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Análises de transcritos de *Paracoccidioides brasiliensis* durante a transição dimórfica de micélio para levedura

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"Nunca deixe que lhe digam
Que não vale a pena Acreditar no sonho que se tem
Ou que seus planos nunca vão dar certo
Ou que você nunca vai ser alguém

Tem gente que machuca os outros
Tem gente que não sabe amar
Mas eu sei que um dia a gente aprende
Se você quiser alguém em quem confiar

Confie em si mesmo

Quem acredita sempre alcança"

Renato Russo

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SUMÁRIO

ABREVIATURAS E SIGLAS

ºC	**Graus centígrados**
%	**Percentagem**
µm	**Micrômetro**
h	**horas**
cDNA	**DNA complementar**
mRNA	**RNA mensageiro**
PCM	**Paracoccidioidomicose**
Pb	***Paracoccidioides brasiliensis***
EST	**Etiqueta de seqüência expressa**
ESTs	**Etiquetas de seqüências expressas**
RT-PCR	**Transcrição reversa - reação da polimerase em cadeia**

RESUMO

O fungo *Paracoccidioides brasiliensis* causa a paracoccidioidomicose (PCM), doença sistêmica humana de maior prevalência na América Latina. O patógeno é um fungo dimórfico que cresce a 37ºC como levedura *in vitro* e nos tecidos do hospedeiro. Em temperaturas inferiores a 26ºC, o fungo cresce como micélio *in vitro* e no meio ambiente. A transição de micélio para levedura é etapa fundametal para o estabelecimento da infecção. Com o objetivo de analisar os genes transcritos do isolado *Pb*01 (ATCC-MYA-826) de *P. brasiliensis* durante a transição dimórfica foi construída uma biblioteca de cDNA a partir de RNAs extraídos durante a transição de micélio para a fase leveduriforme do fungo. Foram obtidas 1107 ESTs (etiquetas de seqüências expressas). Com o objetivo de identificá-las e classificá-las quanto à possível função, todas as ESTs foram comparadas com seqüências de cDNA disponíveis no banco de dados não redundante do GeneBank (http://www.ncbi.nlm.nih.gov), utilizando-se o programa BLAST. Foram identificados genes potencialmente envolvidos no metabolismo celular, produção de energia, síntese protéica, transdução de sinal, defesa celular, biogênese da parede celular, dentre outros. Análises comparativas com o banco de dados do transcriptoma de *P. brasiliensis* (http://www.biomol.unb.br/Pb) revelaram genes ainda não descritos em *P. brasiliensis*. Alguns dos novos genes foram analisados por RT-PCR semiquantitativo, confirmando uma expressão preferencial durante a transição dimórfica. Esses estudos deverão prover informações relevantes para os mecanismos envolvidos na interação patógeno hospedeiro, virulência e patogênese.

ABSTRACT

The fungus *Paracoccidioides brasiliensis* causes the paracoccidioidomycosis (PCM), the major prevalent human systemic mycosis in Latin America. The thermodimorphic pathogen grows as yeast at 37ºC in vitro and host tissues. At temperatures lower than 26ºC the fungus grows as mycelium form both in vitro and in environmental conditions. The transition from mycelium to yeast cells is a crucial event in the establishment of infection. A cDNA library was constructed with RNAs isolated from *P. brasiliensis*, *Pb*01 (ATCC-MYA-826) during mycelium to yeast transition and utilized to analyze the transcriptional profile during the differentiation process. A total of 1107 ESTs (Expressed Sequences Tags) were obtained. The sequences were identified by comparison with non redundant GenBank database (http://www.ncbi.nih.nlm.gov) by using BLAST program. We identified genes potentially related to cell metabolism, energy production, protein synthesis, signal transduction, cell defense and cell wall metabolism, and others. Comparative analyses with *P. brasiliensis* transcriptome database (http://www.biomol.unb.br/Pb) showed a high proportion of new genes. Some new genes were analyzed by semiquantitative RT-PCR and the results confirmed the preferential gene expression during the dimorphic transition. These results showed important information concerning the host-pathogen interactions, pathogenesis and virulence.

I. Introdução

I.1 - *Paracoccidioides brasiliensis*

I.1.1 - Aspectos gerais

Paracoccidioides brasiliensis, um fungo termodimórfico, é o agente etiológico da paracoccidioidomicose (PCM), uma micose sistêmica prevalente na América Latina, ocorrendo principalmente no Brasil, nas regiões Sudeste e Centro-Oeste do país (Camargo *et al.*, 2000; San-Blas & Niño-Vega, 2001). *P. brasiliensis* transita entre as formas miceliana e leveduriforme. A forma miceliana é a infectiva, encontrada na natureza, provavelmente no solo e plantas, e a leveduriforme é encontrada nos tecidos infectados. Evidências indicam que a infecção ocorre por inalação de propágulos aéreos que se convertem na fase leveduriforme, nos pulmões. A transição morfológica entre micélio e levedura, provocada pela mudança de temperatura, constituiu uma etapa essencial para o estabelecimento da infecção e para a fase inicial da interação do fungo com o hospedeiro (San-Blas *et al.*, 2002).

I.1.2 - Classificação Taxonômica

Atualmente o fungo *P. brasiliensis* é classificado como pertencendo ao reino Fungi, ao filo Ascomycota, à ordem Onygenales, à família Onygenaceae, ao gênero *Paracoccicioides* e à espécie *brasiliensis* (San-Blas *et al.*, 2002).

P. brasiliensis é um fungo mitospórico e não apresenta nenhuma forma teleomórfica conhecida (Margulis & Scharwatx, 1998). A sua classificação como pertencendo ao filo Ascomycota foi realizada por Leclerc *et al.* (1994), após comparações filogenéticas entre fungos dimórficos e dermatófitos baseadas em seqüências de DNA da subunidade ribossomal maior (28S). Guého *et al.* (1997), realizaram posteriormente comparações entre seqüências parciais de rRNA de fungos dimórficos, classificando o fungo *P. brasiliensis*, juntamente com os gêneros *Histoplasma*, *Emmonsia* e *Blastomyces*, como pertencentes à família Onygenaceae. Análises filogenéticas realizadas com seqüências da subunidade ribossomal menor de vários fungos reforçaram a tese de que *P. brasiliensis* deveria ser agrupado na ordem Onygenales (Bialek *et al.*, 2000).

I.1.3 - Patogenia

A PCM é uma doença sistêmica, caracterizada por inflamação supurativa, supressão da imunidade celular e altos níveis de anticorpos (San-Blas, 1993). A patogenia da PCM ainda não foi precisamente definida, principalmente pela falta de conhecimento sobre o habitat do agente ecológico (Restrepo, 1985).

A doença apresenta duas formas distintas: a forma aguda, subaguda ou juvenil e a forma crônica ou adulta. A primeira desenvolve-se mais rapidamente e é mais severa que a segunda (Franco *et al.*, 1987; Giraldo *et al.*, 1976; Montenegro, 1986). Em ambos os casos, as funções imunes mediadas por células são alteradas, e na ausência de terapia específica, a mortalidade é alta (Dillon *et al.*, 1986; Londero & Ramos, 1990; Lacaz *et al.*, 1991).

A forma aguda representa apenas 3 a 5% dos casos. É caracterizada por rápido curso (semanas a meses) e por marcar o envolvimento do sistema reticuloendotelial (baço, fígado, nódulos linfáticos e medula óssea). A função imune mediada por células é severamente deprimida nestes pacientes, principalmente em crianças e adultos jovens. Esta é a forma mais severa e a que tem pior prognóstico. As características clínicas são: órgãos do sistema endotelial hipertrofiados, disfunção da medula óssea e com freqüência uma desordem linfoproliferativa ou, se a disseminação severa tiver ocorrido, um episódio de septicemia (Londero & Melo, 1983). Pode ainda ocorrer um hipertrofismo dos nódulos linfáticos mesentéricos que leva à obstrução do intestino e/ou uma síndrome abdominal aguda. Nesta primeira fase da doença os pulmões são raramente os focos primários assim como não existem características clínicas especiais ou manifestações radiológicas (Londero & Melo, 1983). Entretanto, a pesquisa do fungo nas secreções pulmonares é normalmente positiva, indicando que os pulmões também estão envolvidos (Restrepo *et al.*, 1989).

A forma crônica acomete mais de 90% dos pacientes; os homens adultos são os mais afetados. A progressão da doença acontece vagarosamente e pode durar meses ou até anos. Diferentemente dos sintomas da forma aguda, manifestações pulmonares são evidentes em 90% dos adultos com doença crônica (Londero *et al.*, 1978; Londero, 1986; Franco *et al.*, 1989; Londero & Ramos, 1990). Em aproximadamente 25% dos casos, os pulmões são o único sistema clinicamente afetado. Mas em alguns casos, o comprometimento unifocal pulmonar pode ser silencioso e os pacientes doentes são medicados somente após a disseminação, ocorrendo assim, lesões extrapulmonares (forma multifocal) (Restrepo, 1970; Restrepo *et al.*, 1976; Londero, 1986; Franco *et al.*, 1989; Londero & Ramos, 1990;). A forma multifocal envolve freqüentemente outros órgãos como pele, mucosas das vias aéreas superiores, glândulas supra-renais,

tubo digestivo e gânglios linfáticos (Londero, 1986; Tendrich *et al.*, 1991; Brummer *et al.*, 1993). Com menor freqüência, podem ocorrer envolvimento ocular, genital, destruição óssea, e comprometimento dos sistemas nervoso e vascular (Rivitti & Aoki 1999; Severo *et al.*, 2000; Lorenzoni *et al.*, 2002). Os sintomas respiratórios são inespecíficos e incluem tosse e expectoração. Perda de peso, febre e anorexia também são registradas. As lesões visualizadas por raios-X são nodulares, infiltrativas, fibróticas ou cavitárias; são freqüentemente bilaterais e preferencialmente localizadas no centro e porções mais baixas dos pulmões, com os ápices ficando livre da doença (Restrepo *et al.*, 1976; Londero, 1986; Patino *et al.*, 1987; Londero & Ramos, 1990; Campos *et al.*, 1991).

I.1.4 - Epidemiologia

Uma das características da PCM é a sua distribuição geográfica, restrita à América Latina, ocorrendo do México até a Argentina. Entretanto, a doença não ocorre em todos os países dentro destes limites. A maior incidência da doença é observada no Brasil, Colômbia e Venezuela (Wanke & Londero, 1994; Rivitti & Aoki, 1999). Cerca de 80% dos casos são descritos no Brasil (Brummer *et al.* 1993; Coutinho *et al.*, 2002).

No Brasil, a doença ocorre mais frequentemente nas regiões Sul, Sudeste e Centro-Oeste (Blotta *et al.* 1999; Paniago *et al.*, 2003). A PCM ocorre predominantemente em áreas rurais, onde os indivíduos estão possivelmente mais expostos ao agente etiológico, acometendo principalmente indivíduos do sexo masculino entre 30 e 60 anos de idade (Brummer *et al.*, 1993).

Um estudo de análise de óbitos entre os anos de 1980 e 1995, realizado por Coutinho et al (2002), revelou que a PCM é a oitava causa de

mortalidade por doença predominantemente crônica ou repetitiva, entre as infecciosas e parasitárias, e possui a mais elevada taxa de mortalidade entre as micoses sistêmicas. A taxa de mortalidade média anual é de 1,45/milhão de habitantes considerando ambos os sexos, sendo que para homens é de 2,42/milhão e para mulheres 0,43/milhão. No período analisado ocorreu um aumento no número de mulheres desenvolvendo a PCM, em virtude do crescimento desta mão de obra nas atividades rurais (Coutinho *et al.*, 2002).

Nos países onde a doença é endêmica os casos não são distribuídos homogeneamente em todo o território, mas ficam concentrados em áreas próximas a florestas úmidas (tropicais ou subtropicais). Temperaturas amenas entre 17 e 24ºC, florestas abundantes, muitos cursos de água, invernos curtos e verões chuvosos são condições predominantes nos países de alta endemicidade (Restrepo, 1985). Em áreas distantes das florestas poucos casos são relatados, como regiões costais, praias, zonas desérticas e selvas equatoriais. Várias condições têm sido citadas como adversas para o *P. brasiliensis*, como umidade e calor excessivo, aridez, ventos freqüentes, carência de florestas e ausência de rios (Chirife & Del Rio, 1965; Greer & Restrepo, 1975).

O prolongado período de latência, tão característico da PCM, dificulta a determinação precisa do local onde foi adquirida a infecção, fato que levou Borelli (1964) a postular o conceito de reservárea. Reservárea é definida como o local em que todos os fatores conduziram à existência da infecção, isto é, onde os fungos tinham como habitat natural e onde humanos adquirem a infecção primária. Estas áreas são moldadas e limitadas pelo ecossistema (altitude, temperatura, atmosfera, tipo de solo e tipo de vegetação). Áreas de endemicidade, definida como todos os lugares onde a micose é diagnosticada ou reportada ou ambos podem ou não coincidir com a reserva, porque o paciente pode ter sido diagnosticado em

lugar diferente daquele no qual adquiriu a infecção primária (Ajello & Polonelli, 1985).

I.2 - Dimorfismo em fungos patogênicos

O dimorfismo é uma característica da maioria dos fungos patogênicos humanos como *P. brasiliensis, Histoplasma capsulatum, Cryptococcus neoformans, Blastomyces dermatitidis, Coccidioides immitis,* entre outros causadores de micoses sistêmicas. Este processo favorece a instalação dos fungos, os ajudam a resistir às agressões vindas do hospedeiro, é importante para a invasão de tecidos e para o estabelecimento da doença *(*Kurokawa *et al.*, 1998; San-Blas *et al.*, 2002*)*. A patogenicidade está intimamente ligada à transição dimórfica, pois linhagens de *P. brasiliensis*, assim como de *H. capsulatum* e *B. dermatitidis*, que não são capazes de transformar em leveduras não são virulentas (Medoff *et al.*, 1987; San-Blas *et al.*, 2001; Borges-Walmsley *et al.*, 2002; Rooney *et al.*, 2002).

O principal fator responsável pela diferenciação celular de *P. brasiliensis* é a temperatura (San-Blas, 1993). Em temperaturas entre 20ºC e 26ºC, o fungo cresce lentamente na forma miceliana, produzindo colônias pequenas, irregulares, com coloração que vai do branco ao castanho. Microscopicamente, as hifas apresentam-se septadas, multinucleadas, com filamentos finos e ramificados (San-Blas 1993), com esporos terminais ou intercalados (Franco *et al.*, 1989) e duas camadas na parede celular (Carbonell & Rodrigues, 1968). Acredita-se que a fase miceliana seja saprofítica, encontrada na natureza, principalmente no solo e em plantas. A infecção no homem ocorre provavelmente pelo contato desta forma com o

hospedeiro, através da contaminação por via inalatória de propágulos do fungo (Brummer *et al.*, 1993).

Na fase leveduriforme, o fungo apresenta colônias de coloração creme e aspecto cerebriforme, não sendo aderentes ao meio (Carbonell & Rodrigues, 1965). Apresenta crescimento evidente após sete dias de incubação a 37ºC. Microscopicamente as colônias são compostas de células leveduriformes de tamanhos variados, entre 4 a 30 μm, que normalmente são ovais ou alongadas, com dois a cinco núcleos, cromatina e nucléolo evidentes e possuem parede celular refratária espessa e citoplasma contendo lipídios (Lacaz *et al.*, 1991). A principal característica dessa forma é seu aspecto de "roda de leme", uma célula central é circundada por várias células periféricas (Ângulo-Ortega & Pollak, 1971). Este aspecto característico deste microrganismo permite identificar o fungo cultivado *in vitro* e também tem sido utilizado em diagnóstico histológico da doença (Furtado *et al.*, 1967; Lacaz *et al.*, 1991). Células com broto e cadeias curtas de blastoconídios também podem ser observadas (Lacaz *et al.*, 1991).

A transição dimórfica em *P. brasiliensis* ocorre simultaneamente com mudanças na composição da parede celular envolvendo alterações nos polímeros de carboidratos e reorganização de lipídios da membrana, especialmente os glicoesfingolipídios (Levery *et al.*, 1998; Toledo *et al.*, 1999; Vigh *et al.*, 1998). Quando o fungo adota a forma leveduriforme, ocorre um aumento no conteúdo de quitina na parede celular, seguida pela mudança na estrutura anomérica de glucana de β-1,3 para uma cadeia α-1,3 (San Blas & Nino-Vega, 2001). San-Blas (1982) sugere que os fagócitos humanos possam produzir β-1,3 glucanase capaz de digerir somente β-1,3 glucana presente na parede celular da forma miceliana do fungo. Desta forma, a transformação de micélio para levedura no início da infecção protegeria o patógeno contra os mecanismos de defesa do hospedeiro,

devido a incapacidade das enzimas do sistema imune do hospedeiro em digerir α-glucana (San-Blas & San-Blas, 1985), permitindo a instalação do fungo na forma leveduriforme e estabelecendo-se a infecção.

O hormônio feminino 17-β-estradiol tem sido relacionado à diferenciação de *P. brasiliensis*. Estudos mostraram que o hormônio estrogênio foi capaz de inibir a transição de micélio para levedura de maneira dose-dependente *in vitro* (Restrepo *et al.*, 1985), assim como *in vivo* (Aristizabal *et al.*, 1998; Sano *et al.*, 1999). O hormônio induz alterações na síntese protéica em *P. brasiliensis* provavelmente devido à presença da proteína EBP (Estradiol Binding Protein), uma proteína citoplasmática receptora do hormônio que possui interação seletiva a estrogênios (Loose *et al.*, 1983; Clemons *et al.*, 1989). Esta proteína também foi caracterizada em *C. albicans* e em outros fungos (Madani *et al.*, 1994). A EBP foi encontrada preferencialmente expressa durante a fase leveduriforme de *P. brasiliensis* (Felipe *et al.*, 2005). A presença do hormônio tem sido considerada como possível fator de proteção à infecção em mulheres. A maior incidência da PCM em homens adultos leva à hipótese de que fatores hormonais podem ter um papel na patogenicidade da doença (Loose *et al.* 1983; Restrepo, 1984; Stevens, 1989).

Poliaminas são metabólitos que possuem papéis importantes no crescimento celular (Tabor & Tabor, 1984). Elas se ligam a macromoléculas carregadas negativamente, particularmente aos ácidos nucléicos, protegendo-as contra fatores externos (Khan *et al.*, 1992). As poliaminas também estão envolvidas na diferenciação celular de fungos dimórficos, e altos níveis de expressão são encontrados no inicio da diferenciação celular de micélio para levedura (Calvo-Méndez *et al.*, 1987; Inderlied *et al.*, 1980; Martinez-Pacheco *et al.*, 1989; Ruiz-Herrera, 1994). O aumento de poliaminas é acompanhado pelo aumento da atividade da enzima ornitina descarboxilase (ODC), que catalisa a descarboxilação de

ornitina formando putrescina (Nickerson *et al.* 1997). San-Blas *et al.*, (1996) detectaram um aumento na atividade da ODC nas etapas iniciais da transição de micélio para levedura e crescimento das células leveduriformes, ficando constante a atividade da enzima durante o crescimento do fungo na forma miceliana. Em *P. brasiliensis*, a transição dimórfica de micélio para levedura pode ser inibida *in vitro* pela adição de inibidores da ODC (San-Blas *et al.*, 1996).

A proteina poliubiquitina é constituída por vários monômeros de ubiquitina (Ozkaynak *et al.*, 1987), é encontrada em todos os organismos eucariotos e participa do endereçamento de proteínas para a proteólise dentro da célula (Hershko & Ciechanover, 1998). Em *Aspergillus nidulans* e *Candida albicans*, a expressão dos genes ubi é controlada durante o choque térmico e outras condições de estresse (Noventa-Jordao *et al.*, 2000; Roing *et al.*, 2000). Em fungos patogênicos de plantas, como *Tuber borchii*, os genes ubi são diferencialmente expressos durante o dimorfismo (Zeppa *et al.*, 2001). Em *P. brasiliensis*, os genes da poliubiquitina também mostraram uma expressão diferencial durante a diferenciação celular. Goldman *et al.*, (2003) observaram que o gene ubi encontra-se aumentado aproximadamente duas vezes nas primeiras cinco horas de transição, seguindo um nível constante que diminui após 48 horas.

Goldman *et al.* (2003) analisaram a expressão de alguns genes durante a transição dimórfica e detectaram que transcritos de três proteínas de choque térmico (HSP-70, HSP-82 e HSP-104), de um homólogo da delta-9-desaturase (OLE1) e da oxidase alternativa possuem picos de expressão durante a transição de micélio para levedura. Estes resultados sugerem que a estabilização de proteínas e mudanças na organização da membrana são fatores importantes na transição morfológica para a forma leveduriforme a altas temperaturas.

Durante a transição de micélio para levedura existe um aumento no conteúdo de quitina na parede celular (San-Blas & Nino-Vega, 2001). Em *C. neoformans* ocorre um aumento da expressão de quitina sintases durante o crescimento a 37 ^{0}C. Nunes *et al.* (2005) observaram em *P. brasiliensis* um aumento na expressão de vários genes que codificam quitina sintases imediatamente após a elevação da temperatura, enquanto quitinases e endoquitinases apareceram menos reguladas. Interessantemente, outros três genes que codificam quitina sintases não mostraram variação na expressão durante a transição. Este fato sugere que a biossíntese de quitina pode também ocorrer através da ativação de genes alternativos que não respondem ao choque térmico.

A expressão do gene que codifica uma 4-hidroxil-fenil-piruvato dioxigenase (4-HPPD), proteína envolvida no catabolismo de aminoácidos, ocorre na fase inicial da transição morfológica de *P. brasiliensis*, e seu nível de expressão é um dos mais intensos durante esta fase. Este gene pode ser inibido pela adição de NTBC [2-(2-nitro-4-trifluorometilbenzoil)-ciclohexane-1,3-dione], assim como por seus derivados. A inibição de 4-HPPD provoca o bloqueio do crescimento e da diferenciação para a fase leveduriforme do fungo *in vitro* (Nunes *et al.*, 2005).

As vias de sinalização que controlam as mudanças morfológicas em *P. brasiliensis* ainda são pouco compreendidas. Sabe-se que as vias de sinalização através do cAMP e MAP kinase estão relacionadas com a transição morfológica em fungos dimórficos (Lengeler *et al.*, 2000). A via de sinalização através do cAMP mostra-se importante na transição morfológica de *P. brasiliensis*, pois Paris *et al*. (1985) e Borges-Walmsley & Walmsley (2000) demonstraram que a adição exógena do composto inibe a transição de levedura para micélio, mantendo a forma patogênica do fungo. Esta situação difere em *C. albicans*, pois a transição de levedura para micélio é controlada pelo cAMP e este composto exógeno estimula a

produção de pseudohifas, que são potencialmente capazes de invadir células de mamíferos (Rocha *et al.*, 2001).

O sistema Tco (Sistema de dois componentes) é um tipo de sinalização celular que funciona como único ativador para a via MAPK (HOG1) durante estresse em fungos (Bahn *et al.*, 2006). Este sistema é encontrado exclusivamente em procariotos e em eucariotos inferiores como plantas e fungos, mas está ausente em mamíferos (Santos & Shiozaki, 2001; Urao *et al.*, 2001; Catlett *et al.*, 2003). Em *C. neoformans*, o sistema Tco controla a via de sinalização HOG e regula a resposta ao estresse, sensibilidade antifúngica, fatores de virulência e reprodução sexual (Bahn *et al.*, 2006). Nemeck *et al.* (2006) forneceram evidências genéticas que estabelecem o papel central do dimorfismo na patogenicidade. Estes autores descreveram um gene codificante para histidina kinase híbrido (DRK1), que é indispensável para o dimorfismo, expressão de genes de virulência e patogenicidade em *B. dermatitidis* e *H. capsulatum*. DRK1 é um elemento do sistema Tco e o silenciamento da sua expressão provoca marcadamente uma redução da patogenicidade em ambos os fungos.

A via de transdução Ca^{2+}/Calmodulina também parece desempenhar papel na diferenciação celular de *P. brasiliensis*. Esta via está envolvida em vários aspectos no desenvolvimento fúngico, incluindo formação de conídios, extensão da hifa, dimorfismo e patogenicidade (Nunes *et al.*,2005). A calcineurina, uma Ca^{2+}/proteína fosfatase dependente de calmodulina, é essencial para sobrevivência de *C. neoformans* e *C. albicans* a 37 ˚C (Kraus & Heitman, 2003). A expressão da calmodulina e da subunidade regulatória da calcineurina motraram-se aumentadas durante a transição dimórfica em *P. brasiliensis* (Nunes *et al.* 2005), sugerindo importante papel na via de transdução de sinal associada com a morfogênese. Drogas que bloqueiam as quinases dependentes de

Ca^{2+}/Calmodulina inibem a diferenciação de micélio para levedura (Carvalho *et al.*, 2003).

P. brasiliensis produz ATP através da glicólise, fermentação alcoólica e fosforilação oxidativa. Felipe *et al.* (2005) sugerem que na fase miceliana o metabolismo tende a ser mais aeróbico que na fase leveduriforme, pois durante a fase saprofítica, genes que codificam enzimas que participam da fosforilação oxidativa, como a isocitrato desidrogenase e succinil coenzima A sintase, estão altamente expressos. Em contraste, durante a fase leveduriforme o metabolismo tende a ser mais anaeróbico devido aos altos níveis de expressão gênica da álcool desidrogenase, sugerindo que o metabolismo das células leveduriformes favorece a fermentação alcoólica e conseqüente produção de etanol. Durante a transição de micélio para levedura foi observada intensa expressão dos genes que codificam a álcool desidrogenase assim como de outras enzimas envolvidas na produção de etanol a partir de piruvato (Nunes *et al.*, 2005). Esta informação reforça a idéia de que o fungo durante a fase leveduriforme promove o desvio do piruvato da via glicolítica para o metabolismo anaeróbico. Esta observação é consistente com a baixa concentração de oxigênio nos tecidos infectados.

A análise da expressão de genes envolvidos na utilização de enxofre tem sido realizada em *P. brasiliensis* (Andrade *et al.*, 2006; Ferreira *et al.*, 2006) e em outros fungos patogênicos (Marzluf 1997; Thomas & Surdin-Kerjan 1997). Andrade *et al.* (2006) observaram que a diferenciação celular de micélio para levedura e o crescimento leveduriforme em *P. brasiliensis* não acontecem durante a deprivação de enxofre orgânico; enquanto o fungo na fase miceliana é capaz de utilizar tanto enxofre orgânico como inorgânico (Paris *et al.*, 1985). Em *H. capsulatum*, cisteina exógena é requerida como fonte de enxofre orgânico durante a transição morfológica e crescimento leveduriforme (Boguslawski

et al., 1976). Ferreira *et al.* (2006) observaram que dois genes (cisteina dioxigenase e metionina permease) envolvidos na assimilação orgânica de enxofre apresentaram expressão aumentada durante a transição de micelio para levedura e na fase leveduriforme de *P. brasiliensis*. Entretanto os autores também puderam observar que genes envolvidos na mobilização do estoque de enxofre (colina sulfatase) e na assimilação inorgânica (aps kinase e sulfito redutase) estão ativos durante a transição de micélio para levedura e crescimento leveduriforme. Estes resultados sugerem que embora *P. brasiliensis* utilize tanto a via orgânica como a inorgânica, o enxofre inorgânico não é essencial durante a transição de micélio para levedura e crescimento da fase leveduriforme.

II – JUSTIFICATIVA

P. brasiliensis é um fungo termodimórfico que sofre mudanças em sua morfologia sob influência da temperatura, apresentando-se na forma miceliana a 26 ºC no meio ambiente e *in vitro* e na forma leveduriforme a 37 ºC no tecido do hospedeiro e *in vitro* (San-Blas, 1993). Esta característica favorece a instalação dos fungos e os ajudam a resistir às agressões vindas do hospedeiro (Villar *et al.*, 1998**)**. Sendo assim, a patogenicidade está intimamente relacionada à diferenciação celular, pois linhagens de *P. brasiliensis* que não são capazes de transformar na forma leveduriforme não são virulentas (San-Blas & Niño Vega, 2001).

Os eventos moleculares e bioquímicos que levam à transição morfológica em *P. brasiliensis* ainda são pouco compreendidos. Desta forma, a análise de transcritos durante a conversão de micélio para levedura é interessante, pois pode prover novos conhecimentos sobre a biologia de *P. brasiliensis* e permitir a identificação de genes possivelmente associados ao dimorfismo.

III – OBJETIVOS

III.1 - O objetivo geral:

1. Analisar o perfil de expressão gênica do isolado *Pb*01 (ATCC-MYA-826) de *P. brasiliensis* durante a transição da forma miceliana para leveduriforme.

III.2 - Objetivos específicos:

1 - Obtenção de um banco de ESTs de *P. brasiliensis* durante a transição de micélio para levedura.

- Estratégias:
  - ✓ Extração de RNA total de *P. brasiliensis* durante a transição de micélio para levedura;
  - ✓ Construção de uma biblioteca de cDNA a partir do mRNA obtido durante a transição dimórfica;
  - ✓ Seqüênciamento dos clones de cDNA.

2 – Anotação das ESTs obtidas, a fim de identificá-las e classificá-las quanto à possível função.

- Estratégia:
  - ✓ As seqüências foram comparadas com seqüências depositadas em bancos de dados para análise de similaridade.

3 – Identificar genes diferencialmente expressos durante a transição dimórfica e analisar a sua expressão na transição e nas formas estágio específicas.

- Estratégias:

- ✓ Análises comparativas da redundância dos genes entre os bancos de ESTs de micélio e levedura (Projeto Genoma Centro Oeste) e de transição (presente trabalho);
- ✓ Análises de expressão através de Northern blot.

4 – Identificar genes ainda não descritos em *P. brasiliensis*.

- Estratégias:
  - ✓ Análises comparativas entre os bancos de ESTs da transição (presente trabalho) com bancos de ESTs de micélio e levedura (Projeto Genoma Centro Oeste) e banco de dados do NCBI.

5 – Selecionar genes de interesse e analisar a sua expressão durante a transição dimórfica.

- Estratégias:
  - ✓ Realização de RT-PCR semi-quantitativa.

6 – Análises filogenéticas

- Estratégias:
  - ✓ Alinhamento de seqüências de aminoácidos utilizando o programa Clustal X.
  - ✓ Comparação entre as seqüências de aminoácidos utilizando o software Tree View.

IV. MANUSCRITO

Título:

The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals up regulated and novel genes potentially associated to the dimorphic process

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The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals novel and induced genes potentially associated to the dimorphic process

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Abstract

Background

Paracoccidioides brasiliensis is a human pathogen with a broad distribution in Latin America. The fungus is thermally dimorphic with two distinct forms corresponding to completely different lifestyles. Upon elevation of the temperature to that of the mammalian body, the fungus adopts a yeast-like form that is exclusively associated with its pathogenic lifestyle. We describe expressed sequence tags (ESTs) analysis to assess the expression profile of the mycelium to yeast transition. To identify *P. brasiliensis* differentially expressed sequences during conversion we performed a large-scale comparative analysis between *P. brasiliensis* ESTs identified in the transition transcriptome and databases.

Results

Our analysis was based on 1107 ESTs from a transition cDNA library of *P. brasiliensis.* A total of 639 consensus sequences were assembled. Genes of primary metabolism, energy, protein synthesis and fate, cellular transport, biogenesis of cellular components were represented in the transition cDNA library. A considerable number of genes (7.51%) had not been previously reported for *P. brasiliensis* in public databases. Gene expression analysis using in silico EST subtraction revealed that numerous genes were more expressed during the transition phase when compared to the mycelial ESTs [1]. Classes of differentially expressed sequences were selected for further analysis including: genes related to the synthesis/remodeling of the cell wall/membrane. Thirty four genes from this family were induced. Ten genes related to signal transduction were increased. Twelve genes encoding putative virulence factors manifested increased expression. The in silico approach was validated by northern blot and semi-quantitative RT-PCR.

Conclusions

The developmental program of *P. brasiliensis* is characterized by significant differential positive modulation of the cell wall/ membrane related transcripts, and signal transduction proteins, suggesting the related processes important contributors to dimorphism. Also, putative virulence factors are more expressed in the transition process suggesting adaptation to the host of the yeast incoming parasitic phase. Those genes provide ideal candidates for further studies directed at understanding fungal morphogenesis and its regulation.

Background

Paracoccidioides brasiliensis is a dimorphic pathogenic ascomyceteous fungus, endemic to the Latin America that can cause primary disease in humans. In the soil the fungus grows as saprobic mycelium, resulting in the formation of propagules, which initiates the infection in humans when inhaled into the respiratory tract. Subsequently, in the lung, the mycelia propagules develop into yeast cells. The mycelium to yeast transition can be replicated in vitro by growing mycelia in conditions of elevated temperature. The ability of *P. brasiliensis* to grow in the mycelia form in the soil and shift to the yeast form in the host is important for infection and disease. Once introduced into the host, the mycelial propagules have to convert to yeasts, a condition essential for the fungus to survive and proliferate [2, 3].

The morphological transition in *P. brasiliensis* is governed predominantly by the temperature and is preceded by several molecular changes. The identification of genes specifically involved in the mycelium to yeast transition in *P. brasiliensis* has been subject of great interest, since pathogenicity is intimately linked to the dimorphic transition in some fungi [4]. Approaches used in the identification of genes important for the transition process include, for example, the differential expression of *P.*

brasiliensis genes in both fungal phases identified by electronic subtraction and cDNA microarray hybridization, which were employed to search for genes whose expression, displayed statistically significant modulation during the mycelium to yeast transition [5-8].

The biochemical processes that control the morphogenesis of *P. brasiliensis* are just coming to light. The dimorphic transition involves alterations in the cell wall composition and in the structure of carbohydrates polymers [9, 10]. The yeast cells exhibit an energy metabolism biased towards ethanol production through fermentation, whereas mycelium metabolism tends to be more aerobic than that of yeast cells. Also the glyoxylate pathway is more active in the yeast form of *P. brasiliensis* [5]. Hyper expression of some enzymes in the sulphur metabolism pathway in the yeast phase of *P. brasiliensis*, as well as during the transition from mycelium to yeast have been reported, corroborating previous descriptions of the importance of this metabolic pathway to the dimorphic process [6, 8, 11].

Here, we have tested the concept that novel genes involved in *P. brasiliensis* phase transition could be described by applying a transcriptome analysis of cells undergoing mycelium to yeast transition. In this manuscript we describe EST analysis to assess the expression profile of mycelium undergoing yeast transition. This choice of approach distinguishes the present work from previous recently published papers that employed microarray hybridization, electronic subtraction and suppressive subtraction hybridization in order to assess differences using differentiated yeast and mycelium cells [5-8, 12]. Using a custom analysis pipeline for sequences of *P. brasiliensis*, isolate *Pb*01, yeast and mycelium forms [1] we obtained an EST databank web interface [13]. In this study we report the in silico analyses and comparison of ESTs from mycelium undergoing the early transition to yeast with mycelium differentiated cells. Our analysis

revealed 179 genes that are positively modulated during the early transition process, when compared to mycelia. Additionally 48 novel genes were described in the *P. brasiliensis* transition cDNA library. Upon categorization by known databases we have selected MIPS (Munich Center for Protein Sequences) categories for further analyses. Several ESTs were selected for semi-quantitative and quantitative analysis to examine changes in gene expression induced by the temperature induced transition of phases.

Results and Discussion

cDNA library construction, sequencing and sequence annotation

Transcriptome profiling of mycelium undergoing differentiation to yeast cells in *P. brasiliensis* has directed our studies to reveal several uncharacterized genes involved in this process. We performed in this EST-based program the sequencing 2880 randomly selected clones. Of these, 2666 gave readable sequences. 1107 sequences remained after vector and low quality sequences were removed. Of these, 166 consisted of singletons and 473 corresponded to consensus with two or more ESTs. In total, 447761 bp of assembled sequences were obtained corresponding to an average consensus sequence length of 404 bp. The 1107 sequences were annotated. A total of 828 sequences (74.8%) showed significant similarity to known protein sequences (E value $\leq 10^{-4}$) based on BLAST searches and 433 ESTs (39.1%) had unknown function and were classified as hypothetical proteins. 992 sequences (89.6%) gave significant hits to ESTs present in the *P. brasiliensis* transcriptome database [1] or in the GenBank database. In addition, 115 sequences (10.4% of the total) represented novel genes of *P. brasiliensis*.

Description of the ESTs in the transition transcriptome

An overview of the probable adaptations made by *P. brasiliensis* mycelium during morphogenesis can be obtained by analyzing the ESTs in this early stage of cellular differentiation. As shown in Fig. 1, the ESTs were mainly represented as following: a total of 22.11% of the annotated ESTs corresponded to the fungal metabolism; 17.06% of the ESTs were related to the protein synthesis machinery; 10.83% of the transcripts corresponded to homologues encoding transport facilitators; 10.24% corresponded to ESTs related to protein fate; 7.42% to energy; 7.27% to signal transduction proteins; 7.12% were related to the transcription machinery; 6.68% corresponded to transcripts related to the biogenesis of cellular components; 6.38% corresponded to ESTs encoding cell rescue, defense and virulence factors.

Comparison of *P. brasiliensis* ESTs present in the transition library to those described for yeast and mycelium stage specific phases: induced genes identified by in silico EST subtraction

We attempted to determine the putative function of the set of 639 phrap unisequences by searching for homologs in the GenBank non-redundant protein database using BLAST X. We also compared the sequenced ESTs present in the transition library to those present in the mycelium transcriptome database. According to the subtractive analysis, the classification of induced genes was designed for the ESTs that were not previously described in *P. brasiliensis* in databases or that manifested increased expression in the transition library as compared to mycelia transcriptome database [1]. This classification was performed according to the statistical test described by Audic and Claverie [14], with a 99% confidence rate. The comparative analysis of all the ESTs annotated in the transition library is available in Table 1, supplementary material. From the 1107 ESTs identified in this work, 426 of the total corresponded to induced genes in the transition library. From the 426 annotated ESTs, 115 corresponded to novel ESTs,

representing 48 novel classified genes. Table 1 summarizes the results of such comparison. As shown, the majority of transition induced genes (82.12%) was composed of unique sequences or groups of two or three ESTs. Genes with altered expression included those involved in metabolism of amino acids, nitrogen, sulfur, nucleotides, carbohydrates, vitamins and lipids. In addition genes related to energy generation, signal transduction and cell wall biogenesis, were increased. A small subset of genes with elevated expression had unknown function. The largest induced groups of sequences consisted of a total of 24 ESTs with homology to a histidine protein kinase sensor for GlnG regulator, 18 ESTs exhibiting homology to ubiquinone/menaquinone methyltransferase, 11 ESTs with homology to arylsulfatase regulatory protein, 09 ESTs with homology to acidic amino acid permease, 06 ESTs with homology to a HSP 90 and 07 ESTs with homology to aspartyl protease.

Genes involved in sulfur assimilation, have been described as induced in *P. brasiliensis* transition from mycelium to yeast and in yeast differentiated cells [6,8]. Here, we described in the transition transcriptome the induction of a set of genes related to sulphur metabolism, such as, the transcript encoding sulfite reductase (E.C. 1.8.1.2) an enzyme of the sulfur assimilation pathway, leading to cysteine biosynthesis. Sulfite reductase contains a special acidic heme group called siroheme. One of the novel genes detected in the transiton library encodes for an urophorphyrinogen III methylase (E.C 2.1.1.107) homologue to the Met1p of *Saccharomyces cerevisiae*, related to the sirohaem and cobalamin biosynthesis [15,16]. Also, the transcript encoding sulfate permease was induced compared to the mycelia transcriptome. Sulfate is co-transported into the cells in an energy dependent process catalyzed by specific plasma membrane permeases [17]. An arylsufatase regulatory protein probably involved in the regulation of sulfatase genes was described in the transition transcriptome. The transcript in *P.*

brasiliensis has sequence identity to bacterial and fungal arylsulfatases regulatory proteins. Sulfatases catalyze hydrolytic cleavage of sulfate ester bonds, liberating sulfate and the corresponding alcohol [18]. In *Neurospora crassa* arylsulfatase is up regulated by sulfur starvation and appears to function as a mechanism for sulfur scavenging [19]. Also, a thiosulfate sulphurtransferase (TST) (E.C. 2.8.1.1) putatively, a mitochondrial matrix protein that plays roles in formation of iron sulfur proteins, as well as in modification of iron-sulfur proteins [20] was induced in the transition transcriptome. The increase in the expression of genes related to the sulphur metabolism, including the description of novel transcripts corroborates the previous descriptions of the involvement of sulphur metabolism in the transition process of *P. brasiliensis* [6, 8, 11].

The list of induced genes also includes several ESTs encoding proteins related to lipid metabolism, to signal transduction and to carbohydrate metabolism that will be referred below. Also proteases, such the Lon protease putatively related to degradation of damaged or nonnative proteins in the mitochondrial matrix are induced [21]. An aspartyl protease and a zinc metalloprotease were among the transcripts with increased expression. Of special note molecules related to protein fate, such as to glycoslylation and degradation, are abundant in the transition transcriptome, as shown in Table 1.

An overview of genes related to the membrane/cell wall remodeling presenting increased expression in the transition library

We catalogued the ESTs potentially associated with fungal cell wall/membrane synthesis/remodeling described during the mycelium to yeast transition. The Table 2 depicts the ESTs predominantly related to the synthesis of those components. The transcripts with increased expression include those encoding enzymes related to the cell wall carbohydrates biosynthesis and degradation, the transporters of the precursors for

the synthesis of such molecules, enzymes related to protein glycosylation and to the synthesis of membrane lipids.

It is presumed that the dimorphic transition occurs simultaneously with changes in the fungal cell wall composition of such compounds as phospholipids and carbohydrate polymers [3, 10, 22]. In *P. brasiliensis*, lipids, chitin, glucans and proteins are the main constituents of the cell wall in mycelium and yeast cells. The transition transcriptome data suggest that *P. brasiliensis* favors the membrane and cell wall remodeling in the early stages of transition, from mycelium to yeast. Transcription of 34 cell wall/membrane related genes were induced upon temperature shift (Table 2).

In Table 2 and Fig. 2 A, an overview of the induced enzymes and transporters putatively related to the biosynthesis of the carbohydrate compounds of the cell wall, is shown. Many cell wall-related proteins were found among the presently identified ESTs, including molecules related to the chitin synthesis, alpha glucan synthesis and chitin degradation. The main polysaccharide of the yeast cell wall is alpha-glucan, whereas the mycelium contains predominantly beta-glucan [23]. Several genes related to the synthesis of the carbohydrate components of the cell wall were induced in the transition library, in comparison to the mycelium transcriptome database [1]. Those genes include phosphoglucomutase (*pgm*) UDP-Glucose pyrophosphorylase (*ugp1*), and alpha -1,3 glucan synthase (*ags1*), (Table 2, Fig. 2 A), putatively enabling the increase in the synthesis of alpha-1,3 glucan in the yeast incoming cell wall [10]. A novel transcript encoding an alpha-1 glucosidase (GLCase I) was described. It has been suggested that glucosidases are directly involved in the synthesis or processing of beta-1,6 glucan in *S. cerevisiae* [24].

Chitin is the major component of yeast cells in which it comprises (37% to 48%) of the total cell wall components. Of special note is the detection of a novel transcript

encoding an UDP-N-acetyl glucosamine transporter (MNN2), which has been described in *S. cerevisiae.* The cytoplasm is the sole site of sugar nucleotide synthesis and sugar nucleotides must be transported into various organelles in which they are utilized as a donor substrate for sugar chain synthesis. It has been demonstrated that UDP-N-acetyl glucosamine transporter encoded by the YEA4 gene in *S. cerevisiae* is located in the endoplasmic reticulum and is involved in cell wall chitin synthesis in this fungi [25]. GDA1 (guanosine diphosphatase) generates both GMP and UMP required as antiporters for guanosine and uridine sugar transport into the Golgi lumen. Deleted strains of *Kluveromyces lactis* for *gda1* present altered cell wall stability and composition [26]. Chitinase 1 (CTS1) and 3 (CTS3), the latter a novel gene, were induced in the transition library suggesting their role in the remodeling of the cell wall and providing N-acetyl glucosamine for the synthesis of chitin. The DIP5 encoding transcript (acidic amino acid permease) was increased in the transition library and could provide the uptake of glutamate, a precursor required for the synthesis of chitin. We recently described that this transcript is up regulated in *P. brasiliensis* yeast cells during incubation in human blood and is hypothetically related to the cell wall remodeling supposed to occur during osmotic stress [27]. In addition, the induced enzyme HPAT (histidinol phosphate aminotransferase) could also provide glutamate for the synthesis of chitin precursors. Sugar transporters MSTE (monosaccharide transport protein), STL (sugar transport protein), GTT (glucose transporter) were present in the transition transcriptome; the first two genes were present as increased transcripts. The increased expression may permit the fungus to increase uptake of carbohydrates, thus accelerating the synthesis of glucan and chitin (Table 2, Fig. 2A). The *mael* (malate permease) cDNA encoding the transporter for malate is an induced gene in the transition library and could provide the precursor for gluconeogenesis furnishing carbohydrate precursors to the cell wall

components biosynthesis. Also the availability of compounds to the glyoxalate cycle seems to be favored during transition. The MAEL (malate transporter) could provide malate for the glyoxylate cycle. The enzymes (CITA) citrate synthase (E.C.2.3.3.1), (ACO) aconitase (E.C.4.2.1.3), (ICL) isocitrate lyase (E.C.4.1.3.1), and (MDH) malate dehydrogenase (E.C.1.1.1.37) were present in the transition library, indicating that the glyoxalate cycle is functional during the transition from mycelium to yeast. Of note the transcriptome analysis in *P. brasiliensis* showed several pathways that provide substrates for the glyoxalate cycle that is up regulated in the yeast cell, as described previously [5].

Induced transcripts in the transition library also involve those related to the phospholipids synthesis, as well as to ergosterol, as shown in Table 2 and Fig. 2B. The enzyme GFDA (glycerol 3P dehydrogenase) converts DHCP (dihydroxycetona phosphate) in G3P (glycerol 3P). The *gfdA* null mutant of *Aspergillus nidulans* displays reduced G3P levels and an osmoremediable growth defect, which is associated with abnormal hyphal morphology [28]. G3P can be produced by the action of the enzyme GDPD (glycerophosphodiester phosphodiesterase) which promotes the hydrolysis of phosphatidylethanolamine (G3PEtn). Both enzymes are induced in the transition from mycelium to yeast cells, as shown in Table 2 and Fig. 2 B. The ACT (acyltransferase) promotes the addition of acyl groups to G3P generating DG3P (diacylglycerol 3P); this enzyme is described in *P. brasiliensis* in the public databases. The acyl CoA required for the synthesis of DG3P is produced by ACS (acyl- CoA synthetase) which can utilize an acyl group that can be liberated by the action of phospholipases A and B (PLAA LPB1B and respectively); all the ESTs encoding those enzymes are induced in the transition from mycelium to yeast, as described in Fig. 2B and Table 2. Also, DG3P can be produced by GDE1 (diacylgycerol pyrophosphate phosphatase). CDP-diacylglycerol

(CDP-DG) produced from DG3P is the precursor of phospholipids. PSSA (phosphatidylserine synthase) produce phosphatidylserine from CDP-DG, and is a novel transcript described in the present work. The induced transcript of INO1 (myo-inositol-1phoshate synthase), produces myo-inositol 1P the precursor for the synthesis of phosphatidylinositol. The PDR16 (phosphatidylinositol transfer protein), also induced, transports phospholipids from their site of synthesis in the endoplasmic reticulum to the plasma membrane [29].

Polyunsaturated fatty acids (UFA) are major components of the membranes and are produced from monounsaturated fatty acids by several fatty acid desaturases in many fungi. DESA (fatty acid desaturase) was demonstrated to be induced in the transition library suggesting active membrane remodeling during the morphogenetic event in *P. brasiliensis*. The synthesis of ergosterol seems also to be induced during the transition process. ERG 11 (lanosterol 14-alpha demetylase) and ERG 3 (sterol delta 5, 6-desaturase) present transcripts induced in the transiton library (Fig.2B, Table 2).

An overview of induced genes putatively related to signal transduction

We also identified a variety of signal transduction systems in *P. brasiliensis* ongoing differentiation to yeast cells, such as MAPK, serine/threonine protein kinases, signal histidine kinases and two component sensor kinases. The most increased transcript encodes for a histidine protein kinase sensor for GlnG regulator, which presented 24 ESTs in the transition library (Table 1 and Table 2, supplementary material). Novel genes were also those encoding for a two-component sensor kinase (06 ESTs), calcineurin subunit b (02 ESTs), UVSB phosphatidylinositol-3- kinase (01 EST), forkhead associated protein (01 EST), Rho GTPAse activating protein (01 EST). Histidine kinases are signaling transduction proteins that organisms in all three domains of life use to respond to environmental signals and control developmental process [30,

31]. *S. cerevisiae* has a single hybrid histidine kinase, sln1p, which regulates an osmosensing mitogen-activated protein kinase (MAPK) cascade, an oxidative stress-response pathway, and cell wall biosynthesis [32, 33]. *Blastomyces dermatitidis* DRK1 (for dimorphism-regulating histidine kinase) is a conserved hybrid histidine kinase that is indispensable for dimorphism, virulence and pathogenicity [34]. The ESTs encoding the putative histidine kinase induced in the transition library presents some structure domains and sequence of histidine kinase, such as the histidine-containing H-box and an aspartate-containing D-box (data not shown).

The fungal cell wall is an essential cellular boundary that controls many cellular processes. It allows cells to withstand turgor pressure preventing cell lysis. In *S. cerevisiae* a MAPK cascade which is essential in transducing signals to adapt cell wall biosynthesis under a variety of environmental conditions, is activated by the protein kinase C, constituting the PKC cell integrity pathway [35]. A MAPK and PKC proteins were induced in the transition library suggesting their involvement in the cell wall biosynthesis. In addition, calcineurin has been proposed as essential for survival during membrane stress in *Candida albicans* [36]. Also a FHA (forkhead associated) protein and an UVSB phosphatidylinositol-3-kinase were increased in the transition library suggesting the requirement of DNA damage checkpoint kinases in the dimorphic transition of *P. brasiliensis* [37, 38].

In *P. brasiliensis* transition transcriptome it was detected 53 ESTs (4.78% of the total ESTS) encoding for potential signal transduction proteins (see Table 1, supplementary material). From those, 10 are induced transcripts comprehending 06 novel genes, suggesting that the morphological transition in *P. brasiliensis* is mediated by a series of signal transduction systems that control the adaptation to the environment to the fungus survive and proliferate within the host.

Novel genes of *P. brasiliensis* detected in the transition library

Table 3 summarizes the transcripts detected in the transition library that were not present in the *P. brasiliensis* transcriptome [1] or in public databases. A total of 48 novel genes are reported here. Several enzymes related to the general metabolism were described as novel genes. As examples, the orotate phosphoribosyltransferase (URA5) (E.C.2.4.2.10) was present in the transition library. Also a phosphatidylserine synthase (E.C.2.7.8.8) putatively related to the metabolism of phospholipids, as cited above. Enzymes related to protein modification, transport facilitators and signal transduction were also detected as novel genes in the transition library and were discussed before. A novel transcript encodes for a homologue of SamB, related to morphogenesis in ascomycetous fungi [39]. We exploited sequence data to examine the presence of the conserved Zn-finger like domain in the deduced homolog of *P. brasiliensis* (data not shown). It was observed the high conservation of the Zn finger-like domain in SamB, crucial for fungal morphogenesis, as described [39].

Putative virulence factors

Expression analysis can be a valuable first step in virulence genes discovery. Putative virulence factors were selected on basis with homology in other pathogenic microrganisms. With these criteria, we classified 12 induced genes as putative virulence factors of *P. brasiliensis*. Table 4 presents some induced genes, potential virulence factors in *P. brasiliensis*. AGS1 was catalogued as a potential virulence factor, since in *Histoplasma capsulatum* the reduction of its activity by RNA interference or allelic replacement leads to reduction in the fungal ability to colonize lung [40]. Mutants of *Aspergillus fumigatus* in glucanosyltransferases 1 and 2 (gel 1 and 2) have abnormal cell wall compositon and conidiogenesis and reduced virulence in a murine model of

invasive aspergillosis, suggesting that beta(1-3) glucanosyltransfease activity is required for both morphogenesis and virulence in this fungal pathogen [41]. Calcineurin plays a global role in stress responses necessary for fungal cell survival and in this sense can be defined as a virulence factor [42]. Deleted para-aminobenzoic acid synthetase (paba) strains of *A. fumigatus* present complete inability in causing lethal infection in mice [43]. We previously described that the catalase P (CAT P) presents canonical motifs of monofunctional typical catalases, as well as the peroxisome PTS-1 targeting signal and its expression was induced in cells treated with H_2O_2, suggesting its involvement in protecting *P. brasiliensis* yeast cells against exogenously produced peroxides [44]. Secreted products are a common means by which fungi can promote virulence [45, 46]. The aspartyl proteinase (ASP) described in Table 4 is putatively a secreted protease that may facilitate tissue invasion; the same could be hypothesized to the transcript encoding a zinc metalloprotease [46]. Phospholipases are critical for modification and redistribution of lipid substrates, membrane remodeling and microbial virulence. The null mutants and revertant strains for a phospholipase B gene of *C. albicans* present reduced phospolipase A2 activity and attenuated virulence [47]. In addition an inositol phosphosphingolipid phospholipase C (PLC) gene of *C. neoformans* promotes neurotropism of *C. neoformans* depending on the immune status of the host by protecting the fungus from the hostile intracellular environment of phagocytes [48]. Specific adhesins can enable fungal cells to adhere to host cells or the ECM components. We previously demonstrated that he fungal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a potential virulence factor of *P. brasiliensis*, since it can diminish the fungus yeast cells ability to adhere and invade in vitro cultured pneumocytes [49]. Also the mannosyltation of proteins can be related to virulence. The mnn5 mutant of *C. albicans* exhibited attenuated virulence in mice [50]. The transcripts

encoding for a hemolysin like protein of *Candida glabrata* (HLP) and for urease (URE), are possible virulence factors (Table 4). Switching in *C. glabrata* which may provide colonizing populations for rapid response to the changing physiology of the host regulates the hlp expression [51]. Urease which catalyzes the conversion of urea into ammonia is described to contribute to alkalinity at the sites of fungal infection, causing a great damage to the host tissues [52]. Of special note, the up regulation of those potential virulence factors in the transition of mycelium to yeast cells suggests the fungal adaptation to the new conditions to be faced in the host milieu.

Expression profile

We validated the classification of induced transcripts by northern blot analysis, as shown in Figure 3A. The transcripts encoding aspartyl proteinase and sugar transporter protein, were classified as induced in the transition library by electronic northern and according to our experimental northern blot data, were accumulated in mycelium during transition to yeast cells. It has to be emphasized that the in silico analysis of the ESTs redundancy revealed for the transcripts encoding aspartyl protease and sugar transporter protein, 3 ESTs in the mycelium transcriptome database for both; 7 and 5 in the present transition library, respectively and 3 for both, ESTs in the yeast transcriptome database. We also validated 12 novel genes identified in the transition cDNA library, by semi-quantitative RT-PCR, and their expression profiles are shown in Figure 3B. All transcripts were induced upon transition, as demonstrated.

Conclusions

The 1107 ESTs identified in this study represent the first effort to define the *P. brasiliensis* genes present in a cDNA library of the fungal RNA obtained during the

transition from mycelium to yeast. These data increase the number of identified *P. brasiliensis* genes induced during the transition. Annotation of the unisequences revealed that 992 (89.6%) had homologues in the *P. brasiliensis* public databases, and therefore about 115 (10.4%) represent novel genes. Annotation of the ESTs revealed a great repertoire of genes that could function in cell wall/membrane remodeling during the transition process. Also, putative virulence factors, novel transduction signal proteins, novel enzymes related to sulphur metabolism, among others, had been described. Overall these data can help in accelerating research on this important human fungal pathogen.

Methods

Fungal isolate, growth conditions and induction of mycelium to yeast transition

P. brasiliensis, isolate *Pb*01 (ATCC-MYA-826), has been studied at our laboratory. It was grown in Fava-Netto´s medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 4% (w/v) agar, pH 7.2], at 22 ºC, as mycelium. The differentiation was performed in liquid medium (Fava-Netto´s medium) by changing the culture temperature from 22 ºC to 36 ºC for the mycelium to yeast transition, as we previously described [44]. The cells were previously grown in liquid medium for 18 h before changing the incubation temperature, which was maintained for 22h.

RNA extraction and preparation of the cDNA library

Total RNA was purified from *P. brasiliensis* mycelium in transition to yeast cells (see above) using TRIZOL (GIBCO^{TM}, Invitrogen, Carlsbard, CA). The mRNA was purified by using the Poly (A) Quick ^{R} mRNA isolation kit (Stratagene, La Jola, CA). The

cDNA library was constructed in the unidirectional pCMV.SPORT 6 (Invitrogen) according to the manufacturer's instructions, exploiting the *Not* I and *Sal* I restriction sites. The cDNA library was not normalized, i.e., no attempt was made to reduce the redundancy of highly expressed transcripts.

Plasmid isolation and DNA sequencing of the cDNA library

Plasmids constructs were transformed into *Escherichia coli* ElectroMAX™ DH10B cells (Invitrogen). The cDNA library was plated to approximately 200 colonies per plate (150 mm Petri dish). The colonies were randomly selected and transferred to a 96-well polypropylene plate containing LB medium and grown overnight. Plasmid DNA was isolated and purified using Millipore filters (MilliPore®). cDNA inserts were sequenced from the 5´ end by employing standard fluorescence labeling DYE namic™ ET dye terminator kit with the M13 flanking vector primer. Samples were loaded onto a MegaBACE 1000 DNA sequencer (GE Healthcare, Amersham Biosciences), for automated sequence analysis.

EST Processing Pipeline, Annotation and Sequence Analysis

The resulting electropherograms were transferred to the server where the pre-processing took place. ESTs were screened for vector sequences against the UniVec data. The sequences were assembled by using the PHRED/PHRAP/CONSED [53]. EST sequences were pre-processed using the Phred [54] and Crossmatch [55] programs. Only sequences with at least 100 nucleotides and Phred quality greater or equal to 20 were considered for further analysis. A total of 1107 ESTs were selected by these inclusion criteria. The resulting sequences were uploaded to a relational database (MySQL) on a Linux (Fedora Core 3) platform, and processed using a modified version of the PHOREST tool [56]**.** We modified PHOREST to the assembling of the sequences

using the CAP [57] and store the BLAST results of many databases including GenBank non-redundant (nr) database, Cluster of Orthologus Groups (COG), Gene Ontology (GO), MIPS [58], KEGG [59] and some fungi specific databases. In addition, an option to automatically translate EST sequences and compare their frames against the InterPro database [60] was implemented. These modifications allowed easy identification of homolog sequences, as well as the identification of domains and functional sites, which improved the manual annotation process. Similarities with E-values $\leq 10^{-4}$ were considered significant. For comparative analysis the ESTs were grouped in 639 clusters, represented by 166 contigs and 473 singlets. The clusters were compared with *P. brasiliensis* transcriptome database [1] and public databases to identify new transcripts, by using the BLAST program [61]. The ESTs had been submitted to GenBank, under accession numbers EH040628 to EH041734.

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

To assign a differential expression character, the contigs formed with mycelium and the transition ESTs were statistically evaluated using the Audic and Claverie's method [14]. It were considered induced genes in the transition library those that were not previously described in the mycelium transcriptome database [1], as well as those more expressed as determined with a 99% confidence rate. A web site [62] was used to compute the probability of differential regulation.

Northern blot

Northern hybridization was performed with 10μg of total RNA fractioned on a 1.2% agarose-formaldehyde denaturing gel and transferred to a Hybond-N+ nylon membrane (GE Healthcare). The RNAs, corresponding to different times of cellular differentiation,

were hybridized to the correspondent cDNA probes in Rapid-hyb buffer (GE Healthcare) and washed according to the manufacturer´s instructions. Probes were radiolabeled by using Rediprime II Random Prime labeling System (GE Healthcare).

Semi-quantitative RT-PCR analysis (sqRT-PCR)

Semi-quantitative RT-PCR was performed for 12 genes to confirm the presence of new transcripts. Total RNA was extracted from *P. brasiliensis* mycelium in transition to yeast after 22 h of the temperature shift from 22 ºC to 36 ºC, as described. RNAs used for sqRT-PCR were from independent experiments from those used in the cDNA library construction. cDNAs were synthesized by reverse transcription using the Superscript II RNAse H–reverse transcriptase (Invitrogen™, Life Technologies). cDNAs were used for PCR in 30 µl reaction mixture containing specific primers, sense and antisense, as described in Table 5. PCR conditions were: 25-35 cycles at 95 ºC for 1 min; annealing at 55-65 ºC for 2 min; 72 ºC for 1 min. The annealing temperature and the number of PCR cycles were optimized for each experimental condition to ensure linear phase of amplification. Amplicons were analyzed by agarose gels electrophoresis (1%). The analyses of relative differences were performed by using Scion Image Beta 4.03 program [63].

Authors' contributions

KPB prepared the cDNA library, performed the DNA sequencing, the validation experiments, contributed to gene ontology classification and supported the preparation of the figures and tables. AMB contributed to the construction of the cDNA library, to the classification of gene ontology terms, to the data analysis and to the preparation of

the manuscript. CLB contributed to the culture of the fungus, to the construction of the cDNA library, to the classification of gene ontology terms and to the manuscript edition. FPF contributed to the construction of the cDNA library. MSSF contributed to the results discussion and to the manuscript preparation. MGS contributed to the DNA sequencing and to the classification of gene ontology terms. WSM and RBF analyzed the raw sequences and contributed to the construction of the EST database. MP contributed to the analysis of the raw sequences and to the preparation of the manuscript. CMAS designed the project and the database, contributed to the data analysis and to the preparation of the manuscript.

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Figures Legends

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

Figure 2 - The synthesis of the cell wall components from glucose and lipids. Induced transcripts (*), novel transcripts (+), transcripts detected in the transition transcriptome without induction (#) and transcripts present at public databases (o). A- Some steps in the synthesis of glucan and chitin. GLCase 1: Alpha-glucosidase 1; HXK1: hexokinase; PGM: phosphoglucomutase; UGP1: uridine diphosphate glucose pyrophosphorylase; AGS1: alpha glucan synthase; MTLD: mannitol-1-phosphate dehydrogenase; MSTE: monosaccharide transport protein; GTT: glucose transporter protein; STL: sugar transporter protein; CTS 1: chitinase 1; CTS 3: chitinase 3; DIP 5: acidic amino acid permease; MAEL: malate permease; MDH: malate dehydrogenase; CITA: citrate synthase; ACO: aconitase; ICL: isocytrate lyase; MLS: malate synthase; UDPNAG: uridine diphosphate N acetylglucosamine; MNN2: UDPNAG transporter. B- The synthesis of some lipids from the cell membrane. LPL1B: Lysophospholipase; PLAA: phospholipase A2; DHCP: dihydroxycetone phosphate; GFDA: glycerol 3 phosphate dehydrogenase; G3P: glycerol 3 phosphate; G3PEtn: Phosphatidyl ethanolamine; GDPD: glycerophosphodiester phosphodiesterase; ACT: acyltransferase; ACS: acyl-coenzyme A synthethase; Acyl-CoA: acyl-coenzyme A; DGPP: diacylglycerol pyrophosphate; GDE1: diacylglycerol pyrophosphate phosphatase; DG3P: diacylglycerol 3 phosphate; CTP: cytidine triphosphate; PPi: pyrophosphate; CDP-DG: cytidine diphosphate diacylglycerol; PSSA: phosphatidylserine synthase;

PtdSer: phosphatidylserine; PSS2: phosphatidylethanolamine serine transferase; PSD: phosphatidylserine decarboxylase; PtdEtn: phosphatidylethanolamine; PEMT: phosphatidylethanolamine metyltransferase; PtdCho: phosphatidylcholine; INO1: myo-inositol 1 phosphate synthase; Myo-Inol1P: myo-inositol 1phosphate; PtdIns: phosphatidylinositol; PDR16: phosphatidylinositol transfer protein; ERG 11: Lanosterol 14-alpha demetylase; ERG 3: sterol delta 5,6-desaturase.

Figure 3 - **Validation of the classification of induced transcripts in the transition library.** A- Analysis by northern blot was carried out with RNA from mycelium during transition to yeast colleted at 22 h, 48 h and 6 days after the temperature shift. Total RNA was fractionated on a 1.2% formaldehyde agarose gel and hybridized to the cDNA inserts Aspartyl protease *(asp)* and Sugar transporter protein *(stl)*. Ribosomal RNAs are shown as the loading control. The sizes of the transcripts are as follows: *asp* 1.7 kb; *stl* 2.65 kb.

B- Validation of some novel genes of *P. brasiliensis*. Semi-quantitative RT-PCR of RNAs obtained from mycelium in transition to yeast. Semi-quantitative RT-PCR analysis was carried out with specific primers, as described. Gray bars indicate the transcript level for the L34 ribosomal protein and black bars refers to the described new transcript. Numbers associated with the bars indicate fold differences relative to the data for the reference mycelium, which were established by densitometry analysis. Using varied number of cycle numbers, the exponential phase of each primer was determined and used to allow semi-quantitative analysis of the respective reactions. The same amount of cDNA was used for all PCRs. The RNAs used for RT-PCR were obtained from samples of: mycelium (M) and mycelium in transition to yeast after 22 h of the temperature shift (T). Genes and sizes of the respective amplified fragments are as

follows in bp: *dead*: 408; *hlp*: 274; *uvsB*: 318; *cts3*: 268; *gma12*: 152; *mnn2*: 363; *gdpase*: 126; *samB*: 114; *dphs*: 284; *pss*: 281; *glcaseI*: 359; *glnl*: 368.

Tables

Additional files

Additional File 1 - *P. brasiliensis* clusters annotated in the cDNA library.

Table representing the annotated clusters that were generated by sequencing of the cDNA clones. For each cluster the table includes: the unisequences present in each cluster, the function as assigned by BLAST-based similarity, the BLAST subject species, the GenBank ID for the BLAST subject used for functional assignment and the Expect value obtained with each unisequence, the redundancy in the transition library and in the mycelium transcriptome database.

Additional File 2 - *P. brasiliensis* induced transcripts potentially related to signal transduction. Table representing the annotated clusters that were generated by sequencing of the cDNA clones of the transition library. For each cluster the table includes: the unisequences present in each cluster, the function as assigned by BLAST-based similarity, the redundancy in the transition library and in the mycelium transcriptome database.

Figure 1

Figure 2

Figure 3

Table 1 – Induced *P. brasiliensis* ESTs and novel genes generated from the transition library‡.

MIPS Category	Gene Product	Best hit organism/Accession number	e-value	Redundancy	
				M	T
Amino acid metabolism	Histidinol phosphate aminotransferase[b]	*Aspergillus fumigatus*/CAF32122	1e -69	-	1
	Diphthine synthase*[#]	*Aspergillus fumigatus*/CAF32112	1e -38	-	2
	Acetylornithine deacetylase*	*Arabidopsis thaliana*/BP845946	1e-31	-	1
	Gamma-glutamyl phosphate reductase[+]	*Coccidioides immitis*/ EAS33218	1e -21	-	1
	Fumarylacetoacetate hydrolase[b]	*Emericella nidulans*/AAA85778	1e -69	-	2
	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase[b]	*Aspergillus nidulans* /XP_407838	5e -07	-	1
	Anthranilate phosphoribosyltransferase[a]	*Aspergillus nidulans* /XP_407771	2e -44	1	2
	Histidine ammonia lyase*	*Dictyostelium discoideum*/XP_636944	1e -16	-	1
	Glutamate dehydrogenase (NADP(+))*	*Emericella nidulans*/ S04904	5e -06	-	2
Nitrogen and sulfur metabolism	Nitrogen regulatory protein P-II[b]	*Aspergillus nidulans*/XP_408436	5e -37	-	2
	Acetamidase[b]	*Aspergillus nidulans*/XP_405649	2e -53	-	1
	Sulfite reductase NADPH beta subunit[b]	*Aspergillus nidulans*/XP_411737	3e -75	-	1
	Urease, alpha subunit[a]	*Aspergillus fumigatus*/CAE17672	1e -71	1	4
	Cyanate lyase[b]	*Aspergillus nidulans*/XP_411468	6e -46	-	1
	Thiosulfate sulphurtransferase[a]	*Gibberella zeae*/XP_381684	1e -45	1	2
Nucleotide metabolism	Nudix hydrolase family protein*	*Aspergillus nidulans*/XP_409279	1e -19	-	3
	RNA (guanine-N7-) methyltransferase[a]	*Neurospora crassa*/CAF06136	2e -18	1	2
	Adenosine deaminase*	*Aspergillus oryzae*/BAE60718	2e -34	-	4
	Orotate phosphoribosyltransferase*	*Mortierella alpina*/BAD29963	3e -44	-	2
	Pyrimidine 5-nucleotidase[+]	*Aspergillus nidulans*/XP_410462	4e -49	-	2
	TatD DNAse[b]	*Aspergillus nidulans*/XP_408185	4e -10	-	1
	Xanthine dehydrogenase*	*Gibberella zeae*/XP_381737	9e -07	-	1
Phosphate	phnO protein*	*Rhizopus oryzae*/EE002192	4e -116	-	4

metabolism	Inorganic pyrophosphatase[b]	*Coccidioides immitis*/ EAS28880	3e -32	-	1
C-compound and carbohydrate metabolism	Alpha-1,3-glucan synthase[b]	*Paracoccidioides brasiliensis*/AAV52833	8e -68	-	1
	Glucanosyltransferase family protein[a]	*Aspergillus nidulans*/XP_408051	9e -35	1	3
	Chitinase 1[a]	*Aspergillus nidulans*/XP_413527	1e -18	1	2
	Chitinase 3*[#]	*Coccidioides immitis*/AAO88269	7e -40	-	1
	Mannitol-1-phosphate dehydrogenase[a]	*Paracoccidioides brasiliensis*/AAO47089	2e -88	2	3
	Uridine diphosphate glucose pyrophosphorylase[a]	*Emericella nidulans*/AAW49005	3e -69	1	2
	Alpha-glucosidase I*[#]	*Aspergillus fumigatus*/AAR23808	3e -46	-	2
Lipid metabolism	Myo-inositol-1-phosphate synthase[+]	*Aspergillus nidulans*/XP_411762	4e -15	-	1
	Glycerophosphodiester phosphodiesterase[a]	*Aspergillus nidulans*/XP_404274	9e -70	1	4
	Esterase family protein[+]	*Aspergillus nidulans* /XP_412451	2e -13	-	1
	Lysophospholipase[b]	*Aspergillus nidulans*/XP_412885	3e -58	-	1
	Phospholipase A2[+]	*Coccidioides immitis*/ EAS34384	3e-27	-	1
	Peroxisomal hydratase dehydrogenase epimerase[b]	*Aspergillus nidulans*/XP_411248	1e -34	-	4
	Serine esterase[+]	*Aspergillus nidulans*/XP_406618	8e -92	-	3
	Glycerol-3-phosphate dehydrogenase (NAD(P)+)*	*Cryptococcus neoformans*/AAM26266	2e -14	-	1
	Phosphatidylserine synthase*[#]	*Neurospora crassa*/EAA30566	6e -38	-	1
	Fatty acid desaturase[a]	*Neurospora crassa*/EAA29146	4e -48	1	2
	Sterol delta 5,6-desaturase ERG3[b]	*Aspergillus nidulans*/XP_410643	7e -40	-	1
	Lanosterol 14-alpha-demethylase[a]	*Ajellomyces capsulatus*/AAU01158	1e -89	3	4
	Lipoic acid synthase[b]	*Aspergillus nidulans*/XP_413623	8e -40	-	1
Metabolism of vitamins, cofactors and prosthetic groups	Uroporphyrinogen III methylase*	*Rhizopus oryzae*/EE010378	6e-109	-	4
	Ubiquinone/menaquinone biosynthesis methyltransferase UbiE[b]	*Dictyostelium discoideum*/XP_641323	2e-12	-	18
	Para aminobenzoic acid synthetase[b]	*Aspergillus nidulans*/XP_410687	8e -19	-	1

Energy	Phosphoglucomutase[b]	*Neurospora crassa*/EAA34468	5e -73	-	1
	Glyceraldehyde-3-phosphate dehydrogenase[b]	*Paracoccidioides brasiliensis*/AAL34975	7e -70	-	1
	NADH dehydrogenase, 21 kDa subunit[b]	*Aspergillus nidulans*/XP_411113	4e -38	-	1
	Ubiquinol cytochrome c reductase[b]	*Aspergillus nidulans*/XP_408525	6e -39	-	2
	Ferredoxin-like iron-sulfur protein[a]	*Paracoccidioides brasiliensis*/AAP23044	2e -58	2	3
	Protein PET191, mitochondrial precursor[b]	*Gibberella zeae*/XP_388901	4e -11	-	1
	ATP synthase gamma chain[a]	*Aspergillus nidulans*/XP_404389	9e -57	1	3
	ATP synthase subunit 9[a]	*Neurospora crassa*/T43671	8e -22	1	4
	Acyl-coenzyme A synthetase[b]	*Neurospora crassa*/EAA26946	5e -10	-	1
	L-carnitine dehydratase[+]	*Aspergillus nidulans*/XP_408367	1e -62	-	1
	Acetyl CoA hydrolase*	*Aspergillus nidulans*/XP_405684	5e -42	-	1
Cell cycle and DNA processing	Endonuclease III[b]	*Aspergillus nidulans*/XP_411790	1e -06	-	1
	Rad21 protein*	*Neurospora crassa*/EAA34981	6e -17	-	2
	Prohibitin 2[a]	*Aspergillus nidulans*/XP_410210	6e -52	3	5
	Arrestin, N-terminal domain[b]	*Aspergillus nidulans*/XP_404193	2e -56	-	1
	Proliferating Cell Nuclear Antigen (PCNA)*	*Aspergillus nidulans*/XP_404552	3e -36	-	1
	DNA polymerase delta 2 subunit[+]	*Coccidioides immitis*/EAS28821	6e -33	-	2
	DNA polymerase eta[b]	*Aspergillus nidulans*/XP_408815	1e -32	-	1
	Heterokaryon incompatibility protein Het-C[b]	*Aspergillus nidulans*/XP_406304	1e -27	-	2
	Uracil DNA glycosylase*	*Aspergillus fumigatus*/ XP_749743	3e -24	-	1
	Cell division protein 48 (CDC48)[a]	*Coccidioides immitis* /EAS28203	9e -15	2	3
	Chromosome segregation ATPase*	*Coccidioides immitis* /EAS30662	6e -52	-	1
Transcription	DEAD-like helicases superfamily protein*[#]	*Aspergillus nidulans*/XP_410144	3e -55	-	1
	Transcription factor, bromodomain*	*Aspergillus nidulans*/EAA60972	2e -55	-	1
	GatB/YqeY domain protein*	*Aspergillus nidulans*/XP_410874	1e -22	-	1
	snRNA-associated protein, Sm class[b]	*Magnaporthe grisea*/XP_368889	9e -32	-	1

	U6 snRNA-associated Sm-like protein LSm5[b]	*Neurospora crassa*/CAD11394	4e -32	-	1
	Ring type Zinc finger protein*	*Aspergillus nidulans*/XP_411042	1e -12	-	2
	Zinc finger domain protein*	*Aspergillus nidulans*/XP_405585	3e -14	-	2
	tRNA (guanine) methyltransferase[b]	*Aspergillus nidulans*/XP_407082	3e -23	-	3
	Ap-1-like transcription factor[b]	*Aspergillus nidulans*/XP_411679	2e -30	-	1
	Regulator of nonsense transcripts 1 homolog protein[b]	*Neurospora crassa*/XP_323582	5e -15	-	6
	Arylsulfatase regulatory protein*	*Blastocladiella emersonii*/CO964913	1e -138	-	11
	Transcriptional activator protein*	*Coccidioides immitis* /EAS34609	8e -26	-	1
	Nucleolar protein NOP56[a]	*Coccidioides immitis* /EAS36543	8e -84	1	3
Protein Synthesis	40S ribosomal protein S5[a]	*Aspergillus nidulans*/XP_404980	8e -22	8	7
	40S ribosomal protein S13[b]	*Neurospora crassa*/EAA34807	2e -37	-	1
	40S ribosomal protein S26[b]	*Neurospora crassa*/CAA39162	3e -52	-	1
	60S ribosomal protein L2[b]	*Coccidioides immitis*/EAS30555	9e -54	-	1
	60S ribosomal protein L3[a]	*Neurospora crassa*/CAD70371	2e -57	1	2
	60S ribosomal protein L20[a]	*Magnaporthe grisea*/XP_361110	3e -16	3	6
	60S Ribosomal protein L27[a]	*Aspergillus nidulans*/XP_408359	4e -63	1	3
	60S ribosomal protein L43B[b]	*Ustilago maydis*/XP_400133	1e -30	-	1
	60S ribosome subunit biogenesis protein NIP7[b]	*Aspergillus fumigatus*/AAM08680	3e -14	-	1
	Mitochondrial ribosomal protein S19[b]	*Aspergillus nidulans*/XP_404292	5e -19	-	1
	14 kDa mitochondrial ribosomal protein*	*Aspergillus nidulans*/XP_408748	4e -46	-	7
	Translational machinery component protein[b]	*Aspergillus nidulans*/XP_405417	1e -19	-	1
	Translation initiation factor eIF1 subunit Sui1[a]	*Gibberella zeae*/XP_389056	2e -36	2	5
	Translation initiation factor eIF-5A[a]	*Neurospora crassa*/P38672	6e -06	4	4
	Translation initiation factor 3 subunit 2*	*Aspergillus nidulans* /XP_660601	6e -80	-	3
	Isoleucyl-tRNA synthetase[b]	*Aspergillus nidulans*/XP_407499	1e -52	-	2
	GTP-binding GTP1/OBG-family protein[b]	*Aspergillus nidulans*/XP_404829	1e -70	-	1
Protein fate	Cyclophilin like peptidyl prolyl cis-trans isomerase[b]	*Neurospora crassa*/CAD21421	8e -39	-	1

	Cyclophilin seven suppressor 1[b]	*Aspergillus nidulans*/XP_409575	8e -12	-	2
	Peptidyl-prolyl cis-trans isomerase-like 4[a]	*Coccidioides immitis*/ EAS29016	1e -46	1	5
	Rab geranylgeranyl transferase*	*Aspergillus nidulans*/XP_412816	8e -13	-	1
	Protein-L-isoaspartate(D-aspartate)O-methyltransferase[a]	*Aspergillus nidulans*/XP_407601	5e -55	4	5
	COP9 signalosome complex subunit 5[a]	*Aspergillus nidulans*/XP_406266	1e -35	1	2
	Guanosine diphosphatase* [#]	*Aspergillus nidulans*/XP_405219	2e -15	-	1
	Ubiquitin fusion protein[a]	*Schizosaccharomyces pombe*/NP_593923	8e -67	3	3
	Ubiquitin thiolesterase otubain-like protein*	*Aspergillus nidulans*/EAA60354	1e -28	-	1
	Ubiquitin-conjugating enzyme E2[a]	*Gibberella zeae*/XP_388490	1e -29	6	7
	Ubiquitin/S27a fusion protein[a]	*Aspergillus nidulans*/XP_409009	2e -60	7	7
	Lon protease[b]	*Oryza sativa*/AAV59316	1e-05	-	1
	Zinc metalloprotease[b]	*Neurospora crassa*/CAD21161	3e -47	-	1
	Aspartyl protease[a]	*Paracoccidioides brasiliensis*/AAP32823	3e -72	3	7
	26S protease subunit protein[b]	*Aspergillus nidulans*/XP_411125	4e -23	-	1
	Non-ATPase regulatory subunit of the 26S proteasome*	*Aspergillus nidulans*/XP_408912	2e -68	-	1
	26S proteasome regulatory subunit rpn12[b]	*Aspergillus nidulans*/XP_407156	5e -30	-	1
	F-box/LRR-repeat protein 7[b]	*Aspergillus nidulans*/XP_408647	8e -28	-	3
	Peptidase C19 subfamily protein[b]	*Aspergillus nidulans*/XP_412211	7e -08	-	3
	Peptidase M28 domain protein*	*Coccidioides immitis*/EAS33583	1e -22	-	1
	Alpha-1, 2-galactosyltransferase* [#]	*Aspergillus nidulans*/XP_406106	3e -14	-	1
	Oligosaccharyltransferase subunit ribophorin[+]	*Coccidioides immitis*/ EAS29547	9e -37	-	1
	Ring (Really Interesting New Gene) type zinc finger (C3HC4) protein[b]	*Schizosaccharomyces pombe*/CAB08748.1	5e -10	-	1
	Tailless Complex Polypeptide 1 chaperonin, subunit epsilon[b]	*Schizosaccharomyces pombe*/EAA65069	6e -16	-	2
	Mannosyltransferase[b]	*Paracoccidioides brasiliensis*/AAK54761	3e -70	-	1
	Alpha-1, 2-mannosyltransferase[a]	*Neurospora crassa*/CAC18268	1e -29	3	3
Protein with binding function or cofactor requirement	RPEL repeat protein[a]	*Aspergillus nidulans*/XP_407503.1	5e -22	1	3

Transport Facilitation	Mitochondrial carrier protein[b]	*Gibberella zeae*/XP_391004	6e -22	-	3
	Xanthine/uracil/vitamin C permease[+]	*Magnaporthe grisea*/XP_362769	7e -34	-	1
	Sugar transporter protein[a]	*Gibberella zeae*/XP_381006	9e -55	3	5
	Copper transporter family protein[a]	*Gibberella zeae*/XP_380949	1e -28	4	6
	Sulfate permease[b]	*Gibberella zeae*/XP_384418	4e -46	-	1
	Uridine diphosphate N-Acetylglucosamine transporter* [#]	*Neurospora crassa*/T50997	9e -30	-	1
	Monossaccharide transport protein[b]	*Aspergillus nidulans*/XP_408414	8e -55	-	1
	Acidic amino acid permease[a]	*Aspergillus nidulans*/XP_410255	2e -17	9	9
	B-cell receptor-associated protein 31-like protein+	*Aspergillus nidulans*/XP_404956	9e -43	-	1
	Malate permease[b]	*Gibberella zeae*/XP_389995	2e -25	-	2
	Nuclear pore protein 84/107*	*Coccidioides immitis*/EAS31445	2e -13	-	1
	Mitochondrial import receptor subunit Tom20[b]	*Aspergillus nidulans*/XP_404696	9e -40	-	1
	Clathrin adaptor appendage domain protein[b]	*Aspergillus nidulans*/XP_408344	1e -13	-	1
	ER to Golgi transport related protein[b]	*Aspergillus nidulans*/XP_412331	5e -75	-	2
	Vacuolar protein sorting/targeting protein PEP1 precursor[b]	*Coccidioides immitis*/EAS36959	1e -42	-	1
	Regulator of V-ATPase in vacuolar membrane protein*	*Aspergillus nidulans*/XP_404840	9e -59	-	1
	Tctex-1 family protein*	*Aspergillus nidulans*/XP_405470	6e -25	-	2
	Phosphatidylinositol transfer protein[b]	*Aspergillus nidulans*/XP_410990	9e -79	-	1
	Importing beta protein[b]	*Aspergillus nidulans*/XP_410871	6e -71	-	1
	Importin beta N-terminal domain protein*	*Aspergillus nidulans*/XP_410143	1e -44	-	1
	Phox homology (PX) domain protein[b]	*Aspergillus nidulans*/XP_410488	3e -06	-	1
	Nucleoporin SONB[a]	*Aspergillus fumigatus*/ XP_751721	7e -47	1	2
	Exocyst complex component Sec15 protein[a]	*Coccidioides immitis* /EAS37215	4e -65	1	3
Signal Transduction	Two-component sensor kinase*	*Anopheles gambiae*/EAA02130.2	2e-38	-	6
	Histidine protein kinase sensor for GlnG regulator* [#]	*Tetrahymena thermophila*/EAR83219.1	2e-04	-	24
	Protein kinase C conserved region 2[b]	*Aspergillus nidulans*/XP_409761.1	3e -55	-	1
	UVSB Phosphatidylinositol-3 kinase* [#]	*Aspergillus nidulans*/XP_411112.1	1e -29	-	1

	Serine/threonine-protein kinase SAT4[b]	*Aspergillus nidulans* /XP_412967.1	2e -51	-	1
	Mitogen activated protein kinase[b]	*Aspergillus oryzae*/BAD12561	1e -52	-	1
	GTPase, G3E family protein[b]	*Neurospora crassa*/XP_323082	1e -11	-	1
	Rho GTPase activating protein*	*Aspergillus nidulans*/XP_407883.1	3e -49	-	1
	GTP binding protein[b]	*Neurospora crassa*/CAD70888.1	5e -84	-	1
	Calcineurin subunit b*	*Neurospora crassa*/P87072	1e -77	-	2
	Forkhead associated (FHA) protein*	*Gibberella zeae*/XP_389397.1	4e -10	-	1
Cell Rescue, Defense and Virulence	Potential secreted Cu/Zn superoxide dismutase[b]	*Magnaporthe grisea*/XP_360807	8e -11	-	1
	Peroxisomal catalase[a]	*Paracoccidioides brasiliensis*/AAL34518	4e -66	3	4
	Heat shock protein 10, mitochondrial[a]	*Gibberella zeae*/XP_386383	2e -40	1	3
	Heat shock protein 60[b]	*Aspergillus nidulans* /XP_659508	3e -12	-	1
	Heat shock protein 70[b]	*Paracoccidioides brasiliensis*/AAP05987	3e -64	-	2
	Heat shock protein 70[b]	*Emericella nidulans*/CAA67431	7e -39	-	1
	Heat shock protein 90[a]	*Aspergillus nidulans*/EAA59007	4e -38	5	6
	Hemolysin like protein* [#]	*Aspergillus nidulans*/XP_406013	2e -70	-	1
Biogenesis of cellular components	Tubulin alpha 1[b]	*Aspergillus nidulans*/XP_411707	1e -63	-	1
	Tubulin beta-1 chain[b]	*Aspergillus flavus*/P22012	7e -120	-	1
	Actin related protein[b]	*Aspergillus nidulans*/XP_411146	1e -40	-	1
	Histone H3[a]	*Aspergillus fumigatus*/XP_752749	5e -67	11	6
	Histone H2A[a]	*Aspergillus nidulans*/XP_412176	2e -52	7	6
Cell type differentiation	Suppressor of anucleate metulae B protein* [#]	*Aspergillus nidulans*/XP_404215.1	6e -46	-	2
Unclassified	Complex 1 protein (LYR family)*	*Aspergillus nidulans*/XP_408902	8e -32	-	1
	Homolog of translationally controlled tumor protein[a]	*Aspergillus nidulans*/XP_404778	4e -25	3	3
	YCII related domain protein[b]	*Gibberella zeae*/XP_390542	2e -23	-	1

Dimeric alpha-beta barrel domain protein[b]	*Aspergillus nidulans*/XP_406116	1e -23	-	1
Iron-sulfur cluster Isu1-like protein[a]	*Gibberella zeae*/XP_382800	3e -41	5	4

‡Transcripts not detected in the mycelium transcriptome (https//dna.biomol.unb.br/Pb) or those induced as detected according to the Audic and Claverie's method.
[a] Transcript induced in the transition library compared to the mycelium transcriptome database.
[b] Transcripts non detected in the mycelia transcriptome (https//dna.biomol.unb.br/Pb).
***** Novel genes of *P. brasiliensis*.
[#] Transcripts confirmed by semi-quantitative RT-PCR.
[+] Genes not described previously for *P. brasiliensis*, isolate *Pb*01, but present in public databases.

Table 2 - Up regulated *P. brasiliensis* transcripts potentially related to membrane and cell wall synthesis/remodeling.

Gene Product	E.C. number	Annotated function	Predicted redundancy‡	
			M	T
Alpha-glucosidase I* (glcase 1)	3.2.1.106	Single glucose residues remotion from oligossaccharides	-	1
Phosphoglucomutase (pgm)	5.4.2.8	Synthesis of glucose	-	1
UDP-glucose pyrophosphorylase (ugp1)	2.7.7.9	Synthesis of UDP-Glucose	-	2
Alpha-1,3 glucan synthase (ags1)	2.4.1.183	Synthesis of α 1-3-glucan	-	1
Mannitol-1-phosphate dehydrogenase (mtld)	1.1.1.17	Synthesis of fructose 6-phosphate	2	3
Monosaccharide transport protein (mstE)		Low affinity glucose uptake	-	1
Sugar transporter protein (stl1)		Uptake of hexoses	3	5
Chitinase 1(cts1)	3.2.1.14	Hydrolysis of chitin	1	2
Chitinase 3* (cts3)	3.2.1.14	Hydrolysis of chitin	-	1
Acidic amino acid permease (dip5)		Acidic amino acid uptake	9	9
Histidinol phosphate aminotransferase (hpat)	2.6.1.9	Synthesis of L-histidinol phosphate/glutamate	-	1
Malate permease (mael)		Uptake of Malate	-	2
UDP-N-acetylglucosamine transporter* (mnn2)		Required for transport of the chitin precursor to Golgi and Endoplasmic reticulum	-	1
Glucanosyltransferase family protein (gel)	2.4.1.-	Transglucosidase activity	1	3
Rho GTPAse activating protein* (bem3)		Regulation of the beta(1,3)-glucan synthase	-	1
Mannosyltransferase (mnt1)	2.4.1.131	Mannosylation of proteins/lipids	-	1
Alpha-1,2-mannosyltransferase (mnn5)	2.4.1.131	Mannosylation of proteins/lipids	3	3

Guanosine diphosphatase* (gdA1)	3.6.1.42	Synthesis of GMP	-	1
Alpha-1,2 galactosyltransferase* (gma12)	2.4.1.-	Galactose incorporation in N- and O-linked mannoproteins	-	1
Lysophospholipase (lpb1b)	3.1.1.5	Hydrolysis of phospholipids	-	1
Phospholipase A2 (plaA)	3.1.1.4	Hydrolysis of phospholipids	-	1
Glycerol-3-phosphate dehydrogenase* (NADP) (gfdA)	1.1.1.94	Synthesis of Glycerol-3-phosphate.	-	1
Glycerophosphodiester phosphodiesterase (gpdp)	3.1.4.46	Synthesis of choline and ethanolamine	1	4
Acyl-coenzyme A synthetase (acs)	6.2.1.3	Convertion of the fatty acid to acyl-coA for subsequent beta oxidation	-	1
Phosphatidylserine synthase* (pssA)	2.7.8.8	Glycerophospholipid metabolism/ Phosphatidylserine synthesis	-	1
Myo-inositol-1-phosphate synthase (ino1)	5.5.1.4	Synthesis of myo- inositol 1 phosphate	-	1
Phosphatidylinositol transfer protein (pdr16)		Transport of phospholipids from their site of synthesis to cell membranes/Regulator of phospholipid biosynthesis	-	1
Lanosterol 14-alpha-demethylase (erg11)	1.14.13.70	Synthesis of ergosterol	3	4
Sterol delta 5,6-desaturase (erg3)	1.3.3.-	Regulation of ergosterol biosynthesis	-	1
Serine esterase (net1)		Catalysis of the cleavage of fatty acids from membrane lipids	-	3
Peroxisomal hydratase dehydrogenase epimerase (hde)	4.2.1.-	Beta oxidation	-	4
Fatty acid desaturase (desA)	1.14.99.-	Insaturation of acyl group of lipids	1	2
Carnitine dehydratase (caiB)	4.2.1.89	Transport of long-chain fatty acids	-	1
Suppressor of anucleate metulae B protein* (samB)		Morphogenesis regulation	-	1

‡The predicted redundancy was obtained from the transition cDNA library in comparison to mycelia transcriptome database (https://dna.biomol.unb.br/Pb/).
* Novel genes detected in *P. brasiliensis*.

Table 3 – List of novel genes detected in the *P. brasiliensis* transition library.

Functional categories	Gene Product	Best hit/Accession number	e-value	E.C. number
Amino acid metabolism	Diphthine synthase[#]	*Aspergillus fumigatus*/CAF32112	1e -38	2.1.1.98
	Acetylornithine deacetylase	*Arabidopsis thaliana*/BP845946.1	1e-31	3.5.1.16
	Histidine ammonia-lyase	*Dictyostelium discoideum*/XP_636944.1	1e-16	4.3.1.3
	Glutamate dehydrogenase (NADP(+))	*Emericella nidulans*/ S04904	5e-06	1.4.1.4
Nucleotide metabolism	Nudix hydrolase family protein	*Aspergillus nidulans*/XP_409279.1	1e -19	-
	Adenosine deaminase	*Aspergillus oryzae*/BAE60718	2e-34	3.5.4.4
	Orotate phosphoribosyltransferase	*Mortierella alpina*/BAD29963.1	3e-45	2.4.2.10
Phosphate metabolism	phnO protein	*Rhizopus oryzae*/EE002192.1	4e-116	-
C-compound and carbohydrate metabolism	Chitinase 3[#]	*Coccidioides immitis*/AAO88269	7e -40	3.2.1.14
	Alpha-glucosidase I[#]	*Aspergillus fumigatus*/AAR23808	3e -46	3.2.1.106
Lipid metabolism	Glycerol-3-phosphate dehydrogenase (NAD(P)+)	*Cryptococcus neoformans*/AAM26266.1	2e-14	1.1.1.94
	Phosphatidylserine synthase[#]	*Neurospora crassa*/EAA30566.1	6e -38	2.7.8.8
Metabolism of vitamins, cofactors and prosthetic groups	Uroporphyrinogen III methylase	*Rhizopus oryzae*/EE010378.1	6e-109	2.1.1.107
Energy	Xanthine dehydrogenase	*Gibberella zeae*/XP_381737.1	9e-07	1.17.1.4
	Acetyl CoA hydrolase	*Aspergillus nidulans*/XP_405684.1	5e -42	3.1.2.1

Cell cycle and DNA processing	Rad21 region protein	*Neurospora crassa*/EAA34981.1	6e -17	-
	Proliferating Cell Nuclear Antigen (PCNA)	*Aspergillus nidulans*/XP_404552.1	3e -36	-
	Uracil-DNA glycosylase	*Aspergillus fumigatus*/ XP_749743	3e-24	3.2.2.-
	Chromosome segregation ATPase	*Coccidioides immitis* /EAS30662	6e-52	-
Transcription	DEAD-like helicases superfamily protein[#]	*Aspergillus nidulans*/XP_410144.1	3e -55	-
	Transcription factor, bromodomain	*Aspergillus nidulans*/EAA60972	2e -55	-
	GatB/YqeY domain protein	*Aspergillus nidulans*/XP_410874.1	1e -22	-
	Ring type Zinc finger protein	*Aspergillus nidulans*/XP_411042.1	1e -12	-
	Zinc finger domain protein	*Aspergillus nidulans*/XP_405585.1	3e -14	-
	Arylsulfatase regulatory protein	*Blastocladiella emersonii*/CO964913.1	1e-138	-
	Transcriptional activator protein	*Coccidioides immitis* /EAS34609	8e -26	-
Protein Synthesis	14 kDa mitochondrial ribosomal protein	*Aspergillus nidulans*/XP_408748.1	4e -46	-
	Translation initiation factor 3 subunit 2	*Aspergillus nidulans* /XP_660601	6e-80	-
Protein fate	Rab geranylgeranyl transferase	*Aspergillus nidulans*/XP_412816.1	8e -13	2.5.1.60
	Guanosine diphosphatase[#]	*Aspergillus nidulans*/XP_405219.1	2e -15	3.6.1.42
	Ubiquitin thiolesterase otubain-like protein	*Aspergillus nidulans*/EAA60354	1e -28	3.4.-.-
	Non-ATPase regulatory subunit of the 26S proteasome	*Aspergillus nidulans*/XP_408912.1	2e -68	-
	Peptidase M28 domain protein	*Coccidioides immitis*/EAS33583	1e-22	3.4.11.15
	Alpha -1, 2-galactosyltransferase[#]	*Aspergillus nidulans*/XP_406106.1	3e -14	2.4.1.-

Transport Facilitation	Uridine diphosphate N-Acetylglucosamine transporter[#]	*Neurospora crassa*/T50997	9e -30	-
	Nuclear pore protein 84/107	*Coccidioides immitis*/EAS31445.1|	2e-13	-
	Regulator of V-ATPase in vacuolar membrane protein	*Aspergillus nidulans*/XP_404840.1	9e -59	-
	Tctex-1 family protein	*Aspergillus nidulans*/XP_405470.1	6e -25	-
	Importin-beta N-terminal domain	*Aspergillus nidulans*/XP_410143.1	1e -44	-
Signal Transduction	Two-component sensor kinase	*Anopheles gambiae*/EAA02130.2	2e-38	-
	Histidine protein kinase sensor for GlnG regulator[#]	*Tetrahymena thermophila*/EAR83219.1	2e-04	2.7.3.13-
	UVSB Phosphatidylinositol – 3 kinase[#]	*Aspergillus nidulans*/XP_411112.1	1e -29	-
	Rho GTPase activating protein	*Aspergillus nidulans*/XP_407883.1	3e -49	-
	Calcineurin subunit b	*Neurospora crassa*/P87072	1e -77	-
	Forkhead associated (FHA) protein	*Gibberella zeae*/XP_389397.1	4e -10	-
Cell Rescue, Defense and Virulence	Hemolysin like protein[#]	*Aspergillus nidulans*/XP_406013.1	2e -70	-
Cell type differentiation	Suppressor of anucleate metulae B protein[#]	*Aspergillus nidulans*/XP_404215.1	6e -46	-
Unclassified	Complex 1 protein (LYR family)	*Aspergillus nidulans*/XP_408902.1	8e -32	-

[#]Transcripts confirmed by semi-quantitative RT-PCR.

Table 4 - Candidate homologs for virulence factors induced in the cDNA transition library.

Virulence determinant	Function in other fungi	Reference number
Alpha -1,3 glucan synthase (ags1)	Reduction of AGS1 activity reduces the lung colonization by *Histoplasma capuslatum*	[40]
Glucanosyltransferase family protein (gel)	Required for both morphogenesis and virulence in *Aspergillus fumigatus*	[41]
Calcineurin subunit B (canB)	Required for *Candida albicans* virulence and stress resistance	[42]
Para-aminobenzoic acid synthetase (paba)	Essential for *Aspergillus fumigatus* growth in lung tissue	[43]
Peroxisomal catalase (cat P)	Putatively related to the *P. brasiliensis* protection against peroxides	[44]
Aspartyl protease (asp)	Facilitation of pathogenesis in *Candida albicans*	[45]
Zinc metalloprotease (mp)	A elastolytic metalloprotease of *Aspergillus fumigatus* is secreted during fungal invasion of murine lung	[46]
Phosholipase A2 (plaA)	Gene inactivation attenuates virulence in *Candida albicans*	[47]
Glyceraldehyde 3 phosphate dehydrogenase (gapdh)	Recombinant GAPDH and antibodies to GAPDH diminish *P. brasiliensis* yeast binding to and infection of A549 pneumocytes	[49]
Alpha- 1,2 mannosyltransferase (mnn5)	Important for virulence of *Candida albicans*	[50]
Hemolysin like protein (hlp)	Phase specific gene regulated by phenotypic switching in *Candida glabrata*	[51]
Urease (ure)	Required for *Coccidioides posadasii* virulence	[52]

Table 5 - Oligonucleotides primers related to new genes selected for sqRT-PCR analysis.

Sequence name	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
DEAD-like helicases superfamily protein (dead)	GGCCTTCTGAAACGGGGG	GAGCTTCGCAATAGGCCAAG
Hemolysin like protein (hlp)	GGCCTTCTGAAACGGGGG	GAGCTTCGCAATAGGCCAAG
UVSB Phosphatidylinosytol-3-kinase (uvsB)	CTAGCGAATGGCAATATCACT	GATAATGAGGGCATGGTCTC
Chitinase 3 (cts3)	GGAGGAGGATATGTCTCTTG	CTGCTGCCCATCCCTCAG
Alpha 1,2 galactosyltransferase (gma12)	GCTATGTCAACTTCTTCGCG	GAGAGCATGGGCCGACAG
UDP-N-Acetylglucosamine transporter (mnn2)	GCCCTCATTACGTTAACGCA	CATGGATTTTCCTTTGGCACT
Guanosine diphosphatase (gdpase)	GATCTTCCGCTTTCTCGCCA	CTCCTTGACACGGCACTGC
Suppressor of anucleate metulae B protein (samB)	CCAGTGCGCCTACTATAAATG	CAGGCATTCTTCTGGCACTC
Diphitine synthase (dphs)	CTGTTTCGCAGTGTGCCAG	CGTTCCGTAATTGCTTTTCCA
Phosphatidylserine synthase (pss)	GCTGCTCTCGGCGGACTC	CGAAGGAGACCAGATCAGC
Alpha glucosidase I (glcaseI)	CCAGCTGATAGTCCACGGC	CTTGTCCATCCTGTGAAATGC
Histidine protein kinase sensor for GlnG regulator (glnL)	CGTCTGTTGGGGCCGCAG	CATCGGGTAAAACAGCGTATC

Table 1 – *P. brasiliensis* clusters annotated in the cDNA library.

MIPS Category	Gene Product	Best hit/Accession number	e-value	Redundancy	
				M	T
Metabolism	D-amino acid oxidase	*Aspergillus nidulans*/XP_404311	2e -47	5	2
	Histidinol phosphate aminotransferase[b]	*Aspergillus fumigatus*/CAF32122	1e -69	-	1
	Diphthine synthase*[#]	*Aspergillus fumigatus*/CAF32112	1e -38	-	2
	Acetylornithine deacetylase*	*Arabidopsis thaliana*/BP845946	1e -31	-	1
	Glutamic acid decarboxylase 1	*Aspergillus nidulans*/XP_409022	4e -36	1	1
	Gamma-glutamyl phosphate reductase[+]	*Coccidioides immitis*/ EAS33218	1e -21	-	1
	Methionine adenosyltransferase	*Aspergillus oryzae*/ BAE64158	8e -13	2	1
	Acetolactate synthase, regulatory subunit	*Coccidioides immitis*/ EAS33057	2e -64	4	1
	Anthranilate synthase	*Aspergillus terreus*/XP_001210590	5e -22	1	1
	Kynurenine aminotransferase	*Magnaporthe grisea* /XP_360721	1e -38	1	1
	Homogentisate 1,2-dioxygenase	*Coccidioides immitis*/EAS35958	1e -58	13	3
	Fumarylacetoacetate hydrolase[b]	*Emericella nidulans*/AAA85778	1e -69	-	2
	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase[b]	*Aspergillus nidulans* /XP_407838	5e -07	-	1
	Anthranilate phosphoribosyltransferase[a]	*Aspergillus nidulans* /XP_407771	2e -44	1	2
	Histidine ammonia lyase*	*Dictyostelium discoideum*/XP_636944	1e -16	-	1
	Glutamate dehydrogenase (NADP(+))*	*Emericella nidulans*/ S04904	5e -06	-	2
	Ketol-acid reductoisomerase	*Aspergillus nidulans*/XP_406663	3e -68	1	1
	3-Methylcrotonyl-CoA carboxylase non-biotin-containing subunit	*Emericella nidulans*/AAR23111	2e -21	3	1
	Nitrogen regulatory protein P-II[b]	*Aspergillus nidulans*/XP_408436	5e -37	-	2
	Acetamidase[b]	*Aspergillus nidulans*/XP_405649	2e -53	-	1
	Sulfite reductase beta subunit[b]	*Aspergillus nidulans*/XP_411737	3e -75	-	1
	Urease, alpha subunit[a]	*Aspergillus fumigatus*/CAE17672	1e -71	1	4
	Cyanate lyase[b]	*Aspergillus nidulans*/XP_411468	6e -46	-	1
	Thiosulfate sulphurtransferase[a]	*Gibberella zeae*/XP_381684	1e -45	1	2

Phosphoribosylpyrophosphate amidotransferase	*Coccidioides immitis*/ EAS27535	2e -54	6	1
Nudix hydrolase family protein*	*Aspergillus nidulans*/XP_409279	1e -19	-	3
Quinolinate phosphoribosyl transferase	*Aspergillus oryzae*/BAD07264	2e -48	6	1
GMP synthase	*Phaeosphaeria nodorum*/EAT84008	9e -61	1	1
Ribose phosphate diphosphokinase	*Neurospora crassa*/EAA32555	3e -44	1	1
RNA (guanine-N7) methyltransferase[a]	*Neurospora crassa*/CAF06136	2e -18	1	2
Adenine phosphoribosyltransferase 1	*Aspergillus nidulans*/XP_413220	2e -26	2	2
Adenosine deaminase *	*Aspergillus oryzae*/BAE60718	2e -34	-	4
Orotate phosphoribosyltransferase*	*Mortierella alpina*/BAD29963	3e -45	-	2
Nucleoside diphosphate kinase	*Gibberella zeae*/XP_386148	3e -69	7	2
Uracil phosphoribosyltransferase	*Neurospora crassa*/EAA33629	1e -30	2	1
Pyrimidine 5-nucleotidase[+]	*Aspergillus nidulans*/XP_410462	4e -49	-	2
TatD DNAse[b]	*Aspergillus nidulans*/XP_408185	4e -10	-	1
YjgF-like protein	*Aspergillus nidulans*/XP_413217	1e -11	2	1
Arylesterase region protein	*Gibberella zeae* /XP_388094	2e -12	17	1
phnO protein*	*Rhizopus oryzae*/EE002192	4e -116	-	4
Inorganic pyrophosphatase[b]	*Coccidioides immitis*/ EAS28880	3e -32	-	1
Alpha-1,3-glucan synthase[b]	*Paracoccidioides brasiliensis*/AAV52833	8e -68	-	1
Glucanosyltransferase family protein[a]	*Aspergillus nidulans*/XP_408051	9e -35	1	3
Chitinase 1[a]	*Aspergillus nidulans*/XP_413527	1e -18	1	2
Chitinase 3*[#]	*Coccidioides immitis*/AAO88269	7e -40	-	1
Beta-1,3-endoglucanase	*Aspergillus nidulans*/XP_404609	1e -63	4	2
Glycosyl hydrolase family protein	*Aspergillus nidulans*/XP_408041	8e -15	1	1
Glucan 1,3 beta-glucosidase-like protein	*Aspergillus nidulans*/XP_408837	1e -53	7	2
Cell wall organization and biogenesis related protein	*Aspergillus nidulans*/XP_405599	2e -18	1	1
Hydroxyproline-rich glycoprotein	*Zea diploperennis*/CAA45514	4e -07	16	1
Phosphatidylinositol N-acetylglucosaminyltransferase subunit P	*Aspergillus nidulans*/XP_412207	1e -29	3	1

6-phosphogluconolactonase 1	*Aspergillus nidulans*/XP_404422	1e -43	7	4
Transaldolase	*Gibberella zeae*/XP_388899	9e -25	4	2
Malate dehydrogenase	*Paracoccidioides brasiliensis*/AAP37966	6e -81	1	1
Glucose/ribitol dehydrogenase	*Aspergillus nidulans*/XP_409694	1e -08	3	1
Polysaccharide deacetylase family protein	*Aspergillus nidulan/s*XP_410655	1e -96	4	3
Phosphopyruvate hydratase	*Aspergillus oryzae*/Q12560	2e -74	2	1
Uridine diphosphate glucose pyrophosphorylase[a]	*Emericella nidulans*/AAW49005	3e -69	1	2
Alpha-glucosidase I*[#]	*Aspergillus fumigatus*/AAR23808	3e -46	-	1
Mannitol-1-phosphate dehydrogenase[a]	*Paracoccidioides brasiliensis*/AAO47089	2e -88	2	3
Myo-inositol-1-phosphate synthase[+]	*Aspergillus nidulans*/XP_411762	4e -15	-	1
Glycerophosphodiester phosphodiesterase[a]	*Aspergillus nidulans*/XP_404274	9e -70	1	4
Diacylglycerol pyrophosphate phosphatase	*Aspergillus nidulans*/XP_406261	2e -56	1	1
Esterase family protein[+]	*Aspergillus nidulans* /XP_412451	2e -13	-	1
Lysophospholipase[b]	*Aspergillus nidulans*/XP_412885	3e -58	-	1
Phospholipase A2[+]	*Coccidioides immitis*/ EAS34384	3e -27	-	1
3-ketoacyl-CoA thiolase	*Aspergillus fumigatus*/XP_755468	9e -50	2	1
Peroxisomal hydratase dehydrogenase epimerase[b]	*Aspergillus nidulans*/XP_411248	1e -34	-	4
Delta(24) sterol C-methyltransferase	*Gibberella zeae*/XP_382959	1e -74	2	1
Serine esterase[+]	*Aspergillus nidulans*/XP_406618	8e -92	-	3
Glycerol-3-phosphate dehydrogenase (NAD(P)+)*	*Cryptococcus neoformans*/AAM26266	2e -14	-	1
NADH ubiquinone oxidoreductase	*Aspergillus fumigatus* /XP_755823	2e -46	6	1
Phosphatidylserine synthase*[#]	*Neurospora crassa*/EAA30566	6e -38	-	1
3-oxoacyl (acyl-carrier-protein) reductase	*Aspergillus nidulans*/XP_410792	4e -41	9	3
Trans-2-enoyl-CoA reductase	*Aspergillus nidulans*/XP_413538	7e -42	4	1
Fatty acid desaturase[a]	*Neurospora crassa*/EAA29146	4e -48	1	2
Oxysterol binding protein homolog 7	*Magnaporthe grisea*/XP_365104	1e -76	1	1
Sterol delta 5,6-desaturase ERG3[b]	*Aspergillus nidulans*/XP_410643	7e -40	-	1

	Lanosterol 14-alpha-demethylase[a]	*Ajellomyces capsulatus*/AAU01158	1e -89	3	4
	Lipoic acid synthase[b]	*Aspergillus nidulans*/XP_413623	8e -40	-	1
	Uroporphyrinogen III methylase*	*Rhizopus oryzae*/EE010378	6e -109	-	4
	Ubiquinone/menaquinone biosynthesis methlytransferase UbiE[b]	*Dictyostelium discoideum*/XP_641323	2e -12	-	18
	Para aminobenzoic acid synthetase[b]	*Aspergillus nidulans*/XP_410687	8e -19	-	1
	Acid phosphatase	*Aspergillus nidulans*/XP_409104	6e -45	1	1
	Gephyrin	*Aspergillus nidulans*/XP_407915	7e -49	1	1
	Pyridoxine biosynthesis protein pdx1	*Aspergillus nidulans*/XP_411862	7e -51	5	1
	Dephospho-CoA kinase	*Neurospora crassa*/EAA28019	7e -13	2	1
	FabG region protein	*Magnaporthe grisea*/XP_367544	2e -07	1	1
	Isocitrate lyase	*Coccidioides immitis*/AAK72548	6e -65	5	2
	Isocitrate dehydrogenase (NADP+)	*Aspergillus nidulans*/AAK76730	2e -58	2	1
	Aconitase	*Aspergillus terreus*/AAC61778	2e -79	2	1
Energy	Phosphoglucomutase[b]	*Neurospora crassa*/EAA34468	5e -73	-	1
	Enolase 1	*Penicillium chrysogenum*/BAC82549	3e -47	4	3
	Triose phosphate isomerase	*Paracoccidioides brasiliensis*/AAP02959	3e -59	3	1
	Fructose 1,6-biphosphate aldolase 1	*Paracoccidioides brasiliensis*/AAL25625	3e -65	5	2
	Phosphoenolpyruvate carboxylkinase	*Aspergillus nidulans*/XP_406055	3e -53	2	1
	Glyceraldehyde-3-phosphate dehydrogenase[b]	*Paracoccidioides brasiliensis*/AAL34975	7e -70	-	1
	NADH dehydrogenase, 21 kDa subunit[b]	*Aspergillus nidulans*/XP_411113	4e -38	-	1
	Xanthine dehydrogenase*	*Gibberella zeae*/XP_381737	9e -07	-	1
	NADP-cytochrome P450 reductase	*Aspergillus nidulans*/EAA66694	7e -67	3	2
	Ubiquinol cytochrome c reductase[b]	*Aspergillus nidulans*/XP_408525	6e -39	-	2
	Ubiquinol cytochrome c reductase hinge protein	*Aspergillus nidulansg*/XP_409734	2e -19	2	1
	Cytochrome c oxidase subunit Va	*Neurospora crassa*/CAD70919	1e -16	1	1

	NADH-ubiquinone oxidoreductase	*Aspergillus nidulans*/XP_408819	2e -20	2	1
	NADH-ubiquinone oxidoreductase B18 subunit	*Neurospora crassa*/EAA28195	7e -34	1	1
	NADH-ubiquinone oxidoreductase 49 kDa subunit, mitochondrial precursor	*Gibberella zeae*/EAA69636	5e -95	6	2
	Ferredoxin-like iron-sulfur protein [a]	*Paracoccidioides brasiliensis*/AAP23044	2e -58	2	3
	Protein PET191, mitochondrial precursor[b]	*Gibberella zeae*/XP_388901	4e -11	-	1
	Citrate synthase	*Aspergillus nidulans*/EAA59013	1e -62	2	1
	Mitochondrial ATP synthase epsilon chain	*Magnaporthe grisea*/XP_360684	1e -19	5	3
	ATP synthase gamma chain[a]	*Aspergillus nidulans*/XP_404389	9e -57	1	3
	ATP synthase subunit 9[a]	*Neurospora crassa*/T43671	8e -22	1	4
	Vacuolar ATP synthase subunit H	Aspergillus nidulans/XP_405348	1e -34	2	1
	Acyl-coenzyme A synthetase[b]	*Neurospora crassa*/EAA26946	5e -10	-	1
	Phosphoglycerate mutase-like superfamily protein	*Gibberella zeae*/XP_380582	2e -32	5	2
	L-carnitine dehydratase[+]	*Aspergillus nidulans*/XP_408367	1e -62	-	1
	Acetyl CoA hydrolase*	*Aspergillus nidulans*/XP_405684	5e -42	-	1
	2-methylcitrate dehydratase	*Aspergillus nidulans*/XP_410776	4e -73	6	1
	Sorbitol dehydrogenase	*Paracoccidioides brasiliensis*/ AAL25624	2e -28	74	2
Cell cycle and DNA processing	CHPA, a cysteine- and histidine-rich-domain-containing protein	*Emericella nidulans*/AAR23267	5e -56	1	1
	Endonuclease III[b]	*Aspergillus nidulans*/XP_411790	1e -06	-	1
	ATPase involved in DNA repair	*Aspergillus nidulans*/XP_409855	7e -86	1	1
	SGT1-like protein	*Aspergillus nidulans*/XP_412397	4e -33	1	1
	Rad21 protein*	*Neurospora crassa*/EAA34981	6e -17	-	2
	UV excision repair protein (rad23 homolog)	*Aspergillus nidulans*/XP_406441	8e -25	17	1
	Prohibitin 2[a]	*Aspergillus nidulans*/XP_410210	6e -52	3	5
	Wos2 protein (p21)	*Aspergillus nidulans*/XP_411058	1e -07	19	2
	Arrestin, N-terminal domain[b]	*Aspergillus nidulans*/XP_404193	2e -56	-	1
	Proliferating Cell Nuclear Antigen (PCNA)*	*Aspergillus nidulans*/XP_404552	3e -36	-	1

	Single-stranded DNA binding protein	*Magnaporthe grisea*/XP_369938	6e -31	1	1
	DNA polymerase delta 2 subunit[+]	*Coccidioides immitis*/EAS28821	6e-33	-	2
	DNA polymerase eta[b]	*Aspergillus nidulans*/XP_408815	1e -32	-	1
	Heterokaryon incompatibility protein Het-C[b]	*Aspergillus nidulans*/XP_406304	1e -27	-	2
	Uracil DNA glycosylase*	*Aspergillus fumigatus*/ XP_749743	3e -24	-	1
	Cell division protein 48 (CDC48)[a]	*Coccidioides immitis* /EAS28203	9e -15	2	3
	Chromosome segregation ATPase*	*Coccidioides immitis* /EAS30662	6e -52	-	1
	G2-specific protein kinase nimA	*Aspergillus terreus* /XP_001209874	9e -25	1	1
Transcription	DEAD-like helicases superfamily protein*[#]	*Aspergillus nidulans*/XP_410144	3e -55	-	1
	HLA-B associated transcript 4	*Aspergillus nidulans*/XP_408862	4e -09	2	1
	Transcription factor, bromodomain*	*Aspergillus nidulans*/EAA60972	2e -55	-	1
	GatB/YqeY domain protein*	*Aspergillus nidulans*/XP_410874	1e -22	-	1
	RNA polymerase I second-largest subunit	*Aspergillus nidulans*/XP_408070	2e -36	1	1
	Pheromone receptor transcription factor	*Aspergillus nidulans*/XP_412813	2e -32	1	1
	Zn(II)2Cys6 transcriptional activator	*Aspergillus nidulans*/XP_408623	9e -32	1	1
	MATA_HMG-box, class I member of the HMG-box superfamily of DNA-binding proteins	*Aspergillus nidulans*/XP_406099	3e -35	43	1
	tRNA pseudouridine synthase 4	*Aspergillus nidulans*/XP_404209	6e -12	2	1
	Small nuclear ribonucleoprotein D2	*Magnaporthe grisea*/XP_361539	1e -39	1	1
	snRNA-associated protein, Sm class[b]	*Magnaporthe grisea*/XP_368889	9e -32	-	1
	U6 snRNA-associated Sm-like protein LSm5[b]	*Neurospora crassa*/CAD11394	4e -32	-	1
	U6 snRNA-associated Sm-like protein LSm6	*Gibberella zeae*/XP_380634	3e -27	1	1
	tRNA acetyltransferase TAN1	*Aspergillus nidulans*/XP_409026	1e -40	7	1
	tRNA (guanine) methyltransferase[b]	*Aspergillus nidulans*/XP_407082	3e -23	-	3
	Alanine-tRNA ligase	*Coccidioides immitis*/ EAS36927	2e -67	2	2
	Ring type Zinc finger protein*	*Aspergillus nidulans*/XP_411042	1e -12	-	2

	Zinc finger domain protein*	*Aspergillus nidulans*/XP_405585	3e -14	-	2
	Zinc finger domain protein	*Magnaporthe grisea*/XP_370415	9e -06	1	1
	Ap-1-like transcription factor[b]	*Aspergillus nidulans*/XP_411679	2e -30	-	1
	HAPE (CCAAT-binding transcription factor subunit AAB-1)	*Aspergillus oryzae*/BAA25636	2e -12	1	1
	RNA-binding protein	*Aspergillus nidulans*/XP_407876	2e -36	1	1
	RNA-binding protein S1	*Neurospora crassa*/EAA28780	1e -35	2	1
	RNA-binding protein	*Coccidioides immitis*/AAL08969	6e -20	8	1
	Transformer-SR ribonucleoprotein	*Aspergillus nidulans*/XP_410813	1e -22	4	1
	Regulator of nonsense transcripts 1 homolog[b]	*Neurospora crassa*/XP_323582	5e -15	-	6
	Arylsulfatase regulatory protein*	*Blastocladiella emersonii*/CO964913	1e -138	-	11
	Transcriptional activator protein*	*Coccidioides immitis* /EAS34609	8e -26	-	1
	Nucleolar protein NOP56[a]	*Coccidioides immitis* /EAS36543	8e -84	1	3
Protein Synthesis	40s ribosomal protein S2	*Coccidioides immitis*/ EAS29348	6e -82	25	2
	40S ribosomal protein S5[a]	*Aspergillus nidulans*/XP_404980	8e -22	8	7
	40s ribosomal protein S8	*Aspergillus nidulans*/XP_404602	5e -55	13	2
	40S ribosomal protein S9	*Aspergillus nidulans*/XP_408940	1e -87	1	1
	40S ribosomal protein S11B	*Gibberella zeae*/XP_380847	2e -69	2	1
	40S ribosomal protein S12	*Paracoccidioides brasiliensis*/AAO38980	2e -82	15	1
	40S ribosomal protein S13[b]	*Neurospora crassa*/EAA34807	2e -37	-	1
	40S ribosomal protein S19B	*Aspergillus nidulans* /EAA58948	3e -30	3	1
	40s ribosomal protein S21e	*Coccidioides immitis*/ EAS33475	2e -11	8	2
	40S ribosomal protein S26[b]	*Neurospora crassa*/CAA39162	3e -52	-	1
	40S ribosomal protein S30A	*Gibberella zeae*/XP_385091	1e -18	16	1
	60S ribosomal protein L1B	*Aspergillus fumigatus*/CAE47895	8e -55	10	1
	60S ribosomal protein L2[b]	*Coccidioides immitis*/EAS30555	9e -54	-	1
	60S ribosomal protein L3[a]	*Aspergillus fumigatus*/AAM43909	5e -85	1	2

60S ribosomal protein L7, mitochondrial precursor	*Aspergillus nidulans*/XP_406194	7e -32	1	1
60s ribosomal protein L12	*Aspergillus nidulans*/XP_404399	1e -43	1	1
60S ribosomal protein L15B	*Neurospora crassa*/CAD21192	3e -40	2	2
60S ribosomal protein L17	*Paracoccidioides brasiliensis*/AAQ04632	1e -67	5	3
60S ribosomal protein L20[a]	*Magnaporthe grisea*/XP_361110	3e -16	3	6
60S ribosomal protein L20B	*Gibberella zeae*/XP_381692	4e -49	1	1
60 ribosomal protein L23A	*Neurospora crassa*/EAA33841	8e -53	3	1
60S ribosomal protein L23, mitochondrial precursor	*Aspergillus nidulans*/XP_413606	2e -39	1	1
60s ribosomal protein L26	*Schizosaccharomyces pombe*/ CAD37159	2e -30	3	2
60S ribosomal protein L27[a]	*Aspergillus nidulans*/XP_408359	4e -63	1	3
60s ribosomal protein L28	*Coccidioides immitis*/ EAS37227	2e -36	9	4
60S ribosomal protein L29	*Neurospora crassa*/EAA28550	1e -27	2	1
60S ribosomal protein L36	*Gibberella zeae*/XP_381414	3e -33	6	1
60S ribosomal protein L43B[b]	*Ustilago maydis*/XP_400133	1e -30	-	1
60S acidic ribosomal protein P2	*Aspergillus fumigatus*/AAG01801	2e -21	26	3
60S ribosome subunit biogenesis protein NIP7[b]	*Aspergillus fumigatus*/AAM08680	3e -14	-	1
Ribosomal protein P1	*Neurospora crassa*/EAA31448	7e -22	15	4
Ribosomal protein L4B	*Aspergillus nidulans*/XP_412313	3e -60	2	1
Ribosomal protein L10	*Paracoccidioides brasiliensis*/AAO47090	4e -60	1	1
Ribosomal protein L21A	Aspergillus nidulans/XP_408939	1e -54	3	2
Ribosomal protein L31A	*Aspergillus nidulans*/XP_409623	3e -51	6	1
Ribosomal protein L34B	*Aspergillus nidulans*/XP_408659	5e -54	9	3
Ribosomal protein L37	*Emericella nidulan*/AAK17097	1e -34	2	1
Mitochondrial ribosomal protein S19[b]	*Aspergillus nidulans*/XP_404292	5e -19	-	1
Mitochondrial 40S ribosomal protein MRP17	*Aspergillus fumigatus*/CAE47934	5e -35	1	1
Mitochondrial large ribosomal subunit	*Neurospora crassa*/XP_323495	3e -05	1	1
14 kDa mitochondrial ribosomal protein*	*Aspergillus nidulans*/XP_408748	4e -46	-	7

	Eukaryotic initiation factor 4A	*Aspergillus nidulans*/XP_407069	5e -51	9	2
	Translational machinery component protein[b]	*Aspergillus nidulans*/XP_405417	1e -19	-	1
	Translation initiation factor eIF1	*Aspergillus nidulans*/XP_408879	8e -24	4	2
	Translation initiation factor eIF1 subunit Sui1[a]	*Gibberella zeae*/XP_389056	2e -36	2	5
	Translation initiation factor eIF-5A[a]	*Neurospora crassa*/P38672	6e -06	4	4
	Translation initiation factor 3 subunit 2*	*Aspergillus nidulans* /XP_660601	6e -80	-	3
	Translation initiation factor 3 subunit 4	*Aspergillus nidulans*/XP_410154	3e -40	2	1
	Translational elongation factor EF-1 alpha	*Aspergillus nidulans*/XP_405299	4e -31	28	8
	Translation elongation factor 2	*Gibberella zeae*/EAA77131	1e -57	3	2
	Translation elongation factor Tu, mitochondrial	*Aspergillus fumigatus*/CAD27297	1e -14	1	1
	Isoleucyl-tRNA synthetase[b]	*Aspergillus nidulans*/XP_407499	1e -52	-	2
	Peptide chain release factor 2	*Bordetella parapertussis*/CAE37347	3e -71	2	1
	GTP-binding GTP1/OBG family protein[b]	*Aspergillus nidulans*/XP_404829	1e -70	-	1
Protein fate	Protein disulfide isomerase	*Gibberella zeae*/XP_389492	1e -43	4	3
	Cyclophilin like peptidyl prolyl cis-trans isomerase[b]	*Neurospora crassa*/CAD21421	8e -39	-	1
	Cyclophilin seven suppressor 1[b]	*Aspergillus nidulans*/XP_409575	8e -12	-	2
	Peptidyl-prolyl cis-trans isomerase H	*Gibberella zeae*/XP_381060	5e -55	8	1
	Peptidyl-prolyl cis-trans isomerase (FKBP-type)	*Magnaporthe grisea*/XP_362031	1e -44	2	2
	Peptidyl-prolyl cis-trans isomerase E	*Aspergillus nidulans*/XP_410393	3e -33	2	1
	Peptidyl-prolyl cis-trans isomerase (cyclophilin-2)	*Aspergillus nidulans*/XP_412817	2e -41	11	1
	Peptidyl-prolyl cis/trans isomerase (PPIC-type)	*Paracoccidioides brasiliensis*/AAQ83700	4e -38	6	1
	Peptidyl-prolyl cis-trans isomerase-like 4[a]	*Coccidioides immitis*/ EAS29016	1e -46	1	5
	SNF7 domain protein	*Aspergillus nidulans*/XP_409769	1e -52	2	2
	Rab geranylgeranyl transferase*	*Aspergillus nidulans*/XP_412816	8e -13	-	1
	Protein-L-isoaspartate(D-aspartate)O-methyltransferase[a]	*Aspergillus nidulans*/XP_407601	5e -55	4	5
	COP9 signalosome complex subunit 5[a]	*Aspergillus nidulans*/XP_406266	1e -35	1	2

COP9 signalosome complex subunit 7a	*Aspergillus nidulans*/XP_407760	1e -35	3	1
Palmitoyl thioesterase protein	*Aspergillus nidulans*/XP_406634	4e -12	4	1
Oligosaccharyltransferase alpha subunit	*Aspergillus niger*/AAK08631	2e -23	3	1
Guanosine diphosphatase*[#]	*Aspergillus nidulans*/XP_405219	2e -15	-	1
N-acetyltransferase	*Aspergillus nidulans*/XP_409130	3e -17	1	1
Ubiquitin fusion protein[a]	*Schizosaccharomyces pombe*/NP_593923	8e -67	3	3
Ubiquitin thiolesterase otubain like protein*	*Aspergillus nidulans*/ EAA60354	1e -28	-	1
Ubiquitin conjugating enzyme E2[a]	*Gibberella zeae*/XP_388490	1e -29	6	7
Ubiquitin/S27a fusion protein[a]	*Aspergillus nidulans*/XP_409009	2e -60	7	7
Ubiquitin-protein ligase (HUL4)	*Coccidioides immitis* /EAS31641	4e -88	1	1
Polyubiquitin ubi4	*Arabidopsis thaliana*/AAB95252	1e -48	4	3
Lon protease[b]	*Oryza sativa*/AAV59316	1e -05	-	1
Zinc metalloprotease[b]	*Neurospora crassa*/CAD21161	3e -47	-	1
ATP-dependent Clp protease, proteolytic subunit	*Magnaporthe grisea*/XP_370260	4e -37	1	1
Aspartyl protease[a]	*Paracoccidioides brasiliensis*/AAP32823	3e -72	3	7
26S protease subunit protein[b]	*Aspergillus nidulans*/XP_411125	4e -23	-	1
Non-ATPase regulatory subunit of the 26S proteasome*	*Aspergillus nidulans*/XP_408912	2e -68	-	1
26S proteasome regulatory complex component protein	*Aspergillus nidulans*/XP_408920	2e -73	3	1
26s proteasome regulatory subunit rpn12[b]	*Aspergillus nidulans*/XP_407156	5e -30	-	1
Proteasome subunit alpha type 1	*Aspergillus nidulans*/XP_410684	5e -65	4	1
Proteasome subunit alpha type 6	*Candida albicans*/XP_717755	1e -24	1	1
F-box/LRR-repeat protein 7[b]	*Aspergillus nidulans*/XP_408647	8e -28	-	3
Calnexin	*Aspergillus nidulans*/XP_407729	5e -63	5	1
Peptidase C19 subfamily protein[b]	*Aspergillus nidulans*/XP_412211	7e -08	-	3
Peptidase M28 domain protein*	*Coccidioides immitis*/EAS33583	1e -22	-	1
Alpha-1, 2-galactosyltransferase*[#]	*Aspergillus nidulans*/XP_406106	3e -14	-	1
Mitochondrial processing peptidase subunit	*Coccidioides immitis* /EAS32113	2e -40	3	1

	Oligosaccharyltransferase subunit ribophorin[+]	*Coccidioides immitis*/ EAS29547	9e -37	-	1
	Tailless Complex Polypeptide 1 chaperonin, subunit epsilon[b]	*Schizosaccharomyces pombe*/EAA65069	6e -16	-	2
	Mannosyltransferase[b]	*Paracoccidioides brasiliensis*/AAK54761	3e -70	-	1
	Alpha-1, 2-mannosyltransferase[a]	*Neurospora crassa*/CAC18268	1e -29	3	3
	Ring (Really Interesting New Gene) type zinc finger (C3HC4) protein[b]	*Schizosaccharomyces pombe*/CAB08748	5e -10	-	1
Protein with binding function or cofactor requirement	RPEL repeat protein[a]	*Aspergillus nidulans*/XP_407503.1	5e -22	1	3
Transport Facilitation	Mitochondrial carrier protein Ggc1 fragment	*Aspergillus nidulans*/XP_409269	5e -69	1	1
	Mitochondrial carrier protein[b]	*Gibberella zeae*/XP_391004	6e -22	-	3
	Xanthine/uracil/vitamin C permease[+]	*Magnaporthe grisea*/XP_362769	7e -34	-	1
	Sugar (and other) transporter protein	*Aspergillus nidulans*/XP_410859	8e -80	12	3
	Sugar transporter protein[a]	*Gibberella zeae*/XP_381006	9e -55	3	5
	Copper transporter family protein[a]	*Gibberella zeae*/XP_380949	1e -28	4	8
	Copper transport protein-CTR2	*Aspergillus nidulans*/XP_407071	6e -13	2	1
	Sulfate permease[b]	*Gibberella zeae*/XP_384418	4e -46	-	1
	Glucose transporter	*Aspergillus fumigatus*/XP_754226	2e -28	1	1
	Arabinose efflux permease	*Aspergillus nidulans*/XP_407577	6e -18	4	1
	Uridine diphosphate N-Acetylglucosamine transporter*#	*Neurospora crassa*/T50997	9e -30	-	1
	Monossaccharide transport protein[b]	*Aspergillus nidulans*/XP_408414	8e -55	-	1
	Amino acid permease	*Neurospora crassa*/XP_322767	1e -23	8	2
	Acidic amino acid permease[a]	*Aspergillus nidulans*/XP_410255	2e -17	9	9
	B-cell receptor-associated protein 31-like[+]	*Aspergillus nidulans*/XP_404956	9e -43	-	1
	ATP synthase subunit 4	*Paracoccidioides brasiliensis*/AAP22959	2e -79	6	2
	Purine nucleoside permease	*Aspergillus nidulans*/XP_409674	2e -05	1	1

	Malate permease[b]	*Gibberella zeae*/XP_389995	2e -25	-	2
	Nuclear pore protein 84/107*	*Aspergillus nidulans*/XP_405327	3e -07	-	1
	Mitochondrial import receptor subunit Tom20[b]	*Aspergillus nidulans*/XP_404696	9e -40	-	1
	Mitochondrial RNA splicing protein mrs3	*Aspergillus nidulans*/XP_407827	1e -45	3	1
	ADP, ATP carrier-like protein	*Aspergillus nidulans*/EAA58952	3e -101	1	1
	Coatomer protein complex, subunit epsilon	*Magnaporthe grisea*/XP_367110	8e -13	2	1
	Coatomer gamma-2 subunit	*Aspergillus nidulans*/XP_408684	4e -60	1	1
	Clathrin adaptor appendage domain protein[b]	*Aspergillus nidulans*/XP_408344	1e -13	-	1
	ER to Golgi transport related protein[b]	*Aspergillus nidulans*/XP_412331	5e -75	-	2
	Vacuolar protein sorting/targeting protein PEP1 precursor[b]	*Coccidioides immitis*/EAS36959	1e -42	-	1
	Vacuolar protein sorting 29	*Aspergillus nidulans*/ XP_658945	2e -63	11	1
	Regulator of V-ATPase in vacuolar membrane protein*	*Aspergillus nidulans*/XP_404840	9e -59	-	1
	LMBR1 integral membrane protein-like	*Aspergillus nidulans*/XP_408348	9e -14	2	1
	ADP-ribosylation factor 2	*Ajellomyces capsulata*/P34727	2e -63	2	2
	T-snare superfamily protein	*Aspergillus nidulans*/XP_411817	1e -23	3	1
	Tctex-1 family protein*	*Aspergillus nidulans*/XP_405470	6e -25	-	2
	Phosphatidylinositol transfer protein[b]	*Aspergillus nidulans*/XP_410990	9e -79	-	1
	Importing beta protein[b]	*Aspergillus nidulans*/XP_410871	6e -71	-	1
	Importin-beta N-terminal domain protein*	*Aspergillus nidulans*/XP_410143	1e -44	-	1
	Phox homology (PX) domain protein[b]	*Aspergillus nidulans*/XP_410488	3e -06	-	1
	Lysine-specific permease	*Coccidioides immitis*/ EAS34877	4e-46	12	1
	Nucleoporin SONB[a]	*Aspergillus fumigatus*/ XP_751721	7e -47	1	2
	GTP-binding protein ypt1	*Phaeosphaeria nodorum* /EAT86676	3e -63	47	1
	Exocyst complex component Sec15 protein[a]	*Coccidioides immitis* /EAS37215	4e -65	1	3
Signal Transduction	Two-component sensor kinase*	*Anopheles gambiae*/EAA02130	2e -38	-	6
	RACK1-like protein	*Aspergillus nidulans*/EAA59424	9e -94	1	1

	Rhodopsin-like GPCR superfamily protein	*Aspergillus nidulans*/XP_409821	4e -23	1	1
	Histidine protein kinase sensor for GlnG regulator*#	*Tetrahymena thermophila*/EAR83219	2e -04	-	24
	Protein kinase C conserved region 2[b]	*Aspergillus nidulans*/XP_409761	3e -55	-	1
	Anti-silencing factor 1	*Gibberella zeae*/XP_380526	1e -64	4	1
	WD40 domain protein	*Aspergillus nidulans*/XP_405832	2e -25	1	1
	UVSB Phosphatidylinositol-3 kinase*#	*Aspergillus nidulans*/XP_411112	1e -29	-	1
	Serine/threonine-protein kinase SAT4[b]	*Aspergillus nidulans* /XP_412967	2e -51	-	1
	Serine/Threonine protein kinase, catalytic domain	*Aspergillus nidulans*/XP_411674	3e -06	1	1
	Serine/threonine kinase receptor associated protein	*Aspergillus nidulans*/XP_411842	4e -73	1	1
	Serine/threonine protein kinase	*Aspergillus nidulans*/XP_404372	2e -49	2	2
	Protein kinase	*Magnaporthe grisea*/XP_363079	1e -12	1	1
	Mitogen activated protein kinase[b]	*Aspergillus oryzae*/BAD12561	1e -52	-	1
	GTPase, G3E family protein[b]	*Neurospora crassa*/XP_323082	1e -11	-	1
	Rho GTPase activating protein*	*Aspergillus nidulans*/XP_407883	3e -49	-	1
	Rho1 GTPase	*Paracoccidioides brasiliensis*/AAQ93069	7e -82	4	1
	GTP binding protein[b]	*Neurospora crassa*/CAD70888	5e -84	-	1
	Calcineurin subunit b*	*Neurospora crassa*/P87072	1e -77	-	2
	Calmodulin	*Fusarium proliferatum*/ AAL04428	3e -07	5	1
	Forkhead associated (FHA) protein*	*Gibberella zeae*/XP_389397	4e -10	-	1
	Ca^{2+}- binding protein (EF-Hand superfamily)	*Aspergillus nidulans*/XP_404404	2e -36	1	1
	14-3-3-like protein 2	*Paracoccidioides brasiliensis*/ AAR24348	5e -26	4	1
Cell Rescue, Defense and Virulence	Potential secreted Cu/Zn superoxide dismutase[b]	*Magnaporthe grisea*/XP_360807	8e -11	-	1
	Thiol specific antioxidant protein	*Ajellomyces capsulatus*/ AAG31645	1e -71	2	1
	Peroxisomal like protein	*Paracoccidioides brasiliensis*/ AAQ84041	2e -33	2	1
	Peroxisomal catalase[a]	*Paracoccidioides brasiliensis*/AAL34518	4e -66	3	4
	Heat shock protein 10, mitochondrial[a]	*Gibberella zeae*/XP_386383	2e -40	1	3

	Heat shock protein 30	*Aspergillus nidulans*/XP_406667	3e -45	2	1
	Heat shock protein 60, mitochondrial precursor	*Paracoccidioides brasiliensis*/AAC14712	2e -39	1	1
	Heat shock protein 60[b]	*Aspergillus nidulans* /XP_659508	3e -12	-	1
	Heat shock protein 70[b]	*Paracoccidioides brasiliensis*/AAP05987	3e -64	-	2
	Heat shock protein 70	*Paracoccidioides brasiliensis*/AAK66771	4e -81	31	7
	Heat shock protein 70[b]	*Emericella nidulans*/CAA67431	7e -39	-	1
	Heat shock protein 82	*Aspergillus nidulans*/EAA59007	1e -12	8	1
	Heat shock protein 88	*Aspergillus nidulans*/XP_405184	1e -80	9	3
	Heat shock protein 90[a]	*Aspergillus nidulans*/EAA59007	4e -38	5	6
	MDJ1-like protein	*Aspergillus nidulans*/XP_410331	2e -16	4	1
	DNAJ protein	*Coccidioides immitis* / EAS35517	7e -77	14	3
	Hemolysin	*Magnaporthe grisea*/XP_359943	2e -11	3	2
	Hemolysin like protein*[#]	*Aspergillus nidulans*/XP_406013	2e -70	-	1
	Copper-zinc superoxide dismutase	*Paracoccidioides brasiliensis*/AAX13803	2e -24	11	1
	Hemerythrin HHE cation binding domain protein	*Magnaporthe grisea*/XP_360008	1e -05	1	1
	Fasciclin-like protein	*Aspergillus nidulans*/XP_405664	3e -19	3	1
Biogenesis of cellular components	Tubulin alpha 1[b]	*Aspergillus nidulans*/XP_411707	1e -63	-	1
	Tubulin beta-1 chain[b]	*Aspergillus flavus*/P22012	7e -120	-	1
	Actin related protein 3	*Gibberella zeae*/XP_391032	9e -48	2	1
	Actin related protein 2/3 complex, subunit 1A	*Coccidioides immitis*/AAL08969	1e -18	30	1
	Actin related protein[b]	*Aspergillus nidulans*/XP_411146	1e -40	-	1
	Histone H3[a]	*Aspergillus fumigatus*/XP_752749	5e -67	11	6
	Histone H4	*Aspergillus nidulans*/XP_404254	7e -44	24	6
	Histone H2A[a]	*Aspergillus nidulans*/XP_412176	2e -52	7	6
	Histone H2B	*Coccidioides immitis*/EAS36779	4e -28	12	2
	Peroxisomal import complex protein Pex12	*Aspergillus nidulans*/XP_411443	5e -26	2	1

Cell type differentiation	Suppressor of anucleate metulae B protein*[#]	*Aspergillus nidulans*/XP_404215.1	6e -46	-	2
Unclassified	Y20 protein	*Paracoccidioides brasiliensis*/AAL50803	4e -63	4	1
	Predicted membrane protein	*Rattus norvegicus*/XP_345034	6e -05	42	3
	Complex 1 protein (LYR family)*	*Aspergillus nidulans*/XP_408902	8e -32	-	1
	Homolog of translationally controlled tumor protein[a]	*Aspergillus nidulans*/XP_404778	4e -25	3	3
	YCII related domain[b]	*Gibberella zeae*/XP_390542	2e -23	-	1
	27 kDa antigen	*Paracoccidioides brasiliensis*/AAC49615	2e -67	1	1
	Dimeric alpha-beta barrel domain[b]	*Aspergillus nidulans*/XP_406116	1e -23	-	1
	Isopenicillin N synthase and related dioxygenase	*Magnaporthe grisea*/XP_364516	7e -05	2	1
	Iron-sulfur cluster Isu1-like protein[a]	*Gibberella zeae*/XP_382800.1	3e -41	5	4

[a] Transcript up regulated in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie´s method.
[b] Transcripts non detected in the mycelia transcriptome (https//dna.biomol.unb.br/Pb).
* Novel genes detected in *P. brasiliensis*.
[#] Transcripts confirmed by semi-quantitative RT-PCR.
[+]Genes not described previously in *P. brasiliensis* isolate *Pb*01, but present in public databases.

Table 2- *P. brasiliensis* up regulated transcripts potentially related to signal transduction.

Gene product	Putative function	Predicted redundancy‡	
		M	T
Two component sensor kinase* (yehU)	Stress response, drug sensitivity, sexual development and virulence	-	6
Histidine protein kinase sensor for GlnG regulator* (glnL)	Defense against nitrogen limitation	-	24
Protein kinase C conserved region 2 (calB)	Promotes cell-wall stability and increased melanin production	-	1
UVSB phosphatydilinositol-3-kinase* (uvsB)	Required for S-phase progression and recovery from DNA damage	-	1
GTP binding protein (ygr)	Cytoskeleton reorganization	-	1
Serine/threonine-protein kinase SAT4 (sat4)	Involved in cellular potassium uptake	-	1
Rho GTPase activating protein* (bem3)	Regulator of the beta-(1,3)-glucan synthase	-	1
Calcineurin subunit b* (canB)	Survival during a variety of stress conditions	-	2
Mitogen-activated protein kinase (mapkA)	Maintenance of cell integrity	-	1
Forkhead associated (FHA) protein* (fha)	Play important roles in serine/threonine kinase signaling mechanisms	-	1

‡ The predicted redundancy was obtained on basis of the transition cDNA library and from the mycelia transcriptome database (https://dna.biomol.unb.br/Pb/).
* Novel genes detected in *P. brasiliensis*.

V – CONCLUSÕES E PERSPECTIVAS

- Com o objetivo de analisar o perfil transcricional de *P. brasiliensis* durante a diferenciação morfológica foi construída uma biblioteca de cDNA a partir de RNA extraído durante a transição de micélio para levedura.

- As 1107 ESTs geradas foram classificadas quanto a possível função. Genes do metabolismo primário, transcrição, síntese protéica, transporte celular e transdução de sinal foram altamente representados na biblioteca. Esses dados aumentaram o número de genes identificados em *P. brasiliensis* durante a transição que apresentam-se super expressos.

- As ESTs foram comparadas com o banco de dados do transcriptoma de *P. brasiliensis* (http://www.biomol.unb.br/Pb). Esta análise revelou 48 genes ainda não caracterizados em *P. brasiliensis*. Doze novos genes foram analisados por RT-PCR semiquantitativa e todos mostraram uma expressão preferencial durante a transição.

- Genes envolvidos na assimilação do enxofre mostraram-se super expressos durante a transição, sugerindo o envolvimento do metabolismo do enxofre durante o processo de diferenciação de P. *brasiliensis*, como descrito anteriormente.

- Foi observado que vários genes potencialmente relacionados com a síntese de membrana e parede celular aumentavam a sua expressão durante a diferenciação celular de micélio para levedura. Isto sugere que *P. brasiliensis* favorece o remodelamento da membrana e parede celular durante a fase inicial da morfogênese.

- A disponibilidade de compostos para o ciclo do glioxalato mostrou-se favorecida durante a transição. Enzimas que participam do ciclo do glioxalato

foram observadas, indicando que esta via é funcional durante a transição de micélio para levedura.

- Também foi observado que vários genes que codificam proteínas possivelmente relacionadas com a transdução de sinal apresentavam-se super regulados. Isto sugere que a transição morfológica de *P. brasiliensis* é mediada por uma série de sistemas de transdução de sinal que controla a adaptação ao ambiente para a sobrevivência e adaptação do fungo dentro do hospedeiro.

- Genes descritos como fatores de virulência também foram detectados como super expressos, sugerindo a adaptação fúngica às novas condições a serem enfrentadas no ambiente do hospodeiro.

- A análise do transcriptoma de *P. brasiliensis* durante a transição dimórfica de micélio para levedura gerou informações sobre os eventos moleculares que ocorrem durante a diferenciação celular. Estudos posteriores referentes aos novos genes e aos mais expressos podem trazer um melhor esclarecimento sobre suas funções durante a transição dimórfica.

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VII. ANEXO - Normas da revista BMC Microbiology

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Title page

Abstract

Background

Results

Discussion

Conclusions

Methods

List of abbreviations used(if any)

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Acknowledgements

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In press article

3. Kharitonov SA, Barnes PJ: Clinical aspects of exhaled nitric oxide. Eur Respir J, in press.

Published abstract

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Whole issue of journal

7. Ponder B, Johnston S, Chodosh L (Eds): Innovative oncology. In Breast Cancer Res 1998, 10:1-72.

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8. Smith Y (Ed): Proceedings of the First National Conference on Porous Sieves: 27-30 June 1996; Baltimore. Stoneham: Butterworth-Heinemann; 1996.

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Book with institutional author

11. Advisory Committee on Genetic Modification: Annual Report. London; 1999.

PhD thesis

12. Kohavi R: Wrappers for performance enhancement and oblivious decision graphs. PhD thesis. Stanford University, Computer Science Department; 1995.

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