar a 12.000 RPM por 15 minutos.
5. Retirar o sobrenadante e transferir para outro tubo.
6. Colocar isopropanol absoluto na proporção de 1:1.
7. Colocar a 80°C negativos por 2 horas, porém, se for deixado “overnight” o rendimento é melhor.
8. Centrifugar a 12.000 RPM por 10 minutos.
9. Desprezar o sobrenadante e colocar 1ml de Etanol 70%.

10. Centrifugar a 12.000 RPM por 10 minutos.
11. Desprezar o sobrenadante e deixar secar bem em temperatura ambiente ou, se preferir, use a secagem à vácuo.
12. Ressuspender o “pellet” formado em um volume de 50 a 200 l de água tratada com DEPC. Observar a formação de um “gel”, é o nosso RNA total.

3.4.C2. Extração de RNA total do micélio:

1. Macerar totalmente o micélio em nitrogênio líquido utilizando um almofariz com pistilo (queimados a 180°C/8horas). Não deixar faltar nitrogênio na maceração.
2. Colocar rapidamente 200mg do micélio macerado mais o volume de 1 ml de Trizol em um tubo tipo “Eppendorf” de 2ml.
3. Homogeneizar bem e colocar em um Banho Maria a 25°C por 5 minutos.
4. Adicionar em cada tubo 200 l de clorofórmio.
5. Agitar vigorosamente em “vortex” durante 15 segundos
6. Incubar a 25°C por 3 minutos.
7. Centrifugar a 14.000 RPM por 15 minutos.
8. Transferir a fase superior aquosa para um novo tubo e adicionar isopropanol na proporção de 1:1 (v/v).
9. Homogeneizar e colocar a 80°C negativos por 2 horas, ou se preferir “overnight”.
10. Centrifugar a 14.000 RPM por 15 minutos.
11. Desprezar o sobrenadante e colocar 1ml de Etanol 70%.
12. Centrifugar a 14.000 RPM por 5 minutos.

13. Desprezar o sobrenadante e deixar secar bem em temperatura ambiente, ou se preferir, use a secagem à vácuo.

14. Ressuspender o “pellet” formado em um volume de 50 a 200 l de água tratada com DEPC, observar a formação de um “gel”. Este gel é o RNA total.

Para a verificação da integridade do RNA foi utilizado o seguinte procedimento:

Pesar 1,8 gramas de agarose e adicionar 110ml de água destilada.

Fundir a agarose e esfriar a 45°C.

Adicionar 15 ml de tampão MOPS 10x.

Adicionar 25 ml de formaldeído.

Homogeneizar bem e distribuir no suporte para o gel de agarose com o pente adequado (deixar um pouco no frasco e colocar a 50°C, para cobrir os pocinhos posteriormente).

Preparação de cada amostra:

RNA total (10 g)	x,x l.
Formaldeído	3,3 l.
Formamida	10,0 l.
MOPS 10x	2,0 l.
Água qsp	20,0 l
Bromo Fenol Blue	1,0 l

Depois de preparadas as amostras, é necessário desnaturar a 65°C por 15 minutos, colocar no gelo por 5 minutos e aplicar no gel.

O tampão de corrida é o MOPS 1 vez concentrado, porém não é necessário cobrir o gel. Após a aplicação das amostras, realiza-se a eletroforese a 100 volts por 5 minutos. Após este tempo, cobre-se os poços com o restante da agarose e continua-se a corrida a 100 volts até as amostras terem percorrido 7/8 do suporte do gel.

Após a corrida, corar o gel com brometo de etídio (10 g/ l) por 10 minutos e posteriormente deixamos descorar em água destilada “overnight”.

Observar em luz UV. O gel de RNA total tem a característica de possuir 2 bandas bem evidentes e um rastro, sendo que a primeira banda é 2 vezes mais intensa que a segunda banda que correspondem às subunidades (maior e menor) do RNA ribossômico.

3.4.C3. A hibridização do “macroarray”.

A nomenclatura das 15 placas de “contigs” foi definida como descrito a seguir:

Placa 1A de “contigs”, concatenação final; placa 2B de “contigs” concatenação final; placa 3C “contigs” concatenação final e assim sucessivamente até a placa 15O de “contigs” concatenação final. Cada placa foi desnaturada e transferida para duas membranas de náilon em quadruplicata preservando a posição original da placa com os dois controles de DNA genômico depositado superiormente e -tubulina depositado no lado direito da placa para posterior hibridização, seguindo o protocolo abaixo.

3.4.C3i) Desnaturação do cDNA.

1. Deixar descongelar completamente as placas que contêm o DNA plasmidial de interesse. Transferir as placas para a temperatura de 65°C por 5 minutos.
2. Transferir NaOH para uma concentração final de 0,2N para cada poço contendo 100ng/ l de DNA por concentração final. Evitar bolhas nos poços. Colocar as placas a 37°C por 15 minutos.
3. Transferir 1 l da amostra de cada poço para a membrana de náilon (Hibond N+, Amershan) usando o multi-replicador (a placa foi transferida em quadruplicata). Repetir a transferência de mais 1 l/poço. Para passar para outra placa de DNA, lave o multi-replicador da seguinte forma:
 4. Água milliQ autoclavada e esterilizada e etanol absoluto colocados em cubas separadas em seqüência de modo que realize a seguinte bateria de lavagem:
 5. Água/20 segundos por 3 vezes; etanol absoluto/ 10segundos; etanol absoluto novamente e deixar o multi-replicador secar a temperatura ambiente por 1 minuto.
 6. Fixar o DNA na membrana de náilon a 80°C/2 horas ou com luz ultravioleta no aparelho UV Stratalinker 1800 (Stratagene), na função “Auto Cross Link” que tem se mostrado mais eficiente. Estocar as membranas entre papel de filtro à temperatura ambiente.

3.4.C3ii) Hibridização com sonda de cDNA.

Pré-hibridização.

Pré-hibridizar as membranas em cilindros de 30x3,5 cm com 20 ml de solução por 4 horas a 42°C a primeira vez (2 horas as outras vezes).

Solução de pré-hibridização: 5xSSC, 10x solução de Denhardts, 20 mM de Tris-HCl pH 7.5, 1% de SDS, 50% de Formamida e 100 g/ml de DNA de esperma de salmão desnaturado e fragmentado.

3.4.C3iii) Síntese do cDNA (Schummer et al 1999, com modificações.).

Misturar em um tubo tipo “Eppendorf” 6 l de RNA total (30 g) e 1,5 l de Oligo dTV (100 M de estoque). Aquecer 75°C/10 minutos. Centrifugar rapidamente e transferir para o gelo. Adicionar:

5,0 l de First Strand Buffer (5x, Gibco BRL).

2,5 l de DTT solution (100mM).

2,0 l de RNAsin (Promega).

2,5 l de d(ATG)TPs (10mM cada).

5,0 l de ³²P-dCTP (50 Ci.).

a) Aquecer a 42°C por 5 minutos e adicionar 1,25 l de Superscript II (200U/ l). Misturar gentilmente e centrifugar rapidamente. Incubar a 42°C por 20 minutos, centrifugar e adicionar 125 l de dCTP (10mM). Incubar a 42°C por 60 minutos.

b) Centrifugar rapidamente e adicionar 1 l de Poly-A (Amershan 2 g/ l) e denaturar a 94°C por 5 minutos.

- c) Centrifugar e adicionar 1,4 l de NaOH (5N). Incubar a 37°C por 15 minutos.
- d) Centrifugar e adicionar 1,4 l de HCl (4N) e 7 l de Tris-HCl 1M pH 7.5.
- e) Adicionar 19 l do tampão STE (NaCl, Tris-EDTA), misturar bem e purificar a reação em coluna de Sephadex G50 e medir a radioatividade da sonda purificada em um cintilador. A contagem costuma ser de 300.000 a 400.000 CPM, com uma incorporação de 50-75%.

3.4.C3iv) Hibridização.

- a) Preparar 7,5 ml/membrana (Hibond N+, 10 x 15cm) de solução de hibridização. Manter a 65°C em tubos "Falcon". Solução de Hibridização: 5xSSC; 2x solução de Denhardt's, 20mM Tris-HCl pH 7,5; 1% de SDS; 50% de Formamida, 5% de Dextran Sulfato e 100 g/ml de DNA de esperma de salmão desnaturado.
- b) Desnaturar as sondas por 3 minutos a 94°C e transferir para o gelo. Centrifugar rapidamente.
- c) Transferir as sondas para tubos Falcon contendo a solução de hibridização e misturar.
- d) Desprezar a solução de pré-hibridização e adicionar a solução de hibridização com a sonda nas garrafas de hibridização.
- e) Hibridizar por 18 horas a 42°C. NÃO ULTRAPASSAR ESTE TEMPO.

3.4.C3v) Lavagem e exposição das membranas.

Descartar a solução de hibridização contendo a sonda.

Lavar a membrana com 200 ml das soluções pré-aquecidas a seguir:

- I. 2xSSC, 0,1%SDS 15 minutos a 65°C.
- II. 2xSSC, 0,1%SDS 15 minutos a 65°C.

- III. 1xSSC, 0,1%SDS 15 minutos a 65°C.
- IV. 0,1xSSC, 0,1%SDS 15 minutos a 65°C.
- V. 0,1xSSC, 0,1%SDS 15 minutos a 65°C.

Nunca deixar a membrana secar, envolver as membranas com filme plástico.

Expor as membranas em filme de raio X para diagnóstico (T-MAT G/RA 35 por 43cm, Kodak) por 96 horas a 80°C negativos.

3.4.C3vi) Protocolo para retirar a marcação das membranas (“Probe stripping”).

- Colocar SDS 0,1% fervente nas membranas por 5 minutos agitando gentilmente. Repetir mais 2 vezes.
- Envolver as membranas com filme plástico e expor por 96 horas. Se não for detectado nenhum sinal, guardar as membranas a –20°C para reutilizá-las, caso contrário repetir o item anterior de 1 a 3 vezes de acordo com o sinal detectado.

3.4.C4 Captação das imagens.

Foram feitos dois experimentos de hibridização. A primeira hibridização foi feita em 3 etapas, onde foram colocadas as membranas de 1 a 4 de micélio juntas em uma garrafa de hibridização e de 1 a 4 de levedura em outra, depois foram colocadas as membranas de 5 a 10 de micélio e de 5 a 10 de levedura e por último, as membranas de 11 a 15 em suas respectivas garrafas. Depois da hibridização as membranas foram lavadas seguindo o protocolo 8E e expostas em filme de raio X por quatro dias. Após revelação, as membranas foram enviadas o profº Dr. Marcelo

Menossi (Laboratório Genoma Funcional Centro de Biologia Molecular e Eng. Genética Universidade Estadual de Campinas), para expor novamente as membranas e realizar a primeira análise, utilizando o software “Array Vision”. Após a análise foi retirada a marcação das membranas (utilizando o protocolo descrito no item 3.8.F) e reenviadas ao nosso laboratório.

Uma segunda hibridização foi realizada com as primeiras membranas, porém foi feita com as membranas trocadas, isto é, a membrana que foi hibridizada primeiramente com a sonda da levedura, após a lavagem, foi hibridizada com a sonda do micélio. As membranas de 1 a 7 de micélio e de leveduras foram concomitantemente hibridizadas em suas respectivas garrafas de hibridização; Em seguida as membranas de 8 a 15. Após a hibridização as membranas foram lavadas, expostas, como relatado anteriormente, e enviadas novamente para a última análise.

3.4.D Análise experimental e estatística das hibridizações com “macroarrays”

Para a análise do “Macroarray” foi usado o programa “Array Vision”, que identifica mudanças estatisticamente significantes na expressão de genes, por assimilação de um grupo de genes específicos pelo teste “t” de “Student”, onde para cada gene é assinalada uma contagem de base da mudança da expressão gênica relativo ao desvio padrão de repetidas medidas para cada gene (para uma revisão da estatística usada em “microarrays”, ver Nadon e Shoemaker, 2002). Genes com contagem maior que o limiar são avaliados como potencialmente significantes.

Visando a normalização dos valores do experimento, quatro conjuntos diferentes de dados foram gerados: dados brutos, dados normalizados pela mediana, normalizados pela concentração do controle do DNA genômico e normalizados pela concentração do controle do gene da α -tubulina de *P. brasiliensis*. Foi também aplicada uma rigorosa filtragem dos “contigs” que apresentavam repetições discrepantes. Assim, excluímos “contigs” que apresentavam variações de mais de duas vezes nas repetições; foram também excluídos “contigs” em que alguma repetição apresentava mais de duas repetições com sinais abaixo de 10 % do respectivo “background”. Desta forma, a análise estatística foi feita com os oito valores para expressão em levedura e oito valores para expressão em micélio (quatro repetições por membrana *versus* duas membranas). Foi aplicado então um teste “t Student” nesses valores, comparada a diferença entre as médias dos dois grupos com variância presente entre os valores de cada grupo, para assim identificar genes cuja expressão fosse significativamente diferenciada. Foi tomado como valor limite para o teste “t” um valor maior que 6, o que corresponde a uma margem de erro de 1 %.

3.4.E. Utilização da técnica de SSH (“Suppression Subtractive Hybridization”).

Esta é uma poderosa técnica que permite comparar duas “populações” de RNA mensageiro, obtendo genes raramente expressos somente em uma das populações. Visando o estabelecimento desta técnica, utilizamos o kit “PCR-Select cDNA Subtraction Kit” (Clontech).

Primeiramente, ambas as populações de RNAm (0,5 a 2 g) são convertidas para cDNA. A população de nosso interesse, isto é, a que pretendemos subtrair os

transcritos diferencialmente expressos, chamamos de “tester” (no nosso caso a levedura patogênica do *P. brasiliensis*) e a outra população de referência chamamos de “driver” (no nosso caso o micélio do *P. brasiliensis*). O “tester” e o “driver” são digeridos com a enzima *Rsa I* originando moléculas com extremidades abruptas. O cDNA “tester” é dividido em duas alíquotas, onde cada alíquota é ligada a um adaptador diferente. Após a ligação dos adaptadores realizamos duas hibridizações (ver figura 1A e 1B):

1. Na primeira hibridização, exemplificada na figura 1A, a cada uma das duas alíquotas do “tester” (cada uma ligada a um adaptador diferente) é adicionado o cDNA “driver” em excesso originando assim duas misturas que são desnaturadas e no final da hibridização temos 4 possibilidades de híbridos diferentes para cada alíquota de “tester”: somente o “tester” não hibridizado; “tester” hibridizado com ele mesmo; “tester” hibridizado com o “driver” e; “driver” hibridizado com ele mesmo ou somente o “driver”.

2. A segunda hibridização, exemplificada na figura 1B, é feita juntando as duas alíquotas de “tester” que foram hibridizadas anteriormente separadas, e adiciona-se mais “driver”, porém não desnaturamos as amostras, desta forma somente as fitas únicas de cDNA formarão novos híbridos. Estes novos híbridos são fitas duplas de cDNA “tester” com diferentes finais que correspondem aos diferentes adaptadores.

Após as duas hibridizações toda a mistura é submetida a uma reação de PCR para amplificar as seqüências diferencialmente expressas utilizando a técnica de supressão por PCR (ver figura 1C) pois somente serão amplificados fragmentos que possuírem o par correto de adaptadores (possibilidade E, ver figura 1C), o

restante, as seqüências sem adaptadores, adaptadores únicos, e principalmente o par incorreto de adaptadores será suprimido porque os adaptadores se anelarão e formarão a estrutura em forma de panela exemplificada na figura 1C. Os adaptadores foram desenhados de tal forma que eles possuem um pedaço de 22 nucleotídeos complementares. A supressão ocorre quando seqüências complementares estão presentes em cada final da fita simples de cDNA, durante cada passo do anelamento dos “primers” a cinética de hibridização é favorecida para a formação de uma estrutura secundária em forma de “panela” (“pan-like structure”) que evita o anelamento dos “primers” e conseqüentemente a posterior extensão da fita de cDNA. Desta forma, durante a reação de PCR, amplificações inespecíficas são eficientemente suprimidas e amplificações específicas (moléculas diferencialmente expressas) de moléculas de cDNA com adaptadores diferentes em ambos os finais ocorrem normalmente.

Figura 1-A.

Suppression Subtraction Hybridization (SSH)

Figura 1-B.

Figura 1-C.

Figura 1: Esta figura exemplifica as possibilidades de formação de híbridos após a primeira hibridização originando os casos A, B, C e D (figura 1A). Após a segunda hibridização, que origina os híbridos “E” que são os híbridos amplificados exponencialmente (figura 1B e C). Estes últimos são os exclusivamente expressos na população de cDNA “tester” e o “background” restante não será amplificado ou será suprimido pela formação da estrutura em forma de panela (figura 1C).

3.4.F) Complementação do mutante *metR* de *Aspergillus nidulans* pelo gene *METR* de *P. brasiliensis*.

Para realizar a complementação do *MetR* de *P. brasiliensis* em *Aspergillus nidulans* foram realizados os seguintes passos:

Cruzamento da cepa GR5 (PyrG89;PiroA1;Wa3) com a cepa RM119 (metR;ArgB2;AnA1;biA1; cedida gentilmente por Renata Natorff). Deste cruzamento foi originada uma cepa denominada GRM1, com o genótipo (metR; PyrG89;AnA1;biA1).

Foi feita uma construção utilizando a ORF do MetR clonada direcionalmente com Taq DNA polimerase de alta fidelidade (Platinum Taq DNA Polymerase High Fidelity – Invitrogen) pelos “primers” MetR_Fw BamH1 (5’CGG GAT CCG atg cca ttc ctt cgc cct at3’) e MetR_Rev_PstI (5’AAC TGC AGt tac gct ttt ctc ctc tcc aa3’) no plasmídeo pAL3 com o promotor alcA. O plasmídeo pAL3, que coloca o gene a ser clonado (no nosso caso o MetR) sob o controle do promotor da álcool desidrogenase I (ADH1), e o gene pyr4 que complementa a mutação pyrG89 em *A. nidulans* (Waring et al 1989). O gene alcA é reprimido quando *A. nidulans* cresce na presença de glicose, de outro lado é fortemente induzido quando cresce na presença de etanol ou outros álcoois. A treonina, por sua vez é um forte indutor e não é substrato para a ADH1 (Waring et al 1989). Este sistema possibilita clonar genes sob controle do promotor alcA, sendo uma estratégia importante para o controle da expressão e verificação de complementação.

O gene amplificado e a fusão com o promotor alcA foram verificados por sequenciamento e a construção pAL3+MetR foi transformada na cepa GRM1. A complementação foi verificada através do fenótipo de crescimento sem a necessidade da adição de metionina ao meio de cultura, utilizando treonina como indutor do promotor alcA.

3.4.G) Complementação do mutante SCONC de *Aspergillus nidulans* pelo gene SCONC de *P. brasiliensis*.

Para realizar a complementação do *SCONC* de *P. brasiliensis* em *Aspergillus nidulans* foram realizados os seguintes passos:

Foi realizado o cruzamento da cepa GR5 (*pyrG89*; *pyroA4*; *Wa3*) com a cepa R34 (*sconC3*; *ArgB₂*; *pyroA₄*; *yA₂*; cedida gentilmente por Renata Natorff). Deste cruzamento foi originada uma cepa denominada R34pyrG, com o genótipo (*sconC3 pyrG89*).

Para clonar o gene *SCONC* no plasmídio pAL3 (com promotor *alcA*), foram desenhados primers com adaptadores para a endonuclease de restrição *BamHI* para amplificar a região aberta de leitura do gene *SCONC*. A PCR foi realizada com uma enzima de alta fidelidade (Platinum Taq DNA Polymerase High Fidelity – Invitrogen) e o fragmento foi submetido a uma reação de restrição enzimática com a *BamHI*. Os primers utilizados foram SconC Fw : 5'- CGG GAT CCG ATG TCG GCC CCC GAG AAT T-3' e SconC Rev: 5'- CGG GAT CCT TAA CGA TCC TCT GCC CAT T-3'

O plasmídeo pAL3 coloca o gene a ser clonado (*SCONC*) sob o controle do promotor da álcool desidrogenase I (ADH1) e possui o gene *pyr4*, que complementa a mutação *pyrG89* do *A. nidulans* (Waring et al 1989). O gene *alcA* é reprimido quando *A. nidulans* cresce na presença de glicose e é fortemente induzido quando cresce na presença de etanol ou outros álcoois. A treonina, por sua vez, é um forte indutor e não é substrato para a ADH1 (Waring et al 1989). Este sistema possibilita clonar genes sob o controle do promotor *alcA*, sendo uma estratégia importante para o controle da expressão e verificação da complementação.

A ligação foi realizada entre o gene *SCONC* amplificado e o vetor pAL3, previamente digerido com *BamHI* e defosforilado com BAP (Bacterial Alkaline Phosphatase-Invitrogen). A fusão foi verificada por sequenciamento e a construção que continha a orientação correta do inserto e nenhuma alteração na sequência de DNA, foi eletrotransformada em *E. coli* (linhagem DH10B), dando origem ao clone SconC::pAL3. Esta construção foi introduzida em na cepa de *A. nidulans* R34pyrG. Os transformantes foram selecionados pela habilidade de crescerem em meio YAG.

3.4.H) Utilização da técnica de RT-PCR em tempo Real.

Todas as reações e análise de expressão por “Real Time RT-PCR” foram feitas utilizando o aparelho ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). “Taq-manEZ RT-PCR kit” (Applied Biosystems) e/ou síntese de cDNA separadamente foram utilizados nas reações de RT-PCR em tempo real. As condições de termociclagem têm um passo inicial de 50°C por 2 minutos, seguidos da transcrição reversa a 60°C por 30 minutos, desnaturação a 95°C por 5 minutos e seguidos por 40 ciclos de 94°C por 15 segundos e 60°C por 1 minuto. As reações de PCR foram feitas com reagentes “Taq-ManPCR kits”. Os parâmetros das reações foram os seguintes: incubação inicial a 50°C por 2 minutos e posteriormente 95°C por 10 minutos, seguidos por 40 ciclos de 95°C por 15 segundos e 60°C por 1 minuto. Para a checagem da expressão foram desenhadas 30 sondas fluorescentes (ver tabela 1). Para obtermos um controle ideal da expressão gênica, nós primeiro normalizamos com Alfa e Beta-tubulinas. O resultado da expressão dos genes normalizadores foi semelhante, então foi escolhido o gene Alfa tubulina para todas as normalizações. (dados não mostrados). As reações e cálculos foram feitos de acordo com Semighini et al

(2002). As sondas fluorescentes foram sintetizadas pela Applied Biosystems (“Taq-Man”) ou pela Invitrogen (“Lux”), usando softwares adequados e foram baseadas nas seqüências dos genes escolhidos como candidatos a fase-específicos devidamente corrigidos em seus cromatogramas de sequenciamento originais do nosso “EST Genome Project”.

Tabela1) Lista de primers e sondas fluorescentes utilizadas neste trabalho

Primers e sondas	Sequencias	Identidade do "Cluster" de <i>P. brasiliensis</i>	Nome do Locus
*Alpha P-alpha	5'-CCAGAACCAGGCAGTCCAAA-3' 5'-FAM-CACCTGCCTAACAAGATTGGACCAGG5 G-3'	SSHCS001-E11 SSHCS001-E02	AGS1
H82f H82r PH82	5'-ACCGCTGGTGCCGATATTT-3' 5'-GGCCACGAGGTAGGCAGAG-3' 5'-TET-TGATTGGTCAGTTTGGTGTGCGCTTCT-TAMRA3'	PbCC001-B11	HSP82
APS1F APS1R P-APS1	5'-ACTAATCCCGGTAACTCCTTCACA-3' 5'-CGGAGCAGAGAGACCCAAAG-3' 5'-FAM-TCGCCTTCTTATACAGCCC-TAMRA-3'	RC2-Pb5NEW1-154G11	APS1
c101-F c101-R Pc101	5'-AAGCCTTCTCCCCCACCAT-3' 5'-GCCAAAACATAGCCACGA-3' 5'-FAM-TGGCTTGTTTCGAAAGA-TAMRA-3'	PbCC002-A05	C101
c126F c126R Pc126	5'-CGTTGGTAATCCTGGCTTGG-3' 5'-CAACTGCGGTCTCAGGTGG-3' 5'-FAM-ATTGCTTGCCGATATTGCCCTCGC-TAMRA-3'	PbCC002-C06	GSP1
C1363F c1363R Pc1363	5'-ATTTGAGGGAGGCGGAATCC-3' 5'-TTTGGGAACGTCCAGTCTGTT-3' 5'-FAM-CTGCCCTGTACTGACCTC-TAMRA-3'	Contig 1363 RST	MEP1
c1412F c1412R Pc1412	5'-TGTCTGCAGGCGATCGATTA-3' 5'-AGCTACCTGCATCCACTGG-3' 5'-FAM-TCGCAGCACCTCAGCAGCATTCC-TAMRA-3'	PbCC015-F08	TSA1
c162F c162R c162	5'-CTCGACAACGTCCAGGAGAAG-3' 5'-GGGATGACCCTGGCAGAAATG-3' 5'-FAM-AAGGACTTCAGAAATCCA-TAMRA-3'	PbCC002-F06	RHO1
C165F c165R Pc165	5'-TCCCCTATCATCCTGCAAGTGT-3' 5'-CCATTGGGAACGCCCTTC-3' 5'-FAM-CAAAGAAAGCAGCCCC-TAMRA-3'	PbCC002-F09	FBA1
C261F c261R Pc261	5'-CCTACGCGACATGCAGAAATATATC-3' 5'-CGCTGCGCCGAGTCTATC-3' 5'-FAM-CTCCGATTACAGTCATC-TAMRA-3'	PbCC003-F09	LUC7
c32F c32R Pc32	5'-GGCAAAGCCGAAGAAAAAGC-3' 5'-TGTACAGGTAGCCGCTGCC-3' 5'-FAM CGGCATCGCCATC-TAMRA-3'	PbCC001-C08	ILV5
C354F c354R Pc354	5'-CCTACAATATTTCTGGCTCGTTCCT-3' 5'-GGATTGCTCAACAACCTCCTCAT-3' 5'-FAM-ACTCCGGTAATTTTCG-TAMRA-3'	PbCC004-F06	PAL1
c5F c5R Pc5	5'-ACGACCACGGAACTCATACC-3' 5'-GGATCATGCTGAGGCTTTTCCA-3' 5'-FAM-CTGTGCGCATCAATG-TAMRA-3'	PbCC001-A04	ERG25
c71F c71R Pc71	5'-ACCGTTGCCAGCACCATT-3' 5'-AGCCCTCCAGACCATAGACTT-3' 5'-FAM-AGGCCTCTCATTCTC-TAMRA-3'	PbCC001-F11	CIT1
c77F c77R Pc77	5'-GGAGCCGGAAGTCGAATAGG-3' 5'-ACCTACCATTGGAATGTGTGCATA-3' 5'-FAM-CCAAGACTCCAATGGT- TAMRA-3'	PbCC001-G05	C77
C836F c836R Pc836	5'-AACGGTGTTCAGACGATGGT-3' 5'-AACCACCACGACGACCAG-3' 5'-FAM-ACCTCGGCAACATC-TAMRA-3'	PbCC009-F08	PAL2
CHS1F CHS1R P-CHS1	5'-GAATGGGCTTCCGAGATAAATGC-3' 5'-ACCGACACCAACACATCTCAAA-3' 5'-FAM-CCGACATCCAAAGCCT-TAMRA-3'	VP1-Pb50001-178A02	CHS1

Continuação Tabela 1

COT1F COT1R P- COT1	5'-CCTGCTTCATATCCTCATCATGGA-3' 5'- TGACGCCTATACATGACCCTACAA-3' 5'-FAM- CACGTCCAGAGGAATGT-TAMRA-3'	PbCC003-A05	CTR3
FLBA-F FLBA-R P- FLBA	5'-CGGCTAGCAAGACTGTTTCG-3' 5'- TGCTTTTCTTGCTCCTGGATCTC-3' 5'-FAM- CCTGCGAGCTGAACAT-TAMRA-3'	PbCC002-F06 162	FLB1
GCL1F GCL1R P- GCL1	5'-GCATCAGCATCAGCAGCAA-3' 5'- CTGCTTGCTGCTGTTGGTATTGG-3' 5'-FAM- CAGCACGTCAACCCCA-TAMRA-3'	RC2-Pb30001- 119B07	GCL1
Tub2F Tub2R Ptub2	5'-AGCCTTGCGTCGGAACATAG-3' 5'- ACCTCCATCCAGGAACCTTCA-3' 5'-FAM- AGCGTGTAGGTGATCAG-TAMRA-3'	PbCC014-D01	TUB2
CDI1F CDI1R PCDI1	5'-TGGCATGGTCGTGGATAGC-3' 5'- AGGAAACGGCAAAGCAATCTG-3' 5'-FAM- AGCGTGTAGGTGATCAG-3'	PbCC004-C05	CDI1
*Tub1 Ptub1	5'-ACCCAGCTTGAAACAGTGCT-3' 5'- CACGGACCATCAGCCTTCAAACC5G-3'	PbCC001-E05	TUB1
C159F C159R PC159	5'-GCCCCTGGAGGATAAGATAAAGAG-3' 5'- GAGCGAACGAATCGACTTTACTGTA-3' 5'-FAM- CTAACGAAGAGAGAATCCCT-TAMRA-3'	PbCC002-F03	SEP1
P-APS1F P-APS1R S-APS1	5'-ACTAATCCCGGTAAACTCCTTCACA-3' 5'-CGGAGCAGAGAGACCCAAAG-3' 5'-FAM-TCGCCTTCTTATACAGCCC-TAMRA-3'	RC2-Pb5NEW1- 154G11	APS1
P-c1363F P-c1363R S-c1363	5'-ATTTGAGGGAGGCGGAATCC-3' 5'-TTTGGGAACGTCCAGTCTGTT-3' 5'-FAM-CTGCCCTGTACTGACCTC-TAMRA-3'	Contig 1363 RST	MEP1
*P-MAS2161 *S-MAS2161	5' TTCTTGAGCCACCGATTCTCC3' 5'gacctcAGCGCACCGTTAAGGAGG5C3' FAM	MAS2161	METR
*S-SconC_Pb *P-SconC_Pb	5'caaacCAAGCTCGTGACACGAAGGT5TG3' FAM 5'ACGTTCTTGCTGCGGCTTC3'	MAS0244	SCONC

P-primers sem marcação;

S-Sondas fluorescentes marcadas como indicado (TET,6-carboxy-4,7,2',7'-tetrachlorofluorescein;6-FAM,6-carboxyfluorescein;TAMRA,6-carboxy-N,N,N',N'-tetramethylrhodamine);

*Sonda Lux (Invitrogen)

IV-Resultados

4.1. A IDENTIFICAÇÃO DE GENES PREFERENCIALMENTE EXPRESSOS NA FASE LEVEDURIFORME PATOGÊNICA DE *P. brasiliensis*.

Tendo como objetivo a identificação de genes preferencialmente expressos na fase leveduriforme patogênica de *P. brasiliensis*, nós utilizamos dois diferentes procedimentos experimentais: (i) a hibridização diferencial com “macroarrays” e (ii) o SSH.

4.1.1.- Utilização da técnica de “macroarray” na identificação de genes diferencialmente expressos.

Para a construção do “macroarray”, foram escolhidos 1397 “contigs” clusterizados das 8369 ESTs (5’ e 3’), previamente descritas por Goldman et al (2003), tendo sido escolhido um clone representativo para cada “contig”. O inserto de um clone de cDNA representativo de cada um desses “contigs” foi amplificado por PCR e aproximadamente 100 ng foram fixados numa membrana de nylon (4 repetições para cada inserto e 2 repetições para cada membrana) e hibridizados contra o cDNA sintetizado de RNA total, isolado e marcado radioativamente em igual intensidade, de culturas de micélio e de levedura. Após a hibridização e a quantificação dos sinais de hibridização, estes filtros foram completamente limpos da radioatividade e hibridizados contra as membranas invertidas, isto é, a membrana que foi hibridizada pela primeira vez com cDNA de levedura, na segunda vez foi hibridizada com o cDNA de micélio. Assim, obtivemos uma repetição de 8 marcações para cada inserto a ser posteriormente analisado. A

figura 2 mostra as imagens selecionadas destas hibridizações. Os sinais obtidos foram convertidos em intensidade relativa de valores, como descrito na seção 3.4C4 de Materiais e Métodos, quantificados e estatisticamente analisados. Neste trabalho mostramos somente os genes que foram considerados como mais expressos na levedura patogênica de *P. brasiliensis*. A figura 3 mostra o gráfico de dispersão dos 536 genes (do total de 1397 “contigs”) acima dos valores de “background” considerados significativos na análise estatística. Na Tabela 2 são apresentados 38 genes em que a expressão variou de 1,5 a 6,6 vezes na membrana de nylon hibridizada com o cDNA de levedura em comparação aos mesmos genes hibridizados com o cDNA de micélio de *P. brasiliensis*. A busca em banco de dados por possíveis homólogos revelou que 26 destes “contigs” tiveram uma alta significância em seus “e-values” (10^{-19}), 7 dos “contigs” tiveram uma moderada significância ($1e^{-10}$ até $1e^{-19}$) e cinco mostraram uma baixa significância ($1e^{-03}$ até $1e^{-10}$) em seus valores de “e values” nos bancos de dados pesquisados.

4.1.2. Utilização da técnica de SSH na identificação de genes diferencialmente expressos.

Utilizando a técnica de SSH com cDNA de micélio como “driver” e cDNA de levedura como “tester” (ver figura 1A e B) fomos capazes de isolar genes preferencialmente expressos na levedura patogênica de *P. brasiliensis*. Quando os cDNAs originais foram utilizados como sondas para um “Northern Blot” com RNA total de micélio e levedura de *P. brasiliensis*, foram observados sinais de hibridização comparáveis para ambos (ver figura 4A não subtraído). Quando utilizamos como sonda o cDNA subtraído, vimos um rastro forte e disperso na

marcação de RNA de levedura em relação ao micélio (ver figura 4A subtraído), sugerindo uma expressão diferencial e assim validando o procedimento do SSH. A técnica de SSH foi também validada utilizando o método de “real time” PCR com a quantificação da expressão do gene *HSP82*, que foi previamente mostrado como mais expresso na levedura patogênica de *P. brasiliensis* (Goldman et al 2003). O gene *HSP82* mostrou uma expressão de 150 vezes mais no cDNA subtraído em relação ao cDNA não subtraído original (ver figura 4B abaixo), também colaborando assim para a validação do procedimento do SSH.

Figura 2: A membrana 1 A de "contigs" hibridizada com cDNA da levedura do *P. brasiliensis* (esquerda) mostra na posição A05, o "contig" 5; na posição C8 o "contig" 32, na posição F11 o "contig" 71 e na posição G5 o "contig" 77; Membrana 1 A de "contigs" hibridizada com cDNA do micélio do *P. brasiliensis* (direita) mostra na posição A4 o "contig" 4, na posição E3 o "contig" 51 e na posição G3 o "contig" 75 (as setas indicam os "contigs" mais expressos nas suas respectivas membranas).

Figura 3: Gráfico de dispersão da expressão gênica entre levedura e micélio de *P. brasiliensis*. Os pontos representam a média das intensidades dos sinais normalizados (unidades arbitrárias) para 536 genes de *P. brasiliensis*.

Tabela 2 – Análise de “Macroarray” mostrando os “contigs” mais expressos na fase leveduriforme patogênica de *P. brasiliensis*.

Identidade do “cluster” de <i>P. brasiliensis</i>	Homólogo (número de acesso no NCBI)	BLASTevalue/ %identidade/ similaridade	Nº de vezes mais expressos
PbCC001-A04	ERG25 <i>S.cerevisiae</i> NP_011574.1	8e-53/60/78	6.6
PbCC001-C03	CIT1 citrate synthase <i>A. nidulans</i> AAC49728.3	8e-18/70/86	6.0
PbCC002-A05	<i>FBA1</i> aldolase <i>S. cerevisiae</i> NP_012863.1	e-136/65/76	4.7
PbCC005-A06	ILV5 ketol acid reductoisomerase <i>N. Crassa</i> AAB67753.1	2e-82/86/91	4.4
PbCC014-C11	<i>N. crassa</i> predicted protein NCU06390	6e-05/43/61	3.6
PbCC003-F04	<i>N. crassa</i> hypothetical protein NCU05891.1	6e-07/36/49	3.3
PbCC003-B12	<i>N. crassa</i> hypothetical protein NCU04080	2e-10/34/48	3.0
PbCC003-E12	<i>ENO1</i> , enolase <i>A. oryzae</i> Q12560	0.0/86/91	2.9
PbCC001-F06	<i>HIS4</i> , Histone H4 <i>A. nidulans</i> P23751	2e-35/100/100	2.8
PbCC001-D02	<i>EEF3</i> , Translation elongation factor eEF3 <i>C. albicans</i> S25363	e-103/74/89	2.7
PbCC004-A08	<i>RL20</i> , 60S ribosomal protein L20 <i>S. cerevisiae</i> NP_014957.1	8e-67/71/83	2.5
PbCC004-H02	<i>A. nidulans</i> EST c4c08a1.r1	4e-04/86/92	2.5
PbCC005-C08	<i>N. crassa</i> hypothetical protein AL513466	2e-18/30/44	2.4
PbCC001-A10	<i>FCF1</i> , zinc cluster transcription factor Fcr1p <i>C. albicans</i> AF057038	1e-15/64/76	2.3
PbCC001-C10	Unknown		2.2
PbCC004-A04	Probable glutaredoxin <i>N. crassa</i> AL353819	6e-17/52/67	2.1
PbCC004-A09	Unknown		2.0
PbCC004-A10	<i>GCV3</i> , glycine decarboxylase <i>A. thaliana</i> AC004667	8e-30/53/69	2.0
PbCC004-C10	Aminopeptidase B <i>A. niger</i> AJ418036	2e-52/69/80	2.0
PbCC003-G12	Inositol-1-phosphate synthase <i>P. pastoris</i> AF078915	1e-73/48/63	2.0
PbCC007-D02	<i>N. crassa</i> predicted protein NCU02545.1	6e-11/32/52	1.9
PbCC001-H03	Unknown		1.9
PbCC003-E01	Unknown		1.8
PbCC003-C05	<i>SnaK1</i> <i>S. mansoni</i> AF303222	5e-05/28/41	1.8
PbCC002-C05	Putative ribosomal protein <i>A. fumigatus</i> AL807577	9e-56/84/94	1.8
PbCC008-C11	60S ribosomal protein L27A (L29) Y11394	7e-70/79/91	1.8
PbCC001-A12	Unknown		1.8
PbCC003-G06	Conserved hypothetical protein with HesB-like domain <i>S. pombe</i> NC_003423	9e-18/48/64	1.7
PbCC001-D03	Hydroxyproline-rich glycoprotein precursors <i>Z. mays</i> S45164	6e-08/41/44	1.7
PbCC005-D04	Unknown		1.7
PbCC001-A06	Probable snRNP protein <i>N. crassa</i> AL513466	6e-36/71/81	1.7
PbCC006-A01	Unknown		1.7
PbCC004-C05	Cysteine dioxygenase <i>C. elegans</i> U51993	2e-36/48/65	1.7
PbCC007-B07	Hypothetical coiled-coil protein <i>S. pombe</i> AL109957	5e-29/57/74	1.6
PbCC006-E05	<i>N. crassa</i> predicted protein NCU07465.1	1e-06/39/53	1.6
PbCC003-A05	<i>CTR3</i> , High affinity copper transporter protein <i>S. pombe</i>	2e-20/41/57	1.6
PbCC006-A03	<i>HSP70</i> , Heat shock protein 70 X98931	2e-23/91/99	1.5
PbCC004-D04	Hypothetical protein <i>Meorhizobium loti</i> , NC_002678	5e-10/35/52	1.5

Figura 4: Validação da técnica de SSH. A- “Northern blot” utilizando como sonda os cDNAs subtraídos e não subtraído como controle da técnica de SSH. O cDNA subtraído marcou de uma forma generalizada todo o RNA de levedura de *P. brasiliensis* (L, levedura, M, micélio). B-Gráfico mostrando o resultado RT-PCR em tempo real com a sonda HSP82 mostrando um aumento de aproximadamente 150 vezes a expressão no cDNA subtraído.

Após obtermos cDNAs subtraídos e enriquecidos da levedura de *P. brasiliensis*, construímos uma biblioteca dos fragmentos do SSH com cDNA de levedura e sequenciamos 750 ESTs com fragmentos entre 200 a 700 pares de bases. As seqüências produziram 240 “clusters” após análise com o programa CAP3, sendo 112 “contigs” e 128 “singlets” (ver figura 5). Todas as seqüências foram comparadas com o banco não redundante de proteínas do NCBI (www.ncbi.nlm.nih.gov) utilizando a ferramenta blastx, e também com o nosso banco de ESTs de levedura de *P. brasiliensis* (Goldman et al 2003) utilizando

Figura 5: Esta figura sumariza os dados originados com a técnica de SSH.

a ferramenta blastn. Nossa análise revelou 163 novos "clusters" (50 com funções conhecidas e 113 com funções desconhecidas), sendo que 79 foram encontrados em nosso banco de ESTs de *P. brasiliensis* (46 com funções conhecidas e 33 com funções desconhecidas), (para uma visão geral ver figura 5 acima). Os 240 "clusters" foram amplificados por PCR (para os "contigs" somente o mais representativo), fixados em membrana de nylon, foi feita uma hibridização utilizando o cDNA subtraído e amplificado marcado radioativamente. Os insertos que correspondiam aos genes que mostravam maior intensidade de sinal radioativo eram provavelmente aqueles mais representados na biblioteca de SSH. Estes

clones foram selecionados para posterior análise de “real-time” RT-PCR (ver tabela 3).

Alguns destes genes, tais como *ERG25*, *CIT1*, *FBA1*, *ILV5*, *CTR3* (relacionados com metabolismo celular) e *CDI1* (relacionado com o metabolismo do enxofre) foram também detectados pela técnica de “macroarray” (ver tabela 2). Além dos genes mostrados na tabela 3, a biblioteca de SSH contém homólogos aos genes *HSP10*, *-30*, *-70*, *-82* e *-88*, que são importantes para a adaptação dos microorganismos às mudanças de temperatura. O gene *HSP70* (Silva et al 1999) também foi encontrado como mais expresso pela técnica de “macroarray” (ver tabela 2). Outros genes homólogos potencialmente importantes encontrados na biblioteca de SSH, que não estão presentes em nossos bancos de dados de *P. brasiliensis*, foram *MNN4* (relacionado com manosilfosforilação), *PKC1* (que codifica uma proteína quinase AMPc dependente) e *GST1* (que codifica glutathione S transferase). Todos estes genes mostraram uma alta significância em seus “e values” (10^{-19}), com exceção do gene *AGS1* (e-value=3e-16), que mostrou uma significância moderada (1e-10 to 1e-19). Todavia, foi isolado um cluster homólogo a *AGS1* de uma coleção de RST (“Random Sequence Tags”) originada de DNA genômico de *P. brasiliensis* (Nóbrega e Goldman, manuscrito em preparação). As seqüências parciais do gene *AGS1* confirmaram que ele codifica um homólogo do gene que codifica uma α -glucana sintetase de *Aspergillus fumigatus* (e value= 5e⁻²⁵; 70% de identidade).

Tabela 3: Genes identificados utilizando a técnica de SSH e “Macroarray” escolhidos para validação. (Os clones apresentados em negrito foram previamente identificados por Goldman et al 2003).

Identidade do Cluster de <i>P. brasiliensis</i>	Homólogo (N° de acesso ao banco de proteínas do NCBI.	BLASTe-value/% identidade/% similaridade	Critério para a escolha do gene checado por RT-PCR em tempo real
Metabolismo			
SSHCS001-G09 (PbCC010-B11)	<i>ILV5</i> , Branched-chain aminoacid biosynthesis; <i>Ilv5p S. cerevisiae</i> , AAB67753.1	3e-20/73/84	4.4 X no macroarray > 3 fragmentos SSH
PbCC007-B11	<i>LUC7</i> , RNA splicing factor Pad-1 <i>N.crassa</i> CAD21082.1	3e-66/73/77	> 1.6 X no macroarray
SSHCS001-A02 (PbCC012-B10)	<i>CIT1</i> , Citrate synthase <i>A. nidulans</i> , AAC49728.3	9e-61/89/94	> 3 fragmentos SSH; > 6.0 X no macroarray
SSHCS001-B04 (PBCC003-A05)	<i>CTR3</i> , High-affinity copper transporter <i>S. cerevisiae</i> , NC_003421	6e-20/41/57	Forte sinal na hibridização do SSH
SSHCS001-E11 (PbCC002-C06)	<i>GSP1</i> , Small G-protein Gsp1p (<i>C. albicans</i>), AF190700	2e-94/79/82	
SSHCC001-A06 (PbCC001-A04)	<i>ERG25</i> , C-4 methyl sterol oxidase <i>S. cerevisiae</i> NP_011574.1	8e-53/60/78	> 6.6 X no macroarray; Forte sinal na hibridização do SSH
SSHCS001-C08 (PbCC004-F06)	<i>PAL1</i> , Alkaline serine protease Penc2 <i>Penicillium citrinum</i> , AF098517_1	e-157/64/77	Forte sinal na hibridização do SSH
SSHCC001-A12 (PbCC009-F08)	<i>PAL2</i> , Aspartyl aminopeptidase <i>Coccidioides immitis</i> , AF420303	7e-256/75/89	Forte sinal na hibridização do SSH
SSHCC001-D05	<i>AGS1</i> , Putative α -1,3-glucan synthase <i>A. fumigatus</i> , AAL18964.1	3e-16/67/78	Forte sinal na hibridização do SSH
PbCC002-A05 (<i>FBA1</i>)	<i>FBA1</i> aldolase <i>S. cerevisiae</i>	e-136/65/76	> 4.7 X no macroarray
Desconhecidos			
PbCC003-C05 (<i>C101</i>)			> 1.8 X no macroarray
PbCC001-C10 (<i>C77</i>)			> 2.2 X no macroarray
Genes de transdução de sinal			
SSHCS002-C06 (PbCC002-F06)	<i>RHO1</i> , GTPase Rho1 <i>A. fumigatus</i> , AY007297	4e-61/99/99	Forte sinal na hibridização do SSH
Crescimento e Morfogênese			
SSHCC001-H01 (PbCC002-F03)	<i>SEP1</i> , Septin B <i>A. nidulans</i> , AAB41233.1	6e-63/92/95	Forte sinal na hibridização do SSH
SSHCS002-A02 (PbCC006-E07)	<i>FLB1</i> , Developmental regulator <i>flbA A. nidulans</i> , P38093	2e-38/77/86	Forte sinal na hibridização do SSH
Estresse oxidativo			
SSHCS001-G01 (PbCC015-F08)	<i>TSA1</i> , Putative thiol-specific antioxidant protein <i>Tsa1 (Ajellomyces capsulatus)</i> , AAG31645.1	7e-33/89/95	Forte sinal na hibridização do SSH
Metabolismo do enxofre			
RST1363b	<i>MEP1</i> , Methionine permease		Metabolismo do enxofre
PbCS004-F04	<i>APS1</i> , Adenylsulfate kinase		Metabolismo do enxofre
PbCS006-F07	<i>CHS1</i> , Choline sulfatase		Metabolismo do enxofre
PbCS001-E12	<i>GCL1</i> , Glutamate-cysteine ligase		Metabolismo do enxofre
SSHCS001-F05 (PbCC004-C05)	<i>CDI1</i> , Cysteine dioxygenase <i>C. elegans</i> U51993	2e-36/48/65	1.7 X no macroarray > 3 fragmentos SSH; Forte sinal na hibridização do SSH; Metabolismo do enxofre

4.2. VALIDAÇÃO DA EXPRESSÃO DOS GENES IDENTIFICADOS POR “MACROARRAY” E SSH COM “REAL TIME” RT-PCR.

Para a análise de “real-time” RT-PCR, foram desenhadas sondas fluorescentes Taq man (Applied Biosystems) e LUX (invitrogen), ver Tabela 1. Foram desenhadas 13 sondas para genes selecionados pela técnica de SSH (ver tabela 3) e 4 sondas para genes encontrados pela técnica de “macroarray” (c77, c101, *FBA1* e *LUC7*, ver tabela 2). O restante da seleção das 28 sondas apresentadas na tabela 1 foi baseada no grau de expressão observado na fase leveduriforme nas seleções preliminares (ver tabela 3), mas também incluímos genes de *P. brasiliensis* envolvidos no metabolismo de enxofre (*MEP1*, *APS1*, *CHS1* e *GCL1*). Apesar destes genes não aparecerem em nossas investigações, eles tem mostrado influência no estado morfológico de *H. capsulatum* e outros fungos dimórficos (Maresca e Kobayashi, 2000; Hwang et al 2003).

O isolamento do RNA total usado na investigação inicial para o “macroarray” e a biblioteca de SSH foi obtido de leveduras crescidas em meio YPD modificado e micélio crescidos em meio Sabouraud. Para checar se os níveis de expressão estão correlacionados com as condições dos meios de cultura, a validação por “real time” RT-PCR foi feita utilizando uma segunda extração de RNAs de levedura e de micélio crescidos no mesmo meio de cultura a 37°C e a 26°C respectivamente. Assim, foram comparadas a expressão destes genes nas duas temperaturas nos meios YPD, Sabouraud e em meio mínimo. Os resultados foram expressos em número de vezes de expressão da levedura em relação ao micélio de *P. brasiliensis* (ver tabela 4), sendo que o número de transcritos do micélio serviu como referência com o valor fixo de 1.

Os resultados sugerem que muitos dos genes apresentados na tabela 4 podem estar envolvidos na manutenção de *P. brasiliensis* na fase leveduriforme patogênica *in vitro*, porque eles foram altamente expressos na levedura nas três condições de crescimento (ver tabela 4). O cluster desconhecido c101 não foi validado como sendo mais expresso na levedura patogênica de *P. brasiliensis*, pois os seus valores foram abaixo de 1 quando comparados com os valores do micélio. Outros genes apresentaram valores baixos ou próximos a 1 em uma ou duas condições de cultivo (*ILV5*, *CIT1*, *GSP1*, *PAL2*, *FBA1*, *FLB1* e *GCL1*). O mais significativo resultado em relação à maior expressão na levedura de *P. brasiliensis* foram os valores de expressão dos genes da α -1,3-glucana sintetase (*AGS1*) e de uma colina sulfatase (*CHS1*) que mostraram valores de expressão de 82,18 e 61,1 vezes mais expressos na levedura (em YPD modificado). O gene *LUC7* (que codifica um fator de “splicing” de RNA) também foi altamente expresso pela levedura nos três meios de cultura (ver tabela 4) com valores acima de 6 vezes, seguido pelo gene *PAL1* (que codifica uma serina proteinase). Os valores de expressão foram comparáveis em pelo menos duas condições para os genes *ERG25*, *FBA1*, *RHO1*, *SEP1*, *MEP1*, *GCL1* e *CDI1*. Como previamente mostrado por da Fonseca et al (2001), observamos o aumento da expressão dos genes *FBA1* e *ENO1* que codificam a frutose 1-6 bifosfato aldolase e enolase, respectivamente.

Uma observação interessante é que alguns genes mostraram uma alta expressão quando as leveduras foram crescidas em meio mínimo. Este foi o caso dos genes *ILV5*, *CIT1*, *C77*, *RHO1*, *FLB1* e *MEP1*. O gene *RHO1*, envolvido na transdução de sinal e *MEP1*, envolvido no metabolismo de enxofre foram de 4 a 15 vezes mais expressos quando as leveduras foram crescidas em meio mínimo que

em meio completo (ver tabela 4). As flutuações apresentadas nos valores de expressão para todos os genes mostrados na tabela 4 provavelmente refletem diferentes estágios de crescimento e resposta a diferentes estímulos no ambiente de cultura a que foram submetidas as células de leveduras (Hwang et al 2003).

Tabela 4. Expressão em número de vezes relativa de levedura à expressão no micélio de RNAm de *P. brasiliensis* observadas por RT-PCR em tempo real em diferentes condições.

Identidade do cluster de <i>P. brasiliensis</i> (EST cluster)	Expressão relativa em n° de vezes (Levedura e micélio crescidos em meio YPD)	Expressão relativa em n° de vezes (Levedura e micélio crescidos em meio Sabouraud)	Expressão relativa em n° de vezes (Levedura e micélio crescidos em meio mínimo)
Metabolismo			
<i>ILV5</i>	2.95	0.70	5.60
<i>LUC7</i>	27.77	7.02	6.70
<i>CIT1</i>	1.06	3.26	7.30
<i>CTR3</i>	7.37	4.36	2.00
<i>GSP1</i>	3.98	1.40	1.20
<i>ERG25</i>	2.45	2.37	2.00
<i>PAL1</i>	5.07	6.84	6.30
<i>PAL2</i>	4.27	1.68	1.00
<i>AGS1</i>	63.2	82.18	9.20
<i>FBA1</i>	2.7	3.04	1.40
Desconhecidos			
<i>C77</i>	5.09	2.35	11.80
<i>C101</i>	0.06	0.56	0.60
Transdução de sinal			
<i>RHO1</i>	3.17	2.28	30.5
Crescimento e morfogênese			
<i>SEP1</i>	3.75	3.77	3.00
<i>FLB1</i>	1.91	0.94	3.30
Estresse oxidativo			
<i>TSA1</i>	18.81	7.51	2.70
Metabolismo do enxofre			
<i>MEP1</i>	4.23	5.62	29.30
<i>APS1</i>	8.95	4.48	3.00
<i>CHS1</i>	61.10	14.52	26.00
<i>GCL1</i>	1.19	2.38	2.00
<i>CDI1</i>	2.07	1.81	3.00

4.3. Caracterização preliminar da auxotrofia a enxofre orgânico na fase leveduriforme de *P. brasiliensis*.

Paris et al (1985) demonstraram que além da temperatura, fatores nutricionais também estão envolvidos na conversão da forma miceliana para a leveduriforme em *P. brasiliensis*. Estes autores mostraram que a forma miceliana cresce em meio mínimo, utilizando enxofre inorgânico, enquanto a forma leveduriforme cresce somente quando enxofre orgânico é adicionado ao meio. A figura 7 confirma estes dados, mostrando que o isolado Pb18 é auxotrófico para enxofre orgânico quando cultivado a 37°C, somente crescendo quando cultivado em meio mínimo suplementado com enxofre orgânico (cistina, cisteína e metionina). O micélio foi capaz de crescer em ambos os meios de cultura testados (ver figura 7). Essa observação foi ampliada com a caracterização de 79 isolados de *P. brasiliensis* (ver dados suplementares) que apresentam crescimento somente em meio mínimo suplementado com enxofre orgânico.

Como um passo inicial na compreensão deste fenômeno, nós investigamos os dois principais reguladores deste processo, os genes *metR* e *sconC*. Primeiramente, nós isolamos os genes *METR* (AAX47095) e *SCONC* (AAX47094) de *P. brasiliensis* através da avaliação de uma coleção de ESTs descrita por Goldman et al (2003), usando como “queries” MetR e SconC de *A. nidulans*. A região codificadora do gene *METR* tem 819 nucleotídeos, codificando um produto de tradução previsto de 272 aminoácidos com peso molecular e ponto isoelétricos calculados de respectivamente 30,426 kDa e 9,23. A assinatura do domínio básico do “zipper” de leucina (bZIP) está localizada nas posições dos aminoácidos 109 a 123 (ver dados suplementares). A região codificadora do gene *SCONC* tem 510

nucleotídeos, codificando um produto previsto de 169 aminoácidos com peso molecular e ponto isoelétrico calculados de respectivamente 19,551 kDa e 4.39. Os domínios de dimerização e tetramerização da família Skp1 estão localizados nas posições dos aminoácidos 91-168 e 7-72 (ver dados suplementares). As proteínas preditas de *METR* e *SCONC* mostram identidade com homólogos de MetR e SconC de diferentes espécies (Figura 6 A e B).

Visando verificar a funcionalidade dos genes *METR* e *SCONC* de *P. brasiliensis*, eles foram complementados nas mutações correspondentes $\Delta metR$ e *sconC3* de *A. nidulans*. Desta forma, as ORFs de *METR* e *SCONC* foram fusionadas com o promotor *alcA* de *A. nidulans* (Waring et al 1989) no vetor pAL3 que tem *pyr4* como marcador de seleção. O promotor *alcA* é induzido por glicerol, etanol e L-treonina e reprimido por glicose (Waring et al 1989). Estas construções (pAL3::MetR e pAL3::SconC) foram introduzidas nas cepas mutantes GRM1 ($\Delta metR$; *pyrG89*; *biA1*) e R34pyrG (*sconC3*; *pyrG89*) e transformantes selecionados para prototrofia em uridina e uracila. Diversos transformantes foram obtidos nos quais os plasmídios pAL3::MetR e pAL3::SconC integraram ectopicamente em diferentes sítios do genoma de *A. nidulans* e produziram grandes quantidades de RNAm de *METR* e *SCONC* (dados não apresentados). A Figura 8 mostra que a auxotrofia à metionina do mutante $\Delta metR$ foi complementada pelo gene *METR* de *P. brasiliensis*, na figura 8A mostramos que os transformantes, o controle (GRM1+pAL3 vazio) e a cepa GRM1 crescem quando acrescentado todos os suplementos, a figura 8B mostra que na ausência de uridina e uracila a cepa GRM1 não apresenta crescimento, enquanto o controle mostra um pequeno crescimento devido à presença do plasmídeo que tem como marcador o gene *pyrG89* enquanto

que os transformantes cresceram normalmente, A figura 8C mostra que houve a complementação, pois apenas os transformantes cresceram em meio mínimo acrescido de tiamina e biotina, sugerindo que este gene é o homólogo de *metR* em *A. nidulans*. A estratégia de seleção para os transformantes *sconC*⁺ foi a mesma descrita por Natorff et al. (1998). Ela é baseada na sensibilidade de cepas *scon*⁻ ao selenato sob condições de repressão (altas concentrações de metionina).

A.

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P.brasiliensis -----
N.crassa -----
A.nidulans MAEYNGRRAPNFSQYLLDLNAIPSPYDVAMQQQQQQDGFNIDNDLSLFTNTEFFDFDLNLPPFEP
S.cerevisiae -----

P.brasiliensis -----MLNDFN-LASYPPDFQSPITMQAPAY-PLSQLPPF SHNGAPMTS
N.crassa -----MSADFN-FGDFTTTYSPTIPAYPDTLGQLQFIQPNPQAAYPPVS
A.nidulans VEENKHNVNQNSDMDFLDILGGEFGFNVNDYAPQMNISINNQSVPVQNAQFHAVPQSQVPLPNVS
S.cerevisiae ---MSAKQGWKKSTNIDIASRKGMM-VNNLSEHLQNLISDSELGSRLLSLLVSSGNAEELIS

P.brasiliensis GEEG-----HSISQPVSARS SSNVSLASPLSQPAKRKQEAMSPGN--PSMDEAARLAQEEDKRR
N.crassa QHHAS---HHVQHPHQPGYVLSNPQLSGNKRKASDAMSVPPTPGARVMSFEEASRLAAEEDKRR
A.nidulans AQVNQSVNSRATASPSQSSVAAPSTNATSLAAPAPGPKRKHTQKTVPVSVVEEAARIAADEDKRR
S.cerevisiae MINNG-----QDV SQFKKLREPRKGKVAATTAVVVKKEEAPVSTN-----ELDKIKQE---RRR

P.brasiliensis RNTAASARFR IKKKEREKNMERTVKEVTAKNATLEARITQLEMENRWLKNLITEKN-----
N.crassa RNTAASARFR IKKKQREQALES AKEMSEKVTQLEGRIQALETENKWLKGLVTEKHGSKEDILKL
A.nidulans RNTAASARFR VKKMREQALETVDKDTTEKNAALEARVTALELENQWLKNLITEKNK-----
S.cerevisiae KNTASQRFRIRKQKNFENMKNLQNLNTQINKLRDRIEQLNKENEFWKAKLNDIN-----

P.brasiliensis -----SGAIADGDIAGMFDKYRASAEVAQQQPSNGLERRKASTP-----
N.crassa LREFSAHAAKVSKDAADKAEAAADKADAERAREESSFCVSTSSPSSDESVDTDNKKRRKD
A.nidulans -----SAEEGKKAENDISDMFKFLAAQKTE-GERSNGNSKIGVGTV-----
S.cerevisiae -----EIKSLKLLNDIKRRNMGR-----

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B.

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P.brasiliensis MSAPEN---SKITLIPSEDEPDRGIEVERVIER SILIKNMLEDLGDS-----
M.canis MAAAAT---SKITLTSSD---GVDITIDRQVAERSILIKNMLKDLGDS-----
A.nidulans ---MAE---KMLVMTSND---GKNIEVPRDVAERSLLIKNMLEDLGDP-----
N.crassa MAENDERALQKVSLSND---GQIITVDRVVAERSLLIKNLIEDLGDEAVM-----
S.pombe ---MSKIKLISSD---NEEFVVDQLIAERSMLIKNMLEDVGEI-----
S.cerevisiae ---MVT---SNVVLVSGE---GERFTVDKKIAERSILLKNYLNMDHDSNLQNSDSESDSDSETNHKSK

P.brasiliensis -----GEP IPIPNVNAVKLEKVIEWCEHHRNDPPTTND EYDSRRK-TTID EWDQKFMVLVD
M.canis -----GEA IPIPNVNESVLKKVIEWCKHKGDPPTGDDDDVDSRRK-TTID EWDQKFMQVD
A.nidulans -----TEP IPIPNVSENVLSKVLEWCAHHRNDPSSADDD-DSRRK-TTID EWDQKFMQVD
N.crassa -----NEA IPIPNVNEPVLKVVLEWCEHHRKDPPTTTEDEND SRK-STID EWDQKFMQVD
S.pombe -----NVP IPIPNVSSNVLRKVLEWCEHHRNDLYSGTEESDIRLKKSTDID EWDKFMQVD
S.cerevisiae DNNNGDDDDDDDEIVMPVPNVRSVLQKVLEWAEHHR-DSNFPDEDDDDSRKS--APVDSWDREFLQVD

P.brasiliensis QEMLFEIILAANYLDIKALLDVGCKTVANLIK GKSPDIRKTFNIQND FSEQEEA QIRARENEWAEDRSTP
M.canis QEMLFEIILAANYLDIKALLDVGCKTVANMIK GKSP EIRKTFNIQNDFTPEEEDQIRRENEWAEDR---
A.nidulans QEMLFEIILAANYLDIKPLLDIGCKTVANMIK GKSP EIRKTFNIQNDFTPEEEDQIRRENEWAEDR---
N.crassa QEMLFEIILAANYMDIKPLLDVGCKTVANMIK GKSP EIRKTFNITNDFTPEEEDQIRRENEWAEDR---
S.pombe QEMLFEIVLASNYLDIKPLLDTGCKTVANMIRGKSP EIRKTFNIPNDFTPEEEDQIRRENEWAEDR---
S.cerevisiae QEMLYEIILAANYLNKPLLDAGCKVVAEMIRGRSP EIRRTFNIVNDFTPEEEDQIRRENEWAEDR---

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Figura 6 Alinhamento da sequência deduzida de aminoácidos do gene *METR*-AAX47095-(A) e o gene *SCONC*-AY917138- (B) de *P. brasiliensis*: para o gene *METR*, *N. crassa* (CAB98237), *A. nidulans* (AAD38380) e *S. cerevisiae* (NP_012282); para o gene *SCONC* *Microsporium canis* (AAL76231.1), *A. nidulans* (AAB18274.2), *N. crassa* (XP_331383.1), *S. pombe* (T45459) e *S. cerevisiae* (S59793).

O selenato, um análogo tóxico do sulfato, é transportado dentro da célula pela enzima sulfato permease (Arst, 1968). Na presença de metionina, a enzima é reprimida no tipo selvagem, o qual, diferentemente dos mutantes *scon⁻*, é assim resistente ao selenato na presença deste aminoácido. Desta forma, o mutante *sconC3* foi transformado com o plasmídio pAL3::SconC e os transformantes selecionados na base da resistência ao selenato na presença de altas concentrações de metionina no meio de cultura. Como pode ser observado na Figura 9, o gene *SCONC* de *P. brasiliensis* pode complementar o mutante *sconC3*, sugerindo assim que este gene é o homólogo de *A. nidulans*.

Figura 7: Auxotrofia ao enxofre orgânico do isolado Pb18. Este experimento foi repetido com 79 isolados de *P. brasiliensis*, comprovando em todos a mesma auxotrofia para enxofre orgânico (ver dados suplementares).

Figura 8: O gene *METR* do fungo *P. brasiliensis* complementa o mutante *GRM1* de *A. nidulans*. O mutante *GRM1* ($\Delta metR$; *pyrG89*; *biA1*) foi transformado com *METR*::pAL3. Três transformantes (T1, T2 e T3) foram selecionados, a cepa *GRM1* transformada com o vetor pAL3 (controle) e eles foram crescidos em MM + complementos (MM= meio mínimo. UU= uridina e uracila. Met= metionina. Tia= tiamina. Bio= biotina Treo= treonina).

Figura 9: O gene *SCONC* do fungo *P. brasiliensis* complementa o mutante *sconC3* de *A. nidulans*. O mutante R34pyrG (*sconC3*; *pyrG89*) foi transformado com *SconC*::pAL3. Três transformantes (T24, T37 e T45), a cepa R34 (*sconC3*; *argB2*; *pyroA4*; *pyrG89*), a cepa R21 (*pabaA1*) e o pAL3A (cepa R34pyrG transformada com o vetor pAL3) foram crescidos em MM + glicerol + suplementos (arginina, piridoxida, ácido para-aminobenzóico, uridina e uracila) (A) ou MM + glicerol + complementos + 100mMtreonina + 100mMmetionina + 1mM selenato de sódio (B)

4.4. Expressão de RNAm dos genes *METR* e *SCONC* de *P. brasiliensis* durante a transição micélio para levedura em MM+CCM e meio completo YPD.

A mudança de temperatura de 26° para 37° C é a única condição conhecida como ativadora da transição micélio-levedura em *P. brasiliensis*. Nós arbitrariamente classificamos a transição em quatro diferentes estados morfológicos: (i) hifa, (ii) hifa em diferenciação, caracterizada pelo desenvolvimento de células do tipo de clamidosporos, produzidos por intumescimentos intercalares ou laterais nas hifas férteis, (iii) levedura em transformação, caracterizada pela produção de brotamentos múltiplos no clamidosporo e (iv) levedura madura com multibrotamentos. Esta classificação, mostrada na Figura 10, auxiliou no estabelecimento de parâmetros quantitativos visando acessar a transição morfológica em cada ponto. Como pode ser visto na Figura 11, a transição M-L ocorreu mais rapidamente em meio YPD do que em MM+CCM. Na transição M-L em meio YPD, não houve mais hifas em torno de 24 horas enquanto que 5 % das unidades morfológicas já eram leveduras em 48 horas (Figura 11). Na transição em MM+CCM, existiam ainda 25 % de hifas a 48 horas do início da transição enquanto os morfotipos de leveduras apareceram somente a partir de 72 horas do início da transição (em torno de 5 %; Figura 11).

Figura 10: Mudança morfológica induzida pela temperatura, de 26°C para 37°C de *P. brasiliensis*. A classificação acima foi dada arbitrariamente: Hifa; hifa em diferenciação (caracterizada pelo desenvolvimento de células do tipo de clamidosporos, produzidos por intumescimentos intercalares ou laterais nas hifas férteis); levedura em transformação (caracterizada pela produção de brotamentos múltiplos no clamidosporo) e levedura. Foram contadas aleatoriamente 300 unidades morfológicas para cada ponto de temperatura e considerada a média.

Figura 11: Mudança morfológica induzida pela temperatura, de 26°C para 37°C de *P. brasiliensis* comparando meio mínimo + CCM e meio completo (YPD), mostrando que a transição ocorre mais rapidamente em meio completo.

Nós investigamos a expressão do RNAm dos genes *METR* e *SCONC* durante a transição M-L que ocorreu nos meios MM+CCM e YPD (Figura 12). A expressão do RNAm de ambos *SCONC* e *METR* esteve aumentada durante a transição em ambos meios de cultura (Figuras 12). Nós também observamos se a transição dimórfica poderia ter alguma influência na acumulação de RNAm de diversos genes estruturais que estão envolvidos no metabolismo do enxofre. Nós escolhemos cinco genes que participam em diferentes reações no metabolismo do enxofre, tais como, disponibilidade de enxofre (colina sulfatase, *CHS1*, que disponibiliza enxofre a partir de colina-O-sulfato), transporte (metionina permease, *MEP1*), regulação do nível intracelular de cisteína (cisteína dioxigenase, *CDI1*), e a conversão de intermediários na via metabólica (3'-fosfoadenosina-5'-fosfosulfato, PAPS, é convertido pela fosforilação de adenosina-5'-fosfosulfato, APS, que é realizada pela APS quinase, (*APS1*). As tabelas 5 e 6 com os respectivos gráficos 1 e 2, mostram os níveis de acumulação do RNAm durante as transições M-L e L-M. O gene *CHS1* mostra um acúmulo de RNAm muito maior durante a transição M-L do que L-M (compare, 11 a 26 vezes maior durante a transição M-L e somente 4,41 vezes maior 48 horas depois do início da transição L-M). O gene *MEP1* mostra um alto acúmulo de RNAm durante ambas as transições, mas mais elevada durante a transição M-L. O gene *CDI1* foi preferencialmente expresso durante a transição M-L, mostrando um aumento de 6,03 vezes após 120 horas do início da transição L-M, enquanto o gene *GCL1* mostrou níveis constantes de acúmulo de RNAm durante ambas as transições. O gene *APS1* mostrou níveis constantes de expressão do RNAm durante a transição M-L, mas níveis aumentados durante a transição L-M. Estes resultados sugerem que existe algum aumento preferencial da

expressão dos genes envolvidos na via de assimilação orgânica do enxofre durante a transição M-L.

Figura 12: Níveis de RNAm dos genes *METR* (A) e *SCONC* (B) na transição micélio para levedura (ML) do *P. brasiliensis* cultivado em MM+CCM e em meio completo (YPD).

Tabela 5: Transição micélio (26°C / MM+CCM) levedura (37°C / MM+CCM) mostrando os níveis de RNAm visualizados no gráfico 1

	0hs	10hs	24hs	48hs	72hs	120hs	240hs
APS1	1	1,303277	1,915148	0,45282	0,700028	2,346199	3,062384
CDI1	1	6,173533	9,280654	3,784604	4,982867	3,465657	2,997991
GCL1	1	1,147505	1,991017	0,826522	0,915275	1,520716	1,947565
MEP1	1	11,61029	5,093043	1,204267	1,771887	21,1776	29,30198
CHS1	1	1,166541	1,266706	0,534799	0,809	11,06518	26,03776

APS1: Adenosina-5'-fosfosulfato quinase ; CDI1: Cisteína dioxigenase; GCL1: Glutamato cisteína ligase; MEP1: Metionina permease; CHS1: Colina sulfatase

Gráfico 1: Transição micélio (26°C / MM+CCM) levedura (37°C / MM+CCM). Este gráfico foi gerado com os dados da tabela 5 convertidos em Log2 para facilitar a visualização.

Tabela 6: Transição levedura (37°C / MM + CCM) micélio (26°C / MM + CCM)

mostrando os níveis de RNAm, visualizados no gráfico 2.

	0hs	10hs	24hs	48hs	72hs	120hs
APS1	1	4,905233	2,515719	3,704233	6,440775	2,032665
CDI1	1	0,506986	0,770367	0,479036	1,720815	6,033614
GCL1	1	2,231411	0,674419	1,231487	0,868108	0,462681
MEP1	1	3,249071	2,568411	1,787255	2,078119	1,006159
CHS1	1	2,201612	0,611113	4,414699	0,539921	0,674108

APS1: Adenosina-5'-fosfosulfato quinase ; CDI1: Cisteína dioxigenase; GCL1: Glutamato cisteína ligase; MEP1: Metionina permease; CHS1: Colina sulfatase

Gráfico 2: Transição levedura (37°C / MM+CCM) micélio (26°C / MM+CCM). Este gráfico foi gerado com os dados da tabela 6 convertidos em Log2 para facilitar a visualização.

V-Discussão

5.1. IDENTIFICAÇÃO DE GENES MAIS EXPRESSOS NA FASE LEVEDURIFORME PATOGÊNICA DE *P. brasiliensis* ATRAVÉS DE “MACROARRAYS” E SSH.

Os resultados apresentados neste trabalho mostram o uso original das análises de “macroarray” e SSH no exame da expressão gênica na fase patogênica de *P. brasiliensis*. Ambos os procedimentos proporcionaram a identificação de genes com a expressão aumentada na fase de levedura. Recentes estudos têm demonstrado que a transição dimórfica em outros fungos está diretamente associada com a patogenicidade (Braun and Johnson 1997; Csank et al 1998; Leberer et al 1997; Lo et al 1997; Stoldt et al 1997; Yaar et al 1997; Borges-Walmsley et al 2002; Romani et al 2003). Desta forma, a identificação de genes preferencialmente expressos na fase de levedura pode proporcionar maiores informações acerca da patobiologia de *P. brasiliensis*. A estratégia de SSH parcialmente resolve o problema das diferenças em abundância de RNAm através da incorporação de um passo de amplificação que normaliza (equaliza) a abundância da sequência durante o curso da subtração pela cinética normal de hibridização (Diatchenko et al 1996). Como a maior parte dos “clusters” de SSH obtidos neste trabalho (em torno de 68 %) não foram previamente descritos no nosso banco de ESTs (Goldman et al 2003), uma das mais interessantes características do SSH foi conseguida, ou seja a descrição de fragmentos de cDNA relativamente raros. Nas nossas mãos, ambas as análises de “macroarray” e SSH mostraram-se poderosas porque a maior parte dos 21 genes selecionados para validação por “real-time” RT-PCR foram mais expressos na fase de levedura do que

na fase miceliana. Além disso, diversos genes foram concomitantemente identificados em ambas as análises.

Nós classificamos genes preferencialmente expressos na fase leveduriforme em amplas categorias funcionais. Baseado na função de seus ortólogos, nós identificamos genes que estão implicados em diversos destes processos. Uma análise mais profunda desses genes é necessária antes que a sua função possa ser determinada. Contudo, ferramentas de genética clássica, tais como transformação mediada por DNA e modulação da expressão gênica ou por deleção/inativação ou por interferência de RNA ou RNA antisense, não foram ainda desenvolvidas para *P. brasiliensis*. Consequentemente, este tipo de análise genômica pode representar uma alternativa eficiente para identificar genes envolvidos em virulência/patogenicidade e proporcionar uma larga plataforma para uma melhor compreensão da biologia de *P. brasiliensis* e do papel de diversos genes durante a transição dimórfica e no início da virulência/patogenicidade da PCM. Assim que técnicas de inativação genética para este organismo forem desenvolvidas, as funções desses genes aqui identificados poderão ser testadas.

A correlação entre a expressão de genes específicos na forma leveduriforme e a fase morfológica é complicada pelo fato de que uma mudança de temperatura é usada para promover a transição dimórfica. Não foram ainda feitas tentativas em *P. brasiliensis* de obtenção da fase leveduriforme à temperatura ambiente e de seguir a expressão de genes específicos em culturas que não foram ainda submetidas à mudança de temperatura. As condições de crescimento são também uma outra condição ambiental que pode afetar a expressão durante a transição M-L. A identificação de genes preferencialmente expressos na fase leveduriforme é

complicada pelo fato de que condições que induzem a morfogênese também induzirão respostas celulares que não estão necessariamente conectadas com a morfogênese, mas que são necessárias para a adaptação fisiológica ao novo ambiente ou relacionadas à fase de crescimento *in vitro* (Berman e Sudbery 2002; Hwang et al 2003). Nós investigamos a possível influência das condições de crescimento através da verificação da expressão gênica dos genes identificados em RNA do fungo crescido em diferentes meios de cultura. Em geral, embora os níveis de expressão possam ser notavelmente diferentes, tais como *AGS1* e *LUC7* em meio completo e meio mínimo, existe uma tendência consistente que estes genes são mais expressos na fase leveduriforme, proporcionando confiabilidade a estes dados.

A mudança morfológica mais reconhecida em *P. brasiliensis* é mudança em anomeria da glucana predominante que passa de α -1,3- para β -1,3-glucana assim que o fungo adota a forma leveduriforme (San-Blas e Niño-Vega 2001). Desta forma, nós observamos uma expressão muito maior do gene que codifica a α -1,3-glucana sintetase na fase de levedura do que na de micélio. Relatos antigos do estudo da transição M-L em *H. capsulatum* atribuíram um importante papel aos grupos sulfidrílicos, principalmente na forma de cisteína (Maresca and Kobayashi 2000). Hwang et al (2003) ampliaram essas observações mostrando que o gene que codifica a enzima cisteína dioxigenase é mais expressa em levedura do que no micélio. Nós identificamos, em nossa análise de SSH, o gene *CDI1* que codifica a cisteína dioxigenase em *P. brasiliensis*. Foi previamente mostrado que células na fase miceliana de *P. brasiliensis* podem crescer na presença tanto de enxofre orgânico como inorgânico, enquanto que as células de levedura podem crescer

somente em presença de aminoácidos contendo enxofre (Paris et al 1985). Esta auxotrofia ao enxofre sugere que além da temperatura, o enxofre orgânico é um importante estímulo para manter *P. brasiliensis* na fase leveduriforme. Desta forma, nós verificamos que diversos genes envolvidos na assimilação de aminoácidos que contêm enxofre, tais como metionina permease, são mais expressos na fase de levedura do que na de micélio, indicando um possível papel, como em *H. capsulatum*, para o metabolismo do enxofre na manutenção do estado de levedura.

O gene *CIT1* codifica a enzima citrato sintetase que cataliza a primeira reação do ciclo do ácido cítrico, a saber, a condensação de acetil-CoA com oxalacetato para formar citrato. A alta atividade específica de citrato sintase causa um acúmulo de isocitrato e citrato na mitocôndria. Ambos são transportados no citosol onde o citrato é clivado pela ATP:citrato liase, dando origem a Acetil-CoA, o precursor da síntese de ácidos graxos (Lehninger et al 1993). É possível que os altos níveis de expressão do RNAm do gene *CIT1* de *P. brasiliensis* possam refletir um aumento da síntese de ácidos graxos. O mesmo tipo de observação pode ser feito para os níveis aumentados de expressão do gene *ERG25* na fase de levedura. O gene *ERG25* codifica uma C-4 esteroil metil oxidase que participa do primeiro passo enzimático dos três que são necessários para a remoção dos dois grupos C-4 metil que levam à síntese de ergosterol em fungos. É também provável que o aumento na expressão de *ERG25* esteja correlacionado ao aumento do “turnover” da membrana celular. Como nós sugerimos previamente, mudanças na membrana são importantes fatores na transição morfológica a 37°C (Goldman et al 2003); o alto “turnover” de lipídios na membrana celular pode contribuir para isso. Alternativamente, já tem sido demonstrado através de análise global da expressão

gênica em *S. cerevisiae* que os genes envolvidos na biossíntese de ergosterol têm a sua expressão de RNAm aumentada em condições de estresse, tais como estresse oxidativo, e um número de agentes antifúngicos que têm como alvo a biosíntese de ergosterol (Higgins et al 2003; Bammert & Fostel 2000). Permanece a ser determinado se os genes de biosíntese de ergosterol têm a sua expressão de RNAm aumentada a 37 °C, ou se é um efeito específico do gene *ERG25*.

O estresse de altas temperaturas gera estresse oxidativo em *S. cerevisiae*, e células anaeróbicas são diversas ordens de magnitude mais resistentes que células aeróbicas ao “heat shock” a 50 °C (Davidson & Schiestl 2001). A transição de 25° a 37°C em *P. brasiliensis* (e também em *H. capsulatum* and *B. dermatitidis*) é acompanhada por mudanças marcantes na respiração e rápido declínio intracelular nos níveis de ATP, devido ao desligamento da oxidação fosforilativa (Medoff et al 1987; Maresca and Kobayashi 2000). Ainda com o decréscimo na concentração de ATP, o crescimento na fase de levedura a 37°C poderia dar origem à produção de radicais livres de oxigênio devido ao crescimento em temperaturas elevadas. Estas observações sugerem que os sistemas antioxidantes em *P. brasiliensis* poderiam ser extremamente importantes não somente para a sobrevivência em macrófagos, mas também para a detoxificação de radicais livres de oxigênio. Desta forma, a expressão preferencial na fase de levedura dos genes *TSA1* (“thiol-specific antioxidant”) e *GST1*, que codificam respectivamente a tioredoxina peroxidase e a glutathione-S-transferase, poderiam ser usados como antioxidantes visando reduzir as espécies reativas de oxigênio durante o crescimento da forma de levedura. Curiosamente, Choi et al (2003) identificaram Tsa1p como altamente expresso nas hifas de *C. albicans* através de eletroforese de gel bidimensional de proteínas.

As vias de sinalização que controlam as mudanças morfológicas em *P. brasiliensis* são pobremente compreendidas, mas o envolvimento de ambas as vias de transdução de sinal do AMPc e da proteína kinase ativada por mitógenos (MAPK) têm sido relatada em outros fungos dimórficos. Através da análise por SSH, nós identificamos quatro genes, *RHO1*, *SEP1*, *FLB1*, e *PCK1*, que codificam proteínas envolvidas na sinalização celular e no estabelecimento da polaridade (Lengeler et al 2000; Borges and Walmsley 2000; Momany 2002). Interessantemente, *RHO1* foi 10 a 15 vezes mais expressa em levedura crescida em meio mínimo do que em meio completo. Boyce et al (2001) observaram que o homólogo de *CDC42* de *P. marneffei*, *cflA*, é também necessário para a polarização e a determinação da forma celular durante o crescimento como levedura, e *CflA* ativa é necessária para a separação de células-filhas de leveduras. Contudo, *cflA* não é necessário para a transição dimórfica.

Sumarizando, este trabalho proporcionou a identificação de diversos genes que podem estar envolvidos na manutenção da forma patogênica leveduriforme em *P. brasiliensis*.

Uma análise mais aprofundada feita por Nunes et al (2005, ver apêndice D), mostrou a análise de 4692 ESTs através da técnica de “microarray” evidenciando genes mais expressos na transição de micélio para levedura em vários pontos da mudança morfológica (de 5 a 120 hs), esta análise revelou um total de 2583 genes com modulação na expressão de RNAm estatisticamente significativa em um ou mais pontos da transição. Entre as categorias de genes encontradas, o gene 4HPPD (4hidroxifenil piruvato desidrogenase) foi o mais significativamente expresso pela técnica de “microarray” durante a transição micélio para levedura. Foi

utilizado um inibidor da atividade da 4HPPD, mostrando que a transição do micélio para levedura era inibida parcial ou totalmente dependendo da concentração do inibidor no meio de cultivo. Neste trabalho os autores sugerem a possibilidade de uma nova droga no tratamento da PCM e mostram que a técnica de “microarray” pode ajudar na identificação de novos alvos terapêuticos.

5.2. CARACTERIZAÇÃO PRELIMINAR DA AUXOTROFIA A ENXOFRE ORGÂNICO NA FASE LEVEDURIFORME DE *P. brasiliensis*.

Evidências antigas na literatura mostram que *P. brasiliensis* é um auxotrófico condicional para fontes orgânicas de enxofre (Paris et al 1985). Esta auxotrofia ao enxofre orgânico sugere que juntamente com a temperatura, fontes de enxofre orgânico são um importante estímulo para manter a fase leveduriforme patogênica de *P. brasiliensis*. Isso também já foi observado para outro fungo dimórfico, *H. capsulatum* (para uma revisão ver Maresca e Kobayashi 2000). Nós identificamos os dois principais genes reguladores da via metabólica do enxofre em *P. brasiliensis*, *METR* e *SCONC*, e demonstramos a sua funcionalidade através da complementação dos mutantes correspondentes em *A. nidulans*. A proteína MetR é similar às proteínas CYS3 de *N. crassa* e Met4 de *S. cerevisiae*, bem como à proteína CAA94632 de *Schizosaccharomyces pombe*, de função desconhecida (Natorff et al 2003). CYS3 é um membro da família de proteínas de ligação ao DNA bZIP e atua como um homodímero (Kanaan et al 1992). A proteína reconhece a seqüência palindrômica 5'-ATGRYRYCAT-3' (Li and Marzluf, 1996) presente nos

promotores de genes de assimilação de enxofre (Fu et al 1989; Fu and Marzluf, 1990).

O gene *sconC*, que codifica um regulador negativo do metabolismo do enxofre em *A. nidulans*, também foi analisado na transição morfológica de *P. brasiliensis* e é um gene que possui grande similaridade com genes da família SKP1. A especificidade do complexo SCF por uma proteína é determinada pela proteína F-box enquanto a Skp1 é responsável por tornar o substrato alvo para a ubiquitinação (Bai et al 1996; Lyapina et al 1998; Patton et al 1998a; 1998b). O complexo SCF é essencial para muitas funções celulares, incluindo a regulação do ciclo celular (Piotrowska et al 2000). A proteína SconC contém sequências PEST que são comuns a proteínas que estão sujeitas a uma rápida reciclagem. As seqüências PEST são ricas em prolina (P), ácido glutâmico (E), serina (S) e treonina (T) que são alvos para rápida destruição (Rechsteiner e Rogers, 1996). Proteínas que contém a região F-box se ligam a motivos de interações proteína-proteína adicionais (como repetições de WD40 e repetições ricas em leucinas) que reconhecem elementos de seqüência mediadoras de destruição em proteínas alvos (Vodermaier, 2004). Foi encontrado na proteína SconC um motivo de ligação ATP/GTP e uma região P-loop. (Piotrowska, 2000).

Os dados de expressão gênica ao nível de mRNA mostram que *METR* tem uma expressão aumentada durante a transição M-Y em MM+CCM. Curiosamente o gene *metR* é um ativador transcripcional da via de enxofre inorgânico e, todavia, mostra uma super expressão crescente por toda a transição ML na presença de enxofre orgânico. É tentador sugerir a possibilidade de que devido à auxotrofia para o enxofre orgânico durante a fase leveduriforme, o gene *metR* tenha evoluído

outras funções, talvez como indutor transcricional, relacionadas à transição M-L. Estudar efeitos reguladores para o gene *metR* e possíveis interações com outros genes relacionados à via de assimilação de enxofre na transição morfológica de *P. brasiliensis* será um objetivo a ser perseguido, pois traria mais evidências da relação entre a via de assimilação do enxofre e a patogenicidade e/ou virulência da levedura deste fungo.

Nós também avaliamos a expressão de outros genes da via do enxofre durante a transição M-Y. O gene que codifica a metionina permease (*MEP1*), que é uma proteína integral de membrana com 13 domínios putativos hidrofóbicos (THOMAS e SURDIN-KERJAN, 1997). O gene adenilsulfato quinase (*APS1*) participa da via de assimilação de enxofre e, quando deletado em *S. cerevisiae*, confere o fenótipo de auxotrofia a enxofre orgânico e resistência ao selenato (THOMAS e SURDIN-KERJAN, 1997). O gene que codifica a colina sulfatase (*CHS1*), é responsável pela assimilação de sulfato de colina que está presente em plantas e é estocado em muitos fungos, possuindo a função de osmoproteção e também de reserva de enxofre para a célula (Marzluf, 1997). O fungo *Neurospora crassa* utiliza o sulfato de colina como fonte secundária de enxofre. O sulfato de colina exógeno é mantido intacto dentro da célula via uma permease específica, e então é hidrolisado pela CHS1 para produzir uma reserva de enxofre inorgânico (Marzluf, 1997). A expressão de CHS1 é fortemente regulada e depende do aumento da repressão catabólica de enxofre e requer a ação da proteína regulatória positiva CYS3 (Marzluf, 1997). O gene glutamato cisteína ligase (*GCL1*) é responsável pelo primeiro passo da síntese de glutathiona (Huang et al 1988), que é essencial para uma variedade de funções celulares.

Verificamos que estes genes envolvidos na assimilação e metabolismo do enxofre (tabelas 5 e 6; gráficos 1 e 2), estão mais expressos na fase leveduriforme em relação à miceliana de *P. brasiliensis*, indicando uma possível conexão entre a manutenção do estado leveduriforme e o metabolismo do enxofre.

VI-Conclusões

Mostramos a expressão aumentada na levedura patogênica de *P. brasiliensis* de 38 “clusters” pela técnica de “macroarray” variando de 1,5 a 6,6 vezes.

Validamos a técnica de SSH e mostramos uma biblioteca com 240 “clusters”, dentre estes, 46,25% (111 “clusters”) com função desconhecida nos bancos de dados investigados e 13,75% (33 “clusters”) com função desconhecida em *P. brasiliensis*, dando o primeiro passo na procura de genes preferencialmente expressos na levedura patogênica.

Validamos 20 dos 21 genes escolhidos como mais expressos na levedura patogênica de *P. brasiliensis* com a técnica de RT-PCR em tempo real em pelo menos 2 condições de crescimento diferentes.

Mostramos que o estado nutricional influencia de maneira significativa a expressão gênica em *P. brasiliensis*.

Confirmamos a auxotrofia do isolado Pb18 para enxofre orgânico.

Mostramos a complementação dos genes *METR* e *SCONC* de *P. brasiliensis* em mutantes para estes genes em *A. nidulans*.

Fizemos medidas de expressão com a técnica de “real time” RT-PCR em 5 genes, com tendência de expressão preferencial na levedura, possivelmente relacionados com o metabolismo do enxofre em *P. brasiliensis*.

VII-Referências Bibliográficas

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VIII- Datos Suplementares

Tabela adicional: Esta tabela é o resultado final da análise de 79 isolados de *Paracoccidioides brasiliensis* originadas de ambientes distintos (Material Coletado) do Brasil e da Colômbia, mostrando que nenhum dos isolados cresceram somente em meio mínimo, sendo necessária a adição de cistina, cisteína e metionina, confirmando a auxotrofia para todos os isolados.

Isolados Clínicos	Material Coletado	Classificação da Doença	Origem	Crescimento em Meio Mínimo
ATCC76533	ND	Crônica	Colômbia	Nenhum
<i>Cabbasuos armadillo</i>	Tatu		Colômbia	Nenhum
<i>Dasypus armadillo</i>	Tatu		Colômbia	Nenhum
Pb 100	Catarro	Crônica	Brasil	Nenhum
Pb 104	Escrotal	Crônica	Brasil	Nenhum
Pb 106	Catarro	Crônica	Brasil	Nenhum
Pb 1081	ND	ND	Brasil	Nenhum
Pb 1115	ND	ND	Brasil	Nenhum
Pb 112	Catarro	ND	Brasil	Nenhum
Pb 113	Lavagem Brônquica alveolar	Crônica	Brasil	Nenhum
Pb 115	Biópsia de Mucosa	Crônica	Brasil	Nenhum
Pb 18	ND	ND	Brasil	Nenhum
Pb 213	ND	ND	Brasil	Nenhum
Pb 265	ND	ND	Brasil	Nenhum
Pb 319	ND	ND	Brasil	Nenhum
Pb 339	ND	ND	Brasil	Nenhum
Pb 51	Biópsia de Pele	Crônica	Brasil	Nenhum
Pb 52	Biópsia de Pele	HIV/Aguda	Brasil	Nenhum
Pb 54	Biópsia de Pele	Crônica	Brasil	Nenhum
Pb 55	Biópsia de Pele	ND	Brasil	Nenhum
Pb 552	ND	ND	Brasil	Nenhum
Pb 57	Ascitic fluid	Sub aguda	Brasil	Nenhum
Pb 58	Fluído Peritoneal	ND	Brasil	Nenhum
Pb 59	Aspirado Gânglioar	Crônica	Brasil	Nenhum
Pb 590	ND	ND	Brasil	Nenhum
Pb 60	Pele	Aguda	Brasil	Nenhum
Pb 61	Biópsia de Pele	HIV/Aguda	Brasil	Nenhum
Pb 62	Catarro	HIV/Aguda	Brasil	Nenhum
Pb 63	Gânglio	Crônica	Brasil	Nenhum
Pb 65	Catarro	Crônica	Brasil	Nenhum
Pb 66	Biópsia de Pele	Crônica	Brasil	Nenhum
Pb 67	Lesão Bucal	HIV/Aguda	Brasil	Nenhum
Pb 68	Linfonodos	HIV/Aguda	Brasil	Nenhum
Pb 68	ND	Crônica	Colômbia	Nenhum
Pb 71	Abscesso	Aguda	Brasil	Nenhum

Pb 72	Lesão Laríngea	Crônica	Brasil	Nenhum
Pb 74	Lavagem Brônquica alveolar	Crônica	Brasil	Nenhum
Pb 76	Biópsia Duodenal	HIV/Aguda	Brasil	Nenhum
Pb 77	Mucosa	Crônica	Brasil	Nenhum
Pb 808	ND	ND	Brasil	Nenhum
Pb 81	Sangue	Aguda	Brasil	Nenhum
Pb 86	Sangue	HIV/Aguda	Brasil	Nenhum
Pb 88	Gânglio Cervical	HIV/Aguda	Brasil	Nenhum
Pb 93	Fluido Peritoneal	Crônica	Brasil	Nenhum
Pb 95	Catarro	Crônica	Brasil	Nenhum
Pb 97	Sangue	Crônica	Brasil	Nenhum
Pb BAT	Gânglio	Aguda	Brasil	Nenhum
Pb BOAS	Catarro	Crônica	Brasil	Nenhum
Pb BTT	Catarro	Crônica	Brasil	Nenhum
Pb BX305	Biópsia	ND	Brasil	Nenhum
Pb BX479	Biópsia	ND	Brasil	Nenhum
Pb CATA	ND	ND	Brasil	Nenhum
Pb CLAU	ND	ND	Brasil	Nenhum
Pb FAP	Gânglio	Aguda	Brasil	Nenhum
Pb H-45	ND	Crônica	Colombia	Nenhum
Pb H-47	ND	Crônica	Colombia	Nenhum
Pb IBIÁ	Solo		Brasil	Nenhum
Pb IBIÁ T1	Tatu		Brasil	Nenhum
Pb IBIÁ T2	Tatu		Brasil	Nenhum
Pb IBIÁ T3	Tatu		Brasil	Nenhum
Pb OLBA	Gânglio	Sub aguda	Brasil	Nenhum
Pb141	ND	Crônica	Colombia	Nenhum
Pb149	ND	Crônica	Colombia	Nenhum
Pb151	ND	Crônica	Colombia	Nenhum
Pb161	ND	Crônica	Colombia	Nenhum
Pb163	ND	Crônica	Colombia	Nenhum
Pb165	ND	Crônica	Colombia	Nenhum
Pb166	ND	Crônica	Colombia	Nenhum
Pb167	ND	ND	Colombia	Nenhum
Pb168	ND	ND	Colombia	Nenhum
Pb169	ND	ND	Colombia	Nenhum
Pb170	ND	ND	Colombia	Nenhum
Pb171	ND	ND	Colombia	Nenhum
Pb196	ND	ND	Colombia	Nenhum
Pb202	ND	Crônica	Colombia	Nenhum
Pb204	ND	Crônica	Colombia	Nenhum
Pb206	ND	Crônica	Colombia	Nenhum
Pb46	ND	Crônica	Colombia	Nenhum
Pb72	ND	Crônica	Colombia	Nenhum

Seqüência de nucleotídeos do gene *METR* de *P. brasiliensis*.

Depositada no banco de dados do National Center for Biotechnology Information (NCBI).

[<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=61608604>].

LOCUS: AY917139, 819 bp, mRNA, linear PLN 22-MAR-2005

DEFINITION: Paracoccidioides brasiliensis MetR mRNA, complete cds.

ACCESSION (NCBI): **AY917139** (nucleotide).

ACCESSION (NCBI): **AAX47095** (protein)

Atgccattccttcgccctatgatctggcacaacagcaagcggagcggcaagatctattcaatgtggatgctgaattgg
ctctgtttacgaacgcggagcttctggattttgatgctgttggggatctatctgttgacctgcccgtgaaattgaacgcg
tggagggaaccgatgcgcataataaggactctccgagcacggttggcgatggagataataatgtcaagtacctgga
tatgttgaatgacttcaacctggccagctacccgcctgactccaatcacccatcatccatgcaggcaccgcctat
ccgctgtcccagcttccacccccagccacaatggagccccatgacctctggagagtttgggcactcgatttctccc
cagcccgtgtcagcccgatcctcatccaacgtatccttggcatcacctctgtctcaacccgcaaaaaggaaacagga
agcaatgtcccccggaacccaagcatggacgaagccgcgcgactggcacaggaggaagacaaacgccgcc
gcaacacggctgccagtgtcccgttccgcatcaagaagaaggagcgtgaaaagaacatggagcgcaccggttaa
ggaggtcacggccaaaaacgccacactcgaggcccgcatcacccagctcgagatggagaatcgggtggctcaag
aacctgatcacggagaagaatagcgggtccattgctgacggggatattgctggcatgttcgataagtatcgcgcgag
cgcagaggtggcccagcaacaaccgtcgaacggcttggagaggagaaaaagcgtaa.

Seqüência de aminoácidos da proteína *METR* de *P. brasiliensis*.

MPFLRPMIWHNSKRSGKIYSMWMLNWLCLRTRSFDFDAVGDLSDLPVKFERVE
GTD AHNKDSPSTVGDGDNNVKYLDMLNDFNLASYPPDFQSPIITMQAPAYPLSQL
PPPSHNGAPMTSGEFGHSISPQPV SARSSSNVSLASPLSQPAKRKQEAMSPGNP
SMDEAARLAQEEDKRRRNTAASARFRIKKKEREKNMERTVKEVTAKNATLEARIT
QLEMENRWLKNLITEKNSGAIADGDIAGMFDKYRASAEVAQQQPSNGLERRKAX
272 aminoácidos.

Peso em Kdaltons calculado através da ferramenta “**Peptide mass**” fornecida pelo site do “ExPASy Proteomics Server” [http://ca.expasy.org/]. The ExPASy (**Ex**pert **P**rotein **A**nalysis **S**ystem).

peptide masses from your sequence are:

[Theoretical pI: 9.23 / Mw (average mass): 30426.38 Kdaltons.

Identificação do principal domínio proteico da proteína MetR de *P. brasiliensis*.

A identificação do principal domínio protéico foi feita através da ferramenta InterProScan fornecida pelo site [http://www.ebi.ac.uk/InterProScan/]

SEQUENCE: METR CRC64: 0098E466D547BDCB LENGTH: 272 aa			
InterPro IPR004827 Domain	Basic-leucine zipper (bZIP) transcription factor		
	SM00338		BRLZ
	PS00036		BZIP_BASIC
	PS50217		BZIP

SEQUENCE: METR CRC64: 0098E466D547BDCB LENGTH: 272 aa			
InterPro IPR004827 Domain	Basic-leucine zipper (bZIP) transcription factor		
	SMART	SM00338	BRLZ 5.9E-4 [174-234]T
	PROFILE	PS00036	BZIP_BASIC 0.0 [178-192]T
	PROFILE	PS50217	BZIP 9.875 [172-235]T

Seqüência de nucleotídeos do gene *SCONC* de *P. brasiliensis*.

Depositada no banco de dados do National Center for Biotechnology Information (NCBI).

[<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=61608601>].

DEFINITION: *Paracoccidioides brasiliensis* SconC mRNA, complete cds.

ACCESSION (NCBI): **AY917138** (nucleotide).

ACCESSION (NCBI): **AAX47094** (protein)

Atgtcggcccccgagaattctaaaatcacccctgatcccatccgatgagcccgagatggccgtggaatcgaagttgaacgtgtaatcatcgagcgatccatcttgatcaagaacatgctcgaggatctaggtgattctggggaacctattccaattccaatgttaataaagccgtcctcgaaaaggatcatcgaatggtgcgaacatcacaggacgacccaccaaccaccaatgatgaggactacgactcgcgagagaaagaccactgacatcgacgagtgaggacaaaaattcatgctggttgaccaagagatgctttttgagataatcctggccgccaactatcttgatatcaaagccctgctagacgtaggctgcaagaccgtcgccaatttgatcaagggcaagtcgccagatgatatccggaagaccttcaacatccagaacgacttcagcgagcaggaggaggctcaaatccgtgctgagaatgaatgggcagaggatcggttaa.

Seqüência de aminoácidos da proteína SconC de *P. brasiliensis*.

MSAPENSKITLIPSDEPEDGRGIEVERVIIERSILIKNMLEDLGDSGEPPIPI
 NVNKAVLEKVIEWCEHHRNDPPTTNDDEDYDSRRKTTDIDEWDQKFMLVDQEML
 FEIILAANYLDIKALLDVGCKTVANLIKKGKSPDDIRKTFNIQNDFSEQEEAQI
 RAENewaEDR^X 170 aminoácidos.

Peso em Kdaltons calculado através da ferramenta “Peptide mass” fornecida pelo site do “ExPASy Proteomics Server” [http://ca.expasy.org/]. The ExPASy (Expert Protein Analysis System).

peptide masses from your sequence are:

[Theoretical pI: 4.39 / Mw (average mass): 19551.07 Kdaltons.

Identificação do principal domínio proteico da proteína SconC de *P. brasiliensis*.

A identificação do principal domínio protéico foi feita através da ferramenta InterProScan fornecida pelo site [http://www.ebi.ac.uk/InterProScan/]

SEQUENCE: SconC CRC64: AFB9290D0D702361 LENGTH: 169 aa			
InterPro IPR001232 Family	SKP1 component		
	PF01466		Skp1
	PF03931		Skp1_POZ
	SM00512		Skp1
	PS50130		SKP1_NT
	PS50131		SKP1_CT

SEQUENCE: SconC CRC64: AFB9290D0D702361 LENGTH: 169 aa				
InterPro IPR001232 Family	SKP1 component			
	PFAM	PF01466	Skp1	1.4999999999999998E-47 [91-168]T
	PFAM	PF03931	Skp1_POZ	3.1E-19 [7-72]T
	SMART	SM00512	Skp1	9.3000000000000001E-44 [6-118]T
	PROFILE	PS50130	SKP1_NT	22.707 [18-69]T
	PROFILE	PS50131	SKP1_CT	39.751 [89-166]T

IX- Apêndices

APÊNDICE A

Apêndice A – Publicação:

GOLDMAN, G.H., **MARQUES, E.R.**, RIBEIRO, D.C.D., BERNARDES, L.A.S., QUIAPIN, A. C., VITORELLI, P. M., SAVOLDI, M., SEMEGHINI, C.P., OLIVEIRA, R.C., NUNES, L.R., TRAVASSOS, L.R., PUCCIA, R., BATISTA, W.L., FERREIRA, L.E., MOREIRA, J.C., BOGOSSIAN, A.P., TEKAIA, F., NÓBREGA, M. P., NÓBREGA, F.G. AND GOLDMAN, M.H.S. EST analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: Identification of putative homologues of *Candida albicans* virulence/pathogenecity genes. Eukariotic Cell, v. 2(1), p. 34-48, fev 2003.

Expressed Sequence Tag Analysis of the Human Pathogen *Paracoccidioides brasiliensis* Yeast Phase: Identification of Putative Homologues of *Candida albicans* Virulence and Pathogenicity Genes

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Paracoccidioides brasiliensis, a thermodimorphic fungus, is the causative agent of the prevalent systemic mycosis in Latin America, paracoccidioidomycosis. We present here a survey of expressed genes in the yeast pathogenic phase of *P. brasiliensis*. We obtained 13,490 expressed sequence tags from both 5' and 3' ends. Clustering analysis yielded the partial sequences of 4,692 expressed genes that were functionally classified by similarity to known genes. We have identified several *Candida albicans* virulence and pathogenicity homologues in *P. brasiliensis*. Furthermore, we have analyzed the expression of some of these genes during the dimorphic yeast-mycelium-yeast transition by real-time quantitative reverse transcription-PCR. Clustering analysis of the mycelium-yeast transition revealed three groups: (i) RBT, hydrophobin, and isocitrate lyase; (ii) malate dehydrogenase, contigs Pb1067 and Pb1145, GPI, and alternative oxidase; and (iii) ubiquitin, delta-9-desaturase, HSP70, HSP82, and HSP104. The first two groups displayed high mRNA expression in the mycelial phase, whereas the third group showed higher mRNA expression in the yeast phase. Our results suggest the possible conservation of pathogenicity and virulence mechanisms among fungi, expand considerably gene identification in *P. brasiliensis*, and provide a broader basis for further progress in understanding its biological peculiarities.

Paracoccidioides brasiliensis, a thermodimorphic fungus, is the causative agent of the prevalent systemic mycosis in Latin America, paracoccidioidomycosis (PCM). Epidemiological data indicate a broad geographic distribution of this disease in Central and South America, extending from Mexico to Argentina (56). It is estimated that as many as ten million individuals could be infected with *P. brasiliensis*, acquired by the inhalation of airborne microconidia, which reach the pulmonary alveolar epithelium and then transform into the parasitic yeast form (52).

Pathogenicity appears to be intimately linked to the dimorphic transition since strains of *P. brasiliensis* (and also of *Histoplasma capsulatum* and *Blastomyces dermatitidis*) that are unable to transform into yeasts are not virulent (4, 37, 54, 56). Signaling pathways that control the morphological changes in *P. brasiliensis* are poorly understood, but the involvement of

both cyclic AMP (cAMP) and mitogen-activated protein kinase (MAPK) signal transduction pathways have been reported in other dimorphic fungi (6, 30). Exogenous cAMP inhibits *P. brasiliensis* yeast-to-mycelium (Y-M) transition, thus maintaining the pathogenic yeast form (3). This situation differs in *Candida albicans*, in which the Y-M transition is controlled by cAMP and exogenous cAMP stimulates pseudohyphae that are potentially able to invade mammalian cells (53).

The morphological change in *P. brasiliensis* is accompanied by documented changes in the cell wall composition, including migration and reorganization of the membrane lipids, especially glycosphingolipids (GLSs) (31, 61, 65). Furthermore, an increase in the chitin content is observed in the cell wall, followed by a change in the glucan polymer predominant anomery from β -1,3- to α -1,3-glucan as the fungus adopts the yeast form (56). The surface α -glucan may have a role as a protective layer against the host defense mechanisms because of the incapacity of phagocytic cells to digest α -1,3-glucan (55, 56).

The development of paracoccidioidomycosis depends on interactions between fungal and host components (22). The main antigenic component described in *P. brasiliensis* is gp43, an exocellular glycoprotein (57, 59) containing a single oligosac-

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charide chain (1, 12, 49); it elicits a strong humoral response and can be detected in the sera of PCM patients (39). The gp43 is a potential virulence factor as it binds murine laminin, resulting in increased pathogenicity of yeast cells (64). The fungus can also bind to other components of the extracellular matrix, such as fibronectin and collagen (40). Adhesion to host cells and mucosal surfaces is a crucial step in the establishment of *P. brasiliensis* infection. There is a correlation between adherence and virulence, as suggested by measuring fungal adhesion to cultured mammalian epithelial cells, with virulent strains having a greater capacity to adhere (22, 40). An extracellular serine-thiol protease has also been characterized in *P. brasiliensis*; this protease is a probable virulence factor since it can hydrolyze components of the basal membrane, such as fibronectin, laminin, collagen IV, and proteoglycans (9, 50, 51). This enzymatic activity could increase the capacity of *P. brasiliensis* to disseminate, perhaps acting in collaboration with gp43. It is also possible that sialoglycoconjugates exposed at the surface of *P. brasiliensis* cells may also modulate the interactions between fungi and the host tissue (58).

Given the relative lack of information about the *P. brasiliensis* genome and genes expressed in the yeast pathogenic phase, we have undertaken the first global sequence analysis of *P. brasiliensis* expressed genes. Our results reveal the presence of several *C. albicans* virulence and pathogenicity homologues in *P. brasiliensis*. We also analyzed the mRNA expression during the dimorphic transition yeast-mycelium-yeast (Y-M-Y) for some of the identified *P. brasiliensis* genes by real-time quantitative reverse transcription-PCR (RT-PCR). Our results support the importance of genomic analysis in human pathogenic fungi and point to a possible conservation of pathogenic and virulence mechanisms among them.

MATERIALS AND METHODS

RNA extraction and cDNA library construction. *P. brasiliensis* strain 18 (provided by Z. P. Camargo, UNIFESP, Sao Paulo, Brazil) was inoculated (10^6 viable yeast given intraperitoneally) into BALB/c mice. After 20 to 30 days of infection, the mice were sacrificed, and the spleens of these animals were plated as detailed by Pinto et al. (46) in order to obtain the CFU counts. Isolated colonies were expanded in slants of modified YPD medium (0.5% Bacto yeast extract, 0.5% casein peptone, 1.5% glucose; pH 6.3) for 10 days at 37°C. A liquid preculture (4 days, with shaking) was prepared from this growth in YPD (10 ml), and the fungus was then grown in 100 ml of fresh medium for three extra days. The cells were checked microscopically for homogeneity, precipitated by centrifugation ($1,800 \times g$ for 15 min), and immediately mixed with Trizol (Gibco-BRL) for RNA extraction according to the supplier's recommendations. To verify the RNA integrity, 20 µg of RNA from each treatment was fractionated in a 2.2 M formaldehyde–1.2% agarose gel, stained with ethidium bromide, and visualized with UV light. The presence of intact 28S and 18S rRNA bands was used as a criterion to verify if the RNA was intact. RNase-free DNase treatment was done in a final volume of 100 µl containing 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 1 µl of RNasin (40 U/µl; Promega), 10 µl of RNase-free DNase (1 U/µl; Promega or Life Technologies), 2.5 µl of dithiothreitol at 200 mM, and 10 µg of total RNA. The reaction was incubated at 37°C for 60 min and stopped by incubation at 70°C for 30 min. The absence of DNA contamination after the RNase-free DNase treatment was verified by PCR amplification of the *GP43* gene (12). Polyadenylated RNA was purified by using oligo(dT)-cellulose, and a unidirectional cDNA library was constructed in plasmid pCMVSPORT 6.0 by using SuperScript plasmid according to the manufacturer's instructions (Gibco-BRL). The number of clones of the library (transformed into *Escherichia coli* EMDH10B) was ca. 3×10^6 CFU, with an average insert size of 1.6 kb, and 100% of clones had inserts of >200 bp; 10,000 individual colonies were transferred to 110-by-96-well microtiter plates for storage at –80°C.

For the temperature-induced morphological switch in *P. brasiliensis*, mycelial and yeast cells growing in the early exponential phase were induced to undergo

morphological transformation by changing the temperature of incubation from 26 to 37°C. Samples were collected at 0, 5, 10, 24, 48, and 120 h, and from 37 to 26°C samples were collected at 0, 5, 10, 24, and 48 h.

Plasmid DNA extraction and sequencing. Bacterial clones were inoculated into CircleGrow (Bio 101) in a 96-well microtiter plate and incubated in a rotatory shaker at 37°C for 22 h. Plasmid DNA was prepared by standard alkaline lysis procedures and also by the boiling method of Marra et al. (36).

Single-run sequencing was done by using the dideoxy chain termination method, and dye termination chemistry (Applied Biosystems) and analysis was done on ABI 377 and 3100 fluorescence automated sequencers.

Data handling and analysis. A pipeline was built to analyze and assemble the *P. brasiliensis* expressed sequence tag (EST) sequences (The *P. brasiliensis* EST Genome Project is available online [http://143.107.203.68/est/default.html]). Sequences were automatically edited for each EST by using the programs Phred (16, 17), CAP3 (24), Consed (21), and Crossmatch from Phrap (P. Green, http://bozeman.mbt.washington.edu/phrap.html). The sequences were cleaned from the pCMVSPORT 6.0 vector, and ESTs with a minimum size of 300 bases and a Phred quality value of at least 20 were considered for further analysis. Edited sequences were clustered by using the CAP3 program (24). To identify potential homologues to the *P. brasiliensis* genes, the BLASTX and TBLASTX algorithms (2) were used to compare the predicted protein sequences with several databases (see below). The values presented for putative homologues derive from results obtained with sequences of known function as well as with those of unknown function. The database sequence matches (putative homologues) were divided into highly significant ($P \leq 10^{-19}$), moderately significant (1e-10 to 1e-19), weakly significant (1e-03 to 1e-10), and nonsignificant ($>1e-03$) classes (data not shown). When *P* values of $>10^{-3}$ were obtained, they were considered not statistically significant (no significant match).

The classification of functional groups used here was mainly based on that developed at The Institute for Genomic Research (TIGR; Rockville, Md. [http://www.tigr.org/docs/tiger-scripts/egad-scripts/role-report.sp]) (66). The following annotation strategy was used: the clusters were used as query sequences to search the *S. cerevisiae* databank (ftp://ftp.mips.gsf.de/yeast/ORF_SEQ_15_Oct2001/), Gproteome (a compilation of 217,319 entries that contain predicted protein sequences from 41 completely sequenced organisms [60]), and the *Neurospora crassa* and *Schizosaccharomyces pombe* predicted proteins (http://www-genome.wi.mit.edu/annotation/fungi/neurospora and ftp://ftp.sanger.ac.uk/pub/yeast/pombe/CDS_bases) by using the BLASTX program (2). A databank containing *Candida* spp., *H. capsulatum*, and *B. dermatitidis* (2,592 protein sequences) was compiled from the nonredundant protein database at the National Center for Biotechnology Information (NCBI) and BLASTX (2) was used to translate and compare the *P. brasiliensis* cluster query sequences. In addition, the clusters were translated and used as query sequences to search the translated *Pneumocystis carinii* (http://biology.uky.edu/Pc/), *Cryptococcus neoformans* (http://www.genome.ou.edu/cneo.html), *Aspergillus nidulans*, and *N. crassa* EST sequences (http://www.genome.ou.edu/fungal.html) by using the TBLASTX program (2). For all comparisons, BLOSUM62 was used as a matrix, except for GPROTEOME, for which PAM250 was used; for all of the other parameters, such as filtering, BLAST programs with their default settings were used.

PCR and RT-PCRs. All of the PCR and RT-PCRs were performed by using an ABI Prism 7700 sequence detection system (Perkin-Elmer/Applied Biosystems). TaqMan EZ RT-PCR kits (Applied Biosystems) were used for the RT-PCRs. The thermal cycling conditions comprised an initial step at 50°C for 2 min, followed by 30 min at 60°C for RT, 95°C for 5 min, and 40 cycles at 94°C for 20 s and 60°C for 1 min. TaqMan PCR reagent kits were used for the PCRs. The thermal cycling conditions comprised an initial step at 50°C for 2 min, followed by 10 min at 95°C, and 40 cycles at 95°C for 15 seconds and 60°C for 1 min.

In all experiments, appropriate negative controls containing no DNA template or RNA were subjected to the same procedure to exclude or detect any possible contamination or carryover. Each reaction was analyzed at least three times. All reactions were examined by agarose gel electrophoresis to confirm that only one PCR product was synthesized. The results were normalized by using the *C_T* values obtained for the α -tubulin and/or 18S ribosomal gene RNA amplifications run in the same plate. *C_T* is defined as the first amplification cycle in which fluorescence indicating PCR products becomes detectable. The α -tubulin and the 18S RNA are endogenous controls that were used to normalize the samples for differences in the amount of total RNA added to each cDNA reaction. In real-time PCR analysis, quantification is based on the threshold cycle, which is inversely proportional to the logarithm of the initial copy number (62). Real-time PCR was performed with each primer pair and its TaqMan probe by using known amounts of target obtained by serial dilutions of genomic DNA as a template over several orders of magnitude. The initial template amount used in parallel reactions was estimated by assuming a *P. brasiliensis* genome size of 30 Mb. A

FIG. 1. Classification of the 4,692 clusters of *P. brasiliensis* ESTs with BLASTX or TBLASTX E-values $\leq 10^{-3}$ are presented according to the classification developed at TIGR (66). Unclassified are ESTs that show similarity to a sequence with known function but do not fall into any of the classification schemes utilized; hypothetical proteins refer to sequences that show similarity to protein or DNA sequences to which no cellular role has yet been assigned; no match indicates new sequences with no significant similarity to protein or DNA sequences in the databases

linear relationship was obtained by plotting the threshold cycle against the logarithm of the known amount of the initial template. The equation of the line that best fitted the data was determined by regression analysis. The R^2 value was calculated for each data set to estimate the accuracy of the real-time PCR with TaqMan as a quantification method. The amounts of target copies contained in an unknown sample were determined by extrapolation from the linear regression of the standard curve obtained for each primer-TaqMan set (18). For a description of the primers and TaqMan fluorescent probes (Applied Biosystems) used here, see Table 1.

RESULTS

General strategy and data clustering. The EST approach is a rapid and relatively efficient method for quick gene discovery, which may assist studies involving organisms with little or no genetic research history available, as is the case with *P. brasiliensis* and other human fungal pathogens. A unidirectional cDNA library was constructed for the identification of genes expressed in the yeast phase of *P. brasiliensis*. M13 reverse and anchored oligo(dT) (see Materials and Methods) primers were used to generate 5' and 3' sequence information, respectively. We obtained 13,490 ESTs with a minimum length of 300 bases and a Phred quality value of at least 20 (16, 17). We sequenced from the 5' end 6,481 ESTs, whereas 7,009 were sequenced from the 3' end. Clustering by the CAP3 program (24) identified 5,121 singletons and 1,397 contigs. The 913 singleton pairs represented virtual clusters; since they were derived from both ends of the same cDNA clone, the final number of putative *P. brasiliensis* genes identified in our analyses corresponds to the partial sequences of 4,692 expressed genes.

Gene identification and functional categories. The nucleotide data set of clustered sequences was compared to several databases by using the BLASTX and TBLASTX programs (2) with a threshold E-value of $\leq 10^{-3}$. The clusters encoding putative protein sequences that show similarity to products in

our local databanks (see Material and Methods for a description) were classified into functional groups (Fig. 1). During this phase, expert validation was introduced by individual examination of the matches for significance and to assign a functional category (i.e., TIGR classification). The total number of clusters that could be assigned a cellular role was 1,095. Most of the known transcripts belong to housekeeping genes such as those involved in cell metabolism (e.g., amino acid and carbohydrate metabolism [8%]), cell structure (e.g., cell wall and cytoskeleton [5%]) and protein metabolism (4%). The remaining clusters either show similarity to hypothetical proteins (817 clusters) or have no significant similarity to any protein sequence in the databases (no matches, 2,780 clusters). Among the 817 clusters that show similarity to hypothetical proteins, we identified 633 clusters that only show similarity to fungal ESTs. A high percentage of clusters showed no matches with known protein sequences (59%). This most probably reflects the absence in the databases of a completely sequenced genome related to filamentous fungi. Thus, we decided to compare our 2,780 no-match sequences with the predicted proteins of the last version (version 3) of the *N. crassa* genome project (<http://www-genome.wi.mit.edu/annotation/fungi/neurospora>) and *S. pombe* (<ftp://ftp.sanger.ac.uk/pub/yeast/pombe/CDS.bases>) by using BLASTX (2). Among the 2,780 no-match clusters, we were able to identify 2,001 *P. brasiliensis* sequences that display significant similarity to *N. crassa* hypothetical proteins (509 of them also display significant similarity to *S. pombe* hypothetical proteins). Accordingly, the *P. brasiliensis* no-match sequences decreased to 779 sequences (17%), and the hypothetical proteins increased to 2,818 sequences (60%) (see Fig. 1). Thus, we found 17% putative *P. brasiliensis*-specific sequences. The 633 clusters that show similarity only to fungal ESTs plus the 2,001 *P. brasiliensis* sequences that display significant simi-

TABLE 1. List of primers and fluorescent probes used in this study

Primers and probes ^a	Sequence ^b	<i>P. brasiliensis</i> cluster ID ^c
Gpif Gpir PGpi	5'-CGATCTCTATGCCCCGCTACAC-3' 5'-AAGCCCAGGACGTTGGAGT-3' 5'-TET-CCGTCATCGATACCCTCGCCCCCT-TAMRA-3'	PbCC003-D08
H104f H104r PH104	5'-CGAAATCCATGCTCTGGCTC-3' 5'-TGTTTGGCAGCCTCGAGTC-3' 5'-TET-AAAAGGATGCGGCGTCGAAGGC'-TAMRA-3'	PbCC012-B06
H70f H70r PH70	5'-GACCACACCCTCGTTCGTTG-3' 5'-ACTTGATTTTTGGCGGCATC-3' 5'-6FAM-CTTCACAGACACCGAGCGTCTGATCGT-TAMRA-3'	PbCC006-G05
H82f H82r PH82	5'-ACCGCTGGTGCCGATATTT-3' 5'-GGCCACGAGGTAGGCAGAG-3' 5'-TET-TGATTGGTCAGTTTGGTGTGGCTTCT-TAMRA-3'	PbCC001-B11
Hydf Hydr Phyd	5'-GTCCTCGGCACTGTCATCG-3' 5'-AGGCTGAGTTTGGAGCACTGA-3' 5'-TET-CAAGGGCGGCAGCGTCGGT-TAMRA-3'	PbCC004-F01
Isof Isof Piso	5'-TGAAGTCCAGGCCGTCAAA-3' 5'-TGAACGGGCGCTTGGTATA-3' 5'-TET-AATGGTGGCAGGACTCAAGATGGCG-TAMRA-3'	PbCC005-H06
Malf Malr PMal	5'-CCGAGGGAGCCTGCTAGACT-3' 5'-CCCTTCCACCCATCGTTTT-3' 5'-VIC-CCTCCCAGAGACCAAGCACATCCG-TAMRA-3'	PbCC002-G09
Olef Oler POle	5'-CTTCCACCACGAGTTCCCA-3' 5'-ATTTGGTGGGTCGTAAGTGG-3' 5'-VIC-CTACCGCAACGCCATCGAATGGC-TAMRA-3'	PbCC002-E05
Oxif Oxir Poxi	5'-CGCACATCGAAATGCTGAGA-3' 5'-CAGCGCAACATACGAACGG-3' 5'-VIC-CTGGTCCGATTGGGTTCGCTTGG-TAMRA-3'	PbCC006-F12
RBf RBr PRB	5'-GCCGATGTTACGCGGAGAT-3' 5'-CCTTGGCGCTAACAAACACCAA-3' 5'-VIC-ATGCGCGATTCTCCTCGTGGGAT-TAMRA-3'	PbCC008-G03
Tubf Tubr Ptub	5'-TGGCCACTTTCTCTGTCGTTTC-3' 5'-CAGGGTGGCATTGTATGGCT-3' 5'-VIC-TTCGCCCAAAGTCTCCGACACCG-TAMRA-3'	PbCC002-A04
Ubif Ubir Pubi	5'-TCCTCCTGACCAGCAACGTC-3' 5'-ATCAGAAAGGGTGGCACC-3' 5'-VIC-TTTTCGCCGCAAGCAGTTGGAG-TAMRA-3'	PbCC003-E02
Ribf Ribr PRib	5'-CGGAGAGAGGGAGCCTGAGAA-3' 5'-GGGATTGGGTAATTTGCGC-3' 5'-6FAM-CGGCTACCACATCCAAGGAAGGCAG-TAMRA-3'	PbCC012-H11
1067f 1067r P1067	5'-TGTAATGCCCAATCGACACA-3' 5'-AATCGAAGGCATCTCGAATGA-3' 5'-VIC-TGGTCGCAGCACATGACAAGTTGC-TAMRA-3'	PbCC012-A11
1145f 1145r P1145	5'-ATCGCCGTTGCTGTCTCG-3' 5'-GCTGTTCACCGTAATTGCCAG-3' 5'-TET-CTTGCAGTCTCCGTCAGTGCCGCT-TAMRA-3'	PbCC012-H05

^a Grouped by cluster.^b Abbreviations: TET =, 6-carboxy-4,7,2',7'-tetrachlorofluorescein; 6-FAM, 6-carboxyfluorescein; VIC, trademark product from Applied Biosystems; TAMRA, 6-carboxy-*N,N,N',N'*-tetramethylrhodamine.^c ID, identification.

TABLE 2. *P. brasiliensis* clusters similar to *C. albicans* genes involved in virulence and pathogenicity

Gene group and <i>P. brasiliensis</i> cluster ID ^a	<i>C. albicans</i> homologue (NCBI protein accession no.)	Main feature and/or role	BLAST E-value/% identity/% similarity
Metabolic genes			
PbCC003-B07	<i>ADE2</i> (AAC49742)	Nucleotide synthesis	4e-21/58/77
PbCC006-G01	<i>HIS1</i> (P46586)	Amino acid synthesis	9e-57/61/73
PbCC002-G09	<i>MLS1</i> (AAF34695)	Malate synthase, glyoxylate cycle	8e-30/56/69
PbCC009-C01	<i>ICL1</i> (AAF34690)	Isocitrate lyase, glyoxylate cycle	4e-66/74/85
PbCC015-E02	<i>HEM3</i> (094048)	Hemosynthesis	5e-16/39/60
PbCC001-E02			1e-14/41/63
PbCS033-B09	<i>PLB1</i> (AAC72296)	Phospholipase	1e-09/43/55
Cell wall genes			
PbCC011-D01	<i>CHS2</i> (S20538)	Chitin synthase; cell wall synthesis	4e-23/51/73
PbCC013-B07	<i>CHS3</i> (P30573)		2e-21/72/88
PbCS003-C01			3e-31/43/60
PbCS008-H07			5e-30/43/51
PbCC004-H02	<i>HWP1</i> (P46593)	Transglutaminase, adhesion	2e-08/39/50
PbCS001-B02	<i>INT1</i> (P53705)	Integrin, adhesion	1e-49/33/56
PbCS006-A05	<i>MNT1</i> (CAA67930)	Mannosyl transferase	1e-31/55/80
PbCC003-D08	<i>PHR1</i> (AAG16995)	Glycosidase GPI-anchored; cell wall structure	2e-70/49/68
PbCC013-H06	<i>PHR2</i> (AAG16996)		2e-10/35/54
PbCS011-F12	<i>PMT1</i> (AAC31119)	Mannosyl transferase	1e-07/31/48
PbCC004-C02	<i>RBT5</i> (AAG09790)	Secreted cell wall protein, GPI anchor	1e-07/30/47
PbCC008-G03			4e-16/31/45
PbCC002-C04	<i>BGL2</i> (AAA21151)	β -Glucosyltransferase; cell wall synthesis	1e-60/41/57
Signal transduction genes			
PbCS022-F02	<i>CST20</i> (AAB38875)	MEKK kinase hyphal formation	2e-77/77/88
PbCC004-A02	<i>CPP1</i> (P43078)	Phosphatase, hyphal formation	6e-04/44/64
PbCC011-F09	<i>CEK1</i> (A47211)	MAPK, hyphal formation	8e-04/29/48
PbCS023-G05	Protein kinase A (AAF64072)	Hyphal formation	3e-15/43/56
PbCC010-A10	<i>CDC42</i> (AAB69764)	Cell division control protein, hyphal formation	1e-15/64/76
PbCS021-A08	<i>GEF</i> (Q9UQX7)	Ras-like protein, hyphal formation	4e-21/33/54

^a ID, identification.

larity with *N. crassa* and *S. pombe* hypothetical proteins will result in 2,634 *P. brasiliensis* sequences that display similarity only with fungal proteins (which correspond to 56% of the *P. brasiliensis* sequences). The database sequence matches (putative homologs) were divided into highly significant ($P \leq 10^{-19}$ [1,242 ESTs]), moderately significant ($1e-10$ to $1e-19$ [466 ESTs]), weakly significant ($1e-03$ to $1e-10$ [2,195 ESTs]), and not significant ($>1e-03$ [789 ESTs]) classes. The complete list of clusters classified into functional groups is available online (<http://143.107.203.68/est/default.html>).

Identification of putative virulence and pathogenicity genes.

In an attempt to identify additional putative virulence and pathogenicity factors in our *P. brasiliensis* database, we performed a direct BLAST comparison with a collection of about 60 genes that proved to be involved in virulence and pathogenicity in *C. albicans*, the best-studied human pathogenic fungus that displays a dimorphic behavior (7, 15, 23, 42, 44, 45). Sequences from two other dimorphic fungi (*H. capsulatum* and *B. dermatitidis*) have also been used in these comparisons (5, 33, 68). However, although the latter are phylogenetically closer to *P. brasiliensis*, very few genetic determinants of virulence and pathogenicity have been identified in these organisms (5, 33, 68). These analyses allowed us to find 26 *P. brasiliensis* clusters that show *C. albicans* putative homologues (Table 2). These genes were divided into three groups: (i)

metabolic, (ii) cell wall and adhesion, and (iii) transduction signal.

The metabolic genes identified were *ADE2*, *HIS1*, *HEM3*, *MLS1*, *ICL1*, and *PLB1* (Table 2). These genes have already been linked to fungal virulence because the homozygous null strains are attenuated in animal models of candidiasis (42).

Genes involved in the synthesis of the cell wall and in adhesion, such as *CHS2*, *CHS3*, and *BGL2*, were also identified (Table 2). We also identified several homologues that are involved directly or indirectly in adhesion, including *HWP1*, *INT1*, *MNT1*, *PHR1*, *PHR2*, *PMT1*, and *RBT5* (Table 2). Several genes encoding proteins involved in the signal transduction pathway and implicated in the dimorphic switch were also identified (Table 2). We could recognize several factors from the MAPK pathway in *P. brasiliensis*, such as *CST20*, *CPP1*, *CEK1*, and *CDC42*. One of the subunits of protein kinase A and GEF, involved in the cAMP pathway, were also found (Table 2).

In addition to the genes mentioned above, we were able to identify several genes that could play a role either in temperature-induced dimorphic transition and/or in the infection process (for the DNA sequences and annotation, see <http://143.107.203.68/est/default.html>). During the M-Y transition there is an increase in the chitin content in the cell wall (4). Accordingly, we identified a series of chitin synthases (Table 2), along with other genes involved in chitin synthesis, such as

TABLE 3. *P. brasiliensis* clusters that could play a role either in temperature-induced dimorphic transition or in the infection process

Gene or protein type and <i>P. brasiliensis</i> cluster ID ^a	Homologue (NCBI protein accession no.)	Main feature and/or role	BLAST E-value/ % identity/ % similarity
Cell wall genes			
PbCC011-D01	<i>C. albicans</i> (BAA19847)	Chitin synthase regulatory factor	4e-23/51/73
PbCS022-H12	<i>S. cerevisiae</i> CHS6 (NP_012436)	Chitin biosynthesis protein	4e-04/30/49
PbCC002-F03	<i>H. capsulatum</i> (AAG41982)	Chitinase	4e-20/39/50
PbCC012-F03	<i>S. cerevisiae</i> ECM27 (NP_012640)	Protein involved in the cell wall biogenesis and architecture	6e-09/46/71
Heat shock proteins			
PbCC003-E08	<i>S. cerevisiae</i> SIS1 (NP_014391)	Induced by heat shock	5e-07/71/83
PbCC004-A04	<i>S. cerevisiae</i> HFM1 (NP_010978.1)	Heat shock - inducible inhibitor of cell growth	7e-27/48/66
PbCC003-G05	<i>S. cerevisiae</i> STI1 (NP_014670)	Induced by heat shock	2e-23/52/65
Signal transduction genes			
PbCC002-D10	<i>C. albicans</i> PKC1 (P43057)	Protein kinase C	3e-25/55/75
PbCC011-H05	<i>H. capsulatum</i> (AAC27509)	Calmodulin	3e-27/39/62
PbCS011-H09	<i>S. cerevisiae</i> VTC1 (NP_010995)	Protein that acts as a negative regulator of the CDC42 protein	2e-21/68/80
Miscellaneous			
PbCC006-H02	<i>M. grisea</i> (AAK73019)	Ceramide glucosyl synthase	8e-49/48/66
PbCC028-E03	<i>S. cerevisiae</i> YCF1 (NP_010419)	Glutathione S-conjugate vacuolar transporter	2e-14/60/75

^a ID, identification.

chitin synthase regulatory factor, chitin biosynthesis protein, and chitinase (Table 3). We could also recognize a gene that encodes a protein involved in cell wall biogenesis and architecture whose regulation could play a role during the M-Y transition (Table 3). Genes encoding proteins that are induced upon and/or are important for the heat shock response were also recognized. Besides several heat shock proteins (three of which are listed in Table 4), we identified the *S. cerevisiae* *SIS1* homologue, the *S. cerevisiae* *HFM1* homologue that encodes the heat shock-inducible inhibitor of cell growth, and the *S. cerevisiae* *STI1* homologue (Table 3).

Another aspect of the dimorphic switch in *P. brasiliensis* is the change in the composition of the cell membrane lipids. There are documented differences in the composition of GLSs in the cell membrane (31, 61) in the dimorphic transition. GLSs are synthesized from glucosylceramides (GlcCer), and the biosynthesis of GlcCer is catalyzed by glucosylceramide synthase (29). We identified the *P. brasiliensis* ceramide glucosyl synthase homologue (Table 3).

Our knowledge about signal transduction pathways in *P. brasiliensis* is very limited compared to other fungal pathogens. In addition to the genes mentioned in Table 2, we were able to identify the protein kinase C homologue, a calmodulin gene (Table 3), and nine and four genes encoding putative serine-threonine kinases and phosphatases, respectively, with high identity to their respective homologues (data not shown). In addition, we have also seen the *S. cerevisiae* *VTC1* homologue, which encodes a protein that acts as a negative regulator of the *CDC42* protein (Table 3).

Multidrug resistance is becoming an important issue in fungal infections. Several genetic determinants of drug resistance were identified, and most of them encode transporters, mainly of the ATP-binding (ABC) cassette family (for a review, see reference 67). Based on sequence identity, we could identify 90 transporters in our project; 11 of these belong to the family of ABC transporters, whereas one of them is a major facilitator

superfamily transporter. An interesting representative of the ABC family was the cluster PbCS028-E03, which encodes the homologue of the *S. cerevisiae* *YCF1* glutathione S-conjugate vacuolar transporter (Table 3).

Expression analysis of some of the *P. brasiliensis* genes during dimorphic transition. The morphological and functional transition in *P. brasiliensis*, in contrast to other dimorphic fungi such as *C. albicans* that depend on more complex stimuli (15), can be induced by simply shifting the incubation temperatures from 26 to 37°C. To verify the mRNA expression of some of the *C. albicans* homologues and some other selected *P. brasiliensis* genes (see Tables 2 and 4) during dimorphic transition, we isolated RNA from fungi undergoing M-Y and Y-M dimorphic transitions as described in Materials and Methods. As observed in Fig. 2A, there is a progressive transformation of mycelia to spherical or oval yeast cells after 5 to 10 h at 37°C,

TABLE 4. *P. brasiliensis* contigs that were used for the design of TaqMan probes

ID cluster ^a	Contig (BLAST E-value/% identity/ % similarity)
PbCC003-E02	UBI, polyubiquitin <i>C. albicans</i> (e-162/99/99)
PbCC006-F12	OXI, alternative oxidase <i>H. capsulatum</i> (e-151/73/81)
PbCC004-F01	Hydro, hydrophobin precursor <i>A. nidulans</i> (5e-10/40/70)
PbCC008-G03	RBT, <i>RBT5</i> <i>C. albicans</i> (4e-16/31/45)
PbCC006-G05	HSP70, Hsp70 <i>C. albicans</i> (0.0/77/85)
PbCC005-F04	HSP82, Hsp82 <i>H. capsulatum</i> (4e-87/68/72)
PbCC012-B06	HSP104, Hsp104 <i>C. albicans</i> (0.0/55/73)
PbCC003-D08	GPI, <i>PHR1</i> glycosidase GPI-anchored <i>C. albicans</i> (2e-70/49/68)
PbCC005-H06	ISSO, <i>ICL1</i> <i>C. albicans</i> Isocitrate lyase (1e-33/52/68)
PbCC002-G09	MAL, <i>MLS1</i> <i>C. albicans</i> (1e-29/56/69)
PbCC011-G03	OLE1, <i>OLE1</i> <i>S. cerevisiae</i> delta-9-desaturase (0.0/87/91)
PbCC012-A11	Pb1067, unknown
PbCC012-H05	Pb1145, unknown

^a ID, identification.

FIG. 2. Temperature-induced morphological switch in *P. brasiliensis*. Mycelial and yeast cells growing in the early exponential phase were induced to undergo morphological transformation by changing the temperature of incubation. (A) M-Y transition at 26 to 37°C for 0, 5, 10, 24, and 48 h. (B) Y-M transition at 37 to 26°C for 0, 5, 10, 24, and 48 h. Magnifications: the photomicrographs are reproduced at $\times 13$ for 0, 5, and 10 h in the M-Y transition and at $\times 26$ for 24 and 48 h in the M-Y transition, and are reproduced at $\times 26$ for 0, 5, 10, 24, and 48 h in the Y-M transition. The bar at the bottom of each microphotograph represents 10 μm .

and this reaches >50% transformation after 48 and 120 h. Yeast cells start forming pseudohyphae after 5 h of incubation at 26°C; the mycelial transition progresses until reaching >50% transformation after 48 h of incubation at 26°C (Fig. 2B, lower panel).

Real-time RT-PCR with fluorescent TaqMan probes was used to quantify the relative transcript levels of 13 *P. brasiliensis* genes (Table 4). The primers and fluorescent probes designed are shown in Table 1. All of the *P. brasiliensis* RT-PCR products yielded a single band with the expected size, as visualized on agarose gels (data not shown). Figure 3 shows the amplification plots and the respective standard curves for the α -tubulin gene (PbCC004-F01). By using these fluorescent probes, we were able to detect between 1 and 10 copies of each gene. No PCR products were detected in any negative control samples that contained all reagents except for the *P. brasiliensis* genomic DNA, even after 40 rounds of amplification (data not shown). Since there is no ideal control for gene expression, we first compared the α -tubulin and the 18S ribosomal probes as normalizers for the expression of these genes. We have seen no difference with either normalizer. However, we cannot completely exclude the possibility that the α -tubulin and 18S ribo-

somal gene expression are variable during the transition. Accordingly, the calibrator gene used in the expression experiments was the α -tubulin gene (data not shown).

The polyubiquitin gene is induced at transcriptional level in several organisms upon heat shock stress (47). The *P. brasiliensis* *UBI* gene (contig PbCC003-E02) showed an increase of approximately twofold after 5 h, followed by constant levels that decreased after 48 h to the initial levels (Fig. 4A, right panel). In contrast, during the Y-M transition there was a fivefold decrease of mRNA at 5 and 10 h, followed by a steady increase until the initial mRNA levels of the yeast phase at time zero were reached (Fig. 4A, left panel).

To probe mitochondrial gene expression during the transition, we decided to verify the quantitative expression of the *P. brasiliensis* *OXI* gene (contig PbCC006-F12), the homologue of the alternative oxidase. In the M-Y transition, there was a twofold increase of expression during the first 5 h that decreased after 10 to 24 h, followed by a fourfold decrease after 120 h (Fig. 4B, right panel); in the Y-M transition there was an increase of about fivefold after 48 h (Fig. 4B, left panel).

We have chosen two genes, *Pb1067* (contig PbCC012-A11) and *Pb1145* (contig PbCC012-H05) that encode unique pro-

FIG. 3. Amplification plot (A) and the respective standard curve (B) for the α -tubulin gene from *P. brasiliensis*. The upper and lower panels in A and B correspond to the amplification plot and the standard curve for the α -tubulin gene from *P. brasiliensis*. The amplification plot corresponds to PCRs carried out with increasing amounts of genomic DNA, from left to right, 10^7 (σ), 10^6 ($\times$), 10^5 (λ), 10^4 (Θ), 10^3 (ψ), 10^2 ($+$), and 10^1 ($-$) copies (based on an assumed genome size of 30 Mb). The DRn (Δ Rn) is the normalized reporter signal minus the baseline established in the first few cycles of PCR. C_t is defined as the first amplification cycle at which fluorescence indicating PCR products becomes detectable. The data from the amplification plot were used for the construction of the standard curves (i.e., C_t values plotted against the logarithm of the DNA copy number). A linear relationship was obtained by plotting the resulting threshold cycle values (C_t) against the logarithm of the initial template amount. The best-fit line was determined via linear regression analysis. The results are the averages of four determinations. For each gene used for quantitative RT-PCR analyses, a similar amplification plot and standard curve was established.

teins and were classified in the no-match category. In the M-Y transition, the *Pb1067* gene showed a constant mRNA expression after 5 h, increasing ca. 50% after 10 h and decreasing dramatically (25-fold) after 120 h (Fig. 4C, left panel); in the Y-M transition, there was an initial fivefold decrease after 5 h, followed by an eightfold increase (Fig. 4C, right panel). The *Pb1145* gene had an initial increase in the mRNA expression at the first 10 h, followed by a 30-fold decrease after 120 h in the M-Y transition (Fig. 4D, right panel); in the Y-M transition, there was an almost undetectable mRNA expression in the yeast phase that reached a 250-fold increase after 48 h (Fig. 4D, left panel). These results indicate that these two genes are predominantly expressed in the mycelial state.

The *P. brasiliensis* *Hydro* gene (contig PbCC004-F01) showed high similarity with the hydrophobin precursor of *A. nidulans*. The *P. brasiliensis* hydrophobin homologue displayed a decrease of ca. 25- to 30-fold in the mRNA expression after 48 to 120 h in the M-Y transition, whereas there was an increase of ca. 35-fold in the Y-M transition (Fig. 4E, right and

left panels). These results indicate the *Hydro* gene is preferentially expressed in the mycelial state.

We verified the quantitative mRNA expression of the *P. brasiliensis* HSP70, HSP82, and HSP104 genes (Fig. 4F to H). These three genes showed a similar mRNA expression pattern in both transitions. In the M-Y transition, there was an increase of about four to fivefold at 5 h, followed by constant levels of expression and a decrease after 48 and 120 h (Fig. 4F to H, right panels). In the three genes, there was a three- to fourfold decrease in the mRNA expression during the Y-M transition (Fig. 4F to H, left panels). Our results confirm those of Da Silva et al. (13), who had shown that the HSP70 gene is differentially expressed in the transition, with a progressive increase in HSP70 expression during the period of conversion from mycelium to yeast.

We have also chosen two genes that have *C. albicans* homologues involved in adhesion, RBT and GPI (contigs PbCC008-G03 and PbCC003-D08, respectively), a GPI-anchored secreted cell wall protein, and a GPI-anchored glycosidase,

FIG. 4. Quantitation of the mRNA expression of selected genes of *P. brasiliensis* during the M-Y-M transition. The measured quantity of each *P. brasiliensis* gene mRNA in each of the treated samples was normalized by using the C_T values obtained for the α -tubulin RNA amplifications run in the same plate. The relative quantitation of each *P. brasiliensis* gene and α -tubulin gene expression was determined by a standard curve (i.e., C_t values plotted against the logarithm of the DNA copy number). The values represent the number of copies of the cDNAs of each *P. brasiliensis* gene divided by the number of copies of the cDNAs of the α -tubulin gene. The left and right panels represent the M-Y and the Y-M transitions, respectively. (A) UBI gene, ubiquitin; (B) OXI gene, alternative oxidase; (C) contig Pb1067; (D) contig Pb1145; (E) Hydro gene, hydrophobin gene; (F) HSP70 gene; (G) HSP82 gene; (H) HSP104 gene; (I) RBT gene; (J) GPI gene; (K) ISSO gene, isocitrate lyase; (L) MAL gene, malate dehydrogenase; (M) OLE1 gene, delta-9-desaturase. The results are the averages of three repetitions.

FIG. 4—Continued.

linked to the cell wall structure, respectively. Two genes, ISSO and MAL, that encode putative *C. albicans* homologues from genes of the glyoxylate cycle, isocitrate lyase, ISSO, and malate synthase, MAL (contigs PbCC005-H06 and PbCC002-G09, respectively), and the *S. cerevisiae* *OLE1* gene were also selected for further studies.

The *P. brasiliensis* RBT gene showed a 10-fold decrease during the M-Y transition (three times during the first five hours). As for the Y-M transition, we observed a fivefold increase in the mRNA expression during the 5- to 10-h period (Fig. 4I, right and left panels). The GPI gene showed a constant mRNA expression during the first 24 h of M-Y transition (Fig. 4J, left panel), decreasing two- and fourfold after 48 and 120 h, respectively. In the Y-M transition, the GPI mRNA

expression increased ca. 22-fold after 48 h (Fig. 4J, right panel). The ISSO gene showed constant mRNA expression levels in the M-Y transition (Fig. 4K, right panel), while its expression dropped ~7-fold at 5 to 10 h, followed by an increase of 5- and 7-fold at 24 and 48 h, respectively (Fig. 4K, left panel). The MAL gene showed a twofold increase after 5 h, which was kept constant after 24 h and decreased about threefold after 120 h of the M-Y transition; in the Y-M transition, the mRNA levels gradually increased until they reached an approximately sixfold induction after 48 h (Fig. 4K, right panel).

The *P. brasiliensis* *OLE1* gene showed increased mRNA expression in the M-Y transition (about threefold from 5 to 120 h) and constant mRNA expression during the Y-M transition (Fig. 4 M, right and left panels).

FIG. 4—Continued.

Hierarchical clustering is a method of analysis that brings out patterns of gene expression, by grouping genes with similar expression profiles. We decided to cluster the mRNA expression values of *P. brasiliensis* genes during the dimorphic transition Y-M-Y. Thus, mRNA expression values during the M-Y transition (Fig. 5A) clustered three different groups according to their mRNA expression pattern: (i) RBT, Hydro, and ISSO; (ii) MAL, Pb1067, Pb1145, GPI, and OXI; and (iii) UBI, OLE1, HSP70, HSP82, and HSP104. The first two groups

displayed high mRNA expression in the mycelial phase, and a reduced expression during the M-Y transition, whereas the third group had lower mRNA expression in the mycelial phase, with increased expression during the M-Y transition (Fig. 4 and 5A).

Gene clustering of the mRNA expression for the Y-M transition yielded two large groups: (i) HSP82, HSP104, HSP70, ISSO, UBI, Pb1067, and RBT and (ii) OLE1, MAL, OXI, GPI, Hydro, and Pb1145. The first group displayed high mRNA

FIG. 5. Hierarchical clustering showing the gene expression profiles of 13 different *P. brasiliensis* genes during the M-Y (A) and Y-M (B) transitions (Fig. 4). In both experiments, relative levels of each transcript were monitored by real-time RT-PCR with mRNA extracted at the beginning of the differentiation process ($T = 0$) and also at different time points after the temperature-induced switch. These values were used to calculate the relative expression ratios against time $T = 0$. These ratios were log transformed and submitted to a hierarchical clustering algorithm by using the default parameters of the software CLUSTER, kindly provided by M. Eisen and available at <http://www.microarrays.org/software.html>. In both cases, as shown by the scales in the right, a color scheme was used to designate genes that were downregulated (blue) or upregulated (red). The clustering, along with the resulting dendrogram, was displayed by using TREEVIEW, also available at the same URL mentioned above (14).

expression in the yeast state, with lowered expression during the Y-M transition, whereas the second group had lower mRNA expression in the yeast state, with increased expression during the Y-M transition (Fig. 4 and 5B).

DISCUSSION

We have identified in this study the partial sequences of 4,692 nonredundant expressed genes from *P. brasiliensis* yeast phase. By using pulsed-field gel electrophoresis, Cano et al. (8) and Montoya et al. (41) showed that *P. brasiliensis* has between four or five chromosomes, varying in size from 2 to 10 Mb, and the genome size was estimated to be 23 to 20 Mb, suggesting the presence of 10,000 to 15,000 genes. Thus, our work has provided information of ca. 30 to 50% of *P. brasiliensis* genes. Considering that the NCBI nonredundant database contains only over a hundred expressed sequences from *P. brasiliensis*,

our data represent a significant increment in the number of known *P. brasiliensis* genes.

Gene discovery and categorization. Gene discovery in eukaryotes has been usually achieved by sequencing cDNA libraries due to the large amounts of noncoding DNA. The computational approach of identifying genes relies on similarity searches, and the limitation of this approach is apparent when phylum- or species-specific new genes are encountered. In our survey ca. 60% of putative new genes code for hypothetical proteins with no function known but with similarity to described proteins of other organisms. The vast majority of these putative new genes has matches to fungal proteins and is thus phylum specific “maverick” proteins. In the no match category the *P. brasiliensis* specific new sequences classified as unknown represent 17% of the total. The other putative proteins with functions suggested by similarity represent 23% as-

signed to seven broad functional categories (Fig. 1). The genes found represent a very substantial increment in the described *P. brasiliensis* genes.

Virulence and pathogenicity genes. Using *C. albicans* genes already assigned a role in virulence and pathogenicity, we detected a number of *P. brasiliensis* candidates (Table 2). The metabolic genes listed have to be viewed with caution since there is disagreement about including factors associated with metabolic pathways as virulence factors (7, 23). We decided, however, to include metabolic genes as virulence determinants since it was reasoned that there are several other metabolic genes that, when deleted, do not cause reduced virulence in the pathogen. The genes indicated are significant in animal models of fungal disease (43). In *C. albicans*, phagocytosis upregulates the principal enzymes of the glyoxylate cycle, isocitrate lyase (*ICL1*) and malate synthase (*MLS1*). *C. albicans* mutants lacking *ICL1* are markedly less virulent in mice than the wild type (32). Deletion of the *PLB1* gene also results in reduced virulence (28).

Some cell wall biosynthesis and adhesion genes were also found. There is no universal consensus about the causal connection between reduced virulence upon deletion of genes, such as *CHS2*, *CHS3*, and *BGL2*, that encode biosynthetic cell wall enzymes. However, Navarro-Garcia et al. (42) point out that subtle changes in the cell wall could modify adhesion and recognition by the immunological system of the host, thus leading to altered virulence. The putative adhesion genes identified, when deleted in *C. albicans*, resulted in reduced virulence as determined in standard survival curves (42). This finding opens the possibility that conserved mechanisms of adhesion during host infection could be present in *P. brasiliensis* and *C. albicans*.

Signal transduction pathway genes have been implicated in the dimorphic switch and some were found (Table 2). There is a great deal of information about signaling pathways involved in controlling morphological switching, mostly provided by studies about pseudohyphal growth in *S. cerevisiae* (19). These studies have revealed that the signaling pathways are controlled by both cAMP and MAPK pathways (3). The identification of these genes in *P. brasiliensis* suggests that these pathways could be operating in this fungus, probably controlling the morphological switch.

Nonetheless, in spite of all of the information so far obtained, efficient transformation and inactivation systems are yet to be developed for *P. brasiliensis* and, therefore, the unequivocal involvement of these genes in dimorphic transition and/or virulence will require the appropriate experimental demonstration.

Expression analysis of selected *P. brasiliensis* genes during the dimorphic transition. To obtain more accurate data on gene expression in *P. brasiliensis*, we used real-time RT-PCR assays. This approach is preferable over conventional Northern blot analysis because of the narrower linear range associated with radioactively labeled probes. This approach has been successfully used to characterize gene expression of sigma factor genes in *Mycobacterium tuberculosis* (34) and, more recently, in the quantitative expression of ABC transporter encoding genes in *A. nidulans* (48).

As noted by Lambowitz et al. (27), the morphological tran-

sition may be viewed as a heat shock response, followed by cellular adaptation to higher temperatures. Thus, the transition from 25 to 37°C in *H. capsulatum* is accompanied by marked changes in metabolic processes, such as respiration, cysteine metabolism, and rapid decline in intracellular ATP levels, due to uncoupling of oxidative phosphorylation (35). This is possible, because the mitochondria of fungi possess an alternative form of respiration in addition to the cytochrome pathway, which is resistant to cyanide and is mediated by alternative oxidase, an enzyme that transfers electrons from reduced ubiquinone to molecular oxygen (for a review, see reference 63). When electrons produced from the oxidation of NADH flow through the alternative pathway, two of the three sites of energy conservation are bypassed and the level of ATP is decreased. The alternative oxidase expression in *P. brasiliensis* was shown to parallel the behavior of the enzyme in *H. capsulatum*, where the cytochrome system and the alternative oxidase decrease in parallel over the first 24 to 40 h during the M-Y transition (35). The heat shock proteins investigated (HSP70, HSP82 and HSP90) have similar patterns of expression in the dimorphic transition with increased expression during the M-Y transition. The pattern of expression of the polyubiquitin gene suggests that the UBI gene product be directly involved in the increased rate of protein turnover that possibly exists particularly at the M-Y transition. They are certainly important factors in the stabilization of proteins necessary for the morphological transition at a higher temperature.

The RBT-, GPI-, ISSO-, MAL-, and OLE1-like genes were analyzed in their expression since the first four genes have already been shown to be required for fungal virulence (32, 42), whereas the *OLE1* encodes the delta-9-desaturase gene, which is a key enzyme involved in regulating membrane fluidity in animal cells and microorganisms (20). The behavior of the *OLE1* gene is marked by increased expression at the onset of the mycelium-to-yeast phase and a more constant expression in the Y-M transition. The fine regulation of this enzyme is believed to be responsible for major adjustments in the membrane composition in response to temperature or nutritional changes (35, 65). The *H. capsulatum* *OLE1* is expressed in the yeast morphotype of all *H. capsulatum* strains examined but is rendered transcriptionally silent in the mycelia of some but not all strains (20). Evidence suggests that cells exposure to an abrupt increase in temperature causes their membranes to undergo a rapid decrease in molecular order (i.e., fluidity increases) (65). Chatterjee et al. (11) showed that the percentage of cellular unsaturated fatty acids increased when *S. cerevisiae* cells grown at 25°C were shifted to 37°C. Carratù et al. (10) complemented an *S. cerevisiae* mutant that has a disruption in its *OLE1* gene with constructs that expressed the *H. capsulatum* *OLE1* coding region under the control of different promoters. The manipulation of the expression of the *H. capsulatum* *OLE1* gene altered the fatty acid composition of the cell membrane, and the different saturated and unsaturated fatty acid ratios had an important effect on *HSP70* and *HSP82* transcription. The authors of that study proposed that changes in the membrane order are an important factor in determining heat shock gene expression.

The behavior of two hypothetical and apparently species-specific genes, *Pb1067* and *Pb1145*, revealed a predominant expression in the mycelial state, a behavior analogous to that

exhibited by the hydrophobin gene. Fungal hydrophobins are secreted proteins that respond to the external environment. They are normally abundant proteins that are found in aerial hyphae, on the surface of fungal spores and infection structures, or on the surface of fruiting bodies (for a review, see reference 25). Hydrophobins have already been identified in plant pathogenic fungi (26) and in the human pathogen *A. fumigatus* (43).

The hierarchical clustering during the M-Y transition revealed three groups: (i) RBT, Hydro, and ISSO; (ii) MAL, Pb1067, Pb1145, GPI, and OXI; and (iii) UBI, OLE1, HSP70, HSP82, and HSP104. Although groups i and ii suggest that these genes are mainly expressed in the mycelial phase, group iii supports the hypothesis that changes in membrane order and ability to monitor protein folding and degradation are important factors in determining the dynamics of entry into the yeast phase, since *P. brasiliensis* OLE1, HSP70, HSP82, HSP104, and UBI genes display the same expression profile. Expression clustering at the Y-M transition resulted in two groups: (i) HSP82, HSP104, HSP70, ISSO, UBI, Pb1067, and RBT and (ii) OLE1, MAL, OXI, GPI, Hydro, and Pb1145. Most of the genes showed an inverse pattern of clustering in the two transitions (Fig. 5).

The correlation between the expression of specific genes in the yeast form and the morphological phase is complicated by the fact that a temperature shift is used to promote the dimorphic transition. Dissociation of temperature shift responses from morphogenesis in dimorphic fungi was achieved in *Sporothrix schenckii* in a study seeking to correlate certain polysaccharide structures with the Y-M transition (38). Yeast cells were independently obtained either by temperature shift to 37°C or by growing the cells in a synthetic medium with ammonium carbonate in tightened closed flasks with shaking at room temperature. Both cultures yielded yeasts with identical morphology, which synthesized identical polysaccharide structures that differed from those synthesized by the mycelium phase also grown at room temperature. In *P. brasiliensis* no attempts have yet been made to obtain the yeast phase at room temperature and therefore follow the expression of specific genes in cultures that have not been submitted to a temperature shift.

Overall, the results presented herein show, to our knowledge, the first case in which a high-throughput EST analysis was used to examine gene expression in the pathogenic state of *P. brasiliensis*. Classical genetic tools, such as DNA-mediated transformation and modulation of gene expression either by deletion or replacement or inactivation by RNA interference or antisense RNA have not been developed yet for *P. brasiliensis*. Consequently, this type of genomic approach, coupled with transcriptional profiling by microarrays and proteomics may represent an efficient alternative to identify genes involved in virulence or pathogenicity and provide a wider platform for a better understanding of *P. brasiliensis* biology and the role of several genes during the dimorphic transition and the onset of virulence and pathogenicity in PCM.

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APÊNDICE B

Apêndice B – Publicação:

MARQUES. ER, FERREIRA. ME, DRUMMOND. RD, FELIX. JM, MENOSSI. M, SAVOLDI. M, TRAVASSOS. LR, PUCCIA. R, BATISTA. WL, CARVALHO. KC, GOLDMAN. MH, GOLDMAN. GH. Identification of genes preferentially expressed in the pathogenic yeast phase of *Paracoccidioides brasiliensis*, using suppression subtraction hybridization and differential macroarray analysis. **Mol Genet Genomics**. 2004 Jul;271(6):667-77. Epub 2004 May 8.

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Identification of genes preferentially expressed in the pathogenic yeast phase of *Paracoccidioides brasiliensis*, using suppression subtraction hybridization and differential macroarray analysis

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Abstract *Paracoccidioides brasiliensis*, a thermomorphing fungus, is the causative agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in Latin America. Pathogenicity appears to be intimately related to the dimorphic transition from the hyphal to the yeast form, which is induced by a shift from environmental temperature to the temperature of the mammalian host. Little information is available on the *P. brasiliensis* genes that are necessary during the pathogenic phase. We have therefore undertaken Suppression Subtraction Hybridization (SSH) and macroarray analyses with the aim of identifying genes that are preferentially expressed in the yeast phase. Genes identified by both procedures as being more highly expressed in the yeast phase are involved in basic metabolism, signal transduction, growth and morphogenesis, and sulfur metabolism. In order to test whether the observed changes in gene expression reflect the differences between the growth conditions used to

obtain the two morphological forms rather than differences intrinsic to the cell types, we performed real-time RT-PCR experiments using RNAs derived from both yeast cells and mycelia that had been cultured at 37°C and 26°C in either complete medium (YPD or Sabouraud) or minimal medium. Twenty genes, including *AGSI*(α -1,3-glucan synthase) and *TSAl*(thiol-specific antioxidant), were shown to be more highly expressed in the yeast cells than in the hyphae. Although their levels of expression could be different in rich and minimal media, there was a general tendency for these genes to be more highly expressed in the yeast cells.

Keywords *Paracoccidioides brasiliensis* · Dimorphic fungi · Suppression subtraction hybridization · Macroarray analysis · Real-time RT-PCR

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Introduction

The term 'fungal dimorphism' refers to the ability of some fungal pathogens to reversibly switch between yeast (Y) and mycelial (M) forms. *Paracoccidioides brasiliensis*, a thermomorphing fungus, is the causative agent of paracoccidioidomycosis (PCM), the prevalent systemic mycosis in Latin America. Epidemiological data indicate a broad geographic distribution in Central and South America, from Mexico to Argentina (San-Blas and Niño-Veja 2001). It is estimated that as many as ten million individuals may be infected with *P. brasiliensis*. Infection of the human host generally occurs by inhalation of airborne microconidia, which reach the pulmonary alveolar epithelium where they transform into the parasitic yeast form (Restrepo et al. 2001). The dimorphic transition in *P. brasiliensis* occurs simultaneously with changes in cell wall composition involving structural alterations in the carbohydrate polymers, and reorganization of membrane lipids, especially glycos-

phingolipids (GLS) (Leverly et al. 1998, Toledo et al. 1999; Vigh et al. 1998). As the fungus adopts the yeast form, an increase in the chitin content is observed in the cell wall, followed by a change in the anomeric structure of the glucan from a β -1,3-linked polymer to an α -1,3-linked chain (San-Blas and Niño-Veja 2001). The surface α -glucan may act as a protective layer against the host defense mechanisms, in light of the incapacity of host phagocytic cells to digest α -1,3-glucan (San-Blas 1982). The morphological transitions (Y $\rightarrow$ M and vice versa) can be induced in vitro by temperature shifts (see below).

Recently, we reported a survey of the genes expressed in the pathogenic yeast phase of *P. brasiliensis* (Goldman et al. 2003). We obtained 13,490 ESTs from both 5' and 3'-ends of mRNAs, and clustering analysis yielded the partial sequences of 4692 expressed genes, which were functionally classified by similarity to known genes. We have identified several homologues of known *Candida albicans* virulence/pathogenicity factors in *P. brasiliensis*. The transcription of three heat-shock genes (*HSP-70*, *-82* and *-104*), of polyubiquitin (*UBI1*), and of a Δ -9-desaturase homologue (*OLE1*) peaks during the mycelium to yeast (M $\rightarrow$ Y) transition. These results suggested that stabilization of proteins and changes in membrane organization are important factors in the morphological transition to the yeast form at higher temperature.

Pathogenicity appears to be intimately linked to the dimorphic transition (Bialek et al. 2000; San-Blas and Niño-Veja 2001; Borges-Walmsley et al. 2002; Rooney and Klein 2002). In a search for proteins that are differentially expressed in the pathogenic yeast phase of *P. brasiliensis*, da Fonseca et al. (2001) identified and characterized new antigens homologous to catalase, fructose-1,6-biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, and triosephosphate isomerase. Yeast cells preferentially synthesized these antigens. By using differential display, Venancio et al. (2002) were able to isolate two cDNA fragments that were upregulated during the M $\rightarrow$ Y transition. Their predicted products showed no significant similarity to any of the previously described antigens.

Given the present lack of information regarding *P. brasiliensis* genes expressed in the pathogenic yeast phase, we have used Suppression Subtraction Hybridization (SSH) and differential macroarray analyses to identify genes that are preferentially expressed in the yeast cells. Here we report the characterization of several *P. brasiliensis* genes that are more highly expressed in the pathogenic yeast phase than in the mycelial phase.

Materials and methods

RNA extraction

For macroarray and suppression subtraction hybridization, total RNA was extracted from yeast and mycelium of *P. brasiliensis* isolate 18 grown in agitated liquid cultures to near the late log phase. Yeast cells were

cultivated at 36°C in modified YPD (mYPD, 0.5% yeast extract, 0.5% casein peptone, 1.5% glucose) and mycelium was cultured at 26°C in rich (Sabouraud) medium. Yeast cells were initially recovered from the spleens of intraperitoneally infected mice (the same as those used in Goldman et al. 2003), subcultured from mYPD slants in 100 ml of the same medium for 5 days, then transferred to 1 l of fresh medium and grown for another 7 days. Mycelia were obtained by transferring yeast cells subcultured from mYPD slants to liquid Sabouraud (100 ml) for 20 days at 26°C, then transferred to 1 l of the same medium and grown for 12 days. For RT-PCR validation experiments, total RNA was extracted from yeast and mycelium that had been obtained as just described, but were then grown in parallel in the same culture medium (100 ml). We compared the expression of genes from both phases of *P. brasiliensis* cultured in mYPD, Sabouraud and also in modified minimal medium (MM; Restrepo and Jiménez 1980; L-cysteine, and L-methionine were added at 100 mg/l each). RNA extraction and treatment with RNase-free DNase were carried out as described by Goldman et al. (2003). The absence of DNA contamination after treatment with DNase was verified by PCR amplification of the *PbGP43* gene (Cisalpino et al. 1996). Plasmid DNA extraction and sequencing were performed as described by Goldman et al. (2003).

Suppression Subtraction Hybridization (SSH)

All the SSH experiments were done with the *P. brasiliensis* isolate 18. SSH was carried out using double-stranded cDNA derived from the mycelium phase as the driver, and yeast cDNA as the tester. A PCR Select kit (Clontech) was used as recommended by the manufacturer. Briefly, the tester and driver cDNAs were first digested with *Rsa* I, a restriction enzyme with a 4-bp recognition site that yields cDNA fragments with blunt ends. The tester cDNA fragments were divided into two equal aliquots and each one was ligated to a different adapter, resulting in two distinct populations of tester cDNAs. A small amount of each cDNA tester population (approximately 300 ng) was mixed with an excess of driver (2 μ g), and the mixture was heat-denatured and allowed to anneal for 8 h at 68°C. The two samples from the first hybridization were then combined and annealed with an additional, freshly denatured, sample of driver cDNA at 68°C overnight. An initial PCR using adapter primers was performed to amplify those cDNAs that represent differentially expressed genes. A second PCR amplification was carried out using nested adapter primers to minimize non-specific amplification. The cDNA fragments were fractionated by electrophoresis in a 1% agarose gel, excised from the gel and purified by using the Qiagen Plasmid Purification System. The isolated PCR products were then cloned into the cloning vector pCR2.1 TOPO-(Invitrogen) according to the manufacturer's instructions.

Data handling and analysis

A pipeline was designed to analyze and assemble the *P. brasiliensis* SSH sequences. Sequences were automatically edited for each EST using the programs Phred (Ewing et al. 1998; Ewing and Green 1998), CAP3 (Huang and Madan 1999), Consed (Gordon et al. 1998) and Crossmatch from Phrap (P. Green 1996; available at <http://bozeman.mbt.washington.edu/phrap.html>). Sequences originating from the pUC18 vector were filtered out, and only SSH sequences with a quality value of at least 20 were subjected to further analysis. Edited sequences were clustered using the CAP3 program (Huang and Madan 1999). To identify potential homologues to *P. brasiliensis* genes, the BLASTx and BLASTn algorithms (Altschul et al. 1997) were used to compare the predicted protein sequences with those in the NCBI nonredundant and *P. brasiliensis* EST project databases (<http://143.107.203.68/est/default.html>), respectively. The values presented for putative homologues derive from results obtained with sequences of known function as well as those of unknown function. The database sequence matches (putative homologues) were divided into highly significant (e values $\leq 10^{-19}$), moderately significant ($1e-10$ to $1e-19$), weakly significant ($1e-03$ to $1e-10$), and non-significant ($> 1e-03$) classes (data not shown). When e values higher than 10^{-3} were obtained, they were considered not to be statistically significant (no significant match).

Preparation and analysis of cDNA macroarrays

cDNA inserts representing 1397 *P. brasiliensis* genes (Goldman et al. 2003) were amplified by PCR using the M13 reverse and forward primers. The inserts were denatured with 0.2 M NaOH for 15 min at 37°C and then spotted onto Hybond-N filters (AP-Biotech) with a hand-held 96-pin print head tool (V&P Scientific). This tool typically deposited 0.1 μ l of DNA solution, which corresponded to approximately 5 ng of DNA. The DNA was fixed to the filters by baking at 80°C for 2 h, and the filters were then stored at room temperature until used for hybridizations. Each filter carried 96 clones in quadruplicate, giving a total of 384 spots per filter.

cDNA targets were obtained from total RNA isolated from mycelium and yeast phases, as described by Schummer et al. (1999), using [α - 32 P]dCTP. Filters were hybridized, washed and exposed to imaging plates for 96 h, and then scanned in a phosphorimager FLA3000-G (Fujifilm). The radioactive signals were completely removed from the membranes (Sambrook and Russell 2001), and those that had been hybridized with radioactive yeast cDNA were probed with radioactive mycelium cDNA, and vice-versa. The signals were evaluated again, and compared to those observed in the previous hybridization experiment. Signals were quantified using Array vision software (Imaging Research). The local background was subtracted automatically from each

spot. The intensities of the four replicate spots were averaged and used to calculate the expression ratios between yeast and mycelium. To reduce the fluctuations among replica filters due to differences in the experimental conditions, on each membrane, the signal from every spot was normalized using the median of all signals on that membrane; i.e. the median was set to 1 (Schummer et al. 1999). Genes with expression levels close to the background value were discarded and 536 genes (out of 1397) were used for further analysis. Differentially expressed genes were identified with Student's t -test using the $\log_2$ of normalized values from two replicated experiments. A cutoff value of 3.00 for the t -test was chosen ($P < 0.01$).

PCR and real-time RT-PCR

All PCRs and RT-PCRs were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Taq-Man EZ RT-PCR kits (Applied Biosystems) were used for RT-PCR reactions. The thermocycling conditions comprised an initial step at 50°C for 2 min, followed by reverse transcription for 30 min at 60°C and denaturation at 95°C for 5 min, and 40 cycles at 94°C for 20 s and 60°C for 1 min. Taq-Man PCR Reagent kits were used for PCRs. The reaction parameters were as follows: after initial incubation at 50°C for 2 min, and 10 min at 95°C, 40 cycles of 95°C for 15 s and 60°C for 1 min were performed. Since there is no ideal control for gene expression, we first compared the α -tubulin and β -tubulin probes as normalizers for the expression of these genes. We saw no difference between these two normalizers. Accordingly, the calibrator gene used for the expression experiments was the α -tubulin gene (data not shown). The reactions and calculations were performed according to Semighini et al. (2002). Primer and probe sequences are listed in Table 1.

Results

Use of macroarrays to identify differentially expressed genes

Differential macroarray analysis was used as a first approach to isolate genes that are preferentially expressed in the yeast phase of *P. brasiliensis*. PCR fragments of representative clones from 1397 contigs clustered from 8369 ESTs (5' and 3' ends) previously described by Goldman et al. (2003) were spotted on nylon membranes (two copies of each membrane and four repetitions for each clone). Yeast and mycelium cDNA probes were labeled, and equal amounts of radioactively labeled samples were separately hybridized to duplicate nylon membranes. The signals were converted to relative intensity values, quantified and statistically analyzed. Here we will describe only the genes that were more highly expressed in the yeast phase. Figure 1 shows the

Table 1 List of primers and fluorescent probes used in this work

Primers and probes ^a	Sequence (5' to 3')	<i>P. brasiliensis</i> Cluster ID	Locus name
Alpha	CCAGAACCAGGCAGTCCAAA	SSHCS001-E11	<i>AGS1</i>
P-alpha ^b	FAM-CACCTGCCTAACAAGATTGGACCAGG5 G	SSHCS001-E02	
H82f	ACCGCTGGTGCCGATATT	PbCC001-B11	<i>HSP82</i>
H82r	GGCCACGAGGTAGGCAGAG		
PH82	TET-TGATTGGTCAGTTTGGTGTGCGCTTCT-TAMRA		
APS1F	ACTAATCCCGGTAAACTCCTTCACA	RC2-Pb5NEW1-154G11	<i>APS1</i>
APS1R	CGGAGCAGAGAGACCCAAAG		
P-APS1	FAM-TCGCCTTCTTATACAGCCC-TAMRA-		
c101-F	AAGCCTTCTCCCCACCAT	PbCC002-A05	<i>C101</i>
c101-R	GCCAAAACATAGCCCACGA		
Pc101	FAM-TGGCTTGTTCGAAAGA-TAMRA		
c126F	CGTTGGTAATCCTGGCTTGG	PbCC002-C06	<i>GSP1</i>
c126R	CAACTGCGGTCTCAGGTGG		
Pc126	FAM-ATTCGTTGCCGATATTGCCCTCGC-TAMRA		
C1363F	ATTTGAGGGAGGCGGAATCC	Contig 1363 RST	<i>MEP1</i>
c1363R	TTTGGGAACGTCCAGTCTGTT		
Pc1363	FAM-CTGCCCTGTACTGACCTC-TAMRA		
c1412F	TGTCTGCAGGGCATCGATTA	PbCC015-F08	<i>TSA1</i>
c1412R	AGCTACCCTGCATCCACTGG		
Pc1412	FAM-TCGCAGCACCTCAGCAGCATTCC-TAMRA		
c162F	CTCGACAACGTCCAGGAGAAG	PbCC002-F06	<i>RHO1</i>
c162R	GGGATGACCCTGGCAGAAATG		
c162	FAM-AAGGACTTCAGAAATCCA-TAMRA		
C165F	TCCCCTATCATCCTGCAAGTGT	PbCC002-F09	<i>FBA1</i>
c165R	CCATTGGGAACGCCCTTC		
Pc165	FAM-CAAAGAAAGCAGCCCC-TAMRA		
C261F	CCTACGCGACATGCAGAAATATATC	PbCC003-F09	<i>LUC7</i>
c261R	CGCTGCGCCGAGTCTATC		
Pc261	FAM-CTCCGATTACAGTCATC-TAMRA		
c32F	GGCAAAGCCGAAGAAAAGC	PbCC001-C08	<i>ILV5</i>
c32R	TGTACAGGTAGCCGCTGCC		
Pc32	FAM CGGCATCGCCATC-TAMRA		
C354F	CCTACAATATTTCTGGCTCGTTTCCT	PbCC004-F06	<i>PAL1</i>
c354R	GGATTGCTCAACAACCTCCTCAT		
Pc354	FAM-ACTCCGGTAATTTTCG-TAMRA		
c5F	ACGACCACGGAAACTCATACC	PbCC001-A04	<i>ERG25</i>
c5R	GGATCATGCTGAGGCTTTTCCA		
Pc5	FAM-CTGTGCGCATCAATG-TAMRA		
c71F	ACCGTTGCCAGCAACATT	PbCC001-F11	<i>CIT1</i>
c71R	AGCCCTCCCAGACCATAGACTT		
Pc71	FAM-AGGCCTCTCATTCTC-TAMRA		
c77F	GGAGCCGGAAGTCGAATAGG	PbCC001-G05	<i>C77</i>
c77R	ACCTACCATTGGAATGTGTGCATA		
Pc77	FAM-CCAAGACTCCAATGGT-TAMRA		
C836F	AACGGTGTTCAGACGATGGT	PbCC009-F08	<i>PAL2</i>
c836R	AACCACCACGACGACCAG		
Pc836	FAM-ACCTCGGCAACATC-TAMRA		
CHS1F	GAATGGGCTTCCGAGATAAATGC	VP1-Pb50001-178A02	<i>CHS1</i>
CHS1R	ACCGACACCAACACATCTCAAA		
P-CHS1	FAM-CCGACATCCAAAGCCT-TAMRA		
COT1F	CCTGCTTCATATCCTCATCATGGA-	PbCC003-A05	<i>CTR3</i>
COT1R	TGACGCCTATACATGACCCTACAA-		
P-COT1	FAM-CACGTCCAGAGGAATGT-TAMRA-		
FLBA-F	CGGCTAGCAAGACTGTTTCG-	PbCC002-F06 162	<i>FLB1</i>
FLBA-R	TGCTTTTCTTGCTCCTGGATCTC-		
P-FLBA	FAM-CCTGCGAGCTGAACAT-TAMRA-		
GCL1F	GCATCAGCATCAGCAGCAA	RC2-Pb30001-119B07	<i>GCL1</i>
GCL1R	CTGCTTGTGCTGTTGGTATTGG		
P-GCL1	FAM-CAGCACGTCAACCCCA-TAMRA		
Tub2F	AGCCTTGCGTCGGAACATAG	PbCC014-D01	<i>TUB2</i>
Tub2R	ACCTCCATCCAGGAACCTTCA		
Ptub2	FAM-AGCGTGTAGGTGATCAG-TAMRA		
CDI1F	TGGCATGGTCGTGGATAGC	PbCC004-C05	<i>CDI1</i>
CDI1R	AGGAAACGGCAAAAGCAATCTG		
PCDI1	FAM-AGCGTGTAGGTGATCAG		
Tub1	ACCCAGCTTGGAACAGTGCT	PbCC001-E05	<i>TUB1</i>
Ptub1**	CACGGACCATCAGCCTTCAAACC5G		

Table 1 (Contd.)

Primers and probes ^a	Sequence (5' to 3')	<i>P. brasiliensis</i> Cluster ID	Locus name
C159F	GCCCCTGGAGGATAAGATAAAGAG	PbCC002-F03	<i>SEP1</i>
C159R	GAGCGAACGAATCGACTTTACTGTA		
PC159	FAM-CTAACGAAGAGAGAATCCCT-TAMRA		

^aP' primers were fluorescently labeled with the indicated markers (TET, 6-carboxy-4,7,2',7'-tetrachlorofluorescein; 6-FAM, 6-carboxy-fluorescein; TAMRA, 6-carboxy-*N,N,N',N'*-tetramethylrhodamine)

^bProbe Lux (Invitrogen)

scatter correlation graph for 536 genes (out of 1397) that were expressed at levels above the background value. We were able to identify 38 clusters whose expression levels were 1.5–6.6 times higher on the membrane hybridized with the yeast cDNA probe than on that hybridized with the mycelium cDNA probe (Table 2). Homology searches revealed that 26 of these clusters yielded highly significant *e* values ($\leq 10^{-19}$), while seven showed moderately (1e-10 to 1e-19), and five showed weakly (1e-03 to 1e-10) significant *e* values with sequences listed in databases. It is noteworthy that most members of the latter two classes show similarity to hypothetical proteins from *Neurospora crassa* and *Aspergillus nidulans*.

Use of SSH to select preferentially expressed genes

We also performed an SSH analysis using mycelium cDNAs as driver and yeast cDNAs as tester with the aim of isolating genes preferentially expressed in the *P. brasiliensis* yeast phase. When the original cDNA pools were used as probes on Northern blots loaded with yeast and mycelium mRNAs, similar hybridizing signals were detected, while the subtracted cDNAs used as a probe yielded stronger and diffuse hybridizing signals with the yeast mRNA (data not shown). The SSH procedure was also validated using real-time PCR to quantify the expression of the *HSP82* gene, which was previously shown to be overexpressed in the yeast phase (Goldman et al. 2003). *HSP82* RNA was enriched at least 150 fold in the subtracted cDNAs relative to the original cDNA pools (data not shown).

Since our subtracted cDNA population was enriched for cDNA fragments representing mRNAs from the yeast phase, we constructed a library from the SSH subtracted cDNAs and sequenced 750 ESTs whose inserts ranged from 200–700 bp in size. The sequences yielded 240 clusters following analysis with CAP3 (112 contigs and 128 singlets). These sequences were compared with nonredundant NCBI proteins using BLASTx, and with our previous EST collection from *P. brasiliensis* (Goldman et al. 2003) using BLASTn. Our analysis revealed 163 novel clusters (50 with known and 113 with unknown function), while 79 (46 with known and 33 with unknown function) were already represented in our database (Goldman et al. 2003). The most highly expressed sequences were screened by spotting one representative PCR-amplified insert from each of the 240 SSH clusters on nylon membranes and

hybridizing them with radioactively labeled subtracted cDNA. The inserts that corresponded to the genes showing the most intense signals, as well as those highly represented in the library (multiple clones bearing fragments of the same gene), were selected for further expression analysis by real-time RT-PCR (see Table 3). Some of these, such as *ERG25*, *CIT1*, *FBA1*, *ILV5*, *CTR3* (related to cell metabolism) and *CDI1* (related to sulfur metabolism) were also detected by differential macroarray analysis (Table 2). Apart from the clusters listed in Table 3, the SSH library contained homologues of the *HSP10*, -30, -70, -82, and -88 genes, which are important in the adaptation of microorganisms to temperature changes. The *HSP70* heat shock gene (da Silva et al. 1999) was also selected as being more expressed in the yeast phase by the macroarray technique (Table 2). Other potentially important gene homologues found in the SSH library, which were not present in our *P. brasiliensis* database, were: *MNN4* (related to mann-6-phosphorylation), *PKC1* (coding for a cAMP-dependent protein kinase) and *GST1* (coding for a glutathione-S-transferase). All these gene homologues showed highly significant *e* values ($\leq 10^{-19}$), with the exception of *AGS1* (*e*-value = 3e-16), which showed a moderately significant *e* value (1e-10 to 1e-19). However, we have isolated the same gene homologue in a cluster derived from a random sequencing tag collection

Fig. 1 Scatter plot of gene expression in yeast and mycelium. Points represent the average normalized signal intensity (arbitrary units) for 536 *P. brasiliensis* genes

Table 2 Macroarray analysis of contigs more highly expressed in the pathogenic yeast phase than in the mycelial phase of *P. brasiliensis*

<i>P. brasiliensis</i> Cluster ID	Homologue (function, source) and NCBI protein accession number	BLAST e-value (% identity/similarity)	Induction factor
PbCC001-A04	<i>ERG25</i> (C-4 methyl sterol oxidase, <i>S. cerevisiae</i>) NP_011574.1	8e-53 (60/78)	6.6
PbCC001-C03	<i>CIT1</i> (citrate synthase, <i>A. nidulans</i>) AAC49728.3	9e-61 (89/94)	6.0
PbCC002-A05	<i>FBA1</i> (aldolase, <i>S. cerevisiae</i>) NP_012863.1	e-136 (65/76)	4.7
PbCC005-A06	<i>ILV5</i> (Branched-chain amino acid biosynthesis, <i>S. cerevisiae</i>) AAB67753.1	2e-82 (86/91)	4.4
PbCC014-C11	Predicted protein (<i>N. crassa</i>) NCU06390	6e-05 (43/61)	3.6
PbCC003-F04	Hypothetical protein (<i>N. crassa</i>) NCU05891.1	6e-07 (36/49)	3.3
PbCC003-B12	Hypothetical protein (<i>N. crassa</i>) NCU04080	2e-10 (34/48)	3.0
PbCC003-E12	<i>ENO1</i> (enolase, <i>A. oryzae</i>) Q12560	0.0 (86/91)	2.9
PbCC001-F06	<i>HIS4</i> (histone H4, <i>A. nidulans</i>) P23751	2e-35 (100/100)	2.8
PbCC001-D02	<i>EEF3</i> (translation elongation factor eEF3, <i>C. albicans</i>) S25363	e-103 (74/89)	2.7
PbCC004-A08	<i>RL20</i> (60S ribosomal protein L20, <i>S. cerevisiae</i>) NP_014957.1	8e-67 (71/83)	2.5
PbCC004-H02	<i>A. nidulans</i> EST c4c08a1.r1	4e-04 (86/92)	2.5
PbCC005-C08	Hypothetical protein (<i>N. crassa</i>) AL513466	2e-18 (30/44)	2.4
PbCC001-A10	<i>FCF1</i> (zinc cluster transcription factor Fcrlp, <i>C. albicans</i>) AF057038	1e-15 (64/76)	2.3
PbCC001-C10	Unknown		2.2
PbCC004-A04	Probable glutaredoxin (<i>N. crassa</i>) AL353819	6e-17 (52/67)	2.1
PbCC004-A09	Unknown		2.0
PbCC004-A10	<i>GCV3</i> (glycine decarboxylase, <i>A. thaliana</i>) AC004667	8e-30 (53/69)	2.0
PbCC004-C10	Aminopeptidase B (<i>A. niger</i>) AJ418036	2e-52 (69/80)	2.0
PbCC003-G12	Inositol-1-phosphate synthase (<i>P. pastoris</i>) AF078915	1e-73 (48/63)	2.0
PbCC007-D02	Predicted protein (<i>N. crassa</i>) NCU02545.1	6e-11 (32/52)	1.9
PbCC001-H03	Unknown		1.9
PbCC003-E01	Unknown		1.8
PbCC003-C05	Unknown		1.8
PbCC002-C05	Putative ribosomal protein (<i>A. fumigatus</i>) AL807577	9e-56 (84/94)	1.8
PbCC008-C11	60S ribosomal protein L27A (L29) Y11394	7e-70 (79/91)	1.8
PbCC001-A12	Unknown		1.8
PbCC003-G06	Conserved hypothetical protein with HesB-like domain (<i>S. pombe</i>) NC_003423	9e-18 (48/64)	1.7
PbCC001-D03	Hydroxyproline-rich glycoprotein precursor <i>Z. mays</i> S45164	6e-08 (41/44)	1.7
PbCC005-D04	Unknown		1.7
PbCC001-A06	Probable snRNP protein (<i>N. crassa</i>) AL513466	6e-36 (71/81)	1.7
PbCC006-A01	Unknown		1.7
PbCC004-C05	<i>CDII</i> (cysteine dioxygenase, <i>C. elegans</i>) U51993	2e-36 (48/65)	1.7
PbCC007-B07	<i>LUC7</i> (RNA splicing factor Pad-1, <i>N. crassa</i>) CAD21082.1	3e-66 (73/77)	1.6
PbCC006-E05	Predicted protein (<i>N. crassa</i>) NCU07465.1	1e-06 (39/53)	1.6
PbCC003-A05	<i>CTR3</i> (high affinity copper transporter protein, <i>S. cerevisiae</i>) NC_003421	6e-20 (41/57)	1.6
PbCC006-A03	<i>HSP70</i> (heat shock protein 70) X98931	2e-23 (91/99)	1.5
PbCC004-D04	Hypothetical protein (<i>Mesorhizobium loti</i>) NC_002678	5e-10 (35/52)	1.5

(Nobrega and Goldman, manuscript in preparation). Partial sequencing of this gene confirmed that it indeed encodes a putative homologue of the α -glucan synthase gene from *A. fumigatus* (e-value = 5e-25; 70% identity with α -glucan synthase from *A. fumigatus*).

Use of real-time RT-PCR to validate the expression of the genes identified by macroarray and SSH analyses

For real-time RT-PCR analysis, we designed Taq-Man and Lux probes (Tables 1, 2 and 3): 13 for gene homologues selected from the SSH library (Table 2) and 4 from the macroarray analysis, i.e., *C77*, *C101*, *FBA1*, and *LUC7* (Table 3). The selection was based on the degree of expression in the yeast phase in the preliminary selection, but we also included four probes for *P. brasiliensis* gene homologues involved in sulfur metabolism (*MEP1*, *APS1*, *CHS1* and *GCL1*). Although these last genes were not selected in our primary screen, they have been shown to influence the morphological state of

Histoplasma capsulatum and other dimorphic fungi (Maresca and Kobayashi 2000; Hwang et al. 2003).

The total RNA preparations used in the initial screening for both SSH and macroarrays had been isolated from yeast cells grown in mYPD medium and from mycelia grown in Sabouraud medium. To check whether changes in the expression levels of the genes might reflect differences in the growth conditions used to maintain the morphological forms rather than functions required in one or other of the two distinct cell types, validation by RT-PCR experiments was carried out using a second batch of RNAs derived from yeast and mycelium that had been grown in the same medium at 37°C and 26°C, respectively. We compared the expression of these genes at the two temperatures in mYPD, Sabouraud and minimal media.

The results were expressed as the ratio of the transcript number in yeast to that in mycelium, with the transcript copy number in mycelium being set to 1 (Table 3). Our results suggest that many of the genes listed in Table 4 may be involved in the maintenance of

Table 3 *P. brasiliensis* genes chosen for the real-time RT-PCR experiments

<i>P. brasiliensis</i> Cluster ID(EST cluster)	Homologue (NCBI protein accession number)	BLAST e-value (% identity similarity)	Criteria for choosing the gene for RT-PCR analysis
Metabolism			
SSHCS001-G09 (PbCC010-B11)	<i>ILV5</i> (branched-chain aminoacid biosynthesis, <i>S. cerevisiae</i>) AAB67753.1	3e-20 (73/84)	4.4-fold induction in the macroarray; > 3 SSH fragments
PbCC007-B11	<i>LUC7</i> (RNA splicing factor Pad-1, <i>N. crassa</i>) CAD21082.1	3e-66 (73/77)	> 1.6-fold induction in the macroarray
SSHCS001-A02 (PbCC012-B10)	<i>CIT1</i> (citrate synthase, <i>A. nidulans</i>) AAC49728.3	9e-61 (89/94)	> 3 SSH fragments; > 6.0-fold induction in the macroarray
SSHCS001-B04 (PBCC003-A05)	<i>CTR3</i> (high-affinity copper transporter, <i>S. cerevisiae</i>) NC_003421	6e-20 (41/57)	Strong signal in the SSH hybridization
SSHCS001-E11 (PbCC002-C06)	<i>GSP1</i> (small G-protein Gsp1p, <i>C. albicans</i>) AF190700	2e-94 (79/82)	
SSHCC001-A06 (PbCC001-A04)	<i>ERG25</i> (C-4 methyl sterol oxidase, <i>S. cerevisiae</i>) NP_011574.1	8e-53 (60/78)	> 6.6-fold induction in the macroarray; Strong signal in the SSH hybridization
SSHCS001-C08 (PbCC004-F06)	<i>PAL1</i> (alkaline serine protease Penc2, <i>Penicillium citrinum</i>) AF098517.1	e-157/64/77	Strong signal in the SSH hybridization
SSHCC001-A12 (PbCC009-F08)	<i>PAL2</i> (aspartyl aminopeptidase, <i>Coccidioides immitis</i>) AF420303	7e-256 (75/89)	Strong signal in the SSH hybridization
SSHCC001-D05 (not present) ^a	<i>AGS1</i> (putative α -1,3-glucan synthase, <i>A. fumigatus</i>) AAL18964.1	3e-16 (67/78)	Strong signal in the SSH hybridization
PbCC002-A05 (<i>FBAL1</i>)	<i>FBAL1</i> (aldolase, <i>S. cerevisiae</i>) NP_012863.1 Unknown	e-136 (65/76)	> 4.7-fold induction in the macroarray
PbCC003-C05 (<i>C101</i>)			> 1.8-fold induction in the macroarray
PbCC001-C10 (<i>C77</i>)			> 2.2-fold induction in the macroarray
Signal transduction			
SSHCS002-C06 (PbCC002-F06)	<i>RHO1</i> (GTPase Rho1 <i>A. fumigatus</i>) AY007297	4e-61 (99/99)	Strong signal in the SSH hybridization
Growth and morphogenesis			
SSHCC001-H01 (PbCC002-F03)	<i>SEPI</i> (septin B, <i>A. nidulans</i>) AAB41233.1	6e-63 (92/95)	Strong signal in the SSH hybridization
SSHCS002-A02 (PbCC006-E07)	<i>FLB1</i> (developmental regulator <i>flbA</i> , <i>A. nidulans</i>) P38093	2e-38 (77/86)	Strong signal in the SSH hybridization
Oxidative stress			
SSHCS001-G01 (PbCC015-F08)	<i>TSA1</i> (Putative thiol-specific antioxidant protein, <i>Ajellomyces capsulatus</i>) AAG31645.1	7e-33 (89/95)	Strong signal in the SSH hybridization
Sulfur metabolism			
RST1363 ^b	<i>MEP1</i> , Methionine permease		Sulfur metabolism
PbCS004-F04	<i>APST</i> , Adenylsulfate kinase		Sulfur metabolism
PbCS006-F07	<i>CHS1</i> , Choline sulfatase		Sulfur metabolism
PbCS001-E12	<i>GCL1</i> , Glutamate-cysteine ligase		Sulfur metabolism
SSHCS001-F05 (PbCC004-C05)	<i>CDH1</i> (cysteine dioxygenase, <i>C. elegans</i>) U51993	2e-36 (48/65)	1.7-fold induction in the macroarray; > 3 SSH fragments; strong signal in the SSH hybridization; sulfur metabolism

^aA contig derived from a random sequencing tag collection (Nobrega and Goldman, manuscript in preparation)

the *P. brasiliensis* yeast phase in vitro, because they were more highly expressed in the yeast cells grown under three different conditions in different media. The unknown cluster *C101* was not validated as a yeast-specific gene in our experimental conditions, since the ratio of yeast/mycelium ratio was consistently less than 1. Other gene homologues yielded low ratios or ratios near 1 in one or two culture conditions, as seen for *ILV5*, *CIT1*, *GSP1*, *PAL2*, *FBA1*, *FLB1* and *GCL1*. The most impressive results were seen with the homologues of α -1,3-glucan synthase (*AGS1*) and choline sulfatase (*CHS1*), which gave very high yeast/mycelium ratios under all three conditions studied, up to 82.18 and 61.10 times, respectively more highly expressed in the yeast phase (in mYPD). The *LUC7* homologue (predicted to encode an RNA splicing factor) was also abundantly expressed in the yeast phase in all three media (ratios above 6), followed by *PAL1*, which encodes a serine proteinase. The ratios were comparable in at least two conditions for *ERG25*, *FBA1*, *RHO1*, *SEPI*, *MEPI*, *GCL1* and *CDII*. As previously shown by da Fonseca et al. (2001), we also see increased expression of the *FBA1* and *ENO1* genes that code for fructose-1,6-bisphosphate aldolase and enolase, respectively, during the yeast phase.

It is interesting to note that some genes showed much higher yeast/mycelium ratios when the cells were grown in minimal medium than in rich media. This was the case for *ILV5*, *CIT1*, *C77*, *RHO1*, *FLB1* and *MEPI*. The *RHO1* gene, whose product is involved in signal trans-

duction, and *MEPI*, involved in sulfur metabolism, were from 4 to 15 times more highly expressed in minimal medium than in rich medium (Table 4). The fluctuations in the ratios probably reflect different growth stages and responses to diverse stimuli in the culture environments (Hwang et al. 2003).

Discussion

The experiments reported here represent the first application of high-throughput SSH and macroarray analysis to examine gene expression in the pathogenic phase of *P. brasiliensis*. Both procedures enabled us to identify genes that are preferentially expressed in the yeast phase. Recent studies have demonstrated that dimorphic switching is directly associated with pathogenicity (Braun and Johnson 1997; Csank et al. 1998; Leberer et al. 1997; Lo et al. 1997; Stoldt et al. 1997; Yaar et al. 1997; Borges-Walmsley et al. 2002; Romani et al. 2003). Accordingly, the identification of genes preferentially expressed in the yeast phase can be expected to provide information about the pathobiology of *P. brasiliensis*. The SSH strategy overcomes the problem of differences in mRNA abundance by incorporating a hybridization step that normalizes (equalizes) sequence abundance during the course of subtraction by standard hybridization kinetics (Diatchenko et al. 1996). This advantage is reflected in the fact that most of the SSH clusters obtained here (about 68%) were not already present in

Table 4 Relative transcript levels of *P. brasiliensis* genes measured by real-time RT-PCR in different conditions

<i>P. brasiliensis</i> gene Cluster ID (EST cluster)	Relative transcript level in yeast phase ^a		
	Both yeast and mycelium grown in YPD	Both yeast and mycelium grown in Sabouraud medium	Both yeast and mycelium grown in minimal medium
Metabolism			
<i>ILV5</i>	2.95	0.70	5.60
<i>LUC7</i>	27.77	7.02	6.70
<i>CIT1</i>	1.06	3.26	7.30
<i>CTR3</i>	7.37	4.36	2.00
<i>GSP1</i>	3.98	1.40	1.20
<i>ERG25</i>	2.45	2.37	2.00
<i>PAL1</i>	5.07	6.84	6.30
<i>PAL2</i>	4.27	1.68	1.00
<i>AGS1</i>	63.2	82.18	9.20
<i>FBA1</i>	2.7	3.04	1.40
Unknown			
<i>C77</i>	5.09	2.35	11.80
<i>C101</i>	0.06	0.56	0.60
Signal transduction genes			
<i>RHO1</i>	3.17	2.28	30.5
Growth and Morphogenesis			
<i>SEPI</i>	3.75	3.77	3.00
<i>FLB1</i>	1.91	0.94	3.30
Oxidative stress			
<i>TSA1</i>	18.81	7.51	2.70
Sulfur metabolism			
<i>MEPI</i>	4.23	5.62	29.30
<i>APS1</i>	8.95	4.48	3.00
<i>CHS1</i>	61.10	14.52	26.00
<i>GCL1</i>	1.19	2.38	2.00
<i>CDII</i>	2.07	1.81	3.00

^aThe transcript levels of *P. brasiliensis* genes in the yeast phase (YP) were quantitated by RT-PCR, and expressed relative to those of the same transcripts in the mycelial phase (MP). The measured quantity of each mRNA in each sample was normalized using the *C_t* s obtained for the α -tubulin RNA run on the same plate. The relative levels of each transcript and α -tubulin mRNA were determined from a standard curve (i.e., *C_t* s plotted against logarithm of the DNA copy number). The results are averages of three repetitions. The values represent the ratio of the level in YP to that in the mycelial control (set to 1.00)

our EST databank (Goldman et al. 2003). In our hands, both SSH and macroarray analysis proved to be powerful and reliable, since most of the 21 genes selected for validation by real-time RT-PCR were indeed more highly expressed in the yeast phase than in the mycelial phase. Furthermore, several genes were identified by both methods.

We sorted the genes preferentially expressed in the yeast phase into broad functional categories. Based on the function of their orthologues, we have identified genes that are implicated in several cellular processes. A more extensive analysis of these genes will be necessary before their functions can be unequivocally determined. Classical genetic tools, such as DNA-mediated transformation and modulation of gene expression either by deletion/replacement or inactivation by RNA interference or antisense RNA, have not yet been developed for *P. brasiliensis*. The type of genomic approach described here represents an efficient alternative strategy for identifying genes involved in virulence/pathogenicity, and may provide a wider platform for a better understanding of *P. brasiliensis* biology and the role of specific genes during the dimorphic transition and the onset of virulence/pathogenicity in PCM. As soon as gene inactivation techniques for this organism have been developed, the function of the genes identified may be tested directly.

The correlation between the expression of specific genes in the yeast form and the morphological phase is complicated by the fact that a temperature shift must be used to promote the dimorphic transition. In *P. brasiliensis* no attempts have yet been made to induce the switch to the yeast phase at room temperature. Therefore it is not yet possible to follow the expression of specific genes in cultures that have not been submitted to a temperature shift. The growth conditions used to obtain the yeast probe also differ from those used for the source of the mycelial probe, and these differences could themselves affect gene expression. Hence it is possible that genes that are preferentially expressed in the yeast state are not necessarily connected with morphological differentiation, but are required for physiological adaptation to the new environment or related to the growth phase in vitro (Berman and Sudbery 2002; Hwang et al. 2003). We addressed the possible influence of growth conditions by studying the expression of the identified genes in different culture media. In general, although the levels of expression of some of genes, such as *AGS1* and *LUC7*, could be strikingly different in complete and minimal media, there was a consistent tendency for these genes to be more highly expressed in the yeast phase, supporting the idea that most of them are specific for that cell type.

The most obvious biochemical alteration in *P. brasiliensis* as the fungus adopts the yeast form is the switch from β -1,3- to α -1,3-glucan chains in the cell wall (San-Blas and Niño-Vega 2001). Accordingly, we observed a much higher level of expression of the gene for α -1,3-glucan synthase in the yeast phase than in the mycelial phase. Early reports on the mycelium-to-yeast transition

of *H. capsulatum* attributed an important role to sulfhydryl groups, mainly in the form of cysteine (Maresca and Kobayashi 2000). Hwang et al. (2003) extended these results by showing that the gene that encodes cysteine dioxygenase is more highly expressed in yeast than in the mycelium. The *CD11* gene, which encodes a cysteine dioxygenase in *P. brasiliensis*, was also identified in our SSH screen. It has previously been shown that the mycelial phase of *P. brasiliensis* can grow in the presence of both organic and inorganic sources of sulfur, while yeast cells can only grow in the presence of a sulfur-containing amino acid (Paris et al. 1985). This sulfur auxotrophy suggests that, in addition to temperature, organic sulfur is an important stimulus for keeping *P. brasiliensis* in the yeast phase. Accordingly, we verified that several genes involved in the assimilation of sulfur-containing amino acids, such as methionine permease, are more highly expressed in the yeast phase than in the mycelial phase, indicating a possible role for sulfur metabolism in the maintenance of the yeast state, as in the case of *H. capsulatum*.

The *CIT1* gene encodes citrate synthase, which catalyzes the first reaction in the citric acid cycle, the condensation of acetyl-CoA with oxalacetate to form citrate. The high specific activity of citrate synthase causes an accumulation of isocitrate and citrate in the mitochondria. Both acids are transported into the cytosol where citrate is cleaved by ATP:citrate lyase, giving rise to acetyl-CoA, the precursor of fatty acid synthesis (Lehninger et al. 1993). It is possible that the high-level expression of the *CIT1* gene in *P. brasiliensis* yeast cells could reflect an increased requirement for fatty acid synthesis. The same kind of observation can be made regarding the increase in expression of the *ERG25* gene in the yeast phase. The *ERG25* gene encodes the C-4 sterol methyl oxidase that performs the first of the three enzymatic steps leading to ergosterol biosynthesis in fungi. It is also likely that increased *ERG25* activity is involved in cell membrane turnover. As we have suggested previously, changes in the cell membrane are important factors for the morphological transition at 37°C (Goldman et al. 2003); the high turnover of lipids in the cell membrane may contribute to this. It has already been demonstrated by genome-wide expression analysis of *S. cerevisiae* that the genes involved in the ergosterol biosynthesis pathway show increased expression in the presence of stressing conditions, such as oxidative stress, and a number of antifungal agents target ergosterol biosynthesis (Higgins et al. 2003; Bammert and Fostel 2000). It remains to be determined whether all the genes for ergosterol biosynthesis genes are induced at 37°C, or if the effect is specific for the *ERG25* gene.

Lethal heat stress generates oxidative stress in *S. cerevisiae*, and anaerobic cells are several orders of magnitude more resistant than aerobic cells to a 50°C heat shock (Davidson and Schiestl 2001). The transition from 25°C to 37°C in *P. brasiliensis* (and also in *H. capsulatum* and *Blastomyces dermatitidis*) is accompanied by

marked changes in respiration and a rapid decline in intracellular ATP levels, due to uncoupling of oxidative phosphorylation (Medoff et al. 1987; Maresca and Kobayashi 2000). Although the level of ATP synthesis decreases, growth in the yeast phase at 37°C should give rise to increased production of reactive oxygen species (ROS) due to the higher temperature. These observations suggest that antioxidant systems in *P. brasiliensis* could be extremely important, not only for survival in macrophages, but also for detoxification of ROS. Accordingly, the preferential expression in the yeast phase of the *P. brasiliensis* *TSA1* (thiol-specific antioxidant) and *GST1* genes, which code for a thioredoxin peroxidase and a glutathione-S-transferase, respectively, may serve to provide antioxidants to reduce the levels of reactive oxygen species during growth of the yeast. Curiously, Choi et al. (2003) identified *Tsa1p* as highly expressed in the hyphae of *C. albicans* by two-dimensional gel electrophoresis.

The signaling pathways that control the morphological changes in *P. brasiliensis* are poorly understood, but the involvement of both cAMP and mitogen-activated protein kinase (MAPK) signal transduction pathways has been reported in other dimorphic fungi (Lengeler et al. 2000). Exogenous cAMP inhibits the yeast-to-mycelium (Y → M) transition in *P. brasiliensis*, thus maintaining the pathogenic yeast form (Borges-Walmsley and Walmsley 2000). In *Candida albicans*, on the other hand, the Y → M transition is controlled by cAMP and exogenous cAMP stimulates the formation of pseudohyphae (Rocha et al. 2001). By using SSH, we have identified four genes, *RHO1*, *SEP1*, *FLB1* and *PCK1*, which encode proteins involved in cell signaling and polarity establishment (Lengeler et al. 2000; Borges-Walmsley and Walmsley 2000; Momany 2002). Interestingly *RHO1* was expressed at 10- to 15-fold higher levels in minimal medium than in the complete media. Boyce et al. (2001) have observed that the *CDC42* homologue in *Penicillium marneffei*, *cflA*, is also required for polarization and determination of cell shape during yeast-like growth, and active *CflA* is required for the separation of yeast cells. However, correct *cflA* function is not required for dimorphic switching.

In summary, our work has succeeded in identifying several genes that could be involved in the maintenance of the pathogenic yeast phase of *P. brasiliensis*.

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APÊNDICE C

Apêndice C – Publicação.

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Original article

Virulence of *Paracoccidioides brasiliensis* and gp43 expression in isolates bearing known *PbGP43* genotype

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Abstract

Paracoccidioides brasiliensis is the dimorphic fungus responsible for human paracoccidioidomycosis (PCM). We previously observed that *P. brasiliensis* isolates bearing highly polymorphic *PbGP43* of genotype A (Pb2, Pb3 and Pb4) were phylogenetically distant from the others. The *PbGP43* gene encodes an immune dominant diagnostic antigen (gp43), and its polymorphism reflects broader genetic diversity in the species. In the present study, we observed that isolates with *PbGP43* of genotype A showed low virulence when inoculated in B10.A mice by the intraperitoneal, intratracheal and intravenous routes. In vitro studies detected sharp and prolonged down-regulation of *PbGP43* in Pb3 (and not in Pb18 or Pb339) as a result of heat shock at 42 °C and temperature shift to prompt mycelium to yeast transition, which was, however, not disturbed. Differences in transcriptional regulation are possibly a consequence of mutations in the *PbGP43* promoter region, which we here show to be more polymorphic in genotype A isolates. As opposed to Pb3's rapid adaptation to in vitro culture conditions after isolation from the lung, Pb12, the most aggressive isolate tested here, showed slow growth and phase transition in vitro. Interestingly, animals that were highly infected by Pb12 produced small amounts of anti-gp43 antibodies. That was apparently due to down-regulation in *PbGP43* expression. We present the first evidence of transcriptional regulation of gp43 expression, but our results suggest that gene expression is also regulated at the protein and/or secretion levels.

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Keywords: gp43; Virulence; *Paracoccidioides brasiliensis*

1. Introduction

Paracoccidioidomycosis (PCM) in man is a systemic granulomatous mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*. Acute and sub-acute PCM affect both sexes, progress rapidly and disseminate through the lymphatic system. Chronic forms are the most common; they affect predominantly male adults, evolving gradually in

the lungs and possibly involving other organs [1]. The disease is restricted to Latin America, where *P. brasiliensis* has also been isolated from the soil and from nine-banded armadillos [2]. The fungus penetrates the human body through inhalation of propagules, which can only establish infection once they undergo phase transition to yeast in the pulmonary alveolar epithelium [3]. The Balb/c and B.10A strains of isogenic mice have been used to mimic chronic PCM upon intraperitoneal (i.p.), intratracheal (i.t.) or intravenous (i.v.) infection with *P. brasiliensis* (reviewed in [4]). Virulent isolates cause severe PCM forms characterized by inhibition of protective cellular immunity against the infectious agent, resulting in abundant fungal growth, antigenic load, and high antibody titers, which are, however, not protective at this stage of the disease [4].

Abbreviations: CFU, colony-forming units; i.p., intraperitoneal; i.t., intratracheal; i.v., intravenous; M, mycelium; nt, nucleotide; PCM, paracoccidioidomycosis; Y, yeast.

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The gp43 glycoprotein is an immune dominant antigen recognized by most sera of patients with active PCM ([5,6], reviewed in [7]), whose antibody titers decrease with successful treatment [8]. The gp43 also elicits protective cellular immunity, and a conserved T cell epitope, called P10, has been mapped to a 15-mer internal peptide that is compatible with murine [9] and human haplotypes [10]. Additionally, gp43 may be a virulence factor due to its adhesive properties [11,12], and PCM patients have circulating gp43 [13,14]. From a panel of murine anti-gp43 monoclonal antibodies [15] recognizing three different epitopes, one of them was able to modulate infection by *P. brasiliensis* cells coated with laminin in a hamster intratesticular PCM model [16]. The *PbGP43* gene is located in a 1329-bp DNA fragment including two exons, a 78-bp intron, and a leader sequence [17]. Processed gp43 can be abundantly secreted in the supernatant fluid of some aerated cultures grown in rich media [18], but it has also been seen in intracellular compartments and cell wall [19]. However, the question of whether the transcript is not expressed or whether the translated glycoprotein is trapped inside the cell remains unexplored in those cultures containing little or undetectable gp43 released in culture fluids. In vitro, the secreted antigen is, in fact, constituted by a mixture of isoforms with isoelectric points whose values are close but distinct [5] and are characteristic of different isolates [20].

Diversity among *P. brasiliensis* isolates has been reported at many levels [21–28]. Our group detected genetic diversity in the species reflected in the polymorphism of the *PbGP43* gene [29] and the internal transcribed spacer regions of ribosomal DNA [30]. We defined the existence of six *PbGP43* genotypes based on the distribution of 21 substitution sites found in 17 *P. brasiliensis* isolates, which generated predominantly non-synonymous amino acid changes. The most polymorphic genotype showed 14–15 substitution sites that resulted in an increased number of basic amino acids, and therefore, translated peculiarly basic gp43. The isolates bearing these sequences were distant from the others in a maximum-likelihood phylogenetic tree, which was characteristic of clonal or asexual organisms and suggested the existence of genetically isolated groups within *P. brasiliensis* [29].

In the present work, we investigated virulence of *P. brasiliensis* isolates belonging to distinct genetic groups defined by their *PbGP43* sequence, focusing on those showing extensive polymorphism. Virulence was compared in mice inoculated by the i.p., i.t. and i.v. routes. We also addressed questions related to gp43 expression and regulation.

2. Materials and methods

2.1. *P. brasiliensis* isolates and growth conditions

We analyzed eight *P. brasiliensis* isolates, namely (original names in parenthesis), Pb2 (isolate 1925), Pb3 (isolate

608), Pb4 (isolate 1017), Pb5 (isolate AP), Pb18 (isolate 18), Pb8 (isolate 9673), Pb9 (isolate 924) and Pb12 (isolate Argentina), out of 17 samples previously characterized for *PbGP43* polymorphism (find detailed description of the isolates in Ref. [29]). The cultures were maintained in the yeast phase at 36 °C in modified solid YPD (0.5% bacto-yeast extract, 0.5% casein peptone and 1.5% dextrose, pH 6.5). Fungal cells were recovered from infected organs at 36 °C, as described [9], in supplemented BHI plates (BHI, Difco) containing 4% fetal calf serum, 5% spent medium, ampicillin (100 IU/ml) and streptomycin (100 µg/ml). Spent medium was added as growth factor and corresponded to culture supernatant of *P. brasiliensis* B-339 (Pb1) grown for about 7 days in MMCM medium [31] at 36 °C under shaking.

2.2. Pathogenicity of *P. brasiliensis* isolates in mice

The virulence of *P. brasiliensis* isolates was compared after i.p., i.t. and i.v. infections. The fungal cultures used in i.p. infections had been maintained in vitro for 3–4 years. For i.t. and i.v. infections, the samples were previously recovered from the spleen of infected mice in supplemented BHI plates and used after two passages in modified YPD slants. Fungal viability of the cultures (generally over 90%) was verified by trypan blue exclusion in a Neubauer chamber. Groups of 10 male B10.A mice (6–8 weeks old) were infected i.p. or i.t. with 1×10^6 viable yeast cells in 50 µl of phosphate-buffered saline (PBS). The animals were sacrificed after different days post-infection, and the numbers of viable *P. brasiliensis* cells in the spleen, liver and lungs were determined as the number of colony-forming units (CFU) recovered in supplemented BHI plates, as detailed elsewhere [9,31]. Briefly, weighed organs of each mouse were macerated in 1 ml of PBS, and 100 µl were plated in supplemented BHI. CFU for each organ of individual mice were counted, and the CFU per gram of tissue were calculated individually. The results were presented as $\log_{10}$ of the average value $\pm$ standard error. Before sacrificing the animals to remove the organs, blood was taken from each mouse; the serum was separated and stored at –20 °C for further analysis. For mortality curves, *P. brasiliensis* isolates were inoculated i.v. (5×10^6 viable yeasts in up to 100 µl of PBS) in the lateral tail vein of male B10.A mice (eight animals per isolate). Control mice received only PBS. Animal manipulation and maintenance complied with the Brazilian federal guidelines and institutional policies for research with experimental diseases.

2.3. Histopathology

The lungs, spleen, and liver of i.t. and i.p. infected mice were removed from sacrificed animals and a fragment was immediately immersed in 10% buffered formalin, embedded in paraffin and sectioned into 5-µm wide cuts. The sections were stained with hematoxylin–eosin and examined microscopically (Optiphot-2, Nikon, Tokyo, Japan).

2.4. Anti-gp43 antibody response of infected mice

Anti-gp43 antibodies were detected in the sera of infected mice by chemiluminescent ELISA [32], in 96-well plates (Nunc, MaxiSorb surface) coated with 100 ng of gp43 per well and quenched with 5% skimmed milk in PBS. Mouse serum samples were individually tested at 1:100 (in triplicate), and the reactive antibodies were quantified with biotinylated goat anti-mouse IgG (1:1000, Amersham Biosciences) and streptavidin peroxidase (2 µg/ml in PBS). The reactions were developed with luminol (ECL reagent, Amersham Biosciences) and the results recorded in a microplate luminometer (Cambridge Technology, Watertown, MA, Model 7710) were expressed as the mean value of relative luminescence units (RLU), where the values for pre-immune sera (negative controls) were deducted. Sera of gp43-immunized mice were used as positive controls. The gp43 used in the tests was purified from culture supernatants of *P. brasiliensis* B-339 (Pb1) in columns of mAb17c anti-gp43 [15] bound to Affi-Gel 10 (BioRad), as previously described [33].

2.5. Total RNA and protein extraction

We evaluated expression of gp43 and its transcription in total RNA and total protein pools extracted from cultures of Pb3, Pb5, Pb18 and Pb12 in the mycelial and yeast phases, as well as during temperature-induced phase transition from mycelium to yeast. Yeast cells recovered from infected lungs were expanded in modified YPD slants for 7–14 days. They were then subcultured in 100 ml of liquid medium under agitation for 6–12 days at 36 or 26 °C to obtain logarithmically growing cells of, respectively, yeasts (Y) or fully formed mycelium (M). The M precultures were distributed in approximately equal volumes into seven Erlenmeyer flasks containing 120 ml of fresh medium and cultivated for another 13–16 days. At this point, the flasks were shifted to the opposite temperature to induce phase transition, and grown for 0, 5, 10, 24, 48, 72 and 120 h, when the microscopy of the cultures was recorded. The cells were separated from the supernatant by centrifugation (1300 × g). Fourteen (Y) or 28 ml (M to Y) of the homogenized cultures, which corresponded to 200–300 µl of wet packed cells, were spared for quantification of intra- and extracellular gp43. The remaining cell pellet (2–3 ml) was processed for RNA extraction. Culture supernatants were stored at –20 °C until use.

For extraction of intracellular proteins, yeast cell pellets were washed two times in TBS (10 mM Tris–HCl, pH 7.4, 150 mM NaCl) containing proteinase inhibitors (1 mM PMSF and 1 mM *o*-phenanthroline, added by the time of use), mixed with the same volume of glass beads (425–600 µm, Sigma), and double the volume of buffer (400–600 µl), then vigorously agitated in vortex (10 times, 30 s, with 30-s intervals in ice). Cell debris was pelleted by centrifugation (5600 × g) and the supernatant (500 µl) stored in aliquots at –20 °C for further analysis of the intracellular gp43 pool. Hyphae or tran-

sition forms were washed similarly, then packed in aluminum foil and ground in the presence of liquid N₂. The fine white power obtained was mixed with buffer until it was defrosted, then processed as detailed before for yeast, so that the final total volume of intracellular extract was 1 ml. Total proteins were estimated by the adapted Bradford method [34]. For RNA extraction, yeast cells were mechanically broken with glass beads in Trizol (Invitrogen), while the mycelium was ground in the presence of liquid N₂ before addition of Trizol. RNA extraction and RNase-free DNase treatment were carried out as described [35]. The absence of DNA contamination after the RNase-free DNase treatment was verified by PCR amplification of the *PbGP43* gene in the absence of reverse transcriptase.

2.6. Expression analysis of intra- and extracellular gp43

We evaluated the amount of intra- and extracellular gp43 in *P. brasiliensis* cultures by trapping the antigen in Affi-Gel 10 beads coupled to anti-gp43 monoclonal antibody mAb17c [15], which reacts with all gp43 isoforms [18]. The results were analyzed in SDS-PAGE stained gels [36]. Culture supernatants (1 ml, buffered with 200 µl of 6× concentrated PBS) and cell extracts (36 µl, corresponding to cell pellets from 1 ml of culture, buffered with 500 µl of PBS) were incubated with anti-gp43 beads for 30 min in ice, under moderate agitation in Eppendorf tubes. The beads were precipitated by pulse centrifugation, washed with 0.5 M NaCl in PBS (1 ml, three times), and the gp43 eluted with 20 mM citric acid, pH 3.0 (55 µl, two times for 5 min each, in ice). The eluates were recovered by pulse centrifugation, neutralized with 1 M Tris, boiled together in concentrated SDS-PAGE sample buffer until the volume was down to 20 µl and electrophoresed. SDS-PAGE gels were stained with Coomassie brilliant blue, scanned, then destained and impregnated with silver. The gp43 content was estimated by comparison with standard Coomassie brilliant blue and silver-stained gels containing increasing amounts of quantified gp43.

2.7. Real-time reverse-transcribed (RT)-PCR conditions

The RT-PCR reactions were performed in an ABI Prism 7700 Sequence Detection System (Perkin–Elmer Applied Biosystem, USA), using DNA-free total RNA and Taq-Man® EZ RT-PCR kits (Applied Biosystems, USA), as detailed elsewhere [35,37]. The thermal cycling conditions comprised an initial step at 50 °C for 2 min, followed by 30 min at 60 °C for reverse transcription, 5 min at 95 °C, and 40 cycles at 94 °C (20 s) and 60 °C (1 min). Taq-Man® PCR Reagent kits were used for PCR reactions. The thermal cycling was carried out with an initial step at 50 °C (2 min), followed by 10 min at 95 °C, and 40 cycles at 95 °C (15 s) and 60 °C (1 min). An α -tubulin probe was used as calibrator. Primer and probe sequences are listed in Table 1.

Table 1
Primers and probes used in real-time RT-PCR reactions

Pb gene	Primer and probe sequences
α -Tubulin	Rev: 5' ACC CAG CTT GGA AAC AGT GCT 3' Fw: **[6FAM] 5' CAC GGA CCA TCA GCC TTC AAA CCG 5G 3'
β -Tubulin	Rev: 5' TGG CCA CTT TCT CTG TCG TTC 3' Fw: 5' CAG GGT GGC ATT GTA GTA TGG CT 3' probe: 5' [VIC] TTC GCC CAA AGT CTC CGA CAC CG [TAMRA] 3'
PbGP43	Rev: 5' TGT CAC CCT TTT GCC AGT TG 3' Fw: 5' TTC CCA AAA CGG CTT CGA 3' probe: 5' [VIC] ACA GCG GTC ACC GTG GCG C [TAMRA] 3'
PbGP43	Rev: 5' TCC TGC TTG ACG AGG TAC AAT GT 3' Fw: 5' GCT GTG TTC TCG CAT GGG TAT 3' Probe: 5' [6FAM] CTC ACG TCG CAT CTC [TAMRA] 3'

Abbreviations: 6-FAM, 6-carboxyfluorescein; VIC, trademark product from Applied Biosystems; TAMRA, 6-carboxy-*N,N,N',N'*-tetramethylrhodamine, ** Lux Probe (Invitrogen).

2.8. Analysis of the polymorphism in the *PbGP43* promoter

We evaluated polymorphism in the promoter region of the *PbGP43* originally characterized by Cisalpino et al. ([17], GenBank accession number U26160). We sequenced two PCR cloned fragments of 17 isolates exactly as described in our previous work [29]. PCR reactions were carried out with primers sense 2133 (5'-GCG CAT GCC CCA CTC AAT-3') and anti-sense 2137 (5'-CGC CAC GGT GAC CGC TGT-3'), which elongated a 627-bp fragment bearing 321 bp of the non-translated region and part of the *PbGP43* ORF. The sequences were analyzed using the EditSeq, Seqman, Megalign and Protean programs of the Lasergene System (DNAsstar Inc.).

2.9. Statistical analysis

The results were expressed as mean values $\pm$ S.D. Statistical analysis of CFU results was carried out using ANOVA followed by Student–Newman–Keuls (INSTAT software, GraphPad, San Diego, CA). A $P < 0.05$ indicated statistical significance.

3. Results

Virulence in mice of *P. brasiliensis* isolates Pb2, Pb3 and Pb4 (*PbGP43* genotype A, substituted at sites 268, 578, 617, 628, 751, 763, 799, 830, 856, 872, 981, 1082, 1086, 1157, and 1166) was compared with that of Pb5 (genotype D, substituted at nt 589), Pb8, Pb18 (genotype E, substituted at nt 874 and 965), Pb9 and Pb12 (genotype F, substituted at nt 874, 965, 1143) (see Ref. [29] for details on *PbGP43* genotypes).

3.1. Analysis of i.p. infections with in vitro-adapted yeast cells

P. brasiliensis isolates were preliminarily compared according to CFU recovered from the spleen, liver and lungs

in the early stages of i.p. infection (21 days) of B10.A mice inoculated with in vitro-adapted fungal cells (Fig. 1A). Under these experimental conditions, Pb5 was recovered from the spleen at significantly higher CFU ($P < 0.001$) than any other isolate tested. The i.p. infections with Pb5 were rapid and highly disseminated through the peritoneum, as observed macroscopically. Recoveries of Pb2, Pb3 and Pb4 ranged between 206 and 553 mean CFU, notably lower than those of Pb5, or of Pb8, Pb9 and Pb12 (means between 853 and 1677). Dissemination to the lungs was not detected in any animal, while some colonies of each isolate (except Pb3) were recovered from the liver, especially from mice infected with Pb5 (Fig. 1A, C). A small anti-gp43 response was observed in all animals (Fig. 1B), suggesting that gp43 was present in the inoculated cells and/or expressed during proliferation in vivo. Histopathology showed large and confluent granulomas, with numerous yeast cells and neutrophils, which are features more characteristic of the early stages of infection (Fig. 1C).

3.2. Analysis of i.t. and i.v. infections with organ-recovered yeasts

We next compared virulence of *P. brasiliensis* isolates during i.t. and i.v. infections; however, the inocula were normalized by using spleen-recovered yeast cells. We also introduced Pb18 in the study, since this isolate has been widely used in experimental PCM [4]. Intratracheal inoculation mimics the natural infection, while i.v. infection with numerous yeasts exacerbates the disease and consequently shortens the time spent to obtain survival curves.

Fig. 2A shows the number of viable yeast cells recovered from mouse lungs after 30 and 60 days of i.t. inoculation with eight fungal samples recovered from two previous animal passages. Dissemination to the spleen was not detected, while *P. brasiliensis* in the liver could eventually be observed in mice infected with Pb4, Pb5, Pb8, Pb12 and Pb18 (not shown). The lung CFU of Pb18, Pb8 and Pb12 were significantly higher than the others. Pb12, however, was far more aggressive than Pb8 and Pb18 in our experimental conditions. The CFU counts of Pb2, Pb3 and Pb4 (maximum mean of 182) were notably lower than the others. Anti-gp43 antibody levels correlated with fungal load for Pb2, Pb4, Pb18 and Pb8 (Fig. 2B). But the situation was intriguing in mice infected with Pb12, which had copious amounts of viable, actively budding yeasts in their lungs (Fig. 3), however, generally lacked or had reduced anti-gp43 response (Fig. 2B). That was not due to a general failure in the humoral immune response, since immunoblotting of pooled sera from Pb12-infected mice strongly recognized Pb12 antigens (Fig. 2C). Our expression analysis, shown below, suggested that the reduced anti-gp43 response was due to the low level of gp43 expressed in vivo as a result of down-regulation of *PbGP43* in Pb12 highly infected animals.

The histopathology reflected the CFU data shown in Fig. 2A. Pb12-infected mice showed large and confluent granulomas involving extensive areas of the lung, which con-

Fig. 1. **A**, CFU of the *P. brasiliensis* isolates indicated (log scale) recovered from the spleen and liver of B.10A mice 21 days after i.p. infection with 10^6 in vitro-adapted viable yeast cells. **B**, Anti-gp43 antibody levels in sera of individual mice (1:100), tested in triplicate, shown as ELISA mean values of RLU. Values for normal mouse serum samples were deducted. RLU values were about 90,000 for a positive control carried out with serum (diluted at

tained numerous budding yeast cells with an inflammatory cellular infiltration (Fig. 3). The granulomatous inflammation evoked by Pb18 tended to be milder when compared with the damage caused by the most aggressive Pb12. It was characterized by diffuse granulomata containing few fungal cells (generally not viable). Mice infected with Pb3 did not show granulomas or even fungal cells in the fragments studied.

After i.v. infection with fungal cells recovered from one animal passage, Pb2, Pb3 and Pb4 evoked few deaths, while Pb12, isolated from a patient from Argentina with juvenile PCM, killed all the animals by day 60 (Fig. 4A). A second survival experiment was carried out with selected isolates that had been recovered from a series of consecutive animal passages, to allow for maximum reactivation [38]. Pb18, absent in Fig. 4A, was included. Note in Fig. 4B that the mice were killed more quickly by all the isolates; however, the degree of virulence observed in Fig. 4A was unchanged (Pb12 > Pb18 > Pb5 > Pb3).

3.3. Analysis of gp43 expression in yeast cells isolated from lung

The rare anti-gp43 response (Fig. 2B) found in Pb12 highly infected animals (i.t.) called our attention to the situations where fungal load contrasted with anti-gp43 titers. Therefore, we investigated expression of gp43 by Pb12 grown in vitro at 36 °C, using yeasts cells that had just been isolated from lungs (Fig. 2A). We compared it with Pb3 and Pb18, which showed, respectively, low and high virulence, but stimulated production of anti-gp43. We verified the status of gp43 expression at the transcriptional level, using real-time RT-PCR normalized with α -tubulin, and at the protein level in both intracellular extracts and supernatants (Fig. 5, Y). The sensitivity of our detection method was as low as 10–20 ng of gp43. We observed that the Pb12 culture analyzed had minute amounts of *PbGP43* and undetectable amounts of gp43. Pb3 and Pb18 had comparable levels of mRNA transcripts; however, the Pb3 culture was richer in gp43, mostly found in the supernatant, while small amounts were detected only in the intracellular compartment of Pb18. It is of note that the cultures analyzed were composed of fully formed yeasts growing in modified YPD at the log or late log phases, when secretion of gp43 is supposedly higher [39], but they might not be at their secretion peak. However, the low levels of transcripts expressed by Pb12 suggested that *PbGP43* might be down-regulated in vivo. An additional observation is that post-transcriptional events and secretion impairment are probably involved in gp43 expression, considering that comparable mRNA transcript levels of Pb3 and Pb18 resulted in different

1:5000) from a gp43-immunized mouse. In **A** and **B**, error bars indicate standard deviations of mean values. Statistically significant values ($P < 0.001$) are indicated by an asterisk (*). **C**, Histopathology (HE; 100x, upper panel and 400x, lower panel) of a liver from an i.p. infected mouse with Pb5 showing confluent fungal granulomata (arrows) with focal neutrophilic aggregates (arrowhead).

Fig. 2. Mean lung CFU (A, log scale) and anti-gp43 levels (B) of individual B10.A mice 30 and 60 days after i.t. infection with 10^6 organ-recovered viable yeasts of the *P. brasiliensis* isolates indicated. Error bars indicate the standard deviations of mean values. Filled arrows in A point to statistically significant CFU ($P < 0.05$) when Pb12 CFU (plain arrow) were not considered in the analysis. Pb12 values were statistically higher ($P < 0.001$) than all the others. C, Immunoblotting analysis of pooled sera (α -Pb12) from i.t. infected mice with Pb12 (30 and 60 days) or normal mouse serum (nl) and total cell extract from Pb12.

gp43 expression. That was also suggested in the experiments shown in Fig. 5 (M $\rightarrow$ Y) described below.

It was noticeable that lung-recovered Pb12 yeasts (Y) grew more slowly than Pb3 and Pb18 in rich medium, but the three isolates were able to transform into fully formed M within

6–15 days in agitated liquid cultures upon temperature change to 26 °C. Since transformation to yeast is believed to be essential for infection by *P. brasiliensis* to occur, we compared this phenotypic parameter among Pb12, Pb3 and Pb18 by following the in vitro cultures during phase transition from fully formed mycelium after temperature change from 26 to 36 °C. We observed that Pb12 converted more slowly than Pb3 and Pb18 (not shown). Consequently, Pb12 cultures still had a larger proportion of thin, elongated hyphae than that of yeast-like structures after 120 h of growth at 36 °C, while the correspondent Pb3 and Pb18 cultures were mostly composed of budding yeasts.

The transition-phase cultures were analyzed for gp43 expression (Fig. 5, M $\rightarrow$ Y). The initial mycelium culture (0 h) of Pb12 had small levels of *PbGP43* mRNA when compared with Pb3 or Pb18, but there was a significant increase of over 10-fold at 48 and 120 h after temperature shift. The antigen, however, could not be seen in the intra- or extracellular compartments. In Pb18, the mRNA transcript levels did not vary considerably throughout transition, yet gp43 could generally be seen both intra- and extracellularly.

It was interesting to note that Pb3 showed a peculiar pattern of prolonged down-regulation of *PbGP43* during M $\rightarrow$ Y, which was probably evoked by a differentiated mRNA transcription regulation upon increase in temperature. In order to search for polymorphisms in the promoter region of *PbGP43* that could suggest differences in gene regulation, a 306-bp fragment upstream of the starting codon was sequenced in the same 17 isolates used to characterize the *PbGP43* ORF [29]. Although a larger extension of the upstream region of *PbGP43* has not been cloned and sequenced so far, the analysis carried out with the computer program TFSEARCH (<http://www.molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html>) [40] predicted many transcription factor binding sites in that 306-bp fragment. It is of note that the transcription start site has been mapped by primer extension at positions –25, –33, and –35 in four different *P. brasiliensis* isolates (not shown) and that the predicted TATA box (TATAAATA) locates at –80 [17]. In our analysis of promoter polymorphism, two cloned fragments from each isolate were sequenced, and seven informative substitution sites (i.e., seen in at least two isolates) were found (Table 2). They defined five genotypic groups, including the same isolates grouped before in accord with polymorphism in the *PbGP43* ORF [29], except that *P. brasiliensis* showing genotypes E and F (which are similar) had the same promoter sequence (genotype E/F of Table 2).

Isolates Pb2, Pb3 and Pb4 (*PbGP43* genotype A) displayed the most polymorphic sequences, with three substitutions at –104, –120 and –230. We compared the predicted motifs for transcription factors in a sequence substituted at the sites seen in Table 2. Only substitution from T to A at –120 changed the profile, where additional transcription elements were then predicted. When position –120 is substituted by A, five putative transcription elements are created, including two for heat shock factors. It was intriguing to find

Fig. 3. Histopathology (HE; 100 $\times$ and 400 $\times$) of lungs from i.t. infected mice (details in Fig. 2) with the *P. brasiliensis* isolates Pb3, Pb18 and Pb12 indicated. Extensive, confluent, fungal cell-rich granulomata evoked by Pb12 (arrow) in contrast to the more focal involvement seen in a Pb18-infected animal (arrow). No lung involvement evoked by Pb3.

such elements in Pb3, which showed *PbGP43* down-regulation after temperature shift from 26 to 36 °C. However, this result was later confirmed in heat-shocked yeast cells growing at 36 °C (Fig. 6): we observed a sharp decrease in *PbGP43* expression only in Pb3, while in Pb18 (and Pb1, not shown) the mRNA transcript levels remained unaltered after 30 min at 42 °C.

4. Discussion

We found three features that distinguished Pb2, Pb3 and Pb4 from the others: (a) they showed consistently low virulence when inoculated i.p., i.t. and i.v. in B10.A mice; (b) they shared the same pattern of genetic polymorphism in the promoter region of *PbGP43*; (c) *PbGP43* was down-regulated in Pb3 in response to an increase in temperature, but the temperature-induced transition from mycelium to yeast was

not impaired. We present the first evidence of transcriptional regulation of gp43 expression, but our results suggest that gene expression is also regulated at the protein and/or secretion levels. The secretion seemed occasionally compromised, but factors such as protein stability have to be considered. A drop in extracellular gp43 in aging cultures of *P. brasiliensis* B-339 has been associated with proteolysis [39]. We have previously shown that gp43 is resistant to intracellular proteases at neutral pH [15], but stability at the basic pH characteristic of aging cultures [41] has to be better studied, as does pH-regulated gene silencing. It is difficult at this point to assess the role of gp43 in dimorphism, which has previously been addressed by Mattar-Filho et al. [42].

Pb2, Pb3 and Pb4 contain highly polymorphic and phylogenetically distant *PbGP43* sequences [29]. The DNA of these samples also show distinct Southern blot profiles when cleaved with *EcoRI* and probed with *PbLON* [29,43] or

Fig. 4. Survival curves of B10.A mice inoculated i.v. with 5×10^6 viable yeasts of organ-recovered *P. brasiliensis* (indicated), or with PBS (control). In B, inoculated yeasts had been recovered from various consecutive animal passages (in vivo adapted).

cleaved with *Sma*I and probed with the 5' region of *PbGP43* [unpublished results], suggesting that the *PbGP43* polymorphism is reflecting a broader genetic diversity in the species. We have recently shown that a clinical sample (Bt84) and a Brazilian armadillo isolate (T10B1) also share *PbGP43* of genotype A [30], but they were not studied in this work. Polymorphism in the internal transcribed spacer region of the ribosomal DNA was detected in Pb2, Pb4, Bt84 and T10B1 [30]. In the present study, we found the same pattern of nucleotide substitution in the *PbGP43* promoter region of Pb2, Pb3 and Pb4. Substitution at –120 creates several putative transcription elements; however, the link between promoter polymorphism and gene expression will have to be proven by genetic analysis, which is ongoing in our laboratory. It will also be important to analyze the entire 5' intergenic region of *PbGP43*, which has not been characterized yet, in order to look for distant gene repressors or enhancers. gp43 is homologous to fungal β -1,3-glucanases, but not enzymatically active [17], and its biological activity has not been unraveled so far.

Fig. 5. Expression of gp43 and of the *PbGP43* transcript in fully formed yeast (Y) or mycelium (M, 0 h) of *P. brasiliensis*, and during the indicated times of M to Y temperature-induced phase transition of Pb12, Pb3 and Pb18. The columns show the relative *PbGP43* transcript values as *PbGP43* per α -tubulin transcript copy, measured by real-time RT-PCR. Intra- and extra-cellular gp43 corresponding to 1 ml of culture are shown as SDS-PAGE silver-stained bands, from previously Coomassie brilliant-blue-stained/desained gels. Time point 5 h, Pb3 (M to Y) was lost (ND).

It will be interesting to evaluate whether glucanases and gp43 have a conserved pattern of gene regulation.

There are only two previous reports describing correlation between a genetic feature and virulence. One showed higher virulence in mice of two isolates that shared only 17% of RAPD similarity with others [24], and the second observed an association between nucleotide differences in *PbGP43* and virulence of three isolates from different organs of the same armadillo [44]. In both works, the authors used i.p. inoculation of B10.A mice, and infection was recorded at day 28 [44] or followed until day 112 [24]. In the present work, pathogenicity was tested under different conditions. Pb12 was visibly more aggressive in mice inoculated i.t. and i.v.; however, only Pb5 (and not Pb12) was statistically more virulent for i.p. infected animals, despite its being only intermediately

Table 2

Polymorphic nucleotides found in the 5' intergenic region of the *PbGP43* gene from 17 *P. brasiliensis* isolates (Pb1 to Pb17^a and Pb18)

Genotypes		A	B	C	D	E/F
Pb code number		2, 3, 4	15, 16	1	5, 10, 11	6, 8, 9, 12, 13, 14, 17, 18
Sites	Con ^b					
–40	C	•	•	•	•	T
–104	C	G	•	•	•	•
–120	T	A	•	•	•	•
–209	C	•	A	•	•	•
–210	G	•	•	•	T	•
–230	C	A	•	•	•	•
–259	C	•	T	•	•	•

^a As in Ref. [29].^b The consensus nucleotides (con) of the polymorphic sites, numbered as in Cisalpino et al. [17], are shown.

virulent i.t. and i.v. Re-isolated Pb12 continued to evoke a milder i.p. infection than Pb5 (not shown). On the other hand, reactivation was observed in Pb3 after a series of consecutive animal passages. Therefore, although Pb2, Pb3 and Pb4 were consistently less aggressive in comparison with others tested under the same experimental conditions, we believe that features like animal model, route of infection and type of inoculum (degree of in vitro or in vivo adaptation, culture and growth conditions, etc.) might considerably change virulence results, as observed here and elsewhere [4].

Fig. 6. Heat shock effect on transcription of *PbGP43* evaluated by real-time RT-PCR. Pb3 and Pb18 growing yeasts (point 0, 36 °C) were exposed to 42 °C for 30 and 60 min before RNA extraction. The columns show the relative *PbGP43* per β -tubulin transcript copy. Pb1 (B-339) behaved like Pb18. Pb3 viability at 42 °C was assessed by microscopic observation and by overexpression of *PbMDJ1* [43] at time points 30 and 60 min (not shown).

Circulating anti-gp43 was generally detected in the infected animals; however, antibody levels were not strictly correlated with fungal loads. During the fierce i.t. infection caused by Pb12, the antibody response against fungal antigens was high, but gp43 was apparently down-regulated. That probably contributed to the rapid course of infection, considering that the antigen can activate protective cellular immunity in vaccinated mice [9,45], which is mediated by a Th1 lymphocyte response inducing IFN- γ . In human PCM and in mice, the various forms of the disease seem to be associated with the degree of cell-mediated immune suppression, which will depend on the balance between the Th1/Th2 responses [1,4]. The cellular and antibody responses to gp43 seem to vary according to the antigen-presenting cell [46,47]; however, it is not known how these mechanisms will interact during an infection involving the release of many fungal components or different gp43 isoforms. On the other hand, an idiotypic network modulates the immune response to gp43 [48,49], and that will probably be influenced by differences in its antigenicity, especially in the genotype A isoform. In addition, gp43 can bind to mouse laminin and enhance fungal pathogenesis in the hamster testicular model [11,16], but not in the mouse pulmonary model [50]. As observed, the correlation between gp43 and virulence is a complex matter to address, and down-regulation of the antigen in Pb12 is probably not the sole factor involved in its enhanced pathogenicity. Genetic manipulation of *P. brasiliensis* will be essential for future studies on the role of gp43 and other genes in the fungal biology and host–parasite relationship. We believe that this will be possible following the success recently obtained with other dimorphic pathogens [51,52].

The mechanisms involved in down-regulation of gp43 in vivo remain to be investigated, but a correlation with genotype was not observed. In contrast, our results in vitro suggest that transcriptional regulation of gp43 in genotype A isolates might have distinctive features, like that seen for heat-shocked Pb3. If proved experimentally with other genes and isolates of this genetic group, this observation will be helpful to avoid misinterpretation of isolate-related differences in expression results coming from distinct laboratories. This is especially important because two databases of *P. brasiliensis* genes are available today from two different isolates [35,53], and expression analyses are ongoing [54]. In the near future,

overall gene expression will be compared in several lung-recovered isolates studied here using microarrays built with sequence information previously obtained [35]. We hope that the results will shed some light on the comprehension of the *P. brasiliensis* genes involved in pathogenicity.

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APÊNDICE D

Apêndice D – Publicação:

Observação: As figuras coloridas se encontram ao final deste apêndice.

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23 September 2005

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Transcriptome Analysis of *Paracoccidioides brasiliensis* Cells Undergoing Mycelium-to-Yeast Transition

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Paracoccidioides brasiliensis is a thermodimorphic fungus associated with paracoccidioidomycosis (PCM), a systemic mycosis prevalent in South America. In humans, infection starts by inhalation of fungal propagules, which reach the pulmonary epithelium and transform into the yeast parasitic form. Thus, the mycelium-to-yeast transition is of particular interest because conversion to yeast is essential for infection. We have used a *P. brasiliensis* biochip carrying sequences of 4,692 genes from this fungus to monitor gene expression at several time points of the mycelium-to-yeast morphological shift (from 5 to 120 h). The results revealed a total of 2,583 genes that displayed statistically significant modulation in at least one experimental time point. Among the identified gene homologues, some encoded enzymes involved in amino acid catabolism, signal transduction, protein synthesis, cell wall metabolism, genome structure, oxidative stress response, growth control, and development. The expression pattern of 20 genes was independently verified by real-time reverse transcription-PCR, revealing a high degree of correlation between the data obtained with the two methodologies. One gene, encoding 4-hydroxyl-phenyl pyruvate dioxygenase (4-HPPD), was highly overexpressed during the mycelium-to-yeast differentiation, and the use of NTBC [2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione], a specific inhibitor of 4-HPPD activity, as well as that of NTBC derivatives, was able to inhibit growth and differentiation of the pathogenic yeast phase of the fungus in vitro. These data set the stage for further studies involving NTBC and its derivatives as new chemotherapeutic agents against PCM and confirm the potential of array-based approaches to identify new targets for the development of alternative treatments against pathogenic microorganisms.

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Most systemic infections of immunocompetent humans are caused by dimorphic fungi. This group of pathogens includes *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Coccidioides* spp., and thermally regulated dimorphism is their main defining genetic trait (34). *P. brasiliensis* is the causative agent of paracoccidioidomycosis (PCM), a systemic mycosis prevalent in Latin America and with a broad geographic distribution that spans from Mexico to Argentina (47). It is estimated that as many as 10 million individuals are infected with *P. brasiliensis*, acquired by inhalation of airborne microconidia, which reach the pulmonary alveolar epithelium and transform into the parasitic yeast form (43).

Pathogenicity is intimately linked to the dimorphic transition, since strains of *P. brasiliensis* (and also of *H. capsulatum*

and *B. dermatitidis*) that are unable to transform into yeasts are not virulent (3, 38, 44, 47). Signaling pathways that control the morphological changes in *P. brasiliensis* are poorly understood, but the involvement of both cyclic AMP and mitogen-activated protein kinase signal transduction pathways has been reported for other dimorphic fungi (26). The dimorphic transition also involves changes in cell wall composition, such as migration and reorganization of membrane lipids, especially glycosphingolipids (27, 50, 52), and structural alterations in the carbohydrate polymers. As the fungus adopts the yeast form, an increase in chitin content is observed in the cell wall, followed by a change in the glucan anomeric structure from a β -1,3-linked polymer to an α -1,3-glucan (47). The surface α -glucan may play a role against the host defense mechanisms, due to the incapacity of phagocytic cells to digest α -1,3-glucan (46).

The identification of genes specifically involved in the mycelium-to-yeast dimorphic shift has been the subject of great interest, especially after the genome of *P. brasiliensis* was submitted to extensive expressed sequence tag (EST) sequencing, which allows the identification of several *Candida albicans* virulence and pathogenicity homologues (12, 13, 15). In a pre-

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liminary attempt to isolate phase-specific transcripts, Venâncio et al. (51) have employed the technique of differential display, which led to the identification of many differentially transcribed cDNA fragments specific for each phase. With the aid of genomics-based approaches, such as electronic subtraction, suppressive subtraction hybridization, real-time reverse transcription-PCR (RT-PCR), and low-density array hybridizations, further information regarding phase-specific gene expression has been obtained from both mycelium and yeast cells (12, 13, 15, 35). Nonetheless, very few genetic determinants identified in this fungus are known to be directly involved in either phase transition and/or pathogenicity, although the experiments mentioned above identified the up-regulation of genes that may play a role in the structural or metabolic changes that take place during morphogenesis or may be necessary for colonization and survival in the host (44).

For a more comprehensive evaluation of genes that have their expression modulated during the mycelium-to-yeast transition, we performed a large-scale analysis of gene expression in *P. brasiliensis* using a microarray hybridization approach. Since the complete genome of *P. brasiliensis* has not yet been sequenced, we constructed a 4,692-element array containing amplified EST sequences that have been functionally classified by similarity to known genes (15). Using this array, we identified 2,583 genes whose expression displayed statistically significant modulation during the mycelium-to-yeast transition, and analysis of these data allowed us to identify specific genes and gene sets whose induction seem to be related to biochemical and morphological changes associated with transformation into the pathogenic yeast form. These include genes involved in amino acid catabolism, signal transduction, protein synthesis, cell wall metabolism, genome structure, oxidative stress response, growth control, and development. One of these genes, encoding the enzyme 4-hydroxyl-phenyl pyruvate dioxygenase (4-HPPD), has been evaluated as a new potential drug target through the use of NTBC [2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione], a specific 4-HPPD inhibitor (53) that may provide an alternative chemotherapeutic strategy against PCM and other fungus-related diseases.

MATERIALS AND METHODS

Strains, culture medium, and disk diffusion assay. We used *P. brasiliensis* isolate 18, named Pb18 (provided by Z. P. Camargo, UNIFESP, São Paulo, Brazil) throughout the work. Fungal cells were cultivated in modified YPD medium (0.5% yeast extract, 0.5% casein peptone, 1.5% glucose, pH 6.5) at either 37°C (yeast phase) or 26°C (mycelium phase); liquid cultures were shaken at 120 rpm in a rotary shaker. Yeast cells were recovered from the lungs of intratracheally infected mice as described previously (6) and subcultured three successive times in modified YPD slants at 10-day intervals. In order to obtain the fungal mycelium phase, yeast cells from slants were inoculated in 200 ml of the same medium and incubated at 26°C for approximately 9 days under shaking, when 100% conversion was achieved. For analysis of mycelium-to-yeast phase transition cells, equivalent volumes of this initial culture were transferred to Erlenmeyer flasks containing 200 ml of fresh medium, grown for 4 days at 26°C, and shifted to 37°C for temperature-induced phase transition. Cultures were collected at 0, 5, 10, 24, 48, and 120 h of growth for morphological observation and RNA extraction.

For the disk diffusion assay, after standardized growth and harvest (15), 5.0×10^7 *P. brasiliensis* viable yeast cells were resuspended in 2 ml of PGY top-agar medium (Bacto peptone at 5 g/liter, glucose at 10 g/liter, yeast extract at 5 g/liter, and 0.7% agar) and poured onto PGY agar plates (same composition, but agar at 17 g/liter). Blank 6-mm-diameter paper disks (3MM; Whatman) were impregnated with 0.25, 0.5, 1.0, 5.0, 7.5, or 10.0 µg/ml of compound 8 or with 5.0, 10.0,

25.0, 50.0, 100.0, or 200.0 µg/ml of NTBC and all derivatives. After drying, they were placed onto inoculated agar plates. The plates were incubated at 37°C, and the diameters of the inhibition zones were evaluated after 7 days. Each disk diffusion assay was performed twice.

RNA isolation. Yeast cells and mycelium were disrupted with glass beads and by grinding in liquid nitrogen, respectively (15), and immediately mixed with Trizol (Gibco-BRL) for RNA extraction following the supplier's recommendations. To verify the RNA integrity, 20 micrograms of RNA from each treatment was fractionated in 2.2 M formaldehyde, placed on a 1.2% agarose gel, stained with ethidium bromide, and visualized with UV light. The presence of intact 28S and 18S rRNA bands was used as a criterion to verify that the RNA was intact. RNase-free DNase treatment was done in a final volume of 100 µl containing 40 mM Tris-HCl (pH 7.5) and 6 mM MgCl₂, 1 µl of RNasin (40 U/µl; Promega), 10 µl of RNase-free DNase (1 U/µl; Promega or Life Technologies), 2.5 µl of 200 mM dithiothreitol, and 10 µg of total RNA. The reaction was incubated at 37°C for 60 min and stopped by incubating at 70°C for 30 min. The absence of DNA contamination after the RNase-free DNase treatment was verified by PCR amplification of the *GP43* gene using oligonucleotides GP43-2030 (5'-ACAAGTCAACTCGACTA-3') and GP43-2031 (5'-GGGGCAGAG AAGCATCCG-3') (26).

Microarray hybridizations and data analysis. The array consists of cDNA inserts from representative ESTs from 4,692 expressed genes that were functionally classified by similarity to known genes (15). These inserts were PCR amplified with the M13 forward and reverse primers, and the amplified products were purified with a QIAquick 96 PCR purification system (QIAGEN), dried in a Savant Speed Vac system, resuspended in 50% dimethyl sulfoxide at a final concentration of 100 to 200 µg/ml and spotted onto 25- by 75-mm CMT-GAPS coated slides (Corning) using an Affymetrix 427 arrayer, according to the manufacturer's instructions. Those interested in obtaining the *P. brasiliensis* microarrays, please contact the authors for further details.

The RNA samples extracted with Trizol as described above were further purified with aid of an RNeasy kit (QIAGEN) and labeled by incorporation of Cy3- or Cy5-dCTP, as described by Bowtell and Sambrook (4). Briefly, 30 µg of total *P. brasiliensis* RNA was mixed with 2 µg of oligo(dT)12-18 in 20 µl of water and heated to 65°C for 10 min. The tube was briefly chilled on ice and mixed with 4 µl of 5 mM dNTPs, 4 µl of 0.1 M dithiothreitol, 8 µl of 5× first-strand buffer (Invitrogen), and 1.5 µl of Superscript II (200 units/µl) (Invitrogen). The reaction was then incubated at 42°C for 1.5 h and terminated by a 15-min incubation at 65°C. The RNA was degraded by the addition of 1 µl of 10 µg/µl RNase A and incubation at 37°C for 1 h. The resulting first-strand cDNA was purified and concentrated with the aid of a Microcon YM-30 cartridge, and the final volume was adjusted to 29 µl. One µl of 4-µg/µl random primer solution (9-mers) and 4 µl of 10× Klenow buffer were then added to the system. The tube was then heated at 65°C for 5 min and allowed to cool to room temperature for 10 min for primer annealing. Finally, fluorescence incorporation was performed by the addition of 4 µl of 10× low-dNTP solution (5 mM dATP, 5 mM dTTP, 5 mM dGTP, and 2 mM dCTP), 1 µl of Cy3- or Cy5-labeled dCTP (1 mM) (Amersham Biosciences), and 1 µl of the Klenow fragment (50 units/µl) (Invitrogen). The reaction was then incubated at 37°C for 2 h, and the final labeled cDNA was purified in a Microcon YM-30 cartridge (three washes).

Labeled cDNAs were mixed, dried in a Savant speed vacuum, and resuspended in 100 µl of 1× hybridization buffer containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.25 mg/ml salmon sperm DNA, 0.01% sodium dodecyl sulfate (SDS), 50% formamide, and 92.5 ng/µl poly(dT) 25u (Amersham). Arrays were hybridized overnight (42°C) in a Gene-Tac hybridization station (Genomic Solutions, Inc., Ann Arbor, MI) and washed twice (42°C) in 0.5× SSC and 0.01% SDS, which was followed by two washes in 0.06× SSC and 0.01% SDS and two final washes in 0.06× SSC. All washing steps consisted of 1 min of flow followed by 5 min of incubation. Slides were then dried and subjected to fluorescence detection with a GMS 418 array scanner (Affymetrix Inc., Santa Clara, CA). Images were analyzed with the TIGR Spotfinder program (v.2.2.4). All spots with median values lower than the median local background plus 2 standard deviations were flagged and excluded from further analyses. Each time point was analyzed with four independent hybridizations (two pairs of hybridizations with dye swaps within each pair). Since each biochip carried two replicas of the arrayed genes, a total of eight intensity readings were generated for each element in the microarray. The results from each hybridization were submitted to a series of mathematical transformations with the aid of the software TIGR MIDAS v.2.19. These included filtering out all spots whose integrated intensities were below 10,000 a/d units, normalizing between the two channels with the aid of the Lowess algorithm, and regularizing standard deviations of the Cy5/Cy3 ratios across all sectors (blocks) of the array. Finally, replicated slides were submitted to a dye swap consistency check, and the

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FIG. 1. Temperature-induced morphological switch in *P. brasiliensis*. Mycelia growing in the early exponential phase were induced to undergo morphological transformation by changing the temperature of incubation from 26 to 37°C. At the different time points, the morphological units were arbitrarily classified as hyphae, differentiating hyphae (characterized by the development of chlamydospore-like cells produced by intercalary or lateral swellings in the fertile hyphae), transforming yeast (characterized by the production of multiple buds by the chlamydospore), or yeast. Relative quantities of each morphotype were obtained after individually scoring a total of at least 300 morphological units for each time point.

results from each individual experiment were loaded into the software TIGR Multi-Experiment Viewer, v.3.01. Experiments were then normalized, and genes that displayed statistically significant modulation were identified by a one-way analysis of variance, considering a *P* value of <0.01 as a cutoff. Further clustering analyses of the modulated genes have also been performed with the aid of TIGR Multi-Experiment Viewer. For details regarding the use of the TIGR microarray software suite (TM4), see the work of Saeed et al. (45). Raw and normalized data from all microarray hybridizations, as well as the microarray complete annotation file, have been submitted in MIAME (Minimum Information About a Microarray Experiment)-compliant format to NCBI's Gene Expression Omnibus and can be assessed through series number GSE 3238. A tab-delimited file containing the list of significant genes and the averaged expression profile of such genes can be downloaded from <http://143.107.203.68/pbm2ytrans/index.html>.

Real-time PCRs and RT-PCRs. All the real-time PCRs and RT-PCRs were performed using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems). TaqMan EZ RT-PCR kits (Applied Biosystems) were used for RT-PCRs. The thermocycling conditions comprised an initial step at 50°C for 2 min followed by 30 min at 60°C for reverse transcription, 95°C for 5 min, and 40 cycles at 94°C for 20 sec and 60°C for 1 min. TaqMan PCR reagent kits were used for PCRs. The thermocycling conditions comprised an initial step at 50°C for 2 min followed by 10 min at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 1 min. Since there is no ideal control for gene expression, we first compared several genes, such as those encoding α -tubulin, hexokinase, and a translation factor, as normalizers for the expression experiments. We saw no difference by using any of these normalizers. Accordingly, the calibrator gene used along the expression experiments was the α -tubulin gene (data not shown). The reactions and calculations were performed according to the work of Semighini et al. (48). Primer and probe sequences are described in Table S1 in the supplementary

materials at <http://143.107.203.68/pbm2ytrans/index.html>. Correlation coefficients comparing the log₂ ratios for RT-PCR and array data were calculated with the aid of the software Biostat v2.0.

RESULTS

Morphological evaluation of the mycelium-to-yeast transition in *P. brasiliensis*. The temperature shift from 26° to 37°C is the single condition known to trigger the mycelium-to-yeast transition in *P. brasiliensis*. We followed the different steps of the dimorphic transition and established parameters to quantify the different morphotypes that are produced during this process (Fig. 1). We arbitrarily classified the transition in four different morphological states: (i) hyphae; (ii) differentiating hyphae, characterized by the development of chlamydospore-like cells, which are produced by intercalary or lateral swellings in the fertile hyphae; (iii) transforming yeast, characterized by the production of multiple buds by the chlamydospore; and (iv) mature, multibudding yeast. This classification helped us to establish quantitative parameters to assess the morphological transition at each time point. It is clear that a 120-h incubation period is enough to obtain differentiation of the mycelium into yeast forms (over 85% of the morphotypes in the culture are

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FI

TABLE 1. Comparison of gene expression values obtained with microarray hybridization and real-time RT-PCR

Gene product (gene)	Value from array/RT-PCR at ^a :						Correlation coefficient (R_P/R_S) ^b
	5 h	10 h	24 h	48 h	72 h	120 h	
Branched-chain alpha-keto acid dehydrogenase E2 subunit (MAS1216)	0.02/1.57	2.15/2.63	1.36/2.77	3.59/2.99	4.22/4.73	3.89/3.70	0.89/ 0.94
Sulphur transcription regulator <i>METR</i> (MAS2161)	0.72/1.80	1.02/2.15	0.42/1.62	2.69/4.53	2.46/4.30	2.03/2.13	0.88/ 0.94
Alkaline serine protease (MAC0354)	2.92/1.5	−1.27/−0.23	0.10/1.08	1.90/0.27	−0.12/−0.08	1.80/0.46	0.66/ 0.77
Peptide methionine sulfoxide reductase (MAC0725)	0.28/1.68	0.64/2.98	0.01/1.69	1.23/3.34	1.29/6.72	0.74/1.64	0.76 /0.60
Low-affinity hexose transporter <i>HXT3</i> (MAS1077)	−1.27/2.33	0.63/1.92	1.02/3.96	2.79/5.12	4.95/4.99	2.47/6.24	0.74 /0.71
Homogentisate dioxygenase (MAC0559)	−0.65/2.04	0.82/2.65	1.32/4.24	3.95/3.90	1.35/3.38	1.82/2.96	0.74 /0.60
Membrane protein YDL237w (MAS1188)	2.23/1.35	0.16/1.55	−1.28/−0.40	−0.81/0.19	−0.03/3.05	−0.68/0.03	0.50/ 0.71
GPR/FUN34 family protein (MAS2134)	−0.57/1.51	0.09/0.87	0.34/0.67	1.09/4.96	3.30/7.84	2.26/1.23	0.71 /0.50
Alcohol dehydrogenase (MAC0660)	1.5/−2.0	1.95/2.86	1.82/3.41	3.15/5.16	0.91/−0.27	1.32/3.24	0.70 /0.60
Thioredoxin (MAS1661)	1.58/1.43	2.28/1.41	2.37/1.31	0.41/0.79	1.59/1.88	0.57/0.79	0.68 /0.46
Immunoreactive protein precursor (MAS1924)	2.35/0.85	1.10/2.22	3.99/1.99	4.32/0.15	2.79/0.78	−1.92/−2.73	0.66 /0.03
Unknown protein (MAS1735)	−0.63/0.97	0.18/1.89	0.18/1.07	−0.48/2.37	0.63/5.45	0.13/1.25	0.60 /0.55
Unknown protein (MAC0195)	3.02/1.03	0.99/1.06	0.41/−0.61	0.42/−1.72	0.88/2.89	0.48/−0.90	0.41/ 0.60
4-Hydroxyphenylpyruvate dioxygenase (MAC0550)	1.94/1.39	5.76/2.37	4.78/2.94	4.86/3.92	4.53/3.48	3.01/2.76	0.58 /0.37
ADP ribosylation factor-like protein 1 (MAC0849)	−0.35/1.6	0.94/4.85	0.70/0.90	0.95/1.57	1.20/6.25	0.37/−1.24	0.56 /0.54
Unknown protein (MAC0867)	0.03/1.46	0.31/1.44	−0.59/0.56	−0.54/0.54	−0.52/7.21	−0.40/1.26	−0.17/ 0.54
Branched-chain alpha keto acid dehydrogenase E1 (MAS1260)	0.58/1.13	0.72/2.17	0.36/1.29	2.38/1.95	1.58/4.67	2.14/2.17	0.37/ 0.50
Stress-induced protein <i>STII</i> (MAS0665)	2.78/0.69	2.09/1.69	1.28/1.02	0.71/1.01	1.68/1.37	1.33/1.60	−0.17/ 0.09
Unknown protein (MAS2305)	−1.05/1.8	−0.04/−0.03	−0.29/−0.83	−0.70/1.09	0.47/3.29	0.05/−1.71	− 0.002 /−0.08
Peroxiredoxin V protein (MAC1221)	2.05/1.37	0.03/0.56	1.80/0.30	1.80/0.09	−0.07/1.99	2.00/0.14	−0.51/−0.31

^a The values are shown as the $\log_2$ ratios between the values obtained at the indicated experimental time points and the reference value (0 h).

^b We calculated both Pearson's (R_P) and Spearman's (R_S) correlation coefficients for each pair of curves. The former is more adequate for comparisons in which the data assume a normal distribution, while the latter is more appropriate for data sets which do not follow a normal distribution. The higher R value is highlighted in bold. Correlation coefficients were calculated after comparison of the array and RT-PCR data for each gene.

mature yeasts, while those remaining are transforming yeasts). Since gene expression modulations at the earlier stages of the differentiation process might be important in triggering the mycelium-to-yeast transition, we decided to evaluate gene expression variations at each of the time points, which were 5, 10, 24, 48, 72, and 120 h after the temperature shift. Thus, total RNA extracted from these cultures was used to synthesize fluorescence-labeled cDNAs for competitive microarray hybridizations.

Identification of genes that have their expression modulated during the mycelium-to-yeast transition. To assess *P. brasiliensis* gene expression on a large scale, we constructed a microarray carrying PCR-amplified cDNA representatives of 4,692 nonredundant EST clusters obtained from a *P. brasiliensis* (strain Pb18) library (15). Considering that the number of the genes in the *P. brasiliensis* genome has been estimated to be about 8,000 to 10,000, our microarray is covering about 40 to 50% of the expected total number of genes for this fungus. These cDNA amplicons ranged from 0.8 to 1.6 kb in length and have been annotated according to the method of Goldman et al. (15). Further information regarding the specific nomenclature and access codes used to identify the arrayed ESTs discussed in this work can be found at <http://143.107.203.68/pbver2/default.html>.

Microarray hybridizations were carried out with RNA obtained from cells harvested at 5, 10, 24, 48, 72, and 120 h after setting the temperature shift. The RNA obtained from the

original mycelial culture was taken as reference ($t = 0$ h). Statistical analysis of the data set identified a total of 2,583 genes that displayed modulation in at least one experimental time point. To verify the level of reliability of the array-based data, we randomly selected a series of 20 genes and analyzed their respective transcriptional profiles across the mycelium-to-yeast transition by quantitative real-time RT-PCR (Table 1). A direct comparison between the two methodologies was first performed in a simple qualitative way, verifying the number of cases in which the $\log_2$ ratios of gene expression obtained from both methods pointed to the occurrence of either the up- or down-regulation of a gene at a specific time point. As observed in Table 1, only 23 out of 120 time points tested by both analyses failed to reveal a match in the direction of gene expression modulation, indicating an overall qualitative coincidence of ~81% between the array hybridization and real-time RT-PCR data.

We also compared the gene expression variation estimated by these two methodologies by calculating both Pearson's (R_P) and Spearman's (R_S) correlation coefficients for the $\log_2$ ratios obtained by the two approaches. As shown in Table 1, positive correlation was observed for both R_P and R_S in 16 out of 20 genes (80% of the cases). Furthermore, the value of either R_P or R_S was above 0.50 (indicating moderate to strong correlation) in 17 out of 20 genes (85% of the cases). Thus, although we were able to detect some discrepancies between the two methodologies, it seems that our microarray hybridization ap-

FIG. 2. Hierarchical clustering showing the pattern of expression of *P. brasiliensis* genes encoding ribosomal proteins during the mycelium-to-yeast transition. The color code displays the $\log_2(\text{Cy5/Cy3})$ ratio for each time point and has Cy3 as the reference value (hyphae at time point 0).

proach is capable of providing information about *P. brasiliensis* gene expression modulation with a considerably high level of confidence.

Most relevant metabolic aspects of the mycelium-to-yeast transition. A direct analysis of the modulated genes identified through our microarray hybridization experiments allowed us to detect up- and down-regulation of several genes involved in a variety of cellular processes. Their specific modulation is likely to be implicated with the *P. brasiliensis* dimorphic shift, as is their response to heat shock and other environmental stimuli to which the culture has been submitted during the dimorphic transition. Some of these genes have been manually selected from our list of significant genes (available at

<http://143.107.203.68/pbm2ytrans/index.html>) and organized in hierarchical clusters, which are shown in Fig. 2 and 3. F2-3

The first thing to attract our attention was the behavior of genes that encode ribosomal proteins, since coordinated overexpression of approximately 60 of these genes was observed after 24 h of temperature shift (Fig. 2). The same overexpression was observed with the gene encoding an RNA polymerase I transcription factor (MAS0157; see Fig. 5B), indicating that the dimorphic transition is likely to involve intense synthesis of new ribosome particles, affecting the rate of protein synthesis as the fungus transforms into the yeast pathogenic form.

Furthermore, it has been well documented that *P. brasiliensis* yeast cells undergo several changes at the surface level, such

FIG. 3. Hierarchical clustering showing the pattern of expression of *P. brasiliensis* genes encoding proteins involved in chitin synthesis and degradation (A), heat shock proteins (B), and histones (C) during the mycelium-to-yeast transition. The color code displays the $\log_2(\text{Cy5/Cy3})$ ratio for each time point and has Cy3 as the reference value (hyphae at time point 0).

as an increase in cell wall chitin content, in contrast to hyphae (for a review, see reference 3). In fact, our microarray hybridizations identified up-regulation of a series of genes encoding chitin synthases upon temperature shift, whereas chitinases and endochitinases were simultaneously down-regulated (Fig. 3A). Interestingly, our biochip carried three other chitin synthase genes (MAS2882, MAS1009, and MAC1249) that did not show statistically significant variation in expression during the experiment, suggesting that chitin biosynthesis might also occur through the activation of alternative genes that do not respond to heat shock stimuli and/or do not belong to the dimorphic shift regulon. Kraus and coworkers (24) have also shown increased expression of a *Cryptococcus neoformans* chitin synthase gene during growth at 37°C. Niño-Vega et al. (41) have shown that despite the fact that yeast cells contain more chitin than hyphal cells do, the levels of mRNA for chitin synthase genes CHS1, CHS2, CHS4, and CHS5 were higher in hyphal cells than in yeast cells. The ESTs that correspond to CHS1 and CHS2 are not present in our microarray, and those

corresponding to CHS4 and CHS5 (MAS1009 and MAC1249) did not display statistically significant modulation upon analysis, as more expressed during the mycelium-to-yeast transition. It is possible that these four chitin synthase genes are specifically involved in chitin biosynthesis during the saprophytic phase.

Since the dimorphic transition in *P. brasiliensis* is triggered by an increase in temperature, we decided to evaluate the expression of genes encoding heat shock proteins during the mycelium-to-yeast transition. The biochip carried a total of 25 heat shock proteins (including putative heat shock proteins, as predicted by BLAST analyses). Only three of these Hsp genes did not show statistically significant variation throughout our experiment (MAC1157, MAS2388, and MAS0578). The remaining 22 Hsp genes have their expression profiles displayed in Fig. 3B, which shows that about 70% of them are clearly induced early after temperature shift (5 h). However, at time point 10 h, these genes exhibit rather distinct expression patterns. Only two of them, *HSP10* (MAC0138) and *HSP60*

(MAS1469), seem to be up-regulated throughout all analyzed time points. A second group of Hsp genes is characterized by severe down-regulation after 10 h, which occurs during the first 72 h of the differentiation process. Interestingly, this repression pattern seems to cease after 120 h, when their expression mostly returns to constitutive levels.

Alterations in chromatin structure are also likely to be induced by the temperature shift, since transcriptional repression of all histone genes is verified at time point 5 h (Fig. 3C). Interestingly, all histone genes display continued down-regulation until time point 72 h at least, with the exception of histone H4.2, which, coincidentally, has been shown to be under the control of a distinct regulatory mechanism in *Penicillium funiculosum* (2). Down-regulation of histone genes as a function of dimorphism has already been observed in the pathogenic fungus *Cryptococcus neoformans* (49) and may result in significant alterations in the cellular transcriptome, since depletion of histone H4 in *Saccharomyces cerevisiae* induced changes in the expression of approximately 25% of its genes (55). This information is consistent with the observations reported herein, where more than 2,500 genes had their expression modulated during the dimorphic transition. Moreover, down-regulation of histone mRNA levels is known to occur in response to perturbations in cell cycle progression, which might be triggered by the heat shock stimulus and the consequent mycelium-to-yeast dimorphic shift (57).

Detailed analysis of the entire data set of modulated genes (see supplementary materials at <http://143.107.203.68/pbm2ytrans/index.html>) also allowed the identification of up-regulated genes whose products, such as two G-proteins (an α -subunit encoded by MAS0201 and a β -like subunit encoded by MAC0335), two Ser/Thr protein kinases (MAS0755, MAC0845), a cyclic AMP-dependent protein kinase (MAS1886), a 14-3-3-like protein (MAS0350), a Pho-85 cyclin-dependent kinase (MAS0866), a calmodulin-like protein (MAC1225), and the calmodulin-dependent phosphatase calcineurin (MAS2387), might be directly involved in signal transduction, intracellular signaling, and cell cycle progression.

Several genes identified as up-regulated in our experiments are directly involved with pathways that have already been implicated in the mycelium-to-yeast differentiation of dimorphic fungi. For instance, the consistent up-regulation of both the calmodulin gene (MAC1225) and the gene for the regulatory subunit of calcineurin (MAS2387) is likely to influence important morphogenesis-associated signal transduction pathways. In many fungi, Ca^{2+} /calmodulin seems to be involved in various aspects of fungal development, including conidium and appressorium formation, hyphal extension and branching, mycelial dimorphism, photomorphogenesis, and fungal pathogenicity. Calcineurin, a Ca^{2+} /calmodulin-dependent protein phosphatase, is essential for survival of *C. neoformans* and *C. albicans* at 37°C (23). Recently, de Carvalho et al. (9) have shown that several inhibitory drugs of the Ca^{2+} /calmodulin signaling pathway, such as calmidazolium (R24571), trifluoperazine, and W7, were able to inhibit the *P. brasiliensis* mycelium-to-yeast transition.

A series of genes involved in maintenance of the intracellular redox potential and protection against oxidative stress have also been shown to be overexpressed at several time points of the dimorphic shift. These include two indepen-

dent glutaredoxin genes (MAS2032 and MAS0292), three thioredoxin genes (MAS1661, MAS0752, and MAC0975), one peroxiredoxin gene (MAC1221), a thiol-specific antioxidant gene (MAC1412), a methionine sulfoxide reductase gene (MAS0725) and the gene for yeast-specific protein Y20 (MAC1168), which was recently characterized as a flavodoxin-like protein (8).

Cluster analysis of modulated genes. The 2,583 modulated genes have also been analyzed with the aid of a K-means algorithm in an attempt to cluster genes according to the similarities in their expression profiles. Their distribution into 56 distinct clusters shows a large number of genes with minor alterations in their expression levels, while others were more severely up- or down-regulated at one or more steps of the differentiation process (Fig. 4). We focused our attention on the two clusters that seemed to contain genes with the most intense and consistent up-regulation profiles (Fig. 4, clusters 13 and 55 [as counted from left to right, beginning at the top left panel; indicated with bold lines]). In cluster 13, genes were highly expressed after 48 h (Fig. 5A), while cluster 55 contained genes that were overexpressed at all transition time points (Fig. 5B).

In cluster 13, we have observed a series of genes associated with membrane structure, such as *HXT3*, which encodes a low-affinity hexose transporter (MAS2375), as well as the gene encoding the major *P. brasiliensis* immunodominant antigen, *GP43* (2). A member of the GPR/FUN34 family (MAS2134) is also in this cluster. While the exact function of proteins that belong to this family is yet to be established, most of them are predicted to be plasma membrane proteins (1). We also observed up-regulation of the transcription factor gene *METR*, which encodes a zinc finger transcription activator that is essential for the induction of genes involved in the sulfate assimilation pathway (37) and of the gene encoding the flavodoxin-like protein Y20. Finally, cluster 13 shows the up-regulation of the genes coding for subunit E2 of branched-chain α -keto acid dehydrogenase (MAS1216 and MAS0435), involved in oxidative degradation of branched-chain amino acids (Fig. 5A). Interestingly, subunit E1 of this enzyme displays a similar expression profile (see MAC1260 in Table 1 and in the supplementary materials provided at <http://143.107.203.68/pbm2ytrans/index.html>). A direct comparison of the expression profiles from these two genes yielded a correlation coefficient (R_p) of 0.91, as measured by real time RT-PCR.

Cluster 55, on the other hand, contains a series of genes that display consistent overexpression from the very beginning of the dimorphic shift (Fig. 5B). Among the genes found in this cluster, we observed a homologue of an immunoreactive protein precursor from *Coccidioides immitis* (MAS1924), which has been shown to encode a factor that stimulates T-cell response in humans and has been used as a protective vaccine in mice (11). Gene homologues encoding an alcohol dehydrogenase III (MAC1062) and a formamidase (MAS0378) and seven genes encoding unknown proteins (MAC1307, MAC1404, MAS0549, MAS1359, MAS1371, MAS1593, and MAS2393) have also been found within this group of genes. However, the most interesting member of cluster 55 was a gene encoding a homologue of a T-cell-reactive protein from *C. immitis*, which has been shown to encode 4-hydroxyphenyl-pyruvate dehydrogenase (4-HPPD; EC 1.13.11.27) (54, 56), the enzyme involved in

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FIG. 4. Clusters of gene expression generated by the K-means algorithm. The 2,583 genes that showed modulation in expression during the mycelium-to-yeast transition were evaluated by a figure-of-merit algorithm. The obtained results supported their subdivision into 56 clusters, which was achieved with the aid of a K-means algorithm. Groups of genes with similar modulations of gene expression during the mycelium-to-yeast transition are located in each cluster. The figure shows, in the y axis, the variation in the $\log_2(\text{Cy5/Cy3})$ ratios (from -7 to 7), along with the different time points of the mycelium-to-yeast transition (x axis), taking as a reference their respective expression levels at time zero (hyphae). Clusters 13 and 55, containing genes that displayed the most intense and consistent up-regulation profiles, are indicated by bold lines and shown in more detail in Fig. 5.

the second step of aromatic amino acid catabolism, i.e., the conversion of 4-hydroxyphenyl-pyruvate to homogentisate and carbon dioxide in the presence of oxygen and ferrous ion. This gene was recognized by three independent probes spotted in our array (MAC1409, MAC0550, and MAS0502). All of them indicate that the expression of 4-HPPD starts early after temperature shift and might be one of the most intense up-regulations observed during the mycelium-to-yeast differentiation process, reaching an increase in expression of up to 15-fold, as measured by real-time RT-PCR.

NTBC can block *P. brasiliensis* mycelium-to-yeast transition. Clusters 13 and 55 showed that genes encoding enzymes involved in amino acid catabolism, such as branched-chain α -keto acid dehydrogenase (catabolism of branched amino acids) and 4-HPPD (catabolism of phenylalanine and tyrosine), were highly overexpressed during the mycelium-to-yeast transition (Fig. 5A and B). In addition, we have also observed an increase in expression of two other genes encoding proteins that participate in the catabolism of aromatic amino

acids, i.e., homogentisate dioxygenase (MAC0559) and fumarylacetoacetate hydrolase (MAC0670) (see supplementary materials at <http://143.107.203.68/pbm2ytrans/index.html>). Taken together, these results suggested that the activation of some amino acid catabolic pathways could be relevant to *P. brasiliensis* mycelium-to-yeast transition.

Thus, in a preliminary attempt to verify if amino acid catabolism is essential for the mycelium-to-yeast transition, we decided to test the capacity of *P. brasiliensis* hyphae to differentiate in the presence of 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione, also known as NTBC or nitisinone (18, 22). NTBC is a potent specific inhibitor of 4-HPPD and has been used to control the activity of this enzyme in a broad variety of organisms, including bacteria, plants, and humans (5, 30). Adding increasing concentrations of NTBC to the media and submitting *P. brasiliensis* hyphae to growth at 37°C , we verified that NTBC was able to inhibit the dimorphic transition in a dose-dependent manner (Fig. 6). When exposed to low concentrations of the drug (i.e., 5 to 50 $\mu\text{g/ml}$), *P. brasiliensis*

FIG. 5. Hierarchical clustering showing the pattern of expression of *P. brasiliensis* genes contained in clusters 13 (A) and 55 (B), which showed the highest levels of up-regulation during the mycelium-to-yeast transition. Cluster numbering is considered from the upper left corner of Fig. 4. The color code displays the $\log_2(\text{Cy5/Cy3})$ ratio for each time point and has Cy3 as the reference value (hyphae at time point 0).

mycelium was unable to transform into yeast cells, assuming typical pseudohyphal morphology even after 10 days at 37°C. (Fig. 6B to D). However, hyphae could not go through any kind of morphological transition and remained as mycelium when exposed to 100 µg/ml of NTBC (Fig. 6E). Interestingly, we did not observe any effect of 5 to 50 µg/ml of NTBC either on the yeast-to-mycelium transition or on mycelium growth (data not shown), which is consistent with the preferential expression of 4-HPPD in yeast cells.

To verify the direct impact of NTBC on growth of the pathogenic yeast form of *P. brasiliensis*, disk diffusion assays were performed with increasing concentrations of the drug. As observed in Fig. 7A, concentrations above 100 µg/ml of NTBC could clearly inhibit the growth of *P. brasiliensis* yeasts, indicating that this drug may be a good lead for the development of new drugs against this pathogenic fungus. Thus, we extended this approach to evaluate the effect of a series of NTBC derivatives, whose chemical structures are shown in Fig. 7 (compounds 1, 4, and 5 were prepared according to the methods of Wu et al. [53] and Lin et al. [28], while the chemical synthesis of compound 8 will be described elsewhere). The results obtained from the disk diffusion assays showed that the chemical modifications introduced into compounds 1, 4, and 5 had minor impact on *P. brasiliensis* growth compared to the

impact of NTBC. Nonetheless, compound 8 was about 40 times more potent than NTBC in its capacity to inhibit the growth of *P. brasiliensis* pathogenic yeast cells, since the effect of this drug could be clearly observed even at concentrations as low as 5 µg/ml (Fig. 7E, right plate). Compound 8 can also completely

DISCUSSION

The results of the present work demonstrate the use of high-throughput microarray hybridization analysis to examine gene expression during the mycelium-to-yeast transition in the pathogenic fungus *P. brasiliensis*, a process that is intimately related to the pathogenicity of dimorphic fungi. However, the analysis of gene expression during this process involves a temperature shift used to promote the dimorphic transition, making the mycelium-to-yeast differentiation inherently associated with the activation of genes that respond to adaptation to heat shock and other environmental stimuli as well. So far, no attempts have yet been made to obtain *P. brasiliensis* yeast cells at room temperature and thereafter follow the expression of specific genes in cultures that have not been submitted to a heat stress. Nonetheless, several genes identified in this study

FIG. 6. NTBC inhibits *P. brasiliensis* yeast growth and mycelium-to-yeast transition. *P. brasiliensis* mycelium was grown in the absence (a) or presence (b to f) of 5 to 100 µg/ml NTBC for 10 days at 37°C.

corroborate previous analyses regarding the pathobiology of dimorphic fungi and provide new insights into our understanding of *P. brasiliensis* and its adaptation to the environment within the human host.

Our approach differs from those employed in other studies aimed at analyzing the transcriptome of dimorphic fungi, such as the experiments described by Hwang et al. (21) and Kraus et al. (24), who used genomic DNA microarrays to identify differentially expressed genes in the human-pathogenic fungi *H. capsulatum* and *C. neoformans*, respectively. While these authors used whole-genome microarrays, our cDNA microarray has been constructed with EST sequences derived from the yeast pathogenic stage of this fungus, which had previously been clustered and annotated (15), allowing a more precise and direct identification of modulated genes. Moreover, those studies attempted only to compare gene expression between two specific morphological stages of the fungi (yeast versus mycelium), while we chose to analyze transcription modulation from 5 to 120 h into the mycelium-to-yeast dimorphic transition. This allowed us to identify genes that are specifically up- or down-regulated not only at the endpoints of differentiation but also at different time points of the transition process.

This choice of approach also distinguishes the present work from recently published papers that employed in silico electronic subtraction, suppressive subtraction hybridization, and low-density array hybridization to assess transcriptome differences between fully differentiated mycelium and yeast forms of *P. brasiliensis* grown in either complete or minimal medium (12, 13, 15, 35). Although such papers identified a relatively small number of differentially expressed genes compared with the 2,583 genes re-

ported as modulated in the present work, some similarities have been found, reinforcing the involvement of certain metabolic pathways in differentiation of the yeast pathogenic phase of the fungus. For instance, Felipe et al. (13) suggested that the metabolism of mycelium cells tends to be more aerobic than that of yeast cells, considering that, in the saprophytic phase, genes encoding isocitrate dehydrogenase and succinyl-coenzyme A synthase are overexpressed. In contrast, the gene encoding alcohol dehydrogenase I is up-regulated in yeast cells, suggesting that yeast metabolism favors fermentation and consequent production of ethanol. In this study, we have also observed intense overexpression (up to eightfold) of alcohol dehydrogenase I (MAC0060) during the mycelium-to-yeast transition. Moreover, pyruvate decarboxylase, the other enzyme involved in ethanol production from pyruvate also displays an ~2-fold increase at time point 120 h of the differentiation process (see MAS0981 in the supplementary materials at <http://143.107.203.68/pbm2ytrans/index.html>). This information reinforces the idea that yeast cells possess an energy metabolism biased towards ethanol production through fermentation. Surprisingly, however, our microarray data analysis was able to detect only minor alterations in the expression of isocitrate dehydrogenase and succinyl-coenzyme A synthase. A similar situation has been observed with genes of the glyoxylate pathway, whose activation might be related to pathogenicity in dimorphic fungi (31). Our microarray hybridization experiments show no alteration in the expression of isocitrate lyase (MAC1422) at time point 120 h compared to that seen in mycelial cultures, contrary to what has been reported by Felipe and coworkers (13). However, our results show that yeast cells harvested at time point 120 h display an ~2.5-fold

AQ: N

FIG. 7. NTBC derivatives can inhibit *P. brasiliensis* yeast growth. *P. brasiliensis* viable yeast cells were inoculated in solid PGY medium. Blank 6-mm-diameter paper disks were impregnated with 5.0, 10.0, 25.0 $\mu\text{g/ml}$ (from right to left on the first rows on the plates), 50.0, 100.0, 200.0 $\mu\text{g/ml}$ (second rows, from right to left on the plates), or 0 $\mu\text{g/ml}$ (third row) of NTBC (A) or NTBC derivatives (B to D and left plate in E) and placed on top of the previously inoculated agar plates after drying. Plates were incubated at 37°C, and the diameters of the inhibition zones were evaluated after 7 days. Each disk diffusion assay was performed twice. As shown on the right plate in E, compound 8 was also tested at lower concentrations of 0.25, 0.5, 0.75 $\mu\text{g/ml}$ (first row, from right to left on the plate), 5.0, 7.5, 10.0 $\mu\text{g/ml}$ (second row, from right to left on the plate), or 0 $\mu\text{g/ml}$ (third row).

increase in the expression of malate synthase (MAC1234), reinforcing the idea that the glyoxylate pathway is more active in the yeast form of *P. brasiliensis*.

These and other discrepancies might be a result of biological variation associated with the different *P. brasiliensis* strains under consideration, but the use of different culture media and conditions is also likely to exert a great influence on the differential expression of many genes and pathways, as recently demonstrated by Marques and coworkers in their studies involving suppressive subtraction hybridization and macroarray hybridization of *P. brasiliensis* (35). Many results obtained in that work are also consistent with the experiments described herein, such as the yeast-specific overexpression of several Hsp genes and factors involved with oxidative stress response, such as *TSA* (thiol-specific antioxidant) and *GST1* genes, encoding thioredoxin peroxidase and a glutathione-S-transferase, respectively. We have also identified the *Y20* gene as 12 times more expressed at the end of the mycelium-to-yeast transition. The protein encoded by this gene has previously been characterized by Cunha et al. (7) by comparing proteome patterns from both forms of *P. brasiliensis* with two-dimensional gel

electrophoresis. More recently, PbY20 was recognized as a member of the flavin mononucleotide flavodoxin-like WrbA family, which is involved in heat shock and oxidative stress in biological systems (8). Felipe et al. (13) have also shown that *Y20* was preferentially expressed in the yeast phase. These proteins are likely to be used as antioxidants to reduce the reactive oxygen species and their effects during yeast growth. Kraus et al. (24) have shown increased expression of *C. neoformans* genes involved in promoting resistance to reactive oxygen species (catalases and oxidases) during growth at 37°C. Moreover, it remains to be investigated whether *P. brasiliensis* genes involved in promoting resistance to reactive oxygen species are also induced by heat shock and other types of cellular stress as part of a general stress response, as observed in *S. cerevisiae* (14). These observations suggest that antioxidant systems in *P. brasiliensis* and other dimorphic fungi could be extremely important not only for survival in macrophages but also for detoxification of oxygen free radicals that will be much more detrimental at 37°C.

Another interesting set of results includes the observation of increased expression of genes that encode proteins involved in

amino acid catabolism, particularly in the case of aromatic and branched-chain amino acids (BCAAs). Felipe et al. (13) reported that branched-chain aminotransferase, the first enzyme involved in degradation of BCAAs, is preferentially expressed in yeast cells, while we observed intense overexpression of the branched-chain α -keto acid dehydrogenase complex (7.5-fold in the case of MAS0435) responsible for the second (and committed) step of BCAA degradation after 48 h of the mycelium-to-yeast transition. Although an eventual increase in both BCAA and aromatic amino acid degradation might reflect a simple adaptation to the use of these biomolecules as an energy source, it must be noted that such pathways are implicated in other metabolic functions. For instance, BCAAs are required for branched-chain fatty acid synthesis (19, 42), which might be implicated in the phenomena of migration and reorganization of membrane lipids during the dimorphic transition of *P. brasiliensis* (27, 50, 52). More importantly, BCAAs can serve as precursors of fusel alcohols, such as isoamylalcohol, which has been shown to be an important player in a morphogenesis checkpoint that triggers pseudohyphal formation in *S. cerevisiae* (36).

The degradation of aromatic amino acids has also been found to be associated with other metabolic pathways. For example, phenylalanine and its oxidation product, tyrosine, are precursors for the synthesis of melanins, which are thought to play a protective role in the establishment of pathogenicity by several fungi (25). In most fungal species, a specific type of melanin, called eumelanin, is formed by oxidation of diphenolic compounds, such as L-3,4-dihydroxyphenylalanine (L-DOPA), which are polymerized by a copper-dependent diphenol oxidase from the laccase family. *P. brasiliensis* conidia, as well as yeast cells, are capable of synthesizing melanin-like compounds in vitro and in vivo, but the exact type of melanin produced by these cells remains to be fully characterized (16). Although a laccase-like activity has been observed in *P. brasiliensis* cellular extracts, *P. brasiliensis* conidia are able to produce the pigment even in the absence of L-DOPA (16), and a homologue for the highly conserved laccase gene has not been identified, even after extensive EST sequencing of this fungus (12, 13, 15). Thus, the 15-fold up-regulation of 4-HPPD during mycelium-to-yeast transition must be noted, since this enzyme has already been described as the product of *melaA*, which is involved with the synthesis of pyomelanin, a melanin-like pigment typically found in bacteria which is formed through the oxidative-dependent polymerization of homogentisic acid, the end product of 4-HPPD (39).

It has not escaped our attention that the increased expression of 4-HPPD could also be associated with resistance to oxidative attacks, since homogentisic acid has been shown to act as a scavenger of reactive oxygen species or nitric oxide-derived compounds, which might help to block the oxidative burst mediated by macrophages (20). Since nitric oxide seems to be decisive to the success of the host's immune response against *P. brasiliensis* infection (10), the accumulation of a scavenger molecule during the differentiation to the pathogenic yeast form of the fungus, as well as the up-regulation of oxidative stress-related genes, might represent an important adaptation of the pathogen to life within the vertebrate host (3, 17, 40).

Given the lack of classic genetic tools for the study of *P. brasiliensis*, such as DNA-mediated transformation and mod-

ulation of gene expression by gene knockout or RNA interference techniques, the role of 4-HPPD overexpression could be tested due only to the availability of a specific inhibitor for this enzyme. Nitisinone, or NTBC, has been used in humans to treat hereditary type I tyrosinemia, a rare pediatric disease that causes progressive liver failure and liver cancer in young children (29, 32). Our experiments have shown that the presence of increasing concentrations of NTBC in the culture media can affect, in a dose-dependent manner, both the growth of the pathogenic yeast form and its development from mycelium. Interestingly, the yeast-to-mycelium transition is not affected by concentrations of nitisinone that affect both yeast growth and mycelium-to-yeast transition. It remains to be determined if the 4-HPPD inhibitors are directly modulating the developmental transition or are toxic to cells and thus indirectly affecting development. Although we did not demonstrate a direct interaction between NTBC and *P. brasiliensis* 4-HPPD, preliminary data obtained so far show that NTBC exerts a similar inhibitory effect on the growth of other fungal pathogens, such as *Aspergillus fumigatus* and *Candida* spp. (which have been shown to contain 4-HPPD), but fails to inhibit growth and differentiation of *S. cerevisiae* (which lacks 4-HPPD) (G. Goldman et al., unpublished results). Moreover, as mentioned before, NTBC has been shown to display a broad range of activity, interacting with 4-HPPD from many different organisms, including plants, bacteria, and mammals (5, 30).

Although additional studies employing animal models are still necessary to verify whether NTBC and/or NTBC derivatives will be able to eliminate *P. brasiliensis* yeast cells in vivo, the current results raise the perspective that this drug may be an alternative to the treatment of PCM and other fungus-related diseases. If this proves to be the case, the use of NTBC and/or NTBC derivatives shall represent an entirely new approach to treatment, since currently available antifungal agents, such as azoles or equinocandins, affect other fungus-specific biochemical pathways, i.e., ergosterol and β -1,3-glucans in the cell wall biosynthesis (33). The facts that NTBC has already been approved to be used in humans and that its side effects are at least partially known to physicians may foster its eventual use as a new agent for the treatment of fungus-related infections such as PCM. Moreover, the potential of NTBC as a lead to the development of new chemotherapeutic agents has just begun to be exploited, and, judging from the results obtained with the use of compound 8, which appears to be about 40 times more potent than NTBC itself, it is tempting to speculate that derivatives of this drug may develop into a new group of fungicides capable of combating fungal infections through an entirely new pharmacological strategy, the details of which are only beginning to be unraveled.

Taken together, our results provide information regarding the process of gene expression modulation that takes place during the mycelium-to-yeast transition in *P. brasiliensis*. Many of these transcriptional alterations seem to involve genes and pathways that play key roles in the process of cellular differentiation in other fungi, and many of them have been identified as virulence related. Furthermore, since the onset of pathogenicity in dimorphic fungi is directly associated with differentiation into the yeast form, the microarray hybridization analyses also provided information regarding new potential targets to combat *P. brasiliensis* infection. The experiments employing

NTBC and its derivatives as new chemotherapeutic agents against PCM confirm the potential of array-based approaches to identify new targets for the development of alternative treatments against pathogenic microorganisms.

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Figure 2

Figure 3

B

C

Figure 5

B