Universidade Federal de Goiás
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Biologia

Benedito Rodrigues da Silva Neto

Malato sintase de *Paracoccidioides brasiliensis* é uma proteína ligada à superfície que se comporta como uma *anchorless* adesina.

Orientadora: Dra. Maristela Pereira

Goiânia - Goiás
Abril de 2009

Universidade Federal de Goiás
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Biologia

Benedito Rodrigues da Silva Neto

Malato sintase de *Paracoccidioides brasiliensis* é uma proteína ligada à superfície que se comporta como uma *anchorless* adesina.

Orientadora: Dra. Maristela Pereira

Dissertação apresentada ao Programa de Pós-Graduação em Biologia do Instituto de Ciências Biológicas, da Universidade Federal de Goiás como requisito parcial à obtenção do título de Mestre em Biologia Celular e Molecular

Goiânia - Goiás
Abril de 2009

DISSERTAÇÃO DESENVOLVIDA NO LABORATÓRIO DE BIOLOGIA MOLECULAR, DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR, INSTITUTO DE CIÊNCIAS BIOLÓGICAS, UNIVERSIDADE FEDERAL DE GOIÁS, COMO REQUISITO PARCIAL PARA A OBTENÇÃO DO TÍTULO DE MESTRE EM BIOLOGIA CELULAR E MOLECULAR.

APOIO FINANCEIRO: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

BANCA EXAMINADORA

TITULARES

Profa. Dra. Maristela Pereira, Departamento de Bioquímica e Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás.

Profa. Dra. Célia Maria de Almeida Soares, Departamento de Bioquímica e Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás.

Prof. Dr. Henrique Leonel Lenzi, Departamento de Patologia, Instituto Oswaldo, Rio de Janeiro.

SUPLENTES

Prof. Dra. Sílvia Maria Salem Isacc, Departamento de Bioquímica e Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás.

Profa. Dra. Maria José Soares Mendes-Giannini, Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista.

Prof. Dr. André Kipnis, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás.

“Não sei... se a vida é curta
ou longa demais pra nós,
Mas sei que nada do que vivemos tem sentido,
se não tocamos o coração das pessoas.

Muitas vezes basta ser:
Colo que acolhe,
Braço que envolve,
Palavra que conforta,
Silêncio que respeita.

Alegria que contagia,
Lágrima que corre,
Olhar que acaricia,
Desejo que sacia,
Amor que promove.

E isso não é coisa de outro mundo,
é o que dá sentido à vida.
É o que faz com que ela
não seja curta, nem longa demais
Mas que seja intensa,
verdadeira, pura...
Enquanto durar.
Feliz aquele que transfere o que sabe e aprende o que ensina.”
(Cora Coralina)

Ao Autor e Consumador da minha Fé.
Aos meu pais, Osmário Rodrigures da Silva e Aluzair Francisco Dourado, e minha irmã, Laura Maria Gomes Dourado que sempre confiaram, acreditaram e depositaram sua esperança em mim, dedico.

Dedico ainda à Maristela, orientadora
que não hesitou em apoiar e investir seu
tempo em mim, és exemplo de
responsabilidade e compromisso.

AGRADECIMENTOS

À Deus, Tua provisão me faz confiar e faz andar seguro, uma promessa que me sustenta, e um motivo que me faz levantar a cada novo dia.

À Profa. Maristela Pereira, que desde o primeiro dia demonstrou confiança em mim, me orientando e conduzindo pelo caminho do conhecimento.

À Profa. Célia Maria de Almeida Soares, sua capacidade e competência me levaram a chegar ao fim deste trabalho vislumbrando novos horizontes. Grato por todo auxílio e compreensão.

Ao Prof. Lenzi, pela maneira com que me recebeu na FIOCRUZ-RJ, tornastes um exemplo para mim, sua dedicação me inspira cada dia mais a ser não um simples pesquisador, mas de fato um cientista.

À Profa. Zezé, por todo conhecimento transmitido de forma tão graciosa naqueles dias inesquecíveis em Araraquara.

À todos da micologia da UNESP de Araraquara, Julhiany (ainda se lembra do bilhete?), Fernanda, Rô, Marcelo, Aline, Marisa, Tatiane, Roberta, Elaine e Rosângela (até a próxima festa junina!).

À Ester da FIOCRUZ - RJ por toda atenção e contribuição.

Aos professores do Mestrado e do Instituto de Ciências Biológicas, da UFG, que contribuíram tão grandemente para o meu aprendizado.

À professora Sílvia Maria Salem Isacc, por aceitar desafio de última hora.

À Kesser pelos passos iniciais no LBM, sou extremamente grato pela maneira que apoiou e "cobrou" de mim...rs. Viu só, valeu a pena. Muito obrigado!

À Aline e Patrícia Zambuzzi, espero um dia poder retribuir tudo o que fizeram por mim.

À Patrícia Kott, 05.11.2007, origem aeroporto Santa Genoveva – Goiânia; destino Galeão – Rio de Janeiro: Casa Amarela!

À Sarah, minha prima "Tu te tornas eternamente responsável por aquilo que cativas..."

Ao Rogério grande amigo e companheiro de bancada que caminhou junto comigo esses dois anos fazendo soar o velho lema: "Mais vale um mau dia de pesca do que um bom dia de trabalho". E lógico à Flavinha pela torta de limão, rs.

Aos colegas Luiz Augusto (pescador...), Christielly, Lidiane ("oreia" obrigado garotinha!), Moniquinha, Mônica Santiago, Nadya e Lorena (chá de boldo?!rs) que deixaram saudades.

Às colegas de orientadora: Martha, Regilda, Renata, Raquel e Amanda por me suportarem.

Ao Wesley, parceiro nos experimentos finais, um exemplo que quero seguir; ao Rodrigo, quem diria hein, valeu pela força constante! às vezes exagerada em forma de leves tapinhas nas costas...rs; Nathalie, o que dizer quando faltam palavras?...florzinha!

À todos que conviveram de maneira tão agradável no laboratório: Clayton (e ai meu querido!), Alexandre & Mirelle, Elisa, Ronney, Sabrina, Daciene, Juliana, Kelly, Cristina, Ana Flávia, Patrícia Lima (Beijo me liga!), Ívian, Ademar, Leandro, Daiane, Mariana (vamos tirar uma foto?).

À Gleice, pela atenção amizade e carinho prestado durante todas as dúvidas e questões relacionadas à Pós-Graduação; assim como Fernando e Tizuko da Coordenação.

Amo "a" como ama o amor. Não "a" conheço nenhuma outra razão para amar senão amar. Que queres que te diga, além de que te amo, se o que quero dizer-te "logo" é que te amo? (parafraseando Fernando Pessoa).

À Igreja Fonte da Vida que é meu amparo e lugar seguro.

À minha famíla, razão pela qual cheguei até aqui e motivo que me faz acreditar em cada objetivo, cada plano, cada sonho....e é o que me dá força para alcançá-los.

SUMÁRIO

RESUMO	X
ABSTRACT	XI
I – INTRODUÇÃO	12
I.1 - Paracoccidioidomicose	12
I.2 – O fungo *Paracoccidioides brasiliensis*	14
I.2.1 – Aspectos Gerais	14
I.2.2 - Dimorfismo	16
1.2.3 – Transcriptomas de *P. brasiliensis* em condições de contato com hospedeiro	18
I.3 – Ciclo do Glioxalato	20
I.4 – Peroxissomos	23
I.5 – Matriz Extracelular	25
I.6 – Moléculas Envolvidas no Processo de Adesão de Fungos Patogênicos	26
I.7 – Adesinas de *P. brasiliensis*	28
I.8 – Malato sintase	29
I.8.1 – Considerações Gerais	29
I.8.2 – Aspectos da enzima MLS	30
I.8.3 – MLS como Antígeno	32
I.8.4 – MLS de *P. brasiliensis*	33
II - JUSTIFICATIVA	35
III – OBJETIVOS	37
IV - Manuscrito	39
Title and general information	39
Abstract	40
Background	41
Results	42
Discussion	46
Conclusion	49
Methods	49
Author's Contribuitions	57
Acknowledgements	57
References	58
Figure Legends	66
Figures	70
V – CONCLUSÕES	75
VI – PERSPECTIVAS	77
VII – REFERÊNCIAS BIBLIOGRÁFICAS	78
VIII - ANEXOS	90

RESUMO

O fungo de patogênico *Paracoccidioides brasiliensis* agente causador da Paracoccidioidomicose (PCM), uma micose pulmonar adquirida pela inalação de propágulos aéreos do fungo que pode se disseminar a vários órgãos e tecidos levando a uma forma severa da doença. Dentro do hospedeiro, *P. brasiliensis* usa o ciclo do glioxalato (CG) para sobrevivência intracelular. Adesão e invasão das células do hospedeiro são passos essenciais envolvidos na internalização e disseminação do patógeno. Aqui, nós evidênciamos que malato sintase de *P. brasiliensis* (*Pb*MLS) é secretada, e é localizada na parede da célula. *Pb*MLS foi superexpressa em *Escherichia coli*, e o anticorpo policlonal contra esta proteína foi obtido. Usando Microscopia Laser Confocal (CLSM) e análise de *Western blot*, *Pb*MLS foi encontrada no citoplasma e na parede da célula na fase leveduriforme de *P. brasiliensis* nas células mãe e broto. *Pb*MLSr e o respectivo anticorpo policlonal produzido contra esta proteína inibiram a interação de *P. brasiliensis* com células epiteliais A549 cultivads *in vitro*. Estas observações indicariam que MLS associada à parede da célula de *P. brasiliensis* pode estar mediando a ligação do fungo às células, contribuindo assim com a adesão do fungo aos tecidos hospedeiros e para a disseminação da infecção.

ABSTRACT

The pathogenic fungus *Paracoccidioides brasiliensis* causative of Paracoccidioidomycosis (PCM), a pulmonary mycose acquired by inhalation of fungal airborne propagules, which may disseminate to several organs and tissues leading to a severe form of the disease. Adhesion and invasion to host cells are essential steps involved in the internalization and dissemination of pathogens. Inside host, *P. brasiliensis* use the glyoxylate cycle for intracellular survival. Here, we provide evidence that malate synthase of *P. brasiliensis* (*Pb*MLS) is localized on the cell wall, and is secreted. *Pb*MLS was overexpressed in *Escherichia coli*, and polyclonal antibody against this protein was obtained. By using Confocal Laser Scanning Microscopy and Western blot analysis, *Pb*MLS was detected in the cytoplasm and the cell wall of the yeast phase of *P. brasiliensis* of mother and bud yeast cells. *Pb*MLSr and the respective polyclonal antibody produced against this protein inhibited the interaction of *P. brasiliensis* to *in vitro* cultured epithelial cells A549. These observations indicated that cell wall-associated MLS of *P. brasiliensis* could be mediating the binding of fungal cells, thus contributing to the adhesion of fungus to host tissues and to the dissemination of infection.

Introdução

I – INTRODUÇÃO

I.1 – Paracoccidioidomicose

Paracoccidioidomicose (PCM) é uma micose humana sistêmica causada pelo fungo *P. brasiliensis*. A doença foi inicialmente descrita em São Paulo por Adolpho Lutz (Lutz 1908). A PCM é geograficamente restrita à América Latina, sendo o Brasil o maior centro endêmico, onde causa aproximadamente 200 mortes por ano (COUTINHO *et al*., 1998). Nas áreas onde a PCM é altamente endêmica, a incidência da doença é estimada em aproximadamente 1 a 3 casos clínicos para cada 100.000 habitantes por ano (RESTREPO, 1985). Coutinho *et al*. (2002), em estudo de revisão, verificou que, no Brasil, PCM é a oitava causa de morte entre as doenças crônicas e a primeira entre as micoses sistêmicas. A taxa de mortalidade média é de 1,45/milhão de habitantes ao ano, considerando ambos os sexos. No entanto, a PCM atinge preferencialmente trabalhadores rurais do sexo masculino, entre 30 a 60 anos de idade (VILLAR *et al.*, 2000). O trabalho com o solo e plantações em área rural é fator ocupacional predisponente para a aquisição da PCM (FRANCO, 1987). A incidência da doença até à puberdade é a mesma em ambos os sexos, porém na fase adulta, mais de 80% dos pacientes são do sexo masculino (MARTINEZ, 1997). Acredita-se que esse fato seja explicado pela ação protetora que os hormônios estrógenos conferem à mulher (CLEMONS & STEVENS, 1990; SANO *et al.*, 1999), e pela ausência ou menor contato com as fontes de infecção (MARQUES *et al.*, 1983).

A doença é adquirida através da via respiratória, quando propágulos da fase miceliana do fungo são inalados (McEWEN *et al.*, 1987). A partir dos pulmões, o fungo pode se disseminar por todo o corpo, através da corrente sangüínea ou linfática. O

contato inicial do hospedeiro com o fungo aparentemente evolui para uma infecção subclínica ou assintomática (PCM infecção). Se há progressão da infecção, ocorre o estabelecimento da doença, que apresenta duas formas clínicas distintas: a forma aguda ou tipo juvenil e a forma crônica ou adulta (FRANCO *et al*., 1987).

A forma juvenil (aguda ou subaguda) representa 3 a 5% dos casos descritos da doença, sendo a maioria dos pacientes constituídos por crianças ou adultos jovens (REIS *et al*., 1986; BRUMMER *et al*., 1993). O quadro da doença caracteriza-se por um desenvolvimento rápido e por marcante envolvimento de órgãos como baço, fígado, gânglios linfáticos e medula óssea. Apesar de não haver manifestações clínicas ou radiológicas de comprometimento pulmonar evidente, pode-se isolar o fungo do lavado brônquico, evidenciando a participação do pulmão como porta de entrada do patógeno (SHIKANAI-YASUDA *et al*., 2006).

A forma crônica ou adulta representa mais de 90% dos casos, sendo a maioria dos pacientes constituída por homens adultos. Ao contrário da forma aguda, o quadro clínico apresenta um desenvolvimento lento com comprometimento pulmonar evidente (BRUMMER *et al*., 1993). Com freqüência, ocorrem lesões nas mucosas orais e nasais, pele e gânglios linfáticos. Menos freqüentemente, podem ocorrer envolvimento ocular, destruição óssea, e comprometimento dos sistemas nervoso e vascular (SHIKANAI-YASUDA *et al*., 2006).

A PCM é a principal causa de incapacidade e morte entre os trabalhadores rurais durante a idade mais produtiva. A alta freqüência de seqüelas e o índice de mortalidade em PCM são fortemente influenciados por condições econômicas e sociais da população alvo, a maioria representada por trabalhadores rurais de baixa renda e com limitado acesso ao sistema de saúde. Ambos os aspectos contribuem para prejudicar a resposta do hospedeiro à infecção e detecção pelo sistema de saúde de pacientes

infectados nos estágios iniciais da doença (TRAVASSOS *et al*., 2008). O trabalho agrícola é a atividade mais importante nos Estados de Goiás, Mato Grosso, e Mato Grosso do Sul, na região do Cerrado, onde o número de casos tem aumentado (PANIAGO *et al*., 2003).

Nos últimos anos, a PCM tem sido apontada como uma doença oportunista quando associada a pacientes com AIDS; a mortalidade entre esses pacientes tem sido considerada alta (SILVA-VERGARA *et al*., 2003; GOLDANI *et al*., 1991).

I.2 - O FUNGO *Paracoccidioides brasiliensis*

I.2.1 - Aspectos Gerais

P. brasiliensis apresenta forma leveduriforme nos tecidos do hospedeiro ou quando cultivado *in vitro* a 36°C, e forma miceliana (infectiva), em condições saprobióticas no meio ambiente, ou quando cultivado em temperaturas inferiores a 28°C (BAGAGLI *et al*., 2006). Quanto ao habitat, sugere-se que o fungo viva saprobioticamente na natureza, próximo a ambientes de água fresca, tais como rios ou córregos e de forma temporária no solo (BAGAGLI *et al*., 2006; TERÇARIOLI *et al*., 2007), o isolamento do fungo a partir de duas espécies de tatus, o *Dasypus novemcintus* e o *Cabassou centralis* reforça esta hipótese (CORREDOR *et al.,* 2005).

A característica marcante das leveduras de *P. brasiliensis* são os brotamentos múltiplos formados pela evaginação da célula-mãe, onde pode-se observar uma célula central circundada por várias células periféricas dando à célula um aspecto parecido com a roda de leme de um navio. As colônias leveduriformes apresentam-se macroscopicamente com coloração creme, de aspecto cerebriforme, não aderidas ao

meio e com crescimento evidente após sete dias de incubação a 37°C (CARBONELL & RODRIGUES, 1965). As células leveduriformes apresentam morfologia oval ou alongada, em diferentes tamanhos (4 a 30 μm), contendo múltiplos núcleos (2 a 5 por células), nucléolo e cromatina evidentes (LACAZ, 1994). Sua parede celular é espessa (LACAZ *et al*., 1991), a membrana plasmática é composta por três camadas e apresenta múltiplas invaginações que constituem vesículas e estruturas tubulares; também apresenta escasso retículo endoplasmático, vários vacúolos, gotas de lipídios proeminentes, ribossomos e mitocôndrias (BRUMMER *et al.,* 1993).

Os filamentos septados com conídeos terminais ou intercalares são as características que nos permitem identificar a forma miceliana (QUEIROZ-TELLES, 1994; RESTREPO-MORENO, 2003). Macroscopicamente, o fungo produz colônias pequenas, duras, planas, irregulares, de coloração bege, cobertas por micélio aéreo e curto que freqüentemente aderem-se ao ágar (BRUMMER *et al.,* 1993). Quando examinadas ao microscópio óptico, observam-se hifas finas e septadas, com clamidoconidios terminais ou intercalares (FRANCO *et al.,* 1989). Ao microscópio eletrônico, as hifas apresentam um envoltório celular constituído de três camadas: uma interna, a membrana plasmática; uma camada mais espessa, a parede celular, e uma externa de aspecto finamente granular, a membrana basal (MINGUETTI *et al.,* 1985). As hifas são multinucleadas, com cromatinas finas dispersas no nucleoplasma. No citoplasma podem ser vistos ribossomos, mitocôndrias esféricas ou alongadas, retículo endoplasmático e vacúolos (SAMSONOFF *et al.,* 1991).

Análises comparativas de seqüências de genes conservados evolutivamente em fungos sugerem que *P. brasiliensis* se aproximaria filogeneticamente dos ascomicetos (MARGULIS & SCHARWATZ,1998). De acordo com San-Blas *et al*. (2002), em estudo de revisão, *P. brasiliensis* poderia ser agrupado ao filo Ascomycota,

subdivisão Euascomycotina, classe Plectomyceto, sub-classe Euascomycetidae, ordem Onygenales, família Onygenaceae, subfamília Onygenaceae Anamórficos, gênero *Paracoccidioides*, espécie *Paracoccidioides brasiliensis*.

Através da via respiratória, por inalação de propágulos do micélio, como conídios, *P. brasiliensis*, usualmente, alcança o hospedeiro. Esses propágulos se convertem para a fase leveduriforme nos pulmões, de onde podem disseminar-se para diferentes órgãos e tecidos (SAN-BLAS *et al*., 2002). Independentemente do órgão afetado, a PCM usualmente é associada à formação de fibrose, o que pode interferir permanentemente na qualidade de vida dos pacientes (TOBÓN *et al*., 2003). Devido aos diverssos tecidos que podem ser colonizados e infectados pelo fungo, mecanismos que o tornem capaz de aderir, extravasar e invadir barreiras impostas pelos tecidos do hospedeiro devem ter sido desenvolvidos pelo *P. brasiliensis* (MENDES-GIANNINI *et al*., 1994; LENZI *et al*., 2000).

Nos tecidos do hospedeiro o aspecto morfológico do fungo também foi descrito, observando-se que durante a infecção ativa, as lesões contêm um elevado número de leveduras, sendo que 24 – 33% dessas apresentam múltiplos brotamentos (RESTREPO, 2000). Lutz (1908) observou as diferentes formas de *P. brasiliensis* no hospedeiro e em culturas à temperatura ambiente, descrevendo assim, o dimorfismo do fungo.

I.2.2 – Dimorfismo

O dimorfismo é considerado um mecanismo de defesa importante para a adaptação de fungos às condições adversas do hospedeiro humano à invasão de tecidos e ao estabelecimento da doença (KUROKAWA *et al*., 1998; SAN-BLAS *et al*., 2002).

Fatores nutricionais podem interferir no processo dimórfico de *P. brasiliensis*. A adição de soro fetal bovino aos meios de cultura complexo e quimicamente definido permitiu preservar a expressão fenotípica de leveduras, a 25°C (VILLAR *et al.,* 1988), entretanto a temperatura é um dos estímulos mais notórios no dimorfismo de *P. brasiliensis* (SAN-BLAS *et al*., 2002).

A transição da fase miceliana para leveduriforme é o que torna eficaz o processo de instalação do fungo no hospedeiro, assim o estudo de genes/proteínas com expressão diferencial durante a transição dimórfica do fungo, tem sido alvo estudo de diversos pesquisadores. Nesse sentido, Silva *et al.* (1994) avaliaram o processo de transição celular no isolado *Pb*01, mostrando ser caracterizado por alterações na síntese de proteínas durante os estágios iniciais do processo. Cunha *et al*. (1999) detectaram proteínas diferencialmente expressas em *P. brasiliensis* em ambas as fases. Os autores destacaram as proteínas *Pb*M46, similar à enolase (proteína de 46 kDa presente em maior quantidade na fase miceliana) e *Pb*Y20 (proteína de 20 kDa presente somente na fase leveduriforme).

O Projeto Genoma Funcional e Diferencial de *P. brasiliensis*, desenvolvido por pesquisadores da região Centro-Oeste do Brasil, resultou no seqüenciamento de 6.022 genes expressos nas fases miceliana e leveduriforme do isolado *Pb*01, possibilitando a detecção de genes diferencialmente expressos (FELIPE *et al*., 2003; 2005). A diferenciação celular em *P. brasiliensis* requer mudança na temperatura, o que pode ser associado com a resposta ao estresse. Dessa forma, foram identificados 48 transcritos codificando chaperonas ou proteínas envolvidas no processo de estresse, sendo oito desses transcritos diferencialmente expressos. A análise do transcriptoma também revelou alguns prováveis componentes das vias de sinalização e seqüências gênicas consideradas como potenciais alvos para drogas antifúngicas em *P. brasiliensis*, visto

que não possuem homólogos no genoma humano. Entre esses podem ser citados a quitina deacetilase, quitina sintase I, II e IV, isocitrato liase, α-1,3-glicana sintase, β-1,3-glicana sintase, α-1,2-manosiltransferase, urease e malato sintase.

Felipe *et al.* (2003; 2005b) seqüenciaram 25.511 clones derivados de bibliotecas de cDNAs das fases micéliana e leveduriforme, resultando em 6.022 grupos que representam cerca de 80% do genoma total do fungo (FELIPE *et al.,* 2005b). Após categorização pelo COG (categorias de ortólogos funcionais), concluiu-se que 29% desses genes estão envolvidos no metabolismo celular, 12% na transcrição, 10% na síntese de proteínas, 9% na produção de energia e 4% no controle da organização celular. Cerca de 4% do número total de genes anotados estão envolvidos em mecanismos de transdução de sinal e comunicação celular, vias que têm sido relacionadas com a diferenciação celular de fungos dimórficos/patogênicos (FELIPE *et al.,* 2005b).

Nunes *et al*. (2005), através de microarranjos de DNA, avaliaram a expressão de genes de *P. brasiliensis*, isolado *Pb*18, durante a transição de micélio para levedura e identificaram vários genes diferencialmente expressos durante o processo. Estão inclusos entre esses, genes que codificam enzimas envolvidas no metabolismo de aminoácidos, transdução de sinal, síntese de proteínas, metabolismo da parede celular, estrutura do genoma, resposta ao estresse oxidativo, controle do crescimento e desenvolvimento do fungo.

O perfil transcricional de outro isolado de *P. brasiliensis*, isolado *Pb*01 durante a diferenciação morfológica de micélio para levedura foi avaliado por Bastos *et al*. (2007). Vários genes potencialmente relacionados com a síntese de membrana e parede celulares mostraram-se aumentados durante a diferenciação celular de micélio para levedura após 22 horas de indução da transição, sugerindo que *P. brasiliensis* favorece o

remodelamento da membrana e parede celulares nos estágios iniciais da morfogênese. Nesse estudo, genes envolvidos na via de assimilação do enxofre, como a sulfito redutase, mostraram-se super expressos durante a transição, sugerindo o envolvimento do metabolismo do enxofre durante o processo de diferenciação em *P. brasiliensis*. Durante a transição também foi verificada a indução de genes codificantes para enzimas que participam do ciclo do glioxalato, mostrando que essa via metavólica é funcional durante o processo. Genes envolvidos em vias de transdução de sinal tais como MAPK, serina/treonina quinase e histidina quinase, também foram induzidos, sugerindo que a transição morfológica em *P. brasiliensis* é mediada por vias de transdução de sinal que provavelmente controlam a adaptação do fungo ao ambiente externo.

I.2.3-Transcriptomas de *P. brasiliensis* em condições de contato com hospedeiro

Projetos transcriptomas de *P. brasiliensis* têm sido desenvolvidos no sentido de identificar um maior número de genes expressos. Goldman *et al*. (2003) identificaram 4.692 genes expressos em leveduras após infecção em camundongos. Esses genes foram categorizados funcionalmente em: genes envolvidos com resposta à estresse (1%), metabolismo celular (8%), metabolismo de proteínas (4%), síntese de RNA (2%), estrutura celular (5%), divisão celular (1%) e proteínas hipotéticas (60%).

Análises do cDNA de *P. brasiliensis*, isolado de camundongo infectado, realizadas por Bailão *et al*. (2006) mostraram que proteínas envolvidas nos processos de síntese protéica, fatores de elongação e processos pós-traducionais são super expressas nesta condição. Transcritos que codificam tirosinase e aminoácido L-aromático descarboxilase (DDC) foram supra-regulados, sugerindo a ocorrência da síntese ativa de melanina durante o processo infectivo. A tirosinase é a enzima responsável pela

primeira etapa da síntese de melanina (ARREDONDO *et al*., 2005), e está associada com a patogenicidade de algumas infecções microbianas, como a criptococose, causada pelo fungo *Cryptococcus neoformans* (WALTON *et al*., 2005).

Recentemente, o transcriptoma de *P. brasiliensis*, fase leveduriforme, recuperado de fígado de animais experimentais (camundongos B10) foi descrito por Costa *et al*. (2007). Foram seqüenciadas 4.932 ESTs no processo infectivo, sendo 37,47% relacionadas a novos genes e 23,75% pertencentes a genes super expressos. Os genes identificados foram categorizados em processos metabólicos, transporte celular e energia. Do total de ESTs geradas neste estudo, 65,53% das seqüências identificadas, também estavam presentes no transcriptoma de levedura e micélio de células obtidas de cultura *in vitro,* descrito por Felipe *et al*. (2005). A demonstração do perfil gênico das células leveduriformes de *P. brasiliensis* recuperadas de animais infectados é um requisito essencial para o estudo do genoma funcional de modo a esclarecer os mecanismos de patogenicidade e virulência fúngica.

Na fase leveduriforme, o metabolismo é desviado para a produção de etanol, refletindo seu comportamento anaeróbico (RESTREPO *et al.,* 1981). Várias vias que fornecem substratos para o ciclo do glioxalato (CG) são super-reguladas em células leveduriformes de *P. brasiliensis*. A enzima isocitrato liase redireciona o fluxo metabólico utilizando etanol e acetato como fonte com dois carbonos para gerar oxaloacetato, que pode ser reconvertido para glicose (FELIPE *et al.,* 2005a).

I.3 – Ciclo do Glioxalato

A glicose é a fonte de carbono preferencial para muitos organismos, podendo ser convertida em um açúcar contendo cinco carbonos ou ser catabolisada a

acetil-CoA através da glicólise. Entretanto, não é sempre que a glicose está disponível como fonte de carbono para os microrganismos, sendo necessário a atuação de outras vias metabólicas para suprir as necessidades energéticas de acordo com a fonte de carbono disponível no ambiente. Esses microrganismos possuem um sistema regulatório que facilita a conversão de vários nutrientes em componentes do metabolismo central, e dessa forma, levando à produção de energia para transformação de substratos, biossíntese de compostos, e crescimento (COZZONE, 1998).

Uma dessas vias é o Ciclo do Ácido Tricarboxílico (TCA). O TCA resulta na geração de moléculas produtoras de energia, e também atua como reservatório de precursores metabólicos essenciais para a síntese de aminoácidos, ácidos graxos e açúcares. Esse ciclo pode ser regulado, e as enzimas envolvidas podem ser induzidas ou suprimidas de acordo com a necessidade da célula (HAMEL & APPANNA, 2001).

Outra via de grande importância no processo regulatório é o Ciclo do Glioxalato (Figura 01), originalmente descrito por Kornberg e Krebs (1957) em bactérias crescidas em acetato. O ciclo trata-se de uma via alternativa do TCA, oxidando acetato aos ácidos dicarboxílicos succinato, malato e oxaloacetato (FLAVELL *et al.*, 1971). A função do Ciclo do Glioxalato é sintetizar compostos contendo 4 carbonos a partir de compostos contendo 2 carbonos, tais como acetato e etanol (KORNBERG, 1966).

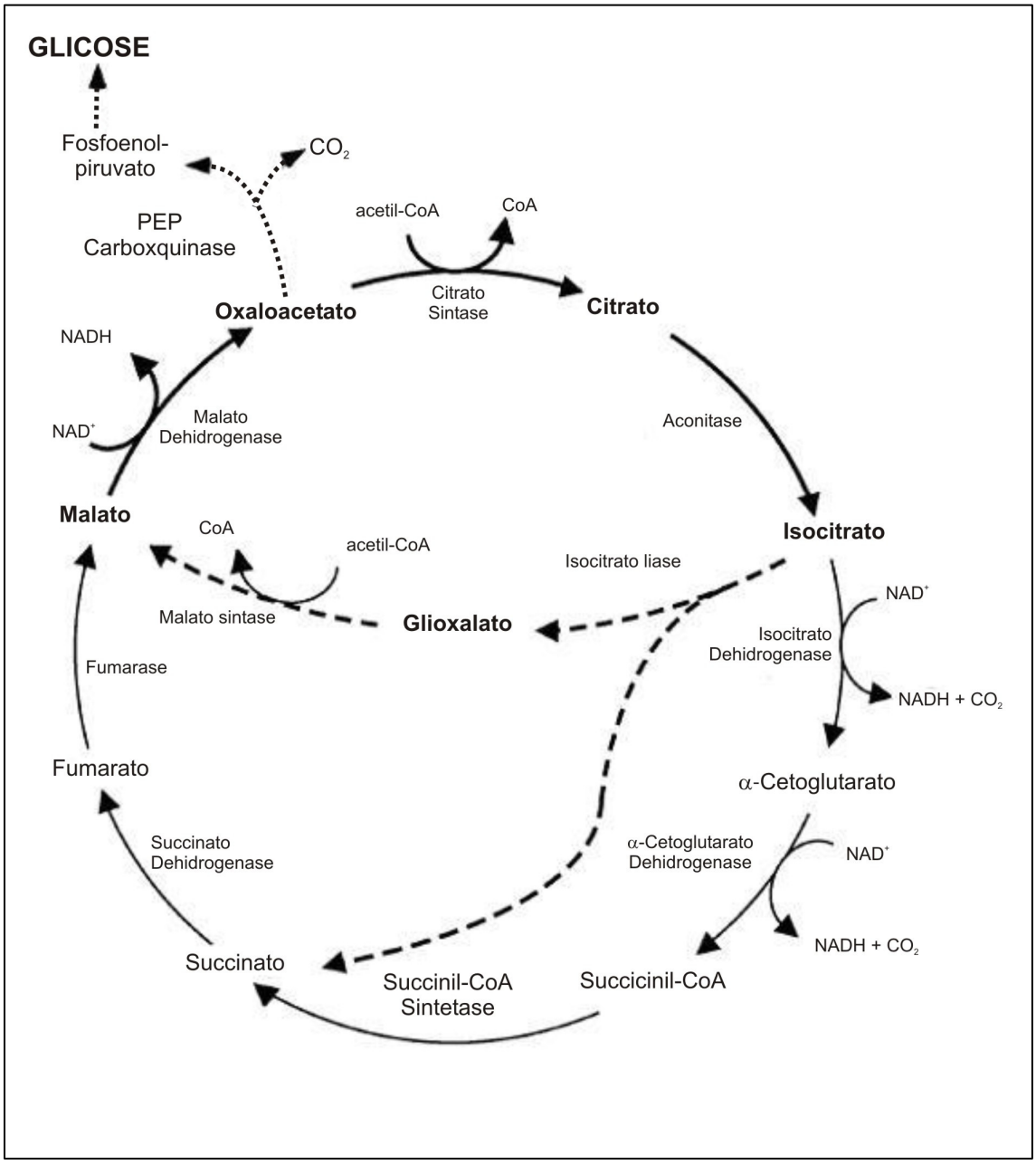


Figura 01 – Esquema representativo do ciclo do ácido tricarboxílico, ciclo do glioxalato e gliconeogênese. Estão evidenciados os passos enzimáticos básicos no ciclo do ácido tricarboxílico (linhas finas), o qual é comum para todos os organismos; passos no ciclo do glioxalato (linhas tracejadas), o qual é comum para microrganismos e plantas; passos compartilhados pelos dois ciclos (linhas grossas); e a reação inicial de gliconeogênese (linhas sombreadas). Fonte: LORENZ & FINK (2002). Life and Death in a Macrophage: Role of the Glyoxylate Cycle in Virulence. *Eukaryotic Cell*. 657-662.

O acetil-CoA entra no TCA, onde é convertido, através de oito passos enzimáticos, em intermediários que alimentam numerosas vias biossintéticas, incluindo as vias para a síntese de aminoácidos, ácidos graxos e glicose (LORENZ & FINK, 2002). Nesse processo participam enzimas comuns ao TCA e também as especificas do Ciclo do Glioxalato, a isocitrato liase (ICL) e a malato sintase (MLS). A enzima ICL catalisa a reação de clivagem do isocitrato a sucinato e glioxalato. Em seguida, a enzima MLS condensa o glioxalato, com acetil coenzima A formando malato (KORNBERG, 1966).

O Ciclo do Glioxalato possui um papel importante na gliconeogênese (KORNBERG & BEEVERS, 1957), visto que o primeiro passo comprometido na gliconeogênese é a conversão de oxaloacetato à fosfoenolpiruvato através da enzima fosfoenolpiruvato carboxiquinase (PEP carboxinase) (Figura 01). Essa enzima e a frutose-1,6-bifosfatase são as mais importantes na gliconeogênese por atuarem como regulatórias na produção de glicose. Em *Saccharomyces cerevisiae*, sob fagocitose, frutose-1,6-bifosfatase (*FBP1*) é altamente induzida (LORENZ & FINK, 2001), validando a idéia de que o primeiro objetivo na indução do Ciclo do Glioxalato é a produção de glicose.

As enzimas do Cilclo do Glioxalato são induzidas após a fagocitose de *C. albicans* e *S. cerevisiae*, possibilitando a sobrevivência desses microrganismos no interior de macrófagos (LORENZ & FINK, 2002). Somente as enzimas exclusivas do ciclo do Glioxalato são induzidas durante o processo de infecção embora existam enzimas comuns ao TCA (MINARD & MCALISTER-HENN, 1991). Entretanto, o mesmo não é observado em resposta a meios condicionados, estresse oxidativo, ou contato com macrófagos inativados por aquecimento (LORENZ & FINK, 2001). De acordo com Cozzone (1998), o macrófago é pobre em compostos carbônicos

complexos, e rico em ácidos graxos e seus produtos de degradação, primeiramente acetil-CoA, os quais são assimilados somente através do Ciclo do Glioxalato, confirmando ser a única rota de síntese de glicose nesse ambiente.

Tem sido descrito que o Ciclo do Glioxalato e as enzimas ICL e MLS são importantes para a virulência de muitos patógenos, como *Candida albicans* (LORENZ & FINK, 2002), *Cryptococcus neoformans* (RUDE *et al.,* 2002) e o fungo fitopatogêncio *Leptosphaeria maculans* (IDNURM & HOWLETT, 2002).

Estudos recentes utilizando isolados de *C. albicans* coletados de três grupos de pacientes apresentando candidíase em regiões diferentes do corpo, mostraram a atividade das enzimas ICL e MLS em todos os casos analisados, confirmando a presença do CG como fator de virulência para o fungo. Entre os três grupos de pacientes analisados, os níveis de atividade dessas enzimas se mostraram alterados dependendo do local da infecção. Estudos utilizando isolado de *C. albicans* proveniente de pacientes diabéticos desenvolvendo candidíase vulvovaginal apresentaram níveis de atividade das enzimas ICL e MLS mais elevados quando comparado a isolado obtido da cavidade orofaríngea dos pacientes HIV/AIDS e da pele de pacientes queimados (LATTIF *et al*., 2006).

I.4 – Peroxissomos

Os peroxissomas são organelos existentes em virtualmente todas as células eucarióticas, compreendendo também os glioxissomas das plantas e dos fungos, os glicossomas dos cinetoplastídeos, e os corpos de Woronin de *Neurospora crassa*. Este último tipo de peroxissoma contém um núcleo cristalóide hexagonal, e está envolvido no estancamento de perdas de citosol em fungos filamentosos (JEDD & CHUA, 2000). São conhecidos apenas alguns seres eucarióticos unicelulares que não possuem

peroxissomas, sendo considerados formas de vida eucariótica primitiva ou descendente de eucariotas com peroxissomas (MULLER, 1969). Os peroxissomas foram descritos pela primeira vez na década de 50 por Rhodin (RHODIN, 1954).

Inicialmente os peroxissomas foram designados por microcorpos (*microbodies*), sendo que essa designação foi alterada para peroxissoma, após a descoberta da respiração baseada no peróxido de hidrogénio, característica dessas organelas (De DUVE & BAUDHUIN, 1966). Os peroxissomas desempenham várias funções metabólicas, como é o caso da oxidação de vários tipos de lípidos (WELLER *et al*., 2003), vários passos da síntese de plasmalogénios e da síntese *de novo* de colesterol, metabolismo de isoprenóides (BIARDI *et al*., 1994), conversão de ácido fitânico a ácido pristânico, oxidação de D-aminoácidos e poliaminas (Weller *et al*., 2003), síntese de ácido docosahexanóico (WANDERS & WATERHAM, 2006), síntese de ácidos biliares e catabolismo de purinas (AZEVEDO *et al*., 2006). Em plantas, o ciclo do glioxilato (sementes) e a fotorrespiração (folhas) ocorre nos peroxissomas (ECKERT & ERDMANN, 2006). Em leveduras, vários passos do ciclo do glioxilato também ocorrem no peroxissoma, bem como a biossíntese de lisina, e a degradação de aminoácidos, metanol, peróxido de hidrogénio e ácidos gordos (MANNAERTS & VAN VELDHOVEN, 1993), sendo que nestes organismos a β-oxidação é um processo exclusivamente peroxissomal (VEENHUIS *et al*., 1987).

Peroxissomos são pequenos microcorpos, normalmente esféricos envoltos por uma única bicamada lipídica (KERSSEN *et al*., 2006), eles apresentam capacidade de replicação por fissão, mas não contém DNA ou aparato de expressão de gene, além de que tem sido elucidado que esles são derivados do retículo endoplasmático, gerando novas questões sobre sua origem endossimbiótica (HOEPFNER *et al*., 2005). Os peroxissomos contêm características de oxidorredutases que transfere elétrons de

reductos diretamente para oxigênio molecular para formar peróxido de hidrogênio e catalase para degradar o peróxido de hidrogênio à água e oxigênio. Peroxissomos contém também enzimas típicas da β-oxidação de ácido graxos (PURDUE & LAZAROW *et al*., 2001).

Proteínas chamadas *peroxins* têm sido identificadas por estratégias genéticas e proteômicas e caracterizadas na biogênese de peroxissomos, algumas responssáveis na topogênese de proteínas peroxissomais. Tem-se investigado também que a membrana peroxissomal e proteínas matriz são sintetizadas em ribossomos livres no citosol e são importadas pós-traducionalmente dentro de organelas pré-existentes (INESHEILAND & EDMAN, 2005).

I.5 – Matriz Extracelular

A matriz extracelular (MEC) é uma rede complexa de macromoléculas que proporciona um arcabouço físico para a estabilização da estrutura tecidual. A MEC é importante para as interações célula a célula e fornece um substrato às mesmas para aderirem e proliferarem, modulando diretamente a forma e funções celulares. A MEC de mamíferos é composta por duas classes principais de macromoléculas: as glicosaminoglicanas (GAG), encontradas normalmente ligadas às proteínas formando proteoglicanas, e as proteínas fibrosas, que desempenham funções estruturais e adesivas, incluindo nessa classe, laminina, fibronectina e colágeno (VERSTREPEN & KLIS, 2006). A composição da MEC varia em diferentes tecidos e durante fases de injúria, inflamação e reparo tecidual (KOTTON *et al*. 2003).

Colágeno é o principal constituinte da MEC e representa um importante alvo para adesão de muitas espécies de microrganismos. Vários tipos de colágeno foram caracterizados, entre eles o colágeno tipo IV, encontrado principalmente na membrana basal e o colágeno tipo I é abundante na matriz intersticial (GIL *et al*., 1996).

Fibronectina é uma glicoproteína dimérica de 440 kDa presente na forma solúvel no plasma sanguíneo e outros fluídos corporais e na forma fibrilar na MEC. A fibronectina provavelmente atua como molécula de adesão em células de mamíferos, um processo que envolve a ligação de receptores específicos da superfície celular com domínios presentes na molécula de fibronectina (MOHRI *et al*., 1996).

Laminina é uma glicoproteína de 900 kDa presente na membrana basal e nos pulmões. Essa glicoproteína pode ser exposta quando o tecido sofre algum dano que pode ser provocado por toxinas bacterianas, drogas ou por processo inflamatório. A interação com laminina é crucial para vários processos biológicos, os quais requerem adesão celular, tais como, diapedese, coesão celular dentro do tecido, metástase de células cancerosas e infecções (BECK *et al*., 1990). De fato, receptores de laminina foram descritos em células que normalmente interagem com a membrana basal, tais como células epiteliais ou endoteliais, células musculares e neurais. Células que extravasam da corrente sanguínea, como por exemplo, macrófagos, leucócitos, e células tumorais, também podem exibir esses receptores (BOUCHARA, 1997).

Tanto o *P. brasiliensis* (MENDES-GIANNINI *et al*., 2000), quanto vários fungos de importância clínica, a exemplo *Cryptococcus neoformans* (GANENDREN *et al*., 2006), *Penicillium marneffei* (SRINOULPRASERT *et al*., 2006), *Histoplasma capsulatum* (McMAHON *et al*., 1995)*, Coccidioides immitis* (HUNG *et al*., 2002) e *C. albicans* (FILLER, 2006), possuem mecanismos que possibilitam sua adesão à MEC, uma vez que esse processo implica em que o patógeno reconheça carboidratos ou

proteínas ligantes na superfície da célula do hospedeiro ou proteínas constituintes da membrana basal (PATTI *et al*., 1994). A adesão de microrganismos patogênicos aos tecidos do hospedeiro é considerada indispensável para o início da colonização e futura disseminação.

I.6 – Moléculas Envolvidas no Processo de Adesão de Fungos Patogênicos

Partindo do princípio de que o primeiro passo para o estabelecimento do processo infectivo nos tecidos do hospedeiro seria a adesão de microrganismos às proteínas da MEC, torna-se necessário o estudo de moléculas que se comportam como adesinas em fungos patogênicos. A habilidade de vários fungos de aderir a uma variedade de superfícies do hospedeiro representa uma característica importante no desenvolvimento da infecção fúngica e na patogênese de micoses. A adesão é mediada por componentes superficiais da célula fúngica chamadas de adesinas, as quais freqüentemente se ligam a componentes da MEC (LENZI *et al*., 1991; PATTI *et al*.,1994).

Em *C. albicans* uma manoproteína de 65 kDa (MP65) foi caracterizada como uma adesina. A propriedade adesiva de MP65 foi evidenciada pelo fato do anticorpo produzido contra MP65 ser capaz de inibir a adesão do fungo à placas de polietileno. Em adição mutante de *C. albicans* que não expressa MP65 na parede celular apresentou menor capacidade de adesão, quando comparado com o tipo selvagem (SANDINI *et al*., 2007).

A atividade da enzima fosfolipase tem sido descrita como fator de aderência de diversos patógenos às células epiteliais. Foi demonstrado que a fosfolipase B1 (PLB1) é um fator de virulência em *Cryptococcus neoformans*, e que estaria envolvido

no início da criptococose pulmonar (SANTANGELO *et al*., 2004). A deleção do gene que codifica PLB1 inibiu significativamente a capacidade de adesão de *C. neoformans* à culturas de pneumócitos (linhagem A549) (GANENDREN *et al*., 2006).

Em *A. fumigatus*, duas proteínas de parede celular de 37 e 72 kDa apresentaram capacidade de ligação à laminina. Em adição outros dois peptídeos de massa molecular de 23 e 30 kDa foram capazes de interagir com fibronectina (PENALVER *et al*., 1996; TRONCHIN *et al*., 1997).

Experimentos de adesão em que o fungo *H. capsulatum* foi colocado em contato com laminina imobilizada mostraram que esse fungo interage com a proteína de maneira rápida e específica; a presença do anticorpo anti-laminina inibiu significantemente a adesão do fungo. A adesão foi mediada possívelmente por uma glicoproteína de 50 kDa identificada na parede celular de *H. capsulatum*. O anticorpo anti-proteína de 50 kDa e o peptídeo IKVAV, presente na molécula de laminina, foram capazez de inibir a adesão do fungo à molécula de laminina (McMAHON *et al*., 1995).

Estudos de interação entre *S. schenckii,* o agente etiológico da esporotricose, e proteínas da MEC mostraram que o fungo foi capaz de aderir à laminina, colágeno e fibronectina, e que essa interação foi mediada por proteínas de 90 e 135 kDa localizadas na superfície do fungo. A interação entre *S. schenckii* e proteínas da MEC foi avaliada através de experimentos de competição entre peptídeos presentes na fibronectina e laminina (LIMA *et al*., 2004).

I.7 – Adesinas de *P. brasiliensis*

O fungo *P. brasiliensis* possui capacidade de aderir, atravessar e invadir barreiras impostas pelos tecidos do hospedeiro (MENDES-GIANNINI *et al*., 2000).

Segundo Hanna *et al*. (2000), a capacidade de aderência e invasão do fungo está relacionada à virulência. Recentes estudos têm caracterizado componentes da MEC envolvidos na interação de *P. brasiliensis* com o hospedeiro (MENDES-GIANNINI *et al*., 2006). A laminina é uma das principais moléculas que promovem a adesão de *P. brasiliensis* facilitando a sua patogenicidade (MENDES-GIANNINI *et al*., 2008).

Vicentini *et al*. (1994) descreveram a primeira adesina de *P. brasiliensis*, a glicoproteína de 43 kDa com a capacidade de interagir com laminina. A gp43 interage com laminina (VICENTINI *et al*., 1994; GESZTESI *et al*., 1996) e fibronectina (MENDES-GIANNINI *et al*., 2006).

P. brasiliensis também apresenta em sua superfície celular duas proteínas com massas moleculares de 19 e 32 kDa que interagem com proteínas da MEC, tais como laminina, fibrinogênio e fibronectina (GONZÁLEZ *et al*., 2005). Ensaios realizados com conídeos de *P. brasiliensis* pré-incubados com anticorpo monoclonal anti-32 kDa demonstraram a inibição da aderência do fungo às proteínas da MEC de maneira dose-dependente (GONZÁLEZ *et al*., 2005). Uma adesina de 30 kDa isolada por Andreotti *et al*. (2005) apresentou capacidade de ligação à laminina, mas não a outros componentes da MEC; a adesão de *P. brasiliensis* às células epiteliais foi inibida após tratamento das células epiteliais com essa proteína.

Enzimas da via glicolítica de *P. brasiliensis*, como a triose fosfato isomerase (TPI) (PEREIRA *et al*., 2007) e gliceraldeído-3-fosfato desidrogenase (GAPDH) (BARBOSA *et al*., 2006), foram também caracterizadas como prováveis adesinas do fungo. Recentemente foi descrita outra molécula presente na superfície do fungo que apresenta capacidade de aderir às proteínas da MEC, a *Pb*Dfg5p. Essa proteína caracterizada como pertencente à família das glicosil hidrolases estaria relacionada com

a formação e manutenção da parede celular de fungos apresentando capacidade de se ligar a laminina, fibronectina, colágenos tipo I e tipo IV (CASTRO *et al*., 2008).

I.8 – Malato sintase

I.8.1 – Considerações gerais

A enzima MLS (EC 2.3.3.9) catalisa a reação de condensação que produz malato a partir de acetil-CoA e glioxalato; essa reação é altamente especifica para esses substratos e requer Mg^{2+} como um cofator (NOGALES *et al.,* 2004). A MLS é uma enzima fundamental do Ciclo do Glioxalato, e também está envolvida na degradação de purinas, convertendo o glioxalato produzido do ácido urico, alantoina e ácido alantoico, em malato (HARTIG *et al*., 1992) que pode participar do TCA ou ciclo do ácido dicarboxilico (DAC) (ORNSTON & ORNSTON, 1969).

As MLSs são classificadas em três famílias baseadas no tamanho da seqüência de aminoácidos. A maioria das MLSs conhecidas de fungos, bactérias e plantas superiores se agrupam no clado das MLSs com tamanho intermediário (HOWARD *et al.*, 2000). Com base nas características das seqüências de aminoácidos, as MLSs podem se dividir ainda, em duas principais famílias, isoformas A (MSA) e G (MSG). MSA, com massa molecular em torno de 65 kDa, ocorre em bactérias, como por exemplo, *E. coli* (KORNBERG, 1966), em fungos, como *S. cerevisiae* (LORENZ & FINK, 2001); e plantas superiores, como o pepino (*Cumcumis sativus*) (EASTMOND & GRAHAM, 2001); MSG, possui massa molecular em torno de 80 kDa, ocorrendo somente em bactérias (NAKAZAWA *et al*., 2005).

I.8.2 – Aspectos da enzima MLS

Em plantas, tem se identificado a presença da enzima MLS em diferentes estágios de crescimento e situações (LEWIS & DOYLE, 2001). Estudos com pepinos (*Cucumis sativus*) mostraram que a MLS apresenta sua atividade alterada durante os diferentes estágios de crescimento da planta (GRAHAM *et al*., 1992). A expressão do gene *MS* nessa planta é influenciada pelo estado metabólico intracelular; sua indução está intimamente relacionada com a baixa concentração de sacarose, glicose e frutose intracelular (GRAHAM *et al*., 1994).

Alguns microrganismos possuem duas classes de MLSs, sendo codificadas por genes diferentes. Exemplificando, em *E. coli*, *aceB* codifica a forma A, a qual é predominante em células crescidas em meio contendo acetato, e *glcB* codifica a forma G, uma proteína monomérica induzida pelo glicolato (MOLINA *et al*., 1994). Em *E. coli*, o *operon aceBAK* contendo os genes ICL (*aceA*) e MLS (*aceB*) é reprimido por glicose e induzido por acetato ou ácidos graxos (CLARK & CRONAN, 1996).

Nakazawa *et al*. (2005) purificaram uma proteína com massa molecular de 116 kDa, como a MLS de *Euglena gracilis* crescida na presença de etanol. Entretanto, sua localização é mitocondrial, e não glioxissomal, como descrito em fungos (SZABO & AVERS, 1969) e plantas superiores (BEEVERS, 1980). Essa localização mitocondrial de EgGCE é confirmada pela presença do peptídeo sinal de endereçamento para a mitocôndria. Em contraste, Woodcock & Merret (1978) isolaram outra MLS de *E. gracilis* crescida na presença de acetato; essa proteína apresenta massa molecular de 175 kDa e está localizada em glioxissomos (LIU *et al*., 1995).

A estrutura tridimensional de MSG de *E. coli* foi elucidada através de Ressonância Magnética Nuclear (NMR) (TUGARINOV *et al*., 2002). MSG é uma

proteína globular multimérica apresentando uma estrutura TIM barril β8/α8. O sítio ativo de MSG está localizado em uma cavidade na interface entre a região C-terminal e os *loops* no final da região C-terminal das folhas β do TIM barril (HOWARD *et al*., 2000). Essa posição do sítio ativo está coerente com as posições descritas em outras enzimas que contêm o TIM barril; o sítio ativo está localizado dentro dos *loops* no final do barril (BRANDEN & TOOZE, 1991). Em *E. coli,* o*s* resíduos conservados do sítio ativo, Glu427 e Asp455, os quais se ligam ao íon magnésio, Arg338 que uni o hidrogênio ao oxigênio aldeídico do glioxalato, e a base catalítica proposta, Asp631, são conservados entre as seqüências de MLSs conhecidas. Os domínios críticos para a atividade de MLS, parece ser o domínio do barril e a ligação C-terminal que cobre o sítio ativo, e que também contribui para a base catalítica proposta (Asp631). O domínio C-terminal é composto por 589 resíduos; o *loop*, formado pelos resíduos 577-588 parece ser flexível, permitindo o movimento do domínio para o restante da enzima. Tal movimento permite a abertura do sítio ativo para entrada do substrato e liberação do produto (HOWARD *et al*., 2000).

Na bactéria marinha *Colwellia maris*, a MLS é descrita como uma enzima dimérica composta por subunidades de 76,3 kDa, que apresenta temperatura ótima de 45º C, característico de enzima mesofílica, e possui parâmetros cinéticos alterados após a redução da temperatura (WATANABE *et al*., 2001). Nas bactérias *Acinetobacter calcoaceticus* e *Corynebacterium glutamicum* MLS se apresenta como uma proteína monomérica com massa molecular de 75 kDa (PARK *et al*., 1986) e 82 kDa (REINSCHEID *et al*., 1994), respectivamente. Em *Fomitopsis palustris*, um fungo basidiomiceto, a MLS apresenta-se na forma de um octâmero, com massa molecular de 520 kDa, distribuídas em oito subunidades de 65 kDa. Essa enzima apresentou

afinidade para ambos os substratos, glioxalato (Km de 45μM) e acetil-CoA (Km de 2,2 μM) (MUNIR *et al*., 2002).

Em sementes de algodão (*Gossypium hirsutm* L.), a enzima MLS se encontra presente no glioxissomo até o período que precede a germinação da planta. Após esse período, ocorre o acúmulo de mRNA nos cotilédones (MAHAN, 2000). No arroz (*Zea* mays), a enzima MLS apresentou atividade aumentada nas pontas das raízes (DIEUAIDE *et al*., 1992).

Kunze *et al*. (2002) mostraram que Mls1p encontrava-se presente no citoplasma quando células de *S. cerevisiae* foram crescidas na presença de etanol. Entretanto, quando as células eram crescidas na presença de ácido oléico, Mls1p apresentou localização peroxissomal. Em *Corynebacterium glutamicum*, MLS e ICL são essenciais para seu crescimento na presença de acetato, pois utiliza preferencialmente, esse substrato como fonte de energia (WENDISCH *et al*., 2000).

I.8.3 – MLS como antígeno

As enzimas do ciclo do Glioxalato, MLS e ICL, tem sido descritas como fatores de virulência em vários patógenos hmanos (OLIVAS, *et al*., 2007; RUDE *et al*., 2002; LORENZ and FINK, 2002), particularmente em *Mycobacterium tuberculosis* (GRAHAM and CLARK-CURTISS, 1999; DUBNAU *et al*.,2002). Genes que codificam essas enzimas são induzidos na fagocitose em *C. albicans* e *S. cerevisiae* (LORENZ and FINK, 2002).

Singh *et al*. (2004) através de amostras de soro de pacientes infectados com HIV antes da manifestação clinica da tuberculose, mostraram o perfil de *M. tuberculosis* e

suas proteínas de filtrado de cultura. Anticorpos para MLS estava presente em 90% dos casos testados, podendo considera-la útil em testes de diagnóstico.

Estudos com pacientes da índia revelaram que MLS é um antígeno imunodominate reconhecido por 80% dos pacientes imunodeficientes, evidenciando que o ensaio de sorodiagnóstico baseado na MLS pode servir como suplemento à microscopia no diagnóstico rápido da tuberculose (ACHKAR *et al*., 2006)

Wanchu *et al*. (2008) mostraram que anti-MLS de *M. tuberculois* tem alta sensibilidade e especificidade para o diagnóstico de tuberculose de pacientes em regiões endêmicas, além de um grande potencial para identificar a tuberculose subclinica em pacientes considerados de alto risco para a doença.

I.8.4 – MLS de *P. brasiliensis*

A seqüência codificante para a *Pb*MLS, foi clonada e caracterizada por Zambuzzi-Carvalho *et al*. (2009). O cDNA apresenta 1882 pares de bases, incluindo uma ORF de 1617 pares de bases. A seqüência deduzida da proteína é constituída por 539 resíduos de aminoácidos com uma massa molecular predita de 60 kDa e p*I* de 8.45. A proteína apresenta assinatura característica da família das MLSs, resíduos catalíticos essenciais para a atividade enzimática, e sinal PTS1 de localização peroxissomal/glioxissomal (ZAMBUZZI-CARVALHO *et al*., 2009).

Análises do cDNA de *P. brasiliensis*, isolado de camundongo infectado, mostraram que proteínas envolvidas nos processos de síntese protéica, fatores de elongação e processos pós-traducionais são super expressas nessa condição (BAILÃO *et al*., 2006)

*Pb*mls tem motivos que poderiam indicar sua regulação por fontes de carbono e nitrogênio. Além disso, todas as enzimas envolvidas no metabolismo de purinas para formar glioxalato foram encontrados em transcriptomas de *P. brasiliensis* (COSTA *et al*., 2007, BASTOS *et al*., 2007).

Os dados sugerem que *Pb*MLS condensa acetyl-CoA de duas fontes de carbono. Na presença de fontes de nitrogênio, o glioxalato produzido do metabolismo de prolina e purina foi condensado com acetil-CoA produzindo malato por ação de *Pb*MLS. O regulamento de *Pb*mls por fontes de carbono e de nitrogênio é reforçado pela presença de motivos reguladores encontrados na região de promotora do gene (ZAMBUZZI-CARVALHO *et al*., 2009)

O estudo do transcriptoma de *P. brasiliensis* tem auxiliado no entendimento global do metabolismo de ambas as formas micélio e levedura (FELIPE *et al.*, 2005a). Em geral, o padrão dos genes induzidos na fase miceliana sugere que essa forma possua um metabolismo aeróbico, ao contrário da forma leveduriforme. Os genes isocitrato dehidrogenase e succinil-CoA sintetase, os quais são os principais pontos regulatórios do ciclo do citrato são induzidos em micélio. (FELIPE *et al.,* 2005a).

No presente trabalho foi demonstrado que *Pb*MLS está associada à parede de *P. brasiliensis*, onde é capaz de se ligar a componentes da matriz extracelular, como fibronectina e colágeno tipo I e IV. Além disso, foi demonstrado que a MLS é capaz de mediar à aderência e internalização de *P. brasiliensis* às células cultivadas *in vitro,* o que sugere o seu papel potencial no estabelecimento da doença.

Justificativa

II – JUSTIFICATIVA

A importância do estudo da PCM não resulta somente de sua prevalência relativamente alta, mas também da severidade de suas formas clínicas, considerando o fato de que essa é a micose sistêmica de maior prevalência na América Latina, com o maior número de relatos de casos no Brasil. A doença varia em severidade, em geral apresenta evolução crônica, de caráter recidivante e pode deixar seqüelas anatômicas e funcionais. O número de doentes que necessitam de assistência médica de longo prazo nas regiões de maior endemicidade é muito grande, tornando a moléstia, por sua prevalência, um importante problema de saúde publica (MARTINEZ, 1997). O coeficiente de mortalidade no período de 1980 a 1995, para o Brasil e para a região Centro-Oeste foi, respectivamente, 1.488 e 2.428/1 milhão de habitantes (COUTINHO *et al*., 1998).

A caracterização de algumas proteínas que aparentemente influenciam na interação com o hospedeiro, conferindo um fenótipo mais patogênico, por permitir ao fungo aderir com maior facilidade aos tecidos do hospedeiro, invadir novos compartimentos, evadir da resposta imune, bem como outras interações hospedeiro-específicas, vem colaborando para o entendimento da instalação do fungo e desenvolvimento da PCM.

A disponibilidade do transcriptoma de *P. brasiliensis* possibilita a obtenção de moléculas presentes no fungo, porém ausentes em humanos, como novos alvos antifúngicos. O Ciclo do Glioxalato não é descrito em humanos assim, acredita-se que inibidores desse ciclo sejam potentes antifúngicos sem efeitos colaterais no homem.

Desta forma, a partir das seqüências completas de cDNA codificantes para a proteína *Pb*MLS, realizamos a expressão heteróloga em *E. coli*, bem como a produção de soro

policlonal contendo anticorpo que reconheça especificamente a proteína nativa, com intuito de obter condições que tornassem possível analisar sua localização celular.

Os recentes estudos de caracterização de proteínas antigênicas, que anteriormente acreditava-se serem apenas enzimas presentes no citoplasma, como moléculas envolvidas na adesão de *P. brasiliensis* aos tecidos do hospedeiro, nos levaram a utilizar a *Pb*MLSr em experimentos de caracterização de adesinas, descritos recentemente na literatura. Deste modo objetivamos avaliar essa molécula com relação à sua capacidade de se ligar a componentes de MEC, culturas de células epiteliais e posteriormente sua influência na adesão e internalização de *P. brasiliensis* em culturas celulares, com o intuito de obter indícios do provável papel funcional da MLS nos primeiros estágios de interação do fungo com tecidos do hospedeiro.

Objetivos

III – OBJETIVOS

III.1 - Purificar a proteína recombinante *Pb*MLS;

- Estratégias:

- ✓ Clonagem do cDNA completo da *Pb*MLS em vetor de expressão (pGEX4-T3 GE Healthcare) e expressão da proteína de fusão no sistema bacteriano *Escherichia coli* (linhagen C43);
- ✓ Purificação da proteína recombinante em condições não desnaturantes através de cromatografia de afinidade.

III.2 - Produzir anticorpos policlonais anti-MLS;

- Estratégia:

- ✓ Inoculação da proteína purificada em coelhos, com adjuvante completo de Freund, pela via sub-cutânea.

III.3 - Localizar *Pb*MLS em células leveduriformes de *P. brasiliensis*;

- Estratégia:

- ✓ Microscopia de varredura laser confocal utilizando o anticorpo anti-*Pb*MLS.
- ✓ *Western blot* utilizando fração citoplasmática e fração de parede celular do fungo.
- ✓ *Western blot* com o filtrado de cultura.

III.4 - Avaliar a capacidade de *Pb*MLS se ligar a componentes da MEC e funcionar como uma adesina;

- Estratégia:

- ✓ *Far-Western blotting* para analizar a capacidade de ligação da proteína recombinante (*Pb*MLSr) aos componentes da MEC (laminina, fibronectina, e colágeno tipo I e tipo IV).
- ✓ ELISA com *Pb*MLSr através de ligação desta aos componentes da MEC (laminina, fibronectina, e colágeno tipo I e tipo IV).
- ✓ A *Pb*MLSr foi submetida à biotinilação utlizando o kit (GE Healthcare, Amersham Pharmacia Biotech).

III.5 - Analisar a reatividade imunológica de *Pb*MLSr;

- Estratégia:

- ✓ ”Immunoblotting” utilizando soros de pacientes com diagnóstico clínico e imunológico de PCM.

III.6 - Avaliar a influência de *Pb*MLSr e do anticorpo policlonal anti-*Pb*MLS, nos processos de adesão e invasão de *P. brasiliensis* às culturas celulares;

- Estratégias:

- ✓ Através da Citometria de Fluxo análisar o número de células leveduriformes de *P. brasiliensis*, após 2 e 5 horas de incubação, aderidas à monocamada de pneumócitos - A549, previamente tratadas com a *Pb*MLS recombinante purificada, em comparação a controles não tratados;
- ✓ Através da Citometria de Fluxo análisar o número de células leveduriformes de *P. brasiliensis*, tratadas previamente com o anticorpo anti-*Pb*MLS, após 2 e 5 horas de incubação com a monocamada de células A549, em comparação com controles de leveduras não tratadas.
- ✓ Microscopia de varredura laser confocal utlizando células leveduriformes de *P. brasiliensis*, aderidas à monocamada de pneumócitos - A549, previamente tratadas com a *Pb*MLSr.

Manuscrito

The malate synthase of *Paracoccidioides brasiliensis* is a linked surface protein behaving as an anchorless adhesin

Benedito Rodrigues da Silva Neto[1], Julhiany de Fátima da Silva[2], Maria José Soares Mendes-Giannini[2], Henrique Leonel Lenzi[3], Célia Maria de Almeida Soares[1], Maristela Pereira[1*]

[1]Laboratório de Biologia Molecular, Departamento de Bioquímica e Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, C.P. 131, 74001-970, Goiânia, GO, Brazil. [2]Laboratório de Micologia Clínica, Universidade Estadual Paulista, UNESP, Araraquara, SP, Brazil. [3]Laboratório de Patologia, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, RJ, Brazil.

Benedito Rodrigues da Silva Neto (bio.neto@gmail.com)

Julhiany de Fátima da Silva (julhiany.silva@gmail.com)

Maria José Soares Mendes-Giannini (gianninimj@gmail.com)

Henrique Leonel Lenzi (henrique.lenzi@gmail.com)

Célia Maria de Almeida Soares (Celia@icb.ufg.br)

Maristela Pereira (mani@icb.ufg.br)

[*]Corresponding author

Maristela Pereira
Laboratório de Biologia Molecular, Departamento de Bioquímica e Biologia Molecular, Instituto de Ciências Biológicas, Campus II, Universidade Federal de Goiás, C.P. 131, 74001-970, Goiânia, GO, Brazil.
E-mail address: mani@icb.ufg.br
Tel./fax: +55 62 35211110

Abstract

Background: The pathogenic fungus *Paracoccidioides brasiliensis* is the agent of paracoccidioidomycosis (PCM), which is a pulmonary mycosis acquired by inhalation of fungal airborne propagules that can disseminate to several organs and tissues leading to a severe form of the disease. Adhesion and invasion to host cells are essential steps involved in the internalization and dissemination of pathogens. Inside host, *P. brasiliensis* may use the glyoxylate cycle for intracellular survival.

Results: Here, we provide evidence that malate synthase of *P. brasiliensis* (*Pb*MLS) is localized on fungal cell surface, and is secreted. *Pb*MLS was overexpressed in *Escherichia coli*, and polyclonal antibody was obtained against this protein. By using Confocal Laser Scanning Microscopy, *Pb*MLS was detected in the cytoplasm and in the cell wall of the mother, but mainly of budding cells of the *P. brasiliensis* yeast phase. *Pb*MLSr and the respective polyclonal antibody produced against this protein inhibited the interaction of *P. brasiliensis* with *in vitro* cultured epithelial cells A549.

Conclusion: These observations indicated that cell wall-associated *Pb*MLS could be mediating the binding of fungal cells to host, thus contributing to the adhesion of fungus to host tissues and to the dissemination of infection, behaving as an anchorless adhesin.

Background

Paracoccidioidomycosis (PCM) is a chronic granulomatous disease involving about 10 million people, characterizing the most important systemic mycosis in Latin America. *Paracoccidioides brasiliensis,* a thermally dimorphic fungus pathogen, is the pulmonary infective agent [1,2]. This initial interaction appears to govern the subsequent mechanisms of innate and acquire immunity, which result in localized infection or overt disease [3].

The mechanisms of adherence and invasion have been studied extensively in pathogenic bacteria [4], and in pathogenic fungi such as *Candida albicans* [5], *Histoplasma capsulatum* [6] and *Aspergillus fumigatus* [7], and *P. brasiliensis* [8,9,10]. Fungi are non-motile eukaryotes that depend on their adhesive properties for selective interaction with the host cells [11]. Molecules of adherence are fundamental in the pathogen-host interaction; during this event, the fungal cell wall is in continual contact with the host and acts as a sieve and reservoir of molecules such as adhesins [12]. The ability of *P. brasiliensis* to adhere to and invade nonprofessional phagocytes or epithelial cells has been recognized in previous studies [13,14,15]. Some adhesins of *P. brasiliensis* such as gp43 [10], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [16], a 30 kDa protein [9], and triosephosphate isomerase (TPI) [17] have been described. Evidence for extracellular localization of some glycolytic enzymes lacking secretion signals on cell-wall anchoring motifs has been reported for some pathogens [18,19]. In addition malate synthase (MLS) is also described as adhesin on *Mycobacterium tuberculosis* [20].

The glyoxylate cycle and its key enzymes isocitrate lyase (ICL) and MLS play a crucial role in the pathogenicity and virulence of various fungi such as the human

pathogens *A. fumigatus* [21], *Cryptococcus neoformans* [22] and *C. albicans* [23,24], the bacterium *M. tuberculosis* [25,26,27] as well as the phytopathogenic fungus *Magnaporthe grisea* [28] and the necrotropic wheat pathogen *Stagonospora nodorum* [29]. It was postulated a relevant role of the glyoxylate cycle in the viability and growth of fungi inside macrophages and, consequently, in the development of a disseminated fungal infection [21]. ICL and MLS have also been considered therapeutic target for development of novel antifungal compounds, since there are no human orthologues. In *P. brasiliensis*, the enzyme MLS (*Pb*MLS) participates in the glyoxylate pathway that enables fungus to assimilate two-carbon compounds from the tricarboxylic acid cycle, and in the allantoin degradation pathway of the purine metabolism allowing the fungus to use nitrogen compounds [30].

Here it is demonstrated that *Pb*MLS is the first fungal MLS localized on the cell surface, interfering with the infection process.

Results

Expression, purification and production of polyclonal antibody to *Pb*MLSr

The cDNA encoding *Pb*MLS was subcloned into the expression vector pET-32a to obtain the recombinant fusion protein. The protein was not present in crude extracts of non induced *E. coli* cells carrying the expression vector (Fig. 1A, lane 1). After induction with IPTG, a 73 kDa recombinant protein was detected in bacterial lysates (Fig. 1A, lane 2). The six-histidine residues fused to the N terminus of the recombinant protein were used to purify the protein from bacterial lysates by nickel-chelate affinity. The recombinant protein was eluted and analyzed by SDS-PAGE (Fig. 1A, lane 3) and His-, Trx-, and S-Tag was removed by cleavage with the enterokinase (Fig. 1A, lane 4).

An aliquot of the purified recombinant protein was used to generate rabbit polyclonal anti-*Pb*MLSr antibody. Western blot confirmed the positive reaction of antibody with the fusion protein (Fig. 1B, lane 1) identifying a protein of 73 kDa. The cleaved recombinant protein was detected as a species of 60 kDa (Fig. 1B, lane 2).

Detection of *Pb*MLS on cell wall extracts, culture filtrates and peroxisomal fraction

To determine the subcellular distribution of *Pb*MLS, a cell wall-enriched, secreted, and peroxissomal fractions purified from *P. brasiliensis* yeast cells were obtained. Crude extract proteins, SDS-extracted cell wall proteins, and extracted cell wall proteins from yeast cells were subjected to SDS–PAGE analysis, blotted onto nylon membrane and reacted to polyclonal anti-*Pb*MLSr antibody. *Pb*MLS was detected in crude extract (Fig. 1B, lane 3), and in SDS-extracted cell wall proteins (Fig. 1B, lane 4), but it was not detected in extracted cell wall proteins (Fig. 1B, lane 5). No cross-reactivity to the pre-immune rabbit serum was evidenced with the samples (Fig. 1C).

To evaluate if *Pb*MLS was secreted to the medium, proteins were extracted from culture filtrates harvested from *P. brasiliensis* growing, 24 and 36 h cultures, (Fig. 1D, lanes 1 and 2, respectively), 7 days (Fig. 1D, lane 3), and 14 days (Fig. 1D, lane 4). The proteins were subjected to SDS–PAGE analysis, blotted onto nylon membrane and reacted to polyclonal anti-*Pb*MLSr antibody. *Pb*MLS was detected in all these preparations (Fig. 1D, lanes 1 to 4). No signal was detected in medium free of cells (Fig. 1D, lane 5). No cross-reactivity to the pre-immune rabbit serum was evidenced with the samples (Fig. 1E). Altogether, these results suggest that *Pb*MLS is weakly binding to cell wall, and is actively secreted in *P. brasiliensis*.

Since *Pb*MLS has the AKL tripeptide, a peroxisomal/glyoxysomal targeting signal PTS1 [31], the presence of the protein was investigated in this cellular compartment. Peroxisomal and mitochondrial fractions purified of *P. brasiliensis* were obtained. The proteins were subjected to SDS–PAGE analysis, blotted onto nylon membrane and reacted to the polyclonal anti-*Pb*MLSr antibody. *Pb*MLS was detected in peroxisomal fraction (Fig. 1F, lane 1) confirming the localization of *Pb*MLS in this organelle. Since *Pb*MLS has not been found in mitochondria, the mitochondrial fraction was used as the negative control (Fig. 1F, lane 2).

Cellular localization of *Pb*MLS by confocal microscopy

To observe the cellular location of *Pb*MLS, *P. brasiliensis* yeast cells were grown in rich medium and visualized by laser confocal microscopy. The expression of *Pb*MLS was highly positive in the budding cells (Fig. 2 B, C and F), while was usually negative (Fig. 2 B and C) or weakly positive (Fig. 2 D) in the mother cells. Although the reactivity was evident inside the cytoplasm of budding cells, it was much more intense on the cell surface (Fig. 2 F). The patterns and intensities of fluorescence spectra of two regions of interest (ROI) were represented in the Figure 2 G.

The localization of *Pb*MLS was also evaluated on *P. brasiliensis* yeast cells grown in medium containing acetate or glucose as sole carbon source. Yeast cells accumulated *Pb*MLS in the presence of acetate (Fig. 3 B) or glucose (Fig. 3 D), but the quantity of *Pb*MLS was higher when the fungus was cultivated in the former condition. This disparity was exemplified by the fluorescence spectra (Fig. 3 E), representative of two ROIs indicated by the arrows 1 and 2 (Fig. 3 B and D). No cross reaction was observed with the pre-immune serum (data not shown).

Binding of *Pb*MLSr to extracellular matrix proteins (ECM) and the reactivity to sera of PCM patients

The ability of the *Pb*MLSr to bind to proteins of ECM was evaluated by Far-Western blot assays. *Pb*MLSr binds to fibronectin, type I and IV collagen, but not to laminin as shown on Fig. 4A, lanes 1, 2, 3 and 4, respectively). Negative controls were obtained incubating *Pb*MLSr with the secondary antibody in the absence of the ECM or *Pb*MLSr with the ECM only (Fig. 4A, lanes 5 and 6, respectively). The reaction between *Pb*MLSr and the antibody anti-*Pb*MLSr was used as a positive control (Fig. 4A, lane 7). The binding between *Pb*MLS and ECM compounds was also evaluated by ELISA assay. The results reinforced that *Pb*MLSr binds to fibronectin, type I and IV collagen (Fig. 4B). Negative controls were performed using *Pb*MLSr (Fig. 4B) or ECM only (data not shown). The positive control was performed using antibody anti-*Pb*MLSr, anti-laminin, anti-fibronectin, anti-colagen I or anti-colagen IV (data not shown).

The reactivity of *Pb*MLSr with three sera of patients with PCM and one serum from healthy individual were reacted in immunoblot assays (Fig. 4C). Strong reactivity was observed with PCM patients sera (Fig. 4C, lanes 1 to 3). No cross-reactivity was observed with control serum (Figure 4C, lane 4). Reaction between *Pb*MLSr and anti-*Pb*MLSr was used as positive control (Fig. 4C, lane 5).

Binding of *Pb*MLSr to pneumocytes

The ability of *Pb*MLSr binding to A549 cells was evaluated. *Pb*MLSr was biotinylated and incubated with A549 cells. After lyses, proteins from A549 cells were electrophoresed by SDS-PAGE, and blotted onto a membrane to perform Western blot with anti-*Pb*MLSr antibody. A positive signal was detected to lysed pulmonary A549 cells treated with biotinylated *Pb*MLSr (Fig. 4D, lane 1). The negative control was

obtained using supernatant of A549 cells untreated with biotinylated protein (Fig. 4D, lane 2).

Inhibitory effect of *Pb*MLSr and of anti-*Pb*MLSr antibody on interaction of *P. brasiliensis* cells with pneumocytes

The infection index was determined by interactions between *P. brasiliensis* yeast cells and A549 pneumocytes, as shown on Figure 5. *P. brasiliensis* yeast cells were treated with the anti-*Pb*MLSr antibody before interaction with pneumocytes, or pneumocytes were treated with *Pb*MLSr before interaction with *P. brasiliensis*. The controls (non treated cells) were used to calculate the percentages of total infection. The interaction was analyzed by flow cytometry. Ten thousand events as monoparametric histograms of log fluorescence, as well as list mode data files were collected to analysis. When *P. brasiliensis* yeast cells treated with antibody anti-*Pb*MLSr were incubated with A549 cells, a decrease in the infection was observed after 2 h and 5 h of incubation (Fig. 5A). Similarly, after treatment of A549 cells with *Pb*MLSr the infection was reduced after 2 h and 5 h of incubation when compared to the values of non treated cells (Fig. 5B). Controls were performed by incubating the pneumocytes with rabbit pre-immune serum or BSA before the addition of A549 cells or yeast cells (Fig. 5A and B, respectively).

Adherence of *P. brasiliensis* to pneumocytes cells

By confocal microscopy, applying antibody anti-*Pb*MLSr, it was verified the adherence capacity to human lung cells of *P. brasiliensis*, showing the participation of *Pb*MLS in this process (Fig. 6 panel, A and B).

Discussion

Our studies showed that *Pb*MLS is a multifunctional protein; besides its enzymatic role as described by Zambuzzi-Carvalho [30], it could participate in the adherence process between the fungus and host cells through its ability to bind fibronectin, type I and type IV collagen. *Pb*MLS was detected in the soluble fraction and culture filtrate of *P. brasiliensis*. Take together ours results suggested that *Pb*MLS is actively secreted by *P. brasiliensis*. In the same way, *M. tuberculosis* MLS has been consistently identified in the culture filtrates of mid-log phase *M. tuberculosis* cultures [32,33,34].

Adherence molecules are important in the pathogen-host interactions. They operate as intercellular adhesion molecules (ICAM) or substrate adhesion molecules (SAM), contributing to cell-cell or cell-ECM adherences, respectively, and are usually exposed on the cellular surface. Successful host tissue colonization by fungus is a complex event, generally involving a ligand (adhesin) encoded by the pathogen and a cell or ECM receptor. The pathogen could interact with three types of host component: secreted cell products, host cell surface, or ECM proteins, such as types I and IV collagen, fibronectin, fibrinogen, and laminin [35]. Here, the potential adhesin property of *Pb*MLS was demonstrated through Far-Western blot, ELISA and binding assays showing that the recombinant protein recognized the ECM proteins fibronectin and types I and IV collagen, as well as, pulmonary epithelial cells. This event indicates that *Pb*MLS can play role in the interaction of the fungus with host components. Studies have reported the capacity of *P. brasiliensis* for adhesion and invasion [9,15]. This is the first enzyme of the glyoxylate cycle to be identified on the fungal surface and released extracellularly which possesses the ability to bind to ECM proteins. The definition of *Pb*MLSr as a surface-exposed ECM-binding protein, with unknown

mechanism of secretion from the cell or of sorting proteins to cellular membrane suggests that the *Pb*MLSr is compatible with anchorless adhesions [36,20]. In these types of adhesions, proteins are reassociated on the cellular surface after been secreted to execute their biological functions [36]. The presence of the *Pb*MLS in the culture filtrate harvested at 24 and 36 h, 7 and 14 days of growth confirmed that it is truly a secreted protein. The presence of *Pb*MLS in SDS-extracted cell wall proteins fraction indicate that *Pb*MLS is associated with the cell surface through weak interactions. Take together these results provide evidence that *Pb*MLS may be transported out of the cell through the cell wall, to be localized on the outer surface of the cell.

Reports have described the presence of some enzymes of the glycolytic pathway on cell surface in *P. brasiliensis* as well as in other pathogens [42,43,18,19,16,17]. The presence of these housekeeping enzymes in unusual locations often correlates with their ability to perform alternative functions such as adherence/invasion of the host cells [43,18]. The ability of anti-adhesin antibodies to confer protection by blocking microbial attachment to host cells is being explored as a vaccination strategy in several microbial diseases [44-48]. The identification of the *Pb*MLS as a probable adhesin has several implications. Understanding the consequences of binding of the *Pb*MLS to the host cells will lead to improved understanding of the initial events during infection and further insights into the role of the *Pb*MLS in the host–pathogen interaction could contribute to design of novel therapeutic strategies for PCM control.

In *P. brasiliensis*, the ICL transcript (*Pbicl*) was up-regulated in yeast cells when compared to the mycelium phase and during the infectious process in mice liver [49,50]. In addition, *Pbicl* and *Pbmls* were up-regulated during the transition from mycelium to yeast [51,52] and in yeast cells upon internalization by murine macrophages [53]. The

results by confocal laser scanning microscopy demonstrated differences in the accumulation of *Pb*MLS among *P. brasiliensis* cells grown in different carbon sources. Accumulation of *Pb*MLS was also higher in *P. brasiliensis* yeast cells than in the mycelial fase (data not shown). These findings were reinforced by the results of Felipe *et al*. [49], which suggested that the glyoxylate cycle is up-regulated in yeast cells [54]. Yeast cells grown on potassium acetate accumulated more *Pb*MLS on the cell membrane than yeast cells grown on glucose. These results are in agreement with those obtained by Zambuzzi-Carvalho *et al*. [30] where *Pbmls* transcript level was higher in yeasts cells grown in two-carbon sources when compared to that one grown on glucose. The high intensity of ROI found in budding cells, mainly in the cellular membrane, suggests that the *Pb*MLS is more metabolically relevant and mainly synthesized by young cells (budding cells). It is unknown that *Pb*MLS takes some part in differentiation and/or maturity processes of *P. brasiliensis* budding cells [51, 55]. In fact, glyoxylate pathway provides a metabolic versatility for *Candida albicans* to utilize alternates substratum for development and differentiation, been involved in the formation of the filamentous state from the single cell state [23]. This process may help *Laccaria bicolor* grow toward the host with the aggressiveness required for mycorrhiza formation [56].

Conclusion

In conclusion, the results showed the presence of the *Pb*MLS in culture filtrate of yeast cells (parasitic phase), its surface location in *P. brasiliensis*, its binding to ECM in Far-Western blot and ELISA assays and to A549 cells membranes. The reduction in adherence of *P. brasiliensis* to A549 cells by anti-*Pb*MLSr suggests that the *Pb*MLS could contribute to active fungal interaction and disease progression in humans by its ability to act as a probable adhesin. In addition, the absence of conventional secretion or cell wall anchoring motifs defines *Pb*MLS as a probable anchorless adhesin that could contribute to virulence by promoting *P. brasiliensis* infection and dissemination.

Methods

P. brasiliensis isolate and growth conditions

The *P. brasiliensis Pb*01 isolate (ATCC-MYA-826) has been previously investigated by our laboratory and was cultivated in semisolid Fava Netto's medium (1.0% 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 glucose and 1.4% w/v agar, pH 7.2) as yeast cells for 7 days at 36°C.

Heterologous expression and purification of the *Pb*MLS recombinant (*Pb*MLSr)

The cDNA encoding to *Pb*MLS was obtained by Zambuzzi-Carvalho *et al*. [30] (GenBank accession number:AAQ75800). *Eco*RI and *Xho*I restriction sites were introduced in oligonucleotides to amplify a 1617 bp cDNA fragment of the *Pbmls*, which encodes a predicted protein of 539 amino acids. The PCR product was subcloned

into the *Eco*RI/*Xho*I sites of the pET-32a(+) expression vector (Novagen, Inc., Madison, Wis.). The resulting plasmid was transferred to *Escherichia coli* BL21 C41 (DE3). Bacteria transformed with the pET-32a-MLS were grown in LB medium supplemented with ampicillim (100 μ/mL) at 37°C until reach the optical density of 0.6 at 600 nm. Synthesis of the recombinant protein was then initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO) to a final concentration of 1 mM to the growing culture and the bacterial extract was pelleted and resuspended in phosphate buffered saline (1 x PBS). After induction, the cells were incubated for 2 h at 37°C with shaking at 200 rpm. Cells were harvested by centrifugation at 10,000 x *g* for 5 min at 4°C. The supernatant was discarded and the cells were resuspended in 1 x PBS buffer. *E coli* cells were incubated for 60 min with lysozyme (100 μg/mL). After addition of 1% v/v Sarcosyl at 4°C, the cells were lysed by extensive sonication. The sample was centrifuged 8,000 x *g* for 15 min at 4°C and 2% v/v Triton was added to the supernatant containing the soluble protein fraction. His-tagged *Pb*MLSr was purified by using the Ni-NTA Spin Kit (Qiagen Inc., Germantown, MD) and the tags were subsequently removed by the addition of EKMax™ Enterokinase (GIBCO™, Invitrogen, Carlsbard, CA).

Antibody production

The purified *Pb*MLSr was used to produce anti-*Pb*MLSr polyclonal antibodies in New Zealand rabbits. The immunization protocol consisted of an initial injection of 300 μg of purified recombinant protein in complete Freund´s adjuvant and two subsequent injections of the same amount of the antigen in incomplete Freund´s adjuvant. Each immunization was followed by an interval of 14 days. After the fourth immunization,

the serum containing the anti-*Pb*MLSr polyclonal antibody was collected and stored at -20ºC.

Western blotting analysis

SDS–PAGE was performed in 12% polyacrylamide gels according to [57]. The proteins were electrophoresed and stained with Coomassie brilliant blue or transferred to a nylon membrane and checked with Ponceau S to determine equal loading. *Pb*MLS, as well as the *Pb*MLSr, were detected with the polyclonal antibody raised against the recombinant protein (diluted 1: 4000). After reaction with alkaline phosphatase anti-mouse immunoglobulin G (IgG) or alkaline phosphatase anti-human IgG, the reaction was developed with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP–NBT).

Cell wall protein extractions

Yeast cells were frozen in liquid nitrogen and disrupted using a pestle and mortar. The procedure was carried out until complete cell rupture, verified by microscopic analysis, and by the failure of cells to grow on Fava Netto's medium. Ground material was lyophilized, and resuspended in 25 μL Tris buffer (50 mM Tris–HCl, pH 7.8) for each milligram of dry weight, as described by [58]. The supernatant was separated from the cell wall fraction by centrifugation at 10,000 × *g* for 10 min at 4°C. The crude extract was kept and a new protein extraction was performed with Tris buffer as described above. The cell wall was extensively washed in solutions with decreasing concentrations of 1 M NaCl to remove any extracellular or cytosolic protein contaminants that could be adhered to the walls through electrostatic forces. Isolated cell walls were treated with SDS–extraction buffer (50 mM Tris–HCl, pH 7.8, 2% w/v

SDS, 100 mM Na–EDTA, and 40 mM β-mercaptoethanol) to extract cell surface-associated proteins, i.e. proteins loosely associated with the cell surface through non-covalent interactions or disulfide bridges (SDS-SW). The proteins from cell wall and from crude extract were quantified according to Bradford [59].

Preparation of culture filtrate proteins

The culture filtrate were processed as described previously [60], with modifications. Briefly, after 24 and 36 h, 7 and 14 days of growth at 37°C with gentle agitation, the culture supernatant were removed from the cells by filtration and the culture filtrate were dialyzed and dried by lyophilization. The protein content of the concentrated culture filtrate was quantified according to Bradford [59].

Preparation of Peroxisomal Fraction

The Peroxisome Isolation Kit (Sigma-Aldrich) was used in the Preparation of Crude Peroxisomal Fraction from cell cultures *P. brasiliensis Pb*01 (~2 x 10^8 cells) by differential centrifugation followed by density gradient centrifugation. Briefly, spheroplasts were obtained at 30°C by lysing the cell wall in 400 U of lyticase (Sigma) for 24 h. Spheroplasts membranes were disrupted using grinder and pestle. After centrifugation for 10 min, the Crude Peroxisomal Fraction was obtained. The organelles were isolated by density gradient centrifugation to get the enriched peroxisomes fraction separated from purified mitochondrial fraction by using the Peroxisome Isolation Kit.

The presence of peroxisomes was determined by measuring the activity of the peroxisomal enzyme marker, catalase (Catalase Assay Kit) (Sigma-Aldrich). Separation

of peroxisomes from mitochondria was determined by measuring the activity of the mitochondrial enzyme marker, cytochrome c oxidase (Cytochrome c Oxidase Assay Kit) (Sigma-Aldrich). In addition, the detection of peroxisomal membrane proteins and their degree of enrichment in the purified fraction was performed by immunoblot using anti-*Pb*MLSr.

Affinity ligand assays

Far-Western blot assays were carried out as previously described [61]. *Pb*MLSr was submitted to SDS-PAGE and blotted onto nylon membrane. Blotted protein was assayed for laminin, fibronectin, type I and type IV collagen, or to PCM patients sera as following. After block for 4 h with 1.5% (w/v) BSA in 10 mM PBS-milk and then washed three times (for 10 min each time) in 10 mM PBS-T, the membranes were incubated with laminin (20 μg/mL), fibronectin (20 μg/mL), or type I and IVcollagen (30 μg/mL), diluted in PBS-T with 2 % BSA for 90 min, and then washed three times (for 10 min each time) in PBS-T. The membranes were incubated for 18 h with rabbit antibodies anti-laminin, anti-fibronectin, or anti-type I and IV collagen in PBS-T with 2 % BSA (diluted 1:100). The blots were washed with PBS-T and incubated with peroxidase-labeled goat anti-rabbit immunoglobulin (diluted 1:1000). The blots were washed with PBS-T, and the reactive signals were developed with hydrogen peroxide and diaminobenzidine (Sigma-Aldrich) as the chromogenic reagent. The positive control was obtained by incubating the *Pb*MLSr with the polyclonal anti-*Pb*MLSr antibody (diluted 1:500), and the reaction was developed as described above.

ELISA analysis

ELISA was accomplished as previously described by Mendes-Giannini *et al*. [8] with modifications. Briefly, Polypropylene 96-well microtiter ELISA plates were sensitized with extracellular matrix (ECM) proteins (10 μg/mL), overnight at 4°C. After blocking with 2% w/v BSA, 10% v/v SFB and 1% w/v milk, the incubation was followed with *Pb*MLSr (5 μg/mL) for 2 h at 37°C in triplicate wells. The reaction was developed using buffer citrate pH 4.9 conjugated with o-phenylenediamine as chromogenic substrate. Negative controls were performed using *Pb*MLSr or ECM only. Positive controls were performed using antibody anti-*Pb*MLSr, anti-laminin, anti-fibronectin, anti-colagen I or anti-colagen IV. The absorbance was measured at 490 nm and the results analyzed by using Software Microcal ™ Origin ™ Version 5.0 Copyright© [62].

Inhibition assay of interaction of *P. brasiliensis* with epithelial cells by using *Pb*MLSr and antibody anti-*Pb*MLSr

A549 pneumocytes were incubated for 1 h at 37°C with *Pb*MLSr (50 μg/mL), diluted in 10 mM PBS. After this incubation period, the cells were washed three times in PBS and 10^6 yeast forms of *P. brasiliensis* were added. Incubation was performed for 2 and 5 h at 37°C, to allow the invasion and internalization, respectively, as described previously [9,15,13]. Four control experiments were performed: A549 cells not preincubated with the *Pb*MLSr; *P. brasiliensis* yeast cells not preincubated with the anti-*Pb*MLSr antibody; pneumocytes preincubated with BSA (25 μg/mL) and *P. brasiliensis* yeast cells preincubated with rabbit pre-immune serum. The percentage of infected cells, or not, was obtained by flow cytometry (BD FACSCanto) (BD Biosciences, Hialeah, FL). Adhesion index was achieved by multiplying the mean number of attached yeast cells per pneumocyte by the percentage of infected cells. The infection index (adherence plus

internalization) was determined by the number of total fungi interacting with the epithelial cells 5 h after addition of the yeast cells, as previously described [15,13]. The mean and S.D. of at least three independent experiments were determined. Statistical analysis was calculated by using ANOVA (*F* test followed by Duncan test). *P* values of 0.05 or less were considered statistically significant.

Biotinylation of protein

*Pb*MLSr was biotinylated with the ECL protein biotinylation kit (GE Healthcare, Amersham Biosciences) as recommended by the manufacturer. Monolayers of A549 cells were incubated with the biotinylated proteins at 37°C overnight and washed with PBS, to remove unbound protein. Next, double-distilled water was added and the cells were incubated for 4 h at 25°C, to obtain total lysis. The lysates were centrifuged at 1,400 × *g* for 5 min, and the supernatant was submitted to electrophoresis by SDS-PAGE. Proteins in the gel were blotted onto a membrane of nylon; membrane strips were incubated with blocking buffer for 4 h at 25°C. Then was incubated for 1h with streptavidin-HRP. Control containing *Pb*MLSr was revealed with the Catalyzed Signal Amplification (CSA) System kit (DAKO). The negative control was developed with the supernatant of A549 cells after lyses (without incubation with the biotinylated protein).

Confocal analysis

The cellular localization of the *Pb*MLS was performed as described by Batista *et al*. [63] and Lenzi *et al*. [64] for confocal laser scanning microscopy (CLSM). Briefly, the cells growing in different sources of carbon were fixed in paraformaldeído 4% for 1h, washed and centrifuged. After permeabilization with Triton X-100, the cells were washed in PBS and incubated in blocking solution (2.5% BSA, 1% skim milk, 8% fetal

calf serum) for 20 min (Fernandes da Silva, 1988). The primary antibody anti-*Pb*MLSr diluted (1:100) was added overnight at 4°C. After washing three times with PBS, the cells were incubated with secondary antibody (Alexa Fluor 488 anti-rabbit Molecular Probes 1:700) for 1 hour. Before mounting in 90% glycerol in PBS, adjusted to pH 8.5, containing antifading agent (p-phenylenediamine 1 g/L) (Sigma-Aldrich), the cells were stained with Evans blue (1/10000 in 0.01M PBS). The specimens were analyzed by laser confocal microscopy (LSM 510-META, Zeiss).

Cultive of cells A549 in lamínula and adhesion of *P. brasiliensis* by confocal analysis

The adhesion total (infection and invasion) assays were accomplished in plates of 24 holes containing coverslipes at the bottom. In all the tests, a cellular suspension of 10^6 cells/mL was standardized. After the tripsinization of the cell suspension, 0,2 mL of this was retreat of the bottle, and diluted in 1,8 mL of HAM F12 medium. Cells were counted in hemocytometer after several dilutions until the definition of appropriate concentration. After, 0,5 mL of the adjusted concentration of cells was spared in each hole of the plates and incubated at 36.5°C for 24 h. The cellular monolayers formed on the coverslips were used for adhesion, invasion and total infection tests. PBS was poured in the wells, and 300 µL of 0.5% Triton in PBS (PBS-T) was used to permeabilize the cells for 10 min at room temperature. After three washings with frozen PBS-T, the monolayers were incubated for 1 hour with 300 µL antibody anti-*Pb*MLSr (1/50 in PBS + 3% skimmed milk + 1%BSA). Three washes with frozen PBS-T were again done, and subsequently the monolayers were incubated with 300 µL of TRITC-rabbit antibody and mouse anti-IgG (1/400) for 1 hour, followed by new three washes with frozen PBS-T.

Flow cytometry assay analysis

All flow cytometry analyses were performed on a BD FACSCanto (BD Biosciences) using an air-cooled argon-ion laser tuned at 488 nm and 115 mW. The flow rate was kept at approximately 10,000 events (cells), and green fluorescence was amplified logarithmically. Ten thousand events were collected as monoparametric histograms of log fluorescence, as well as list mode data files. The data were analyzed by FACSDiva Software (BD Biosciences) and Origin Software [61].

Statistical analysis

Results are expressed as the mean ± SE of the mean of three independent experiments. Statistical analysis was performed using ANOVA (F-test followed by Duncan test). P-values of 0.05 or less were considered statistically significant.

Author´s contributions

BRSN realized all assays. JFS and MJSMG participated of adhesion and infection assays. HLL participated of confocal assays. BRSN, MJSMG, HLL, CMAS and MP contributed to the preparation of the manuscript. MP conceived, design and coordinated the study. All authors contributed to discussion of results. All the authors have read and approved the final manuscript.

Acknowledgements

This work at Universidade Federal de Goiás was supported by grants from Ministério de Ciência e Tecnologia (MCT), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), and by International Foundation for Science (IFS), Stockholm, Sweden, through a grant to Maristela Pereira. B.R.S.N. was supported by a fellowship from Coordenação de Aperfeiçoamento de Ensino Superior (CAPES).

References

1. Rippon JW: **Dimorphism in pathogenic fungi.** *Crit Ver Microbiol* 1980, **8**:49-97.

2. Brummer E, Castaneda E, Restrepo A: **Paracoccidioidomycosis: an update.** *Clin Microbiol Microbiol* 1993, **6:**89-117.

3. Pina, A, Bernadino S, Calich VLG: **Alveolar macrophages from susceptible mice are more competent than those of resistant mice to control initial *Paracoccidioides brasiliensis* infection.** *J Leukoc Biol* 2008, **83:**1088-99.

4. Tart RC, Van IR: **Identification of the surface component of *Streptococcus defectivus* that mediates extracellular matrix adherence**. *Infect Immun* 1993, **61:**4994-5000.

5. Li F, Palecek SP: **Distinct domains of the *Candida albicans* adhesin Eap1p mediate cell–cell and cell–substrate interactions.** *Microbiol* 2008, **154:**1193-1203.

6. McMahon JP, Wheat J, Sobel ME, Pasula R, Downing JF, Martin WJ: **Laminin Binds to *Histoplasma capsulatum* A Possible Mechanism of Dissemination.** *J Clin Invest* 1995, **96:**1010-1017.

7. Paris S, Boisvieux-Ulrich E, Crestani B, Houcine O, Taramelli D, Lombardi L, Latgé JP: **Internalization of *Aspergillus fumigatus* conidia by epithelial and endothelial cells.** *Infec Immun* 1997, **4:**1510-1514.

8. Mendes-Giannini MJ, Andreotti PF, Vincenzi LR, Monteiro Da Silva JL, Lenzi HL, Benard G, Zancope- Oliveira R, De Matos Guedes HL, Soares CP: **Binding of extracellular matrix proteins to *Paracoccidioides brasiliensis.*** *Microb Infect* 2006, **8:**1550-9.

9. Andreotti PF, Monteiro Da Silva JL, Bailão AM, Soares CM, Benard G, Soares CP, Mendes- Giannini MJ: **Isolation and partial characterization of a 30 kDa adhesin from *Paracoccidioides brasiliensis*.** *Microb Infect* 2005, **7:**875-81.

10. Vicentini AP, Gesztesi JL, Franco MF, De Souza W, De Moraes JZ, Travassos LR : **Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis.** *Infect Immun* 1994, **62:**1465-9.

11. Dranginis AM, Rauceo JM, Coronado JE, Lipke PN: **A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions.** *Microbiol Mol Biol Rev* 2007, **71:**282-94.

12. Gonzalez A, Lenzi HL, Motta EM, Caputo L, Sahaza JH, Cock AM, Ruiz AC, Restrepo A, Cano LE: **Expression of adhesion molecules in lungs of mice infected with *Paracoccidioides brasiliensis* conidia.** *Microb Infect* 2005, **7:**666-73.

13. Hanna SA, Monteiro da Silva JL, Mendes-Giannini MJ: **Adherence and intracellular parasitism of *Paracoccidioides brasiliensis* in Vero cells.** *Microbes Infect* 2000, **2:**877-84.

14. Mendes-Giannini MJS, Taylor ML, Bouchara JB, Burger E, Calich VLG, Escalante ED: **Pathogenesis II: fungal responses to host responses: interaction of host cells with fungi.** *Med Mycol* 2000, **38:**113-23.

15. Mendes-Giannini MJ, Hanna SA, da Silva JL, Andreotti PF, Vincenzi LR, Benard G, Lenzi HL, Soares CP: **Invasion of epithelial mammalian cells by *Paracoccidioides brasiliensis* leads to cytoskeletal rearrangement and apoptosis of the host cell.** *Microbes Infect* 2004, **6:**882-891.

16. Barbosa MS, Bao SN, Andreotti PF, de Faria FP, Felipe MS, dos Santos Feitosa L, Mendes-Giannini MJ, Soares CM: **Glyceraldehyde 3-phosphate dehydrogenase of *Paracoccidioides brasiliensis* is a cell surface protein, involved in fungal adhesion to extracellular matrix proteins and interaction with cells.** *Infect Immun* 2006, **74:**382-389.

17. Pereira LA, Báo SN, Barbosa MS, da Silva JL, Felipe MS, Santana JM, Mendes-Giannini MJ, de Almeida Soares CM: **Analysis of the *Paracoccidioides brasiliensis* Triosephosphate Isomerase suggests the potential for adhesin function.** *FEMS Yeast Res* 2007, **7:**1381-1388.

18. Pancholi V, Chhatwal GS: **Housekeeping enzymes as virulence factors for pathogens.** *Int J Med Microbiol* 2003, **293:**391-401.

19. Ling E, Feldman G, Portnoi M, Dagan R, Overweg K, Mulholland F, Chalifa-Caspi V, Wells J, Mizrachi-Nebenzahl Y: **Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse.** *Clin Exp Immunol* 2004, **138:**290-298.

20. Kinhikar AG, Vargas D, Li H, Mahaffey SB, Hinds L, Belisle JT, Laal S: ***Mycobacterium tuberculosis* malate synthase is a laminin-binding adhesin.** *Mol Microbiol* 2006, **60:**999-1013.

21. Olivas I, Royuela M, Romero B, Monteiro MC, Mínguez JM, Laborda F, De Lucas JR: **Ability to grow on lipids accounts for the fully virulent phenotype in neutropenic mice of *Aspergillus fumigatus* null mutants in the key glyoxylate cycle enzymes.** *Fungal Genet Biol* 2007, **45:**45-60.

22. Rude TH, Toffaletti DL, Cox G, Perfect JR: **Relatioship of the glyoxylate pathway to the pathogenesis of *Cryptococcus neoformas*.** *Infect Immun* 2002, **70:**5684-5694.

23. Lorenz MC, Fink GR: **The glyoxylate cycle is required for fungal virulence.** *Nature* 2001, **412:**83-86.

24. Lorenz MC, Fink GR: **Life and death in a macrophage: Role oh the glyoxylate cycle in virulence.** *Eukaryot Cell* 2002, **1:**657-662.

25. McKinney JD, Höner zu Bentrup K, Muñoz-Elías EJ, Miczak A, Chen B, Chan WT, Swenson D, Sacchettini JC, Jacobs WR Jr, Russell DG: **Persistence of *Mycobacterium***

tuberculosis **in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase**. *Nature* 2000, **406:**735-8.

26. Muñoz-Elías EJ, McKinney JD: ***Mycobacterium tuberculosis*** **isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence.** *Nat Med* 2005, **11:**638-44.

27. Gould TA, van de Langemheen H, Muñoz-Elías EJ, McKinney JD, Sacchettini JC: **Dual role of isocitrate lyase 1 in the glyoxylate and methylcitrate cycles in *Mycobacterium tuberculosis*.** *Mol Microbiol* 2006, **61:**940-7.

28. Wang ZX, Brämer CO, Steinbüchel A: **The glyoxylate bypass of *Ralstonia eutropha*.** *FEMS Microbiol Lett* 2003, **228:**63-71.

29. Solomon PS, Lee RC, Greer Wilson TJ, Oliver RP: **Pathogenicity of *Stagonospora nodorum* requires malate synthase.** *Mol Microbiol* 2004, **53:**1065-1073.

30. Zambuzzi-Carvalho PF, Cruz AHS, Santos-Silva LK, Goes AM, Soares CM, Pereira M: **The malate synthase of *Paracoccidioides brasiliensis* is required in the glyoxylate cycle and in the allantoin degradation pathway.** *Med Mycol* 2009, **4:**1-12.

31. Gould SJ, Keller GA, Subramani S: **Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase.** *J Cell Biol* 1987, **105:**2923-2931.

32. Laal S, Samanich KM, Sonnenberg MG, Zolla-Pazner S, Phadtare JM, Belisle, JT: **Human humoral responses to antigens of *Mycobacterium tuberculosis*: immunodominance of high-molecular-mass antigens.** *Clin Diagn Lab Immunol* 1997, **4:**49-56.

33. Sonnenberg MG, Belisle JT: **Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, Nterminal amino acid sequencing and electrospray mass spectrometry.** *Infect Immun* 1997, **65:**4515-4524.

34. Samanich K, Belisle JT, Laal S: **Homogeneity of antibody responses in tuberculosis patients.** *Infect Immun* 2001, **69:**4600-4609.

35. Mendes-Giannini MJ, Monteiro da Silva JL, de Fátima da Silva J, Donofrio FC, Miranda ET, Andreotti PF, Soares CP: **Interactions of *Paracoccidioides brasiliensis* with host cells: recent advances.** *Mycopathologia* 2008, **165:**237-248.

36. Chhatwal GS: **Anchorless adhesins and invasins of Gram-positive bacteria: a new class of virulence factors.** *Trends Microbiol* 2002, **10:**205-8.

37. Johnson TL, Olsen LJ: **Building new models for peroxisome biogenesis.** *Plant Physiol* 2001, **127:**731-9.

38. Eckert JH, Erdmann R: **Peroxisome biogenesis.** *Rev Physiol Biochem Pharmacol* 2003, **147:**75-121.

39. Schliebs W, Kunau WH: **Peroxisome membrane biogenesis: the stage is set.** *CurrBiol* 2004, **14:**397-9.

40. Baker A, Sparkes IA: **Peroxisome protein import: some answers, more questions.** *Curr Opin Plant Biol* 2005, **8:**640-7.

41. Heiland I, Erdmann R: **Biogenesis of peroxisomes. Topogenesis of the peroxisomal membrane and matrix proteins.** *FEBS J* 2005, **272:**2362-72.

42. Bergmann S, Rohde M, Chhatwal GS, Hammerschmidt S: **Alpha-enolase of *Streptococcus pneumoniae* is a plasmin (ogen)-binding protein displayed on the bacterial cell surface**. *Mol Microbiol* 2001, **40:**1273-1287.

43. Dallo SF, Kannan TR, Blaylock MW, Baseman JB: **Elongation factor Tu and E1 beta subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in *Mycoplasma pneumoniae*.** *Mol Microbiol* 2002, **46:**1041-1051.

44. Rennermalm A, Li YH, Bohaufs L, Jarstrand C, Brauner A, Brennan FR, Flock JI: **Antibodies against a truncated *Staphylococcus aureus* fibronectinbinding protein protect against dissemination of infection in the rat.** *Vaccine* 2001, **19:**3376-3383.

45. Ofek I, Hasty DL, Sharon N: **Anti-adhesion therapy of bacterial diseases: prospects and problems.** *FEMS Immunol Med Microbiol* 2003, **38:**181-191.

46. Vidal Pessolani MC, Marques MA, Reddy VM, Locht C, Menozzi FD: **Systemic dissemination in tuberculosis and leprosy: do mycobacterial adhesins play a role?** *Microbes Infect* 2003, **5:**677-684.

47. Carvalho HM, Teel LD, Kokai-Kun JF, O'Brien AD: **Antibody against the carboxyl terminus of intimin alpha reduces enteropathogenic *Escherichia coli* adherence to tissue culture cells and subsequent induction of actin polymerization.** *Infect Immun* 2005, **73:**2541-2546.

48. Williams A, Reljic R, Naylor I, Clark SO, Falero-Diaz G, Singh M, Challacombe S, Marsh PD, Ivanyi J: **Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs.** *Immunology* 2004, **111:**328-333.

49. Felipe MS, Andrade RV, Arraes FB, Nicola AM, Maranhão AQ, Torres FA, Silva-Pereira I, Poças-Fonseca MJ, Campos EG, Moraes LM, Andrade PA, Tavares AH, Silva SS, Kyaw CM, Souza DP, Pereira M, Jesuíno RS, Andrade EV, Parente JA, Oliveira GS, Barbosa MS, Martins NF, Fachin AL, Cardoso RS, Passos GA, Almeida NF, Walter ME, Soares CM, Carvalho MJ, Brígido MM, PbGenome Network: **Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells.** *J Biol Chem* 2005, **280:**24706-24714.

50. Costa M, Borges CL, Bailão AM, Meirelles GV, Mendonça YA, Dantas SF, de Faria FP, Felipe MS, Molinari-Madlum EE, Mendes-Giannini MJ, Fiuza RB, Martins WS, Pereira M, Soares CM: **Transcriptome profiling of *Paracoccidioides brasiliensis***

yeast-phase cells recovered from infected mice brings new insights into fungal response upon host interaction. *Microbyology* 2007, **153:**4194-4207.

51. Bastos KP, Bailão AM, Borges CL, Faria FP, Felipe MS, Silva MG, Martins WS, Fiúza RB, Pereira M, Soares CM: **The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals novel and induced genes potentially associated to the dimorphic process**. *BMC Microbiol* 2007, **10:**7-29.

52. Nunes LR, Costa de Oliveira R, Leite DB, da Silva VS, dos Reis Marques E, da Silva Ferreira ME, Ribeiro DC, de Souza Bernardes LA, Goldman MH, Puccia R, Travassos LR, Batista WL, Nóbrega MP, Nóbrega FG, Yang DY, de Bragança Pereira CA, Goldman GH: **Transcriptome analysis of *Paracoccidioides brasiliensis* cells undergoing mycelium-to-yeast transition.** *Eukaryot Cell* 2005, **4:**2115-2128.

53. Derengowski LS, Tavares AH, Silva S, Procópio LS, Felipe MSS, Silva-Pereira I: **Upregulation of glyoxylate cycle genes upon *Paracoccidioides brasiliensis* internalization by murine macrophages and *in vitro* nutritional stress condition.** *Med Mycol* 2008, **46:**125-134.

54. Monteiro JP, Clemons KV, Mirels LF, Coller JA, Wu TD, Shankar J, Lopes CR, Stevens DA: **Genomic DNA microarray comparison of gene expression patterns in *Paracoccidioides brasiliensis* mycelia and yeasts *in vitro*.** *Microbiology, i*n press.

55. Goldman GH, dos Reis Marques E, Duarte Ribeiro DC, de Souza Bernardes LA, Quiapin AC, Vitorelli PM, Savoldi M, Semighini CP, de Oliveira RC, Nunes LR, Travassos LR, Puccia R, Batista WL, Ferreira LE, Moreira JC, Bogossian AP, Tekaia F, Nobrega MP, Nobrega FG, Goldman MH**: Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of**

putative homologues of *Candida albicans* virulence and pathogenicity genes. *Eukaryot Cell* 2003, **2:** 34-48.

56. Balasubramanian S, Kim S-J, Podila GP: **Differential expression of malate synthase gene during the preinfection stage of symbiosis in the ectomycorrhizal fungus *Laccaria bicolor*.** *New Phytol* 2002, **154:**517-527.

57. Laemmli UK: **Cleavage of structural proteins during the assembly of head of bacteriophage T_4.** *Nature* 1970, **2227:**680-685.

58. Damveld RA, Arentshorst M, VanKuyk PA, Klis FM, van den Hondel CA, Ram AF: **Characterization of CwpA, a putative glycosylphosphatidylinositolanchored cell wall mannoprotein in the filamentous fungus *Aspergillus niger*.** *Fungal Genet Biol* 2005, **42:**873-885.

59. Bradford MM: **A dye binding assay for protein**. *Anal Biochem* 1976, **72:**248-254.

60. Singh KK, Dong Y, Belisle JT, Harder J, Arora VK, Laal S: **Antigens of *Mycobacterium tuberculosis* recognized by antibodies during incipient, subclinical tuberculosis**. *Clin Diagn Lab Immunol* 2005, **12:**354-358.

61. Guichet A, Copeland JW, Erdelyi M, Hlousek D, Zavorszky P, Ho J, Brown S, Percival-Smith A, Krause HM, Ephrussi A: **The nuclear receptor homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors.** *Nature* 1997, **385:**548-552.

62. **MICROCAL ITC** Data Analysis in Origin^R. Tutorial Guide. 5.0; MicroCal: 1998.[http://www.microcalorimetry.com].

63. Batista WL, Matsuo AL, Ganiko L, Barros TF, Veiga TR, Freymüller E, Puccia R: **The *PbMDJ1* gene belongs to a conserved *MDJ1/LON* locus in thermodimorphic pathogenic fungi and encodes a heat shock protein that localizes to both the**

mitochondria and cell wall of *Paracoccidioides brasiliensis*. *Eukaryot Cell* 2006, **5:**379-390.

64. Lenzi HL, Pelajo-Machado M, Vale BS, Panasco MS: **Microscopia de Varredura Laser Confocal: Princípios e Aplicações Biomédicas.** *Newslab* 1996, **16:**62-71.

Figures Legends

Figure 1: Localization of *Pb*MLSr. **(A)** SDS-PAGE analysis of *Pb*MLSr. *E. coli* BL21 C41 cells harboring the pET-32a-MLS plasmid were grown at 37°C to an OD_{600} of 0.6 and harvested before (lane 1) and after induction with 1 mM IPTG (lane 2). The cells were lysed by sonication, and the recombinant His-, Trx-, and S-Tagged *Pb*MLS was isolated by affinity chromatography (lane 3). Tags were removed by EKMax™ Enterokinase digestion (lane 4). **(B)** Western blots of fusion *Pb*MLSr (lane 1), cleaved *Pb*MLSr (lane 2), crude extract proteins from yeast cells (lane 3), SDS-extracted yeast cell wall proteins (lane 4), and yeast cell wall proteins (lane 5). Proteins were probed with anti-*Pb*MLSr antibody or with pre-immune rabbit **(C)**. **(D)** Western blots of proteins of culture filtrate of *P. brasiliensis* yeast cells harvested on 24 h (lane 1), 36 h (lane 2), 7 days (lane 3), and 14 days (lane 4) of culture, and culture filtrate without *P. brasiliensis* as negative control (lane 5). Proteins were probed with anti-*Pb*MLSr antibody or with pre-immune rabbit **(E)**. **(F)** Western blots of peroxisomal fraction (lane 1) and mitochondrial fraction (lane 2) proteins from *P. brasiliensis* yeast cells were probed with anti-*Pb*MLSr antibody. Molecular mass markers are indicated at the side.

Figure 2: Localization of *Pb*MLS by confocal laser scanning microscopy in *P. brasiliensis* yeast cells. Differential accumulation of *Pb*MLS on surface of budding cells is easily seen in **B, C** and **F**. The images **A** and **E** represent the differential interference contrast (DIC) of **B** and **F** images, respectively. The image **C** corresponds to a three-dimensional reconstruction of immunofluorescent tomographic image, showing the accumulation of *Pb*MLS only on the budding cells and not in the mother, as is also observed in images **B** and **F**. The image **G** displays the fluorescence pattern and intensity of two regions of interest (ROI) specified by the arrows 1 and 2 in the image

F, indicating that the fluorescence is more intense on the cell surface (2) than in the cytoplasm of budding cells (1). The image **D** shows a mother cell positive to *Pb*MLS on the cellular surface and the formation, in culture, of budding cells also expressing *Pb*MLS.

Figure 3: Localization of *Pb*MLS by confocal laser scanning microscopy in *P. brasiliensis* yeast cells growing in different carbon sources. The same groups of cells grown in the presence of potassium acetate (images **A** and **B**) or glucose (imagens **C** and **D**) as sole carbon source are shown, side by side, by differential interference contrast microscopy (DIC) and confocal immunofluorescence. In both situations, the accumulation of *Pb*MLS was restricted to the budding cells. The graphic in **E** displays, comparatively, the immunofluorescence patterns and intensities of two regions of interest (ROI 1 and 2), corresponding to the arrows 1 and 2. The date indicate that, in the same labeling conditions, the budding cells cultivated on potassium acetate accumulate *Pb*MLS more intensely on the cell surface than those grown on glucose.

Figure 4: **(A)** Binding of *Pb*MLSr to ECM by Far-Western blot. *Pb*MLSr (0.5 μg) was subjected to SDS-PAGE and electroblotted. Membranes were reacted with fibronectin (lane 1) and types I and IV collagen (lanes 2 and 3, respectively) and laminin (lane 4), and subsequently incubated with rabbit IgG anti-fibronectin, mouse anti-type I and anti-type IV collagen antibodies, and anti-laminin, respectively. Peroxidase-conjugated anti-rabbit and anti-mouse IgG reveal the reactions. Negative control was obtained by incubating the *Pb*MLSr with peroxidase-conjugated anti-rabbit IgG (lane 5), and *Pb*MLSr with ECM (lane 6). Positive control was obtained by incubating the *Pb*MLSr with polyclonal anti-*Pb*MLSr antibody (lane 7). **(B)** Binding of *Pb*MLSr to ECM

fibronectin, types I and IV collagen (10 μg/mL). The interaction was revealed by ELISA with peroxidase-conjugated streptavidin. The results were expressed in absorbance units. The negative controls were performed using *Pb*MLSr only. **(C)** Reactivity of the *Pb*MLSr to PCM patient sera. 1.0 μg of purified *Pb*MLSr was electrophoresed and reacted to patients sera with PCM, diluted 1:100 (lanes 1 to 3) and to control sera, diluted 1:100 (lane 4). The positive control was obtained by incubating the *Pb*MLSr with its polyclonal antibody (lane 5). After reaction to the anti-human IgG alkaline phosphatase-coupled antibody (diluted 1:2000), the reaction was developed with BCIP–NBT. **(D)** Biotinylation assay by Western blot. Lysed A549 cells incubated with biotinylated *Pb*MLSr (lane 1); Lysed A549 cells (lane 2) as negative control.

Figure 5: Interaction of *P. brasiliensis* yeast forms with pneumocytes. The interaction was assayed by indirect immunofluorescence and analyzed by flow cytometry. **(A)** *P. brasiliensis* yeast cells were pretreated for 1 h with anti-*Pb*MLSr polyclonal antibody (diluted 1:100), and control cells were pretreated with rabbit pre-immune serum. **(B)** A549 cells were pretreated for 1 h with 25 μg/mL of *Pb*MLSr, and control pneumocytes were pretreated for 1 h with 25 μg/mL of BSA. Adhesion of *P. brasiliensis* to pneumocytes was analyzed 2 h after the treatments. Infection (adhesion plus internalization) of *P. brasiliensis* to pneumocytes was analyzed 5 h after the treatments.

Figure 6: Interaction of *P. brasiliensis* yeast forms with pneumocytes. The interaction was assayed by confocal laser scanning microscopy. A549 cells were pretreated for 1 h anti-*Pb*MLSr. The arrows indicate the presence of the protein *Pb*MLS of the pathogen in interaction with A549 surface.

FIGURE 1

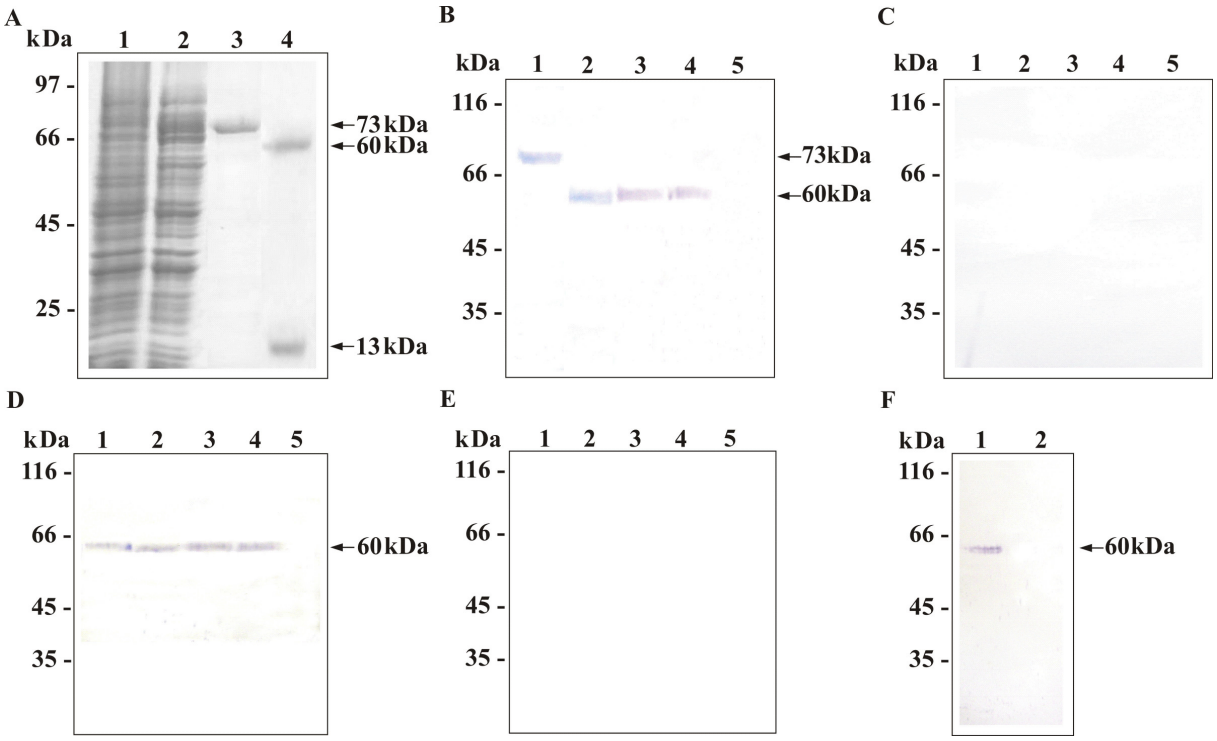

A
kDa 1 2 3 4
97 -
66 -
45 -
25 -
←73 kDa
←60 kDa
←13 kDa
B
kDa 1 2 3 4 5
116 -
66 -
45 -
35 -
←73kDa
←60kDa
C
kDa 1 2 3 4 5
116 -
66 -
45 -
35 -
D
kDa 1 2 3 4 5
116 -
66 -
45 -
35 -
←60 kDa
E
kDa 1 2 3 4 5
116 -
66 -
45 -
35 -
F
kDa 1 2
116 -
66 -
45 -
35 -
←60kDa

FIGURE 2

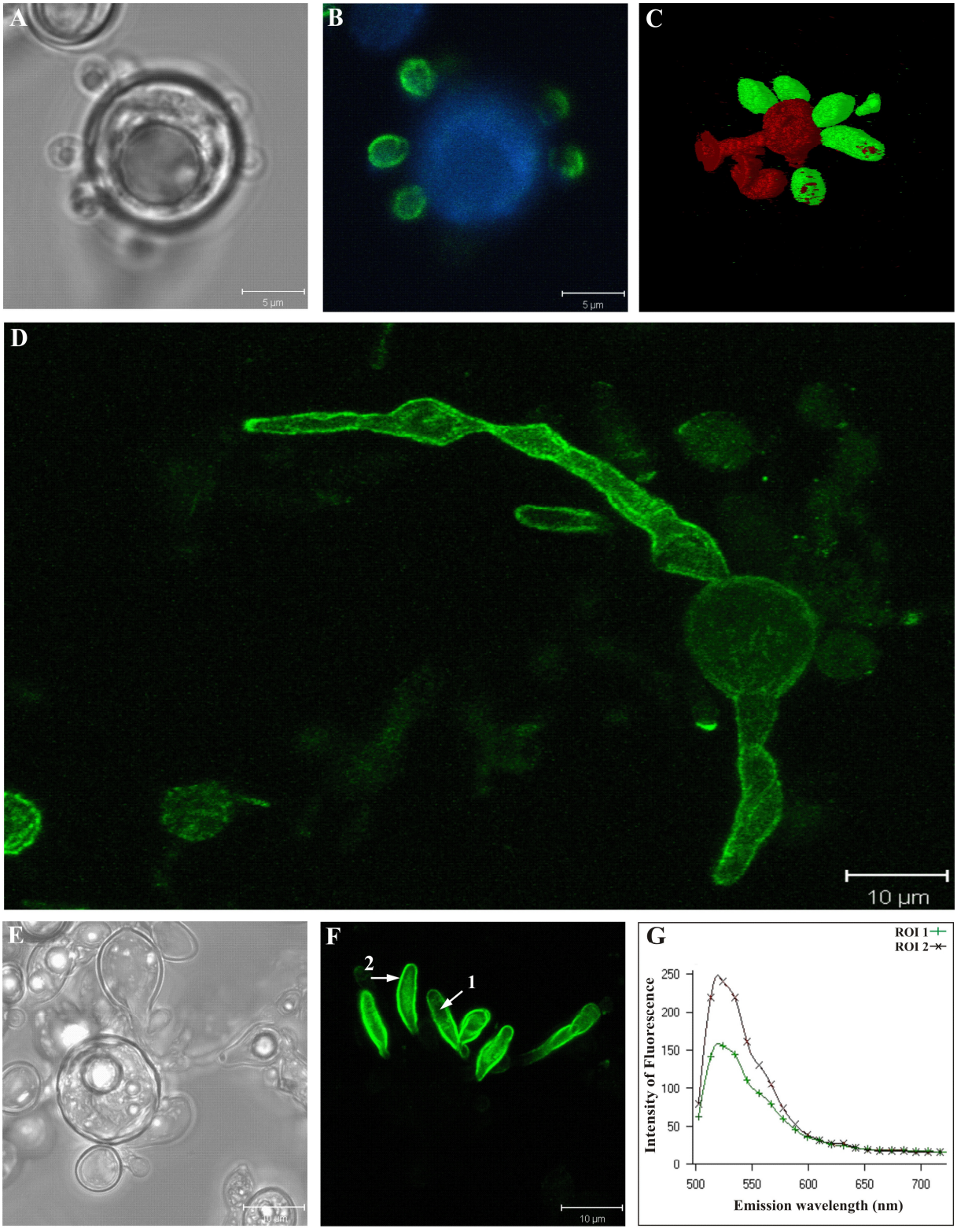

A
5 µm
B
5 µm
C
D
10 µm
E
F
2
1
10 µm
G
ROI 1
ROI 2
Intensity of Fluorescence
250
200
150
100
50
0
500
550
600
650
700
Emission wavelength (nm)

FIGURE 3

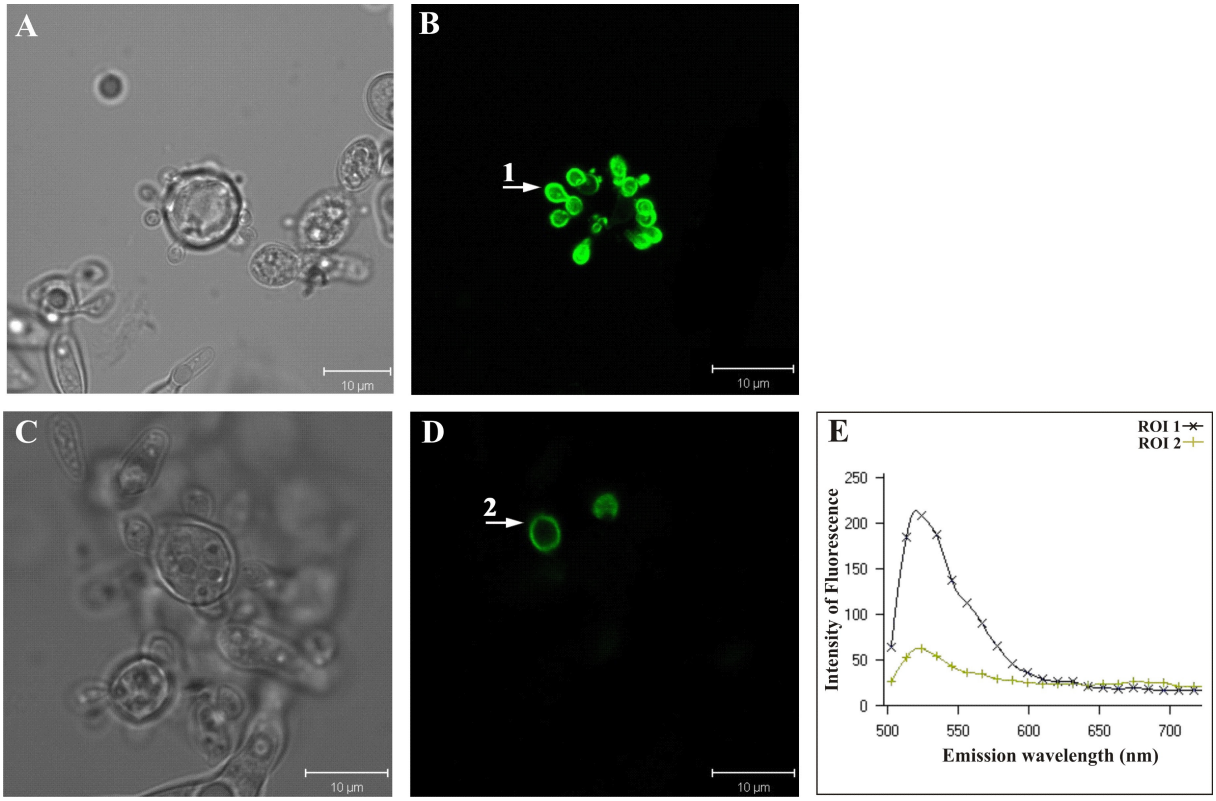

A
B
1
10 µm
10 µm
C
D
2
10 µm
10 µm
E
ROI 1
ROI 2
Intensity of Fluorescence
250
200
150
100
50
0
500
550
600
650
700
Emission wavelength (nm)

FIGURE 4

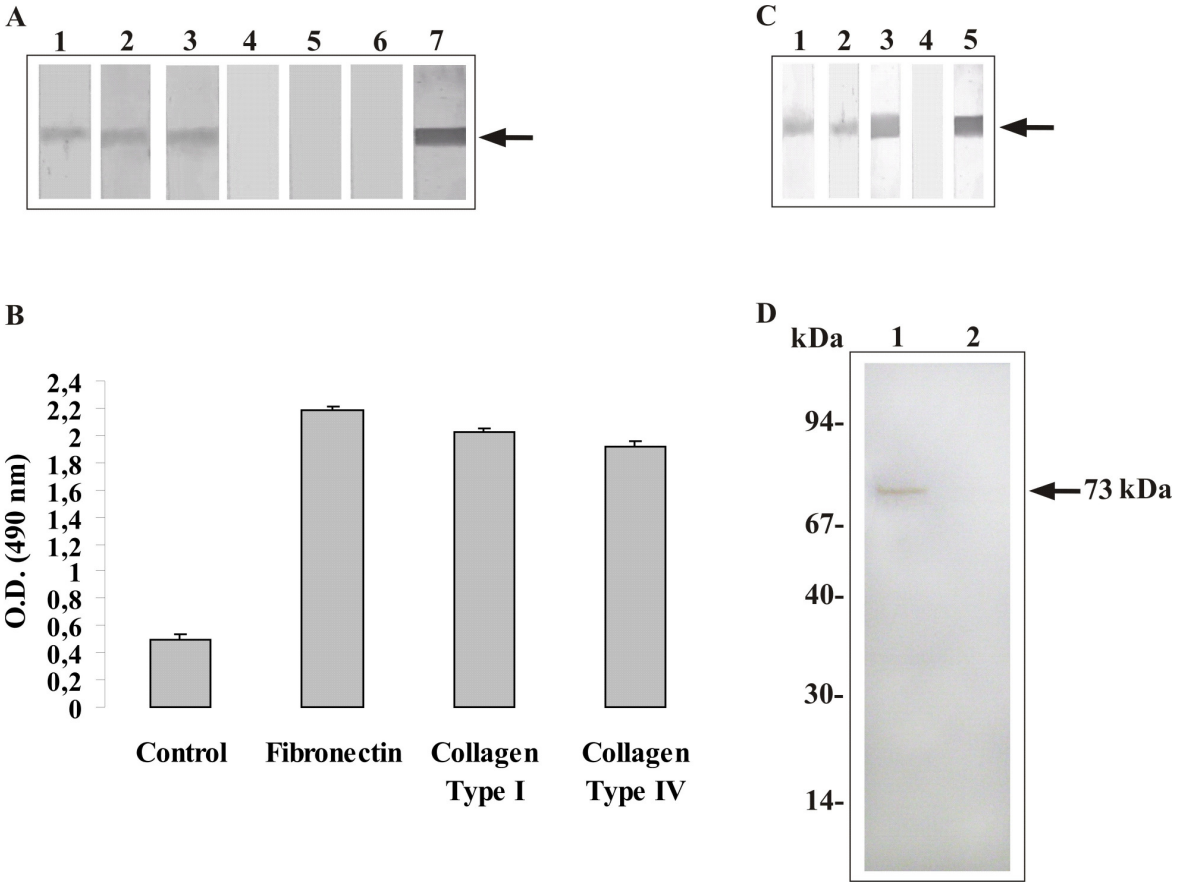
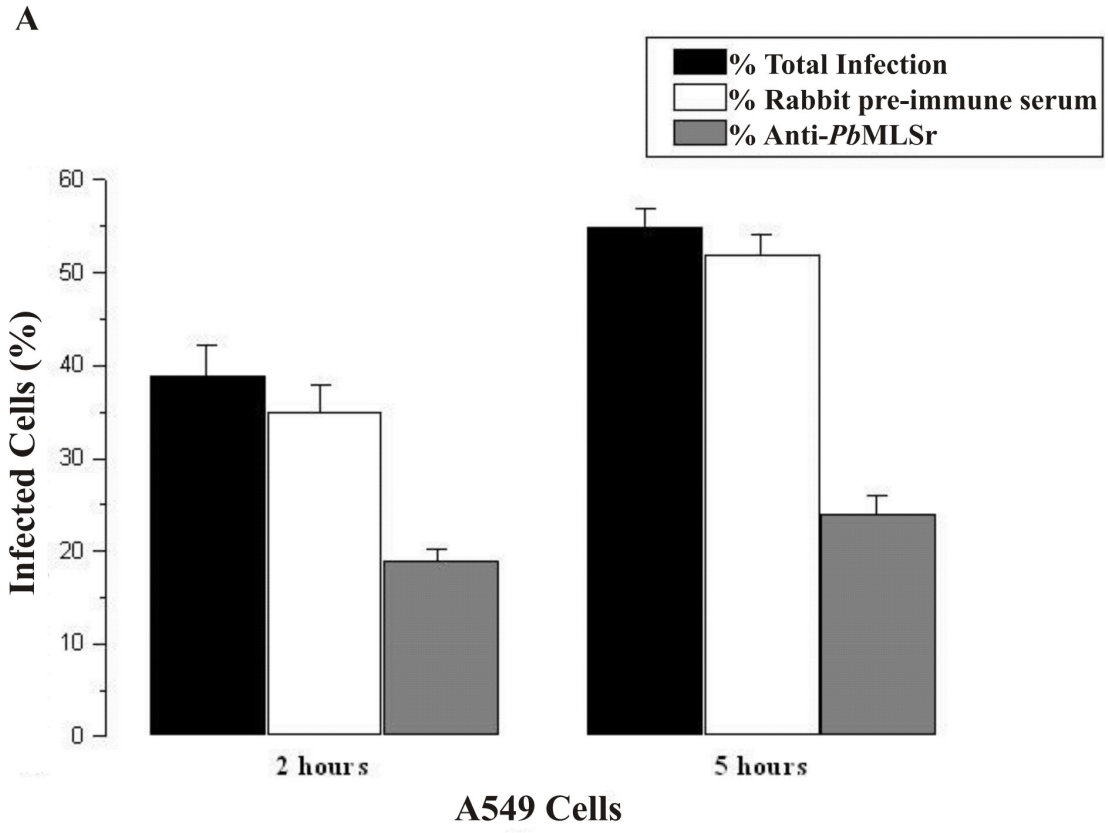

A
1 2 3 4 5 6 7
C
1 2 3 4 5
B
O.D. (490 nm)
2,4
2,2
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Control
Fibronectin
Collagen Type I
Collagen Type IV
D
kDa
1
2
94-
67-
40-
30-
14-
73 kDa

FIGURE 5


A
% Total Infection
% Rabbit pre-immune serum
% Anti-*Pb*MLSr
Infected Cells (%)
60
50
40
30
20
10
0
2 hours
5 hours
A549 Cells

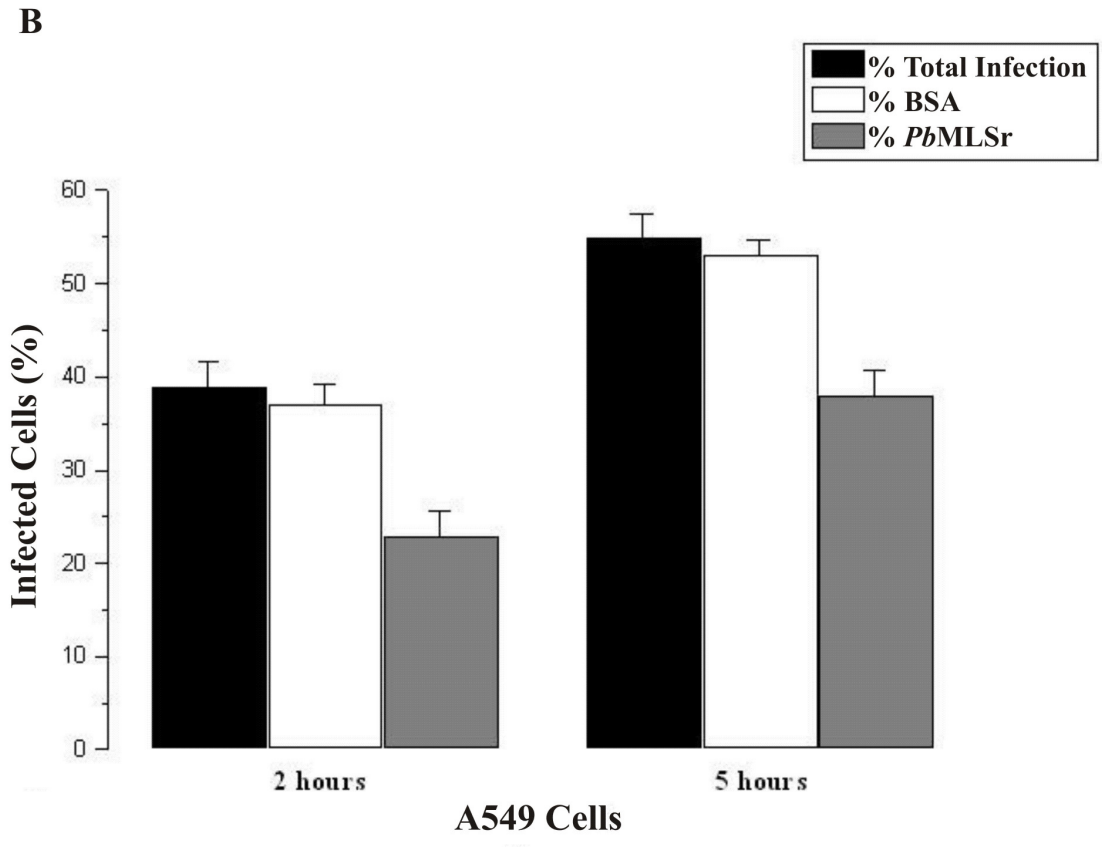
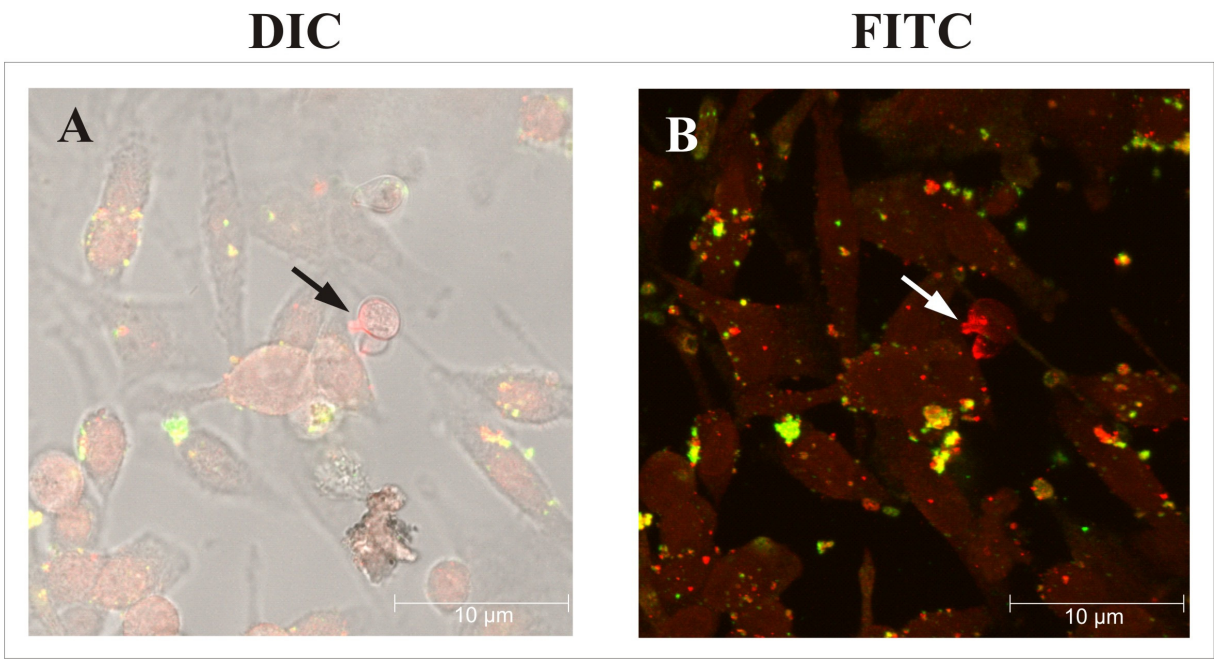

B
% Total Infection
% BSA
% *Pb*MLSr
Infected Cells (%)
60
50
40
30
20
10
0
2 hours
5 hours
A549 Cells

FIGURE 6



Conclusões

V – CONCLUSÕES

Apartir da expressão heteróloga e purificação da proteína recombinante foi possível a obtenção de um anticorpo policlonal que reconhece especificamente a *Pb*MLS nativa do fungo.

Com o intuito de se definir a localização celular da *Pb*MLS, realizamos inicialmente experimentos de *Western blotting*, utilizando extrato de proteínas presentes na porção mais superficial da parede celular e Filtrado de Cultura do fungo. Os resultados mostraram a presença de *Pb*MLS na Fração Citoplasmática assim como Fração de Parede, e na porção secretada de células leveduriformes de *P. brasiliensis*. A evidência da localização desta proteína na superfície do fungo foi confirmada posteriormente com a realização de experimentos de microscopia confocal de células leveduriformes, mostrando que a *Pb*MLS está presente na parede celular, bem como no citoplasma do fungo.

A técnica de microscopia de varredura laser confocal nos permitiu realizar a citolocalização da *Pb*MLS em células leveduriformes crescidas na presença de acetato de potássio, que apresentaram uma intensidade de fluorescência maior do que células leveduriformes crescidas em glicose, indicando a utilização de fontes de dois carbonos no Ciclo do Glioxalato. *Pb*MLS foi visualizada na superfície celular, indicando que *Pb*MLS também esta localizada na parede da célula.

Através dos resultados obtidos em experimentos de *Western blotting* de afinidade foi mostrado que a *Pb*MLSr foi capaz de se ligar à fibronectina e colágeno tipo I e tipo IV.

A *Pb*MLSr foi reconhecida durante os ensaios sorológicos por soros pacientes portadores de PCM e não de indivíduos saudáveis, mostrando assim que a

*Pb*MLSr poderá ser utilizada juntamente com outras proteínas imunoreativas do fungo *P. brasiliensis* para futuros imunodiagnósticos da PCM.

O comportamento de adesina para *Pb*MLS foi avaliado também pela interação de *Pb*MLSr com culturas celulares de pneumócitos A549. Foi verificado que a proteína biotinilada se liga às células da monocamada e é detectada por *Western blotting* com extrato total da cultura, mesmo após lavagem com tampão fosfato seguida de posterior lise osmótica, reforçando seu papel como molécula de adesão.

Ensaios com células leveduriformes de *P. brasiliensis Pb*01 mostraram inibição da adesão, e conseqüentemente internalização do fungo em culturas celulares de pneumócitos A549 pré-incubadas com anticorpo policlonal anti-*Pb*MLS ou *Pb*MLSr, sugerindo sua influência na adesão e invasão do fungo a tecidos, etapas primordiais no processo infectivo de *P. brasiliensis*. Nesse sentido a *Pb*MLS pode ser compreendida como um possível fator de virulência do fungo.

Nesse trabalho nós caracterizamos uma nova adesina de *P. brasiliensis*. Os dados sugerem que *Pb*MLS está potencialmente envolvida nos mecanismos de adesão e colonização, requeridos durante o processo infectivo de *P. brasiliensis*. Esses dados podem levar a uma melhor compreensão da interação de *P. brasiliensis* com tecidos do hospedeiro e da patogênese da PCM.

Perspectivas

VI – PERSPECTIVAS

- ✓ Caracterizar enzimaticamente a *Pb*MLSr;
- ✓ Avaliar a inibição de *Pb*MLSr;
- ✓ Mapear os epítopos de *Pb*MLS, que efetivamente interagem com as proteínas da MEC;
- ✓ Avaliar o potencial papel protetor de *Pb*MLS contra infecção por *P. brasiliensis*, através de modelos animais;
- ✓ Estudar as prováveis interações de *Pb*MLS, com outras proteínas de *P. brasiliensis*, utilizando a metodologia do duplo-híbrido (em desenvolvimento);
- ✓ Resolver a estrutura de *Pb*MLS, através de cristalografia e modelagem;
- ✓ Realizar testes de vacinação com *Pb*MLSr.

Referências Bibliográficas

VII – REFERÊNCIAS BIBLIOGRÁFICAS

Andreotti, P.F., Monteiro, Da Silva J.L., Bailão, A.M., De Almeida Soares, C.M., Benard, G., Soares C.P., Mendes- Giannini, M.J.S. (2005) Isolation and partial characterization of a 30 kDa adhesin from *Paracoccidioides brasiliensis*. *Microb Infect.* **7**:875–81.

Arredondo, M., Nunez, M.T. (2005) Iron and copper metabolism, *Mol. Aspects Med.* **26**:313 e 327.

Achkar, J.M., Dong, Y., Holzman, R.S., Belisle, J., Kourbeti, I.S., Sherpa, T., Condos, R., Rom, W.N., Laal, S. (2006) *Mycobacterium tuberculosis* Malate Synthase- and MPT51-Based Serodiagnostic Assay as an Adjunct to Rapid Identification of Pulmonary Tuberculosis. *Clinical and vaccine immunilogy.* **13**: 1291–1293.

Azevedo, J.E., Costa-Rodrigues, J., Guimaraes, C.P., Oliveira, M.E., Sa-Miranda, C. 2004, Protein translocation across the peroxisomal membrane, Cell Biochem Biophys, 41, 451-468.

Bagagli, E., Bosco, S. M., Theodoro, R. C., Franco, M. (2006) Phylogenetic and evolutionary aspects of *Paracoccidioides brasiliensis* reveal a long coexistence with animal hosts that explain several biological features of the pathogen. *Infect Genet Evol* **6**: 344-51.

Bailão, A.M., Schrank, A., Borges, C.L., et al. (2006) Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. *Microbes Infect*; **12-13**: 2686–2697.

Barbosa, M.S., Bao, S.N., Andreotti, P.F., *et al*. (2006) Glyceraldehyde 3-phosphate dehydrogenase of *Paracoccidioides brasiliensis* is a cell surface protein involved in fungal adhesion to extracellular matrix proteins and interaction with cells. *Infect Immun* **74**:382–389.

Beevers, H. (1980) The role of the glyoxylate cycle. *The Biochemistry of Plants*. **4**:117-130, Academic Press.

Bastos, K.P., Bailão, A.M., Borges, C.L., *et al*. (2007) The transcriptome analysis of early morphogenesis in *Paracoccidioides brasiliensis* mycelium reveals novel and induced genes potentially associated to the dimorphic process. *BMC Microbiol*; **10**: 7-29.

Beck, K., Hunter, I., Engel, J. (1990) Structure and function of laminin; anatomy of a multidomain glycoprotein. *FASEB J* **4**:148-160.

Biardi, L., Sreedhar, A., Zokaei, A., Vartak, N.B., Bozeat, R.L., Shackelford, J.E., Keller, G.A., Krisans, S.K. 1994, Mevalonate kinase is predominantly localized in

peroxisomes and is defective in patients with peroxisome deficiency disorders, J Biol Chem, 269, 1197-1205.

Bouchara, J.P., Sánchez, M., Chevailler, A., Marot-Leblond, A., Lissitzky, J.C., Tronchin, G., Chabasse, D. (1997) Sialic acid-dependent recognition of laminin and fibronogen by *Aspergillus fumigatus* conidia. *Infect Immun* **65**:2717-2724.

Branden, C., & Tooze, J. (1991) Introduction to protein structure. *Garland Publishing Inc.* New York.

Brummer, E., Castaneda, E., Restrepo, A. (1993) Paracoccidioidomycosis: an update. *Clin.Microbiol. Microbiol. Rev*; **6**: 89-117.

Carbonell, L.M, Rodríguez, J. (1965) Transformation of mycelial and yeast forms of *Paracoccidioides brasiliensis* in cultures and in experimental inoculations. *J. Bacteriol.* **90**: 504-510.

Castro, N.S., Barbosa, M.S., Maia, Z.A., Báo, S.N., Felipe, M.S., Santana, J.M., Soares Mendes-Giannini, M.J., Pereira, M., de Almeida Soares, C.M. (2008) Characterization of *Paracoccidioides brasiliensis* PbDfg5p, a cell-wall protein implicated in filamentous growth. *Yeast* **25(2)**:141-54.

Clarck, D.P., Cronan, J.E. JR. (1996) Two carbon compounds and fatty acids as carbon sources. In *Escherichia coli* and *Salmonella typhimurium*. *Cellular and Molecular Biology*. p. 343-357.

Clemons, K. V., Stevens, D. A. (1990) Interactions of mammalian steroid hormones with *Paracoccidioides brasiliensis*: estradiol receptor binding and mediation of cellular functions. *Interciencia*. **15**:206-208.

Corredor, G.G., Peralta, L.A., Castaño, J.H., Zuluaga, J.S., Henao, B., Arango, M., Tabares, A.M., Matute, D.R., McEwen, J.G., Restrepo, A. (2005) The naked-tailed armadillo *Cabassous centralis* (Miller 1899): a new host to *Paracoccidioides brasiliensis*. Molecular identification of the isolate. *Med Mycol* **43**:275-280.

Costa, M., Borges, C.L., Bailão, A.M., *et al*. (2007) Transcriptome profiling of *Paracoccidioides brasiliensis* yeast-phase cells recovered from infected mice brings new insights into fungal response upon host interaction. *Microbyology.* **153**: 4194-4207.

Coutinho, Z., Silva, D., Lázera, M., Petri, V., Sabroza, P. C., Wanke, B. (1998) Mortalidade por paracoccidioidomicose. Brasil 1980-1995. II Congreso Brasileiro de Micologia, Rio de Janeiro, Brasil. April 1998. Rio de Janeiro: Sociedade Brasileira de Micologia: Abstract A57.

Coutinho, Z., Silva, D., Lazera, M., Oliveira, R.M., Sabroza, P. C., Wanke, B. (2002) Paracoccidioidomycosis mortality in Brasil 1980-1995. *Cad. Saúde Pública.* **18**:1441-1454.

Cozzone, A.J. (1998) Regulation of acetate metabolism by protein phsphorytion in enteric bacteria. *Annu. Rev. Microbiol.* **52:**127–164.

Cunha, A.F., Sousa, M.V., Silva, S.P., Jesuino, R.S., Soares, C.M. A., Felipe, M.S. (1999) Identification, N-terminal region sequencing and similarity analysis of differentially expressed proteins in *Paracoccidioides brasiliensis*. *Med Mycol* **37**: 115-121.

De Duve, C., Baudhuin, P. 1966, Peroxisomes (microbodies and related particles), Physiol Rev, 46, 323-357.

Dieuaide, M., Brouquises, R., Pradet, A.,Raymond, P. (1992) Increased fatty acid β-oxidation alter glucosa starvation in maize root tips. *Plant Physiol.* **99**:595-600.

Eastmond, P.J., Graham, I.A. Re-examining the role of the glyoxylate cycle in oilseeds. *Trend Plant Sci.* **6**:72-78.

Eckert, J.H., Erdmann, R. 2003, Peroxisome biogenesis, Rev Physiol Biochem Pharmacol, 147, 75-121.

Felipe, M.S., Andrade, R.V., Petrofeza, S.S., Maranhão, A.Q., Torres, F.A., Albuquerque, P., Arraes, F.B., Arruda, M., Azevedo, M.O., Baptista, A.J., Bataus, L.A., Borges, C.L., Campos, E.G., Cruz, M.R., Daher, B.S., Dantas, A., Ferreira, M.A., Ghil, G.V., Jesuino, R.S., Kyaw, C.M., Leitão, L., Martins, C.R., Moraes, L.M., Neves, E.O., Nicola, A.M., Alves, E.S., Parente, J.A., Pereira, M., Poças-Fonseca, M.J., Resende, R., Ribeiro, B.M., Saldanha, R.R., Santos, S.C., Silva-Pereira, I., Silva, M.A., Silveira, E., Simões, I.C., Soares, R.B., Souza, D.P., De-Souza, M.T., Andrade, E.V., Xavier, M.A., Veiga, H.P., Venancio, E.J., Carvalho, M.J., Oliveira, A.G., Inoue, M.K., Almeida, N.F., Walter, M.E., Soares, C.M.A., Brígido, M.M. (2003) Transcriptome characterization of the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis* by EST analysis. *Yeast*. **20**:263-271.

Felipe, M.S., Andrade, R.V., Arraes, F.B., Nicola, A.M., Maranhão, A.Q., Torres, F.A., Silva-Pereira, I., Poças-Fonseca, M.J., Campos, E.G., Moraes, L.M., Andrade, P.A., Tavares, A.H., Silva, S.S., Kyaw, C.M., Souza, D.P., Pereira, M., Jesuíno, R.S., Andrade, E.V., Parente, J.A., Oliveira, G.S., Barbosa, M.S., Martins, N.F., Fachin, A.L., Cardoso, R.S., Passos, G.A., Almeida, N.F., Walter, M.E., Soares, C.M.A., Carvalho, M.J., Brígido, M.M. (2005) Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. *J Biol Chem* **280**(26):24706-24714.

Felipe, M.S.S., Torres, F.A.G., Maranhão, A.Q., Silva-Pereira, I., Poças-Fonseca, M.J.,Campos, E.G., Moraes, L.M.P., Arraes, F.B.M., Carvalho, M.J.A., Andrade, R.V., Nicola, A.M., Teixeira, M.M., Jesuíno, R.S.A., Pereira, M., Soares, C.M.A., Brígido, M.M. (2005b) MiniReview Functional genome ofthe human patogenic fungus *Paracoccidioides brasiliemsis*. *FEMS Immunology and Medical Microbiology*.

Felipe, M.S., Andrade, R.V., Arraes, F.B., *et al*. (2005) Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. *J Biol Chem*. **280**: 24706-24714.

Filler Scott G. (2006) *Candida*-host cell receptor-ligand interactions. Current Opinion in Microbiology. **9**:333-339.

Flavel, R.B., Woodward, D.O. (1971) The concurrent regulation of metabolically related enzymes. The Krebs cycle and glyoxylate shunt enzymes in *Neurospora. Eur. J. Biochem.* **17**(2):284-291.
Franco, M. (1987) Host-parasite relationship in paracoccidioidomycosis. *J. Med. Vet. Mycol.* **25,** 5–18.

Franco, M., Sano, A., Kera, K., Nishimura, K., Takeo, K., Miyaji, M. (1989) Clamydospore formation by *Paracoccidioides brasiliensis* mycelial form. *Rev. Inst. Med. Trop. São Paulo*. **31**:151-157.

Ganendren, R., Carter, E., Sorrell, T., Widmer, F., Wright, L. (2006) Phospholipase B activity enhances adhesion of *Cryptococcus neoformans* to a human lung epithelial cell line. *Microbes Infect*. **8**(4):1006-15.

Gesztesi J. L, Puccia R, Travassos L. R, Vicentini A. P, de Moraes J. Z, Franco M. F, Lopes J. D. (1996) Monoclonal antibodies against the 43KDa glycoprotein from *Paracoccidioides brasiliensis* modulate lamininmediated fungal adhesion to epithelial cells and pathogenesis. *Hybridoma*. **15**(6):415-22.

Gil, M.L., Peñalver, M.C., López-Ribot, J.L., O'Connor, J.E. and Martinez, J.P. (1996) Binding of extracellular matrix proteins to *Aspergillus fumigatus* conidia. *Infect Immun*. **64**: 5239-5247.

Goldani, L. Z., Coelho, I. C., Machado, A. A. (1991) Paracoccidioidomycosis and AIDS. *Scand. J. Infect. Dis*. **23**:393.

Goldman G.H, dos Reis Marques E., Duarte Ribeiro D.C., de Souza Bernardes L.A., Quiapin A.C., Vitorelli P.M., Savoldi M., Semighini C.P., de Oliveira R.C., Nunes L.R., Travassos L.R., Puccia R., Batista W.L., Ferreira L.E., Moreira J.C., Bogossian A.P., Tekaia F., Nobrega M.P., Nobrega F.G., Goldman M.H. (2003) Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. *Eukaryot Cell.* **2**: 34-48.

Gonzalez, A., Lenzi, H.L., Motta, E.M., Caputo, L., Sahaza, J.H., Cock, A.M., Ruiz, A.C., Restrepo, A., Cano, L.E. (2005) Expression of adhesion molecules in lungs of mice infected with *Paracoccidioides brasiliensis* conidia. *Microb Infect*. **7**:666–73.

Graham, I.A., Leaver, C.J., Smith, S.M. (1992) Induction of Malate synthase gene expression in senescent and detached organs of cucumber. *The Plant Cell*. **3:**349–357.

Graham, I.A.; Denby, K.J., Leaver, C.J. (1994) Carbon catabolite repression regulates glyoxylate cycle gene expression in cucumber. *The Plant Cell*. **6:**761–772.

Graham, J.E. and Clark-Curtiss, J.E. (1999) Identification of *Mycobacterium tuberculosis* RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). *Proc. Natl. Acad*. Sci. **96**: 11554–11559.

Hamel, R.D. & Appana, V.D. (2001) Modulation of TCA cycle enzymes and aluminum stress in *Pseudomonas fluorescens*. *Journal of Inorganic Biochemistry*. **87:**1-8.

Hanna, S.A., Monteiro, da Silva J.L., Mendes-Giannini, M.J.. (2000) Adherence and intracellular parasitism of *Paracoccidioides brasiliensis* in Vero cells. *Microbes Infect*. **2**:877–84.

Hartig, A., Simon, M.M., Schuster, T., Daughert, J.R; Yoo, H.S,; Cooper, T. G. (1992) Differentially regulated malate synthase genes participein carbon and nitrogen metabolism of *Saccharomyces crevisiae*. *Nucleic Acids Re.* **20**:5677-5686.

Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P., Tabak, H.F. 2005, Contribution of the endoplasmic reticulum to peroxisome formation, Cell, 122, 85-95.

HOWARD, B.R.; ENDRIZZI, J.A.; REMINGTON, S.J. (2000) Crystal structure of *Escherichia coli* malate synthase G complexed with magnesium and glyoxylate at 2.0 Å resolution: Mechanistic Implications. *Biochemistry*. **39**:3156–3168.

Hung, C.Y., Seshan, K.R., Reichard, U. and Cole, G.T. (2002) A parasitic phase-especific adhesin of *Coccidioides immitis* contributes to the virulence of this respiratory fungal pathogen. *Infect Immun*. **7**: 3443-3456.

Idnurm, A., Howlett, B.J. (2002) Isocitrate lyase is essencial for phatogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). *Eukarryotic cell*. **1**:719-724.

Jedd, G., Chua, N.H. 2000, A new self-assembled peroxisomal vesicle required for efficient resealing of the plasma membrane, Nat Cell Biol, 2, 226-231.

Kerssen, D., Hambruch, E., Klaas, W., Platta, H.W., de Kruijff, B., Erdmann, R., Kunau, W.H., Schliebs, W. 2006, Membrane association of the cycling peroxisome import receptor Pex5p, J Biol Chem, 281, 27003-27015.

Kornberg, H.L. and Krebs, H.A. (1957) *Nature*. **199**: 988.

Kornberg, H.L. and Beevers, H. (1957) A mechanism of convertion of fat to carbohydrate in castor beans. *Nature*. **180**: 988.

Kornberg, H.L. (1966) The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochem.* **99:** 1.

Kottom, T. J., J. R. Kohler, C F. Jr. Thomas, G. R. Fink, and A. H. Limper. (2003) Lung epithelial cells and extracellular matrix components induce expression of *Pneumocystis carinii* STE20, a gene complementing the mating and pseudohyphal growth defects of *ste20* mutant yest. *Infect. Immun*. **71**: 6463-6471.

Kurokawa C.S., Sugizaki M.F., Peracoli M.T. (1998) Virulence factors in fungi of systemic mycoses. Rev. Inst. *Med. Trop*. Sao Paulo **40**: 125-135.

Kunze, M., Kragler, F., Binder, M., Hartig, A., Gurvitz, A. (2002) Targeting of malate synthase 1 to the peroxisomes of *Saccharomyces cerevisiae* cells depends on growth on oleic acid medium. *Eur. J. Biochem*. **269**: 915-922.

Lacaz, C. S., PORTO E., MARTINS, J. E. C. (1991) Paracoccidioidomicosis, *In: Micologia Médica*, 8th ed. Sarvier Editora, p.248-261.

Lacaz, C. S. (1994) *Paracoccidioides brasiliensis*: morphology, evolutionary cycle; maintenance during saprophytic life; biology, virulence, taxonomy. *In*: Franco, M.

Lattif, A.A., Prasad, R., Banerjee, U., Gupta, N., Mohammad, S. and Baquer, N. Z. (2006) The glyoxylate cycle enzymes activities in the pathogenic isolates of *Candida albicans* obtained from HIV/AIDS, diabetic and burn patients. *Mycoses.* **49**:85-90.

Lenzi, H.L., Calich, V.L.G., Mendes-Giannini, M.J.S., Xidiex, C.F., Miyaji, M., Mota, E.M., Machado, M.P., Restrepo, A. (2000) Two patterns of extracellular matrix expression in experimental paracoccidioidomycosis. *Med. Mycol*. **38**: 115-119.

Lenzi, H.L., Lenzi, J.A., Kerr, I.B.; Antunes, S.L.; Mota, E.M.;
OLIVEIRA, D.N. (1991) Extracellular matrix in parasitic and infectious diseases. *Mem. Inst. Oswaldo Cruz***.**86: 77-90.

Lewis, C.E. & Doyle, J. (2001) Phylogenetic utility of the nuclear gene malate synthase in the palm family (*Arecaceae*). *Molecular Phylogenetics abd Evolution*. **19**:(3)409-420.

Lima O.C, Bouchara J.P, Renier G, Marot-Leblond A, Chabasse D, Lopes-Bezerra L.M. (2004) Immunofluorescence and flow cytometry analysis of fibronectin and laminin binding to *Sporothrix schenckii* yeast cells and conidia. *Microb Pathog*. **37**(3):131-40.

Liu, F., Thatcher, J.D., Barral, J.M. Epstein, H.F. (1995) Bifunctional glyoxylate cycle protein of *Caenorhabditis elegans*: a developmentally regulated protein of intestine and muscle. *Dev. Biol.* **169**:399-414.

Lorenz, M.C. & Fink, G.R. 2001. The glyoxylate cycle is required for fungal virulence. *Nature*. **412**:(6842):83–86.

Lorenz, M.C. & Fink, G.R. 2002. Life and death in a macrophage: Role oh the glyoxylate cycle in virulence. *Eukaryotic Cell*. **1**:(5):657-662.

Lutz, A. (1908) Uma mycose pseudo-coccidica localizada na boca e observada no Brasil: "Contribuição ao conhecimento das hiphoblastomycoses americanas". *Brás Méd* **22**:121-124.

Mahan, J.R. (2000) Thermal dependence of malate synthase activity and its relationship to the thermal dependence of seedling emergence. *J. Agric. Food Chem.* **48**(10):4544-4549.

Mannaerts, G.P., Van Veldhoven, P.P. 1993, Metabolic pathways in mammalian peroxisomes, Biochimie, 75, 147-158.

Margulis, L., Schwartz, K.V. (1998) Five kingdoms, an illustrated guide to the phyla of life on Earth. WH Feeman & Co.

Marques, S.A., Franco, M.F., Mendes, R.P., (1983) Aspectos epidemiológicos da paracoccidioidomicose na área endêmica de Botucatu. (São Paulo-Brasil*). Rev Inst Med trop.* **25**:87-92.

Martinez, R. (1997) Blastomicose-Sul-Americana (Paracoccidioidomicose)-Etioepidemiologia e Ecologia. *In*: Veronesi, R., Focaccia, R. Tratado de Infectologia. Vol II. Ateneu. 1082.

McMahon, J.P., Wheat, J., Sobel, M.E., Pasula, R., Downing, J.F., Martin, W.J. (1995) Laminin Binds to Histoplasma capsulatum A Possible Mechanism of Dissemination. *The Journal of Clinical Investigation, Inc* **96**: 1010-1017.

McEwen, J. G., Restrepo, B.I., Salazar, M.E., Restrepo, A. (1987) Nuclear staining of *Paracoccidioides brasiliensis*. *J. Med.Vet. Mycol*. **25**:343-345.

Mendes-Gianinni, M.J.S., Ricci, L.C., Uemura, M., Toscano, E. and Arns, C.W. (1994) Infection and apparent invasion of Vero cells by *Paracoccidioides brasiliensis*. *J. Med. Vet. Mycol*. **32**: 189-195.

Mendes-Giannini, M.J.S., Taylor, M.L., Bouchara, J.B., Burger, E., Calich, V.L.G., Escalante, E.D., *et al*. (2000) Pathogenesis II: fungal responses to host responses: interaction of host cells with fungi. *Med Mycol*.**38**: 113–23.

Mendes-Giannini, M.J., Andreotti, P.F., Vincenzi, L.R., Monteiro Da Silva, J.L., Lenzi, H.L., Benard, G., Zancope- Oliveira, R., De Matos Guedes, H.L., Soares, C.P. (2006) Binding of extracellular matrix proteins to *Paracoccidioides brasiliensis. Microb Infect*. **8**:1550–9.

Mendes-Giannini, M.J., Monteiro da Silva, J.L., de Fátima da Silva, J., Donofrio, F.C., Miranda, E.T., Andreotti, P.F., Soares, C.P. (2008) Interactions of *Paracoccidioides brasiliensis* with host cells: recent advances. *Mycopathologia* **165**:237–248.

Minard, K.I. & Mcalister-Henn, L. (1991) Isolation, nucleotidesequenceanalysis, and discruption of the MDH2 gene from *Saccharomyces cerevisiae*: evidence for three isoenzymes of yeast malate dehydrogenase. *Mol. Cell. Biol*.**11**(1):370–380.

Minguetti, G., Queiroz Telles, F., Hofmeister, R.M., Freitas, O.T., Lameira, R.F. (1985) Scanning electron microscopic studies of *Paracoccidioides brasiliensis* in the yeast phase. *Sabouraudia.* **23**: 443-446.

Mohri H. (1996) Fibronectin and integrin interactions. *J Invest Med*. **44**: 429-41.

Molina, I.; Pellicer, M. T.; Badia, J.; Aguilar, J.; Baldoma, L. (1994) Molecular characterization of *Escherichia coli* malate synthase G. Differentiation with the malate synthase A isoenzyme. *Eur. J. Biochemistry*. **224**:541-548.

Munir, E.; Takefumi, H.; Mikio, S. (2002) Purification and Characterization of Malate Synthase from the Glucose-grown Wood-rotting Basidiomycete *Fomitopsis palustris*. *Biosci., Biotechmol., Biochem*. **66**:576-581.

Muller, M. 1969, Peroxisomes of protozoa, Ann N Y Acad Sci, 168, 291-301.

Nakazawa, M., Minami, T., Teramura, K., Kumamoto, S., Hanato, S., Takenaka, S., Ueda, M., Inui, H., Nakano, Y., Miyatake, K. (2005) Molecular characterization of a bifunctional glyoxylate cycle enzyme, malate synthase/isocitrate lyase, in *Euglena gracilis. Comparative Biochemistry and Physiology*. **141**:445-452.

Nogales, J., Guijo, M.I, Quesada, A., Merchán, F. (2004) Functional analysis of the malate synthase from *Clamydomonas reinhardtii*. *Planta.* **219:**325-331.

Nunes, L.R., Costa de Oliveira, R., Leite, D.B., da Silva, V.S., dos Reis Marques. E., da Silva Ferreira, M.E., Ribeiro, D.C., de Souza Bernardes, L.A., Goldman, M.H., Puccia, R., Travassos, L.R., Batista, W.L., Nóbrega, M.P., Nóbrega, F.G., Yang, D.Y., de Braganca Pereira, C.A., Goldman, G.H. (2005) Transcriptome analysis of *Paracoccidioides brasiliensis* cells undergoing mycelium-to-yeast transition. *Eukaryot Cell* **4**: 2115-2128.

Olivas, I. *et al*., (2007) Ability to grow on lipids accounts for the fully virulent phenotype in neutropenic mice of Aspergillus fumigatus null mutants in the key glyoxylate cycle enzymes, *Fungal Genet.Biol*. doi:10.1016/j.fgb.2007.05.002
Ornston LN, Ornston MK. (1969) Regulation of glyoxylate metabolism in *Escherichia coli* K-12.*J. Bacteriol*. **98**: 1098-1108.

Paniago, A.M., Aguiar, J.I., Aguiar, E.S., Pereira, G.R., Londero, A.T. (2003) Paracoccidioidomicose: estudo clínico e epidemilógico de 422 casos observados no estado do Mato Grosso do Sul. *Rev. Soc. Bras. Med. Trop*. **36**: 455-459.

Patti, J.M., Allen, B.L., Mcgavin, M.J.; Höök, M. (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu. Rev. Microbiol***. 48** :
585-617.

Park, J.B., Chae, H.Z. and Kim, Y.S. (1986) Purification and characterization of malate synthase from *Acinetobacter calcoaceticus* grown on malonate. *Korean Biochem. J.* **19:**235-241.

Peñalver, M.C., O'Connor, J.E., Martinez, J.P. and Gil, M.L. (1996) Binding of human fibronectin to *Aspergillus fumigatus* conidia. *Infect Immun*. **64:**1146-1153.

Pereira, L.A., Báo, S.N., Nair Barbosa, M.S., Monteiro da Silva, J.L., Felipe, M.S.S., Santana, J.M., Mendes-Giannini, M.J.S., & Soares, C.M.A. (2007) Analysis of the *Paracoccidioides brasiliensis* Triosephosphate Isomerase suggests the potential for adhesin function. *FEMS Yeast Res*.**7:** 1381-1388.

Purdue, P.E., Lazarow, P.B. 2001, Peroxisome biogenesis, Annu Rev Cell Dev Biol, 17, 701-752.

Queiroz-Telles, F. (1994) *Paracoccidioides brasiliensis* ultrastructural findings. *In*: Franco, M., Lacaz, C. S., Restrepo-Moreno, A., Del-Negro, G. (Eds.), Paracoccidioidomycosis, CRC Press, Boca Ratón, Chapter 3:27-47.

Reis, V.L.L., Rolla, V.C., Rangel, C.A., Sampaio, M.B.N.O., Tavares, W., Praxedes, H., Rocha, W.B. (1986) Paracoccidiodomicose disseminada aguda com extenso derrame pleural. *Arq Bras Med* **60**:275-80.

Reinscheid, D.J., Eikmanns, B.J., and Sahm, H. (1994) Malate synthae from *Corynebaterium glutamicum*: sequence analysis of the gene and biochemical characterization of enzyme. *Microbiology.* **40**:3099-3108.

Restrepo, A., Bedout, C., Cano, L.E., Arango, M.D. and Bedoya, V. (1981) *SABOURAUDIA*. **19:** 295-300.

Restrepo, A. (1985) The ecology of *Paracoccidioides brasiliensis*: a puzzle still unsolved. *J. Med. Vet. Mycol.* **23**:323-334.

Restrepo, A. (2000) Morphological aspects of *Paracoccidioides brasiliensis* in lymph nodes: implications for the prolonged latency of paracoccidioidomycosis? *Med. Mycol.* **38**:317-322.

Restrepo-Moreno, A. (2003) Paracoccidioidomycosis. In: Dismukes, W.E., Pappas, P.G., Sobel, J. (Eds.), *Clinical Mycology*. Oxford University Press, New York, pp. 328–345.

Rhodin, J. 1954, Correlation of ultrastructural organization and function in normal and experimentally changed peroxisomal convoluted tubule cells of the mouse kidney, Stockholm University, Aktiebolaget Godvil, Stockholm, Sweden.

Rude, T.H., Toffaletti, D.L., Cox, G.M., Perfect, J.R. (2002) Relatioship of the glyoxylate pathway to the pathogenesis of *Cryptococcus neoforms*. *Infect. Immun.* **70**:(10):5684 – 5694.

Samsonoff, W. A., Salazar, M. E. Mckee, M. L., Restrepo, A., Cano, L. E., Edwards, M. R. (1991) Scanning electron microscopy of the conidia produced by the mycelial form of *Paracoccidioides brasiliensis. Mycopathol*.**114**:9-5.

San-Blas, G., Niño-Vega, G., Iturriaga, T. (2002) *Paracoccidioides brasiliensis* and paracoccidioidomycosis: molecular approaches to morphogenesis, diagnosis, epidemiology, taxonomy and genetics. *Med. Mycol*. **40:**225-242.

Sandini S, La Valle R, De Bernardis F, Macri C, Cassone A. (2007) The 65 kDa mannoprotein gene of *Candida albicans* encodes a putative beta-glucanase adhesin required for hyphal morphogenesis and experimental pathogenicity. *Cell Microbiol*. (5):1223-38.

Sano, A., Nishimura, K., Myaji, M. (1999) The research encouragement award. Effects of sex hormone on sexual difference of experimental paracoccidioidomycosis. *Nippon Ishinkin Gakkai Zasshi.* **40**:1-8.

Santangelo R., Zoellner H, Sorrell T. Wilson C, Donald C, Djordjevic J., Shounan Y. Wright L. (2004) Role of extracellular phospholipases and mononuclear phagocytes in dissemination of cryptococcosis in a murine model, *Infect. Immun*. **72**: 2229 -2239.

Silva, S. P., Felipe, M. S. S., Pereira, M., Azevedo, M. O., Soares, C. M. A. (1994) Phase transition and stage-specific protein synthesis in the dimorphic fungus *Paracoccidioides brasiliensis. Exp. Mycol*. **18**:294-299.

Silva-Vergara, M.L., Teixeira, A.C., Curi, V.G., Costa Júnior, J.C., Vanunce, R., Carmo, W.M., Silva, M.R. (2003) Paracoccidioidomicosis associated with human immunodeficiency vírus infection. Repost 10 cases. Med Mycol. **41**(3): 259-63.

Shikanai-Yasuda, M.A., Telles Filho, F. de Q., Mendes, R.P., Colombo, A.L., Moretti, M.L. (2006) Guidelines in paracoccidioidomycosis. *Rev Soc Bras Med Trop* **39**(3):297-310.

Singh, R. Green, M.R. (1993) Sequence-specificity binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase, *Science* **259**: 365-368.

Srinoulprasert, Y., Kongtawelert, P., Chaiyaroj, S.C. (2006) Chondroitin sulfate B and heparin mediate adhesion of *Penicillium marneffei* conidia to host extracellular matrices. *Microb Pathog*. Mar;**40**(3):126-32.

Szabo, A.S., Avers, C.J. (1969) Some aspects of regulation of peroxisomes and mitochondria in yeast. *Ann N Y Acad Sci*. **19**: 302-312.

Terçarioli, G.R., Bagagli, E., Reis, G.M., Theodoro, R.C., Bosco, S.M., Macoris, S.A., Richini-Pereira, V.B. (2007) Ecological study of *Paracoccidioides brasiliensis* in soil: growth ability, conidia production and molecular detection. *BMC Microbiol* **7**:92.

Tobón, A. M., Agudelo, C. A., Osorio, M. L., Alvarez, D. L., Arango, M., Cano, L. E., Restrepo A. (2003) Residual pulmonary abnormalities in adult patients with chronic paracoccidioidomycosis: prolonged follow-up after itraconazole therapy. *Clin Infect Dis*. **1;37**(7):898-904.

Travassos, L.R., Rodrigues, E.G., Iwai, L.K., Taborda, C.P. (2008) Attempts at a peptide vaccine against paracoccidioidomycosis, adjuvant to chemotherapy. *Mycopathologia*. doi:10.1007/s11046-007-9056-1.

Tronchin,G., Esnault, K., Renier, G., Filmon, R., Chabasse, D. and Bouchara, J.P. (1997) Expression and identification of a laminin-binding protein in *Aspergillus fumigatus* conidia. *Infect Immun*. **65**: 9-15.

Tugarinov, V., Muthandiram, R., Ayed, A., Kay, L.E. (2002) Four-dimensional NMR Spectroscopy of a 723-residue protein: chemical shit assigments and secondary struture of malate synthase. *J.Am. Chem. Soc.***124**:10025–10035.

Veenhuis, M., Mateblowski, M.,. Kunau, W.H., Harder, W. 1987, Proliferation of microbodies in Saccharomyces cerevisiae, Yeast, 3, 77-84.

Verstrepen, K.J., and Klis, F.M. (2006) Flocculation, adhesion and biofilm formation in yeasts. *Molecular Microbiology* **60**(1):5-15.

Vicentini, A.P., Gesztesi, J.L., Franco, M.F., De Souza, W., De Moraes, J.Z., Travassos, L.R., *et al*. (1994) Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. *Infect Immun*. **62**:1465–9.

Villar, L.A., Salazar, M.E., Restrepo, A. (1988) Morphological study of a variant of *Paracoccidioides brasiliensis* that exists in the yeast form at room temperature. *J Med Vet Mycol.* **26**:269-276.

Villar, L. A., Tobón A., Calle, D., Rosero, D.S., Gómez, B. L., Restrepo, A. (2000) Central nervous system paracoccidioidomyscosis. Report of a case successfully treated with itraconazol. *Scielo*.**42**:231-234.

Walton F.J., Idnurm A., Heitman J., (2005) Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans*. *Mol. Microbiol*. **57**:1381-1396.

Wanchu, A., Dong, Y., Sethi, S., Myneedu, V.P., Nadas, A., *et al*. (2008) Biomarkers for Clinical and Incipient Tuberculosis: Performance in a TB-Endemic Country. *PLoS ONE* **3**(4): e2071.

Wanders, R.J., Waterham, H.R. 2006, Peroxisomal disorders: the single peroxisomal enzyme deficiencies, Biochim Biophys Acta, 1763, 1707-1720.

Watanabe, S., Takada, Y. and Fukunaga, N. (2001) Purification and characterization of a cold-adapted isocitrate lyase and malate synthase from *Colwellia maris*, a psychrophilic bacterium. *Biosci. Biotechnol. Biochem.* **65**:1095-1103.

Wendisch, V.F., De Graaf, A.A., Sahm, H., Eikmanns, B.J. (2000) Quantitative determination of metabolic fluxes during coutilization of two carbon sources: comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose. *J Bacteriol*. **182**(11):3088-96.

Weller, S., Gould, S.J., Valle, D., 2003 Peroxisome biogenesis disorders, Annu Rev Genomics Hum Genet, 4, 165-211.

Woodcock, E., Merrett, M.J. (1978) Purification and immunochemical characterization of malate synthase from *Euglena gracilis*. *Biochem. J.* **173**:95-101.

Zambuzzi-Carvalho, P.F., Cruz, A.H.S., Santos-Silva, L.K., Goes, A.M., Soares, C.M.A., and Pereira, P. (2009) The malate synthase of Paracoccidioides brasiliensis is required in the glyoxylate cycle and in the allantoin degradation pathway. In press.

Anexos

VIII – ANEXOS

Molecular Microbiology

Edited by:
John D. Helmann

Print ISSN: 0950-382X
Online ISSN: 1365-2958
Frequency: Twice Monthly
Current Volume: 71 / 2009
ISI Journal Citation Reports® Ranking: 2007: 10/94 (Microbiology); 43/263 (Biochemistry & Molecular Biology)
Impact Factor: 5.462

Top**Author Guidelines**

Did you know... *Molecular Microbiology* has no page charges?

Online submission and review.

Molecular Microbiology is published twice a month and is covered by Wiley-Blackwell's *Accepted Articles* and *Early View* services. The Journal invites high quality, original research articles reporting the results of molecular studies of eukaryotic microorganisms and of prokaryotes (bacteria and archaea) and their viruses. Studies that include genetic, biochemical, biophysical, bioinformatic and structural analyses leading to a deeper understanding of the molecular principles underlying basic physiological processes or mechanisms are considered as potentially suitable for publication in the Journal. Topics that are considered appropriate include gene expression and regulation, pathogenicity and virulence, physiology and metabolism, synthesis of macromolecules (proteins, nucleic acids, lipids, polysaccharides, etc.), cell biology and subcellular organization, membrane biogenesis and function, traffic and transport, cell-cell communication and signalling pathways, evolution and gene transfer.

MicroReviews Short reviews are published in areas of particular interest and current importance. Many, but not all, reviews are invited; authors wishing to submit a review should refer to the instructions available at http://www.blackwellpublishing.com/pdf/mmiMicroReview.pdf

MicroMeetings reports are short, succinct, personal accounts of the most recent and exciting developments in a particular aspect of microbial molecular biology, as presented at a recent symposium, workshop, colloquium or congress. *MicroMeetings* are written by one or more participants at the meeting. Authors wishing to submit a *MicroMeeting* report should first get in touch with Tony Pugsley, who will decide whether the chosen topic is suitable. Further information on the format and content of *MicroMeetings* is available at http://www.blackwellpublishing.com/pdf/mmiMicroMeeting.pdf

MicroCommentaries are invited articles designed to bring the details of one or more research or review papers published in *Molecular Microbiology* to as broad a readership as possible. They include additional background information and are designed to stimulate the reader to investigate the subject further. Further information on the format and content of *MicroCommentaries* is available at http://www.blackwellpublishing.com/pdf/mmiMicroCommentary.pdf

The cost of publishing colour images will be waived for authors of *MicroReviews, MicroCommentaries* and *MicroMeetings*, who will also, on request to the editors, receive free colour reproduction in the next original article they publish in *Molecular Microbiology*.

Editorial Policy
The Journal publishes studies on prokaryotic and eukaryotic microorganisms. *Molecular Microbiology* particularly encourages studies on organisms where new insights are emerging from the application of molecular approaches. Papers must be broad in scope and lead to general and substantial advances in our understanding of a biological process. Papers reporting incremental advances are not appropriate. Papers should be at the cutting edge of research on any given organism. Comparative studies are not appropriate unless they lead to new biological insights. Manuscripts reporting genome, transcriptome or proteome-wide screening approaches will only be considered suitable for publication if these components are an integral but not the predominant part of a deeper, mechanistic study (see Mol. Microbiol. 52:311-312 (2004) for an Editorial discussing this policy). Genome mapping/sequence papers must lead to novel biological insights or provide the definitive resource. Nucleotide sequence data are welcome as part of a larger study which leads to mechanistic insights. *Molecular Microbiology* does not normally publish primary sequence data in the form of a figure. Instead, such data should be referred to in the text by citing the database accession number (see Supporting Information and Data Deposition).

Submission of Manuscripts
All submissions must be electronic at http://mc.manuscriptcentral.com/mmi. Paper submissions will be returned to the authors for resubmission. A user ID and password are required and can be obtained on the first use of the site. Full instructions are provided. All file types are supported but the following types are recommended: text in Microsoft Word or generic rich text format (RTF) and figures in EPS or TIFF. Macintosh users are advised to add the correct three-letter filename suffix. At the time of initial submission, a single PDF file may be uploaded containing both text and figures. Full instructions for online submission are available via the 'Instructions and Forms' button on the login page at: http://mc.manuscriptcentral.com/mmi

Full instructions and support are available online from the submission site via the Get Help Now button. You can also email for online submission support support@scholarone.com. When submitting online, authors are encouraged to use the option to suggest the names of potential referees and/or referees the author would prefer were not used to review the manuscript. Please do not duplicate your submission by submitting online and by post.

Molecular Microbiology has a large editorial team with experience in all areas of research covered by the Journal. Authors may submit their manuscript to any Editor but should generally choose the one whose interests are closes to their own:

- Bonnie Bassler (Bacterial Gene Regulation and Signal Transduction)
- Steve Bell (Archaea, Replication and Recombination)
- Brendan Cormack (Fungal Molecular Biology and Pathogenesis)
- Peggy Cotter (Bacterial Pathogens)
- Alan Cowman (Parasitology)
- Kenn Gerdes (Bacterial Cell Biology, Translation, and Accessory Elements)
- John Helmann (Editor-in-Chief; Bacterial Gene Regulation, Physiology, Structural Biology)
- John D. McKinney (Bacterial Pathogens and Pathogenesis)
- Tracy Palmer (Membrane Structure, Protein Traffic, and Transport)
- Tony Pugsley (*MicroReviews* and *MicroCommentaries*)

Please note that:

1. All manuscripts (including *MicroReviews*) on eukaryotic parasites must be submitted to Alan Cowman.
2. All other *MicroReviews* must be submitted to Tony Pugsley.
3. All manuscripts on pathogenic fungi must be submitted to Brendan Cormack.
4. All manuscripts on Archaea must be submitted to Steve Bell.

An editor might reassign a paper if there is a conflict of interest, if another editor would be more appropriate or if the chosen editor cannot handle the manuscript in a timely fashion. Authors will normally be asked to approve the change of editor before it is enacted. The temporary absence of an editor will be indicated alongside the editor's name on the online manuscript submission page. Authors can still select this editor, knowing that their manuscript will not be processed until after the date indicated, or they can select another editor from the list. Authors should take note of other details indicated and should act accordingly. If in doubt, authors should submit their manuscripts to John Helmann who will assign them to appropriate editors.

When submitting a manuscript, please suggest six potential reviewers, together with their contact details (including email) and area of expertise.

Every effort will be made to ensure rapid publication. Authors will usually receive reviewers comments within four weeks. The acceptance date will be the date at which the correctly formatted paper with any associated information required, arrives at the production office.

No page charges will be levied.

Papers must be submitted exclusively to *Molecular Microbiology* and are accepted on the understanding that they have not been, and will not be, published elsewhere. If any part of a study submitted to *Molecular Microbiology* is resubmitted at a later date, the manuscript must be sent to the editor who originally handled it. If accepted, papers become copyright of the Journal. Authors must give signed consent to publication in a letter sent with the paper, but permission to use material elsewhere (e.g. in review articles) will normally be granted on request.

All authors are expected to adhere to the Vancouver guidelines outlining best practice. These can be found at www.nejm.org/general/text/requirements/1.htm

Presentation of Manuscripts

Title Page
The title page should include the names of authors, affiliations and the address to which all correspondence and proofs should be sent. Telephone and fax numbers should also be supplied, along with E-mail addresses. Present addresses of authors should appear as a footnote. A running title of not more than 50 characters should be provided together with up to six key words for indexing purposes.

Summary
All papers must normally include a summary not exceeding 200 words.

Text
Papers should be as concise as possible, compatible with clarity and completeness. The main text should be subdivided into *Introduction, Results, Discussion and Experimental procedures*. The *Results* and *Discussion* sections may be combined and can include additional subheadings. *Experimental procedures* should be sufficiently detailed to enable the experiments to be reproduced. Files should be formatted as double-spaced and have no hard returns within paragraphs. All pages must be numbered consecutively. Line numbers should be added to the text prior to electronic submission to facilitate refereeing. Please type your text consistently, e.g. take care to distinguish between 1 (one) and l (lower-case L) and 0 (zero) and O (capital O), etc. Tables and figure legends should follow the main text. Footnotes should be

avoided. Standard abbreviations should be as recommended in *Quantities, Units, and Symbols* (The Royal Society, 1988). Abbreviations of non-standard terms should follow, in parentheses, their first full usage.

Tables
Tables should be typed as text, using tabs to align columns. The use of table editors should be avoided. Do not use graphics software to create tables.

Mathematics
Inline equations should be typed as text. The use of graphics programs and equation editors should be avoided. Displayed equations are rekeyed by our typesetter.

Figures
Please send us digital versions of your figures if at all possible (EPS and TIFF files only). Please consult our web site for further details (http://www.blackwellpublishing.com/bauthor/illustration.asp). Please note that your paper will go through production more quickly if the figures do not have to be relabelled or redrawn. Please ensure that electronic artwork is prepared such that, after reduction to fit across one or two columns or two-thirds width (80 mm, 169 mm or 110 mm respectively) as required, all lettering will be clear and easy to read, i.e. no labels should be too large or too small. Subpanels of multipanel figures should be labelled with upper-case roman letters (A, B, etc.) at the top left-hand corner of each panel. Avoid using tints if possible; if they are essential to the understanding of the figure, try to make them coarse.

It is the policy of *Molecular Microbiology* for authors to pay the full cost for the reproduction of their colour artwork (for exceptions see *'MicroReview'* section). However, if the authors cannot meet these charges, and the editors feel that colour figures are essential for a manuscript, then it may be possible to waive part of these charges. Please note that if there is colour artwork in your article, Blackwell Publishing require you to complete and return a colour work agreement form before your article can be published. This form can be downloaded as a pdf from the internet. The web address for the form is: http://www.blackwellpublishing.com/pdf/SN_Sub2000_X_CoW.pdf

If you are unable to access the internet, or are unable to download the form, please contact the Production Editor at mmi@wiley.com, and they will be able to email or fax a form to you.

Cover Photographs
Photographs suitable for the cover of Molecular Microbiology are welcomed by the editors. It is preferable, but not essential, that these should be related to submitted papers.

References
Authors should use the system illustrated below. Only full articles which have been published or are in press may be included in the reference list. In the text, unpublished or submitted studies should be referred to as such (e.g. J.M. Smith, unpublished), or as a personal communication. It is the authors responsibility to obtain permission from colleagues to include their work as a personal communication. In the text, references should be inserted in parentheses in date order, as follows: (Pugsley, 1996; Matsunaga *et al*., 1997). The reference list should be in alphabetical order according to the first named author. Papers with two authors should follow those of the first named author, arranged in alphabetical order according to the name of the second author. Articles with more than two authors should follow those of the first named author in chronological order. The title of the article must be included. For papers with up to seven authors, the names of all authors should be listed. For papers with eight or more authors, the first six names should be listed, followed by '*et al*.'. Standard abbreviations of journal titles should be used, as in the *Index Medicus*. The following provide examples:

Pugsley, A.P. (1996) Multimers of the precursor of a type IV pilin like component of the general secretory pathway are unrelated to pili. *Mol Microbiol* **20:** 1235-1245.

McGowan, S.J., Sebaihias, M., OLeary, S., Hardie, K.R., Williams, P., Stewart, G.S.A.B., et al. (1997) Analysis of the carbapenem of Erwinia carotovora: definition of the antibiotic biosynthetic genes and evidence for a novel ß-lactam resistant mechanism. *Mol Microbiol* **26:** 545-556.

Higgins, C.F., Causton, H.C., Dance, G.S.G., and Mudd, E.A. (1993) The role of the 30 end in mRNA stability and decay. In Control of Messenger RNA Stability. Belasco, J.G., and Brawerman, G. (eds). San Diego: Academic Press, pp. 13-30.

References to material available on the World Wide Web can be given, but only if the information is available without charge to readers on an official site. Authors will be asked to provide electronic copies of the cited material for inclusion on the *Molecular Microbiology* web site at the discretion of the Editors. The format for citations is as follows:

Beckleheimer, J. (1994). How do you cite URLs in a bibliography? [WWW document]. URL http://www.nrlssc.navy.mil/meta/bibliography.html.

Reference Management and Formatting
We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting. EndNote reference styles can be searched for here:
http://www.endnote.com/support/enstyles.asp

Reference Manager reference styles can be searched for here:
http://www.refman.com/support/rmstyles.asp

Genetic Nomenclature
Standard genetic nomenclature should be used. For further information, including relevant websites, authors should

refer to the Genetic Nomenclature Guide in *Trends in Genetics* (Elsevier Science Ltd, 1995). For other detailed information, authors should consult Bachman (*Microbiol Rev* **47:** 180-230, 1983) for *E. coli* K-12: Sanderson and Roth (*Microbiol Rev* **47:** 310-453, 1983) for *Salmonella typhimurium*. Holloway *et al*. (*Microbiol Rev* **43:** 73-102, 1979) for *Bacillus subtilis*; Perkins *et al*. (*Microbiol Rev* **46:** 426-570, 1982) for *Neurospora crassa*; and the *Handbook of Genetics* Vol. 1 (R. C. King, ed., Plenum Press, 1974) for *Saccharomyces cerevisiae*.

Nomenclature for DNA restriction and modification enzymes and their genes should follow Roberts *et al.* (*Nucleic Acids Res* **31:** 1805-1812, 2003).

Copyright Transfer Agreement
Authors will be required to sign a Copyright Transfer Agreement (CTA) for all papers accepted for publication. Signature of the CTA is a condition of publication and papers will not be passed to the publisher for production unless a signed form has been received. Please note that signature of the Copyright Transfer Agreement does not affect ownership of copyright in the material. (Government employees need to complete the Author Warranty sections, although copyright in such cases does not need to be assigned). After submission authors will retain the right to publish their paper in various medium/circumstances (please see the form for further details). To assist authors an appropriate form will be supplied by the editorial office. Alternatively, authors may like to download a copy of the form at www.wiley.com/go/ctaaglobal.

OnlineOpen
OnlineOpen is a pay-to-publish service from Wiley-Blackwell that offers authors whose papers are accepted for publication the opportunity to pay up-front for their manuscript to become open access (i.e. free for all to view and download) via the Wiley Interscience website. Each OnlineOpen article will be subject to a one-off fee of $3000, excluding colour charges, to be met by or on behalf of the Author in advance of publication. Upon online publication, the article (both full-text and PDF versions) will be available to all for viewing and can be downloaded free of charge. The print version of the article will also be branded as OnlineOpen and will draw attention to the fact that the paper can be downloaded for free via the Wiley Interscience service.

Any authors wishing to send their paper OnlineOpen will be required to complete the combined payment and copyright licence form available from our website at: http://www.blackwellpublishing.com/pdf/MMI_OOF.pdf

(Please note this form is for use with OnlineOpen material ONLY.)

Once complete this form should be sent to the Production Office along with the rest of the manuscript materials at the time of acceptance or as soon as possible after that (preferably within 24 hours to avoid any delays in processing). Do not inform the Editorial Office that you intend to publish your paper OnlineOpen before acceptance.

The copyright statement for OnlineOpen authors will read:

Publication Options for NIH-funded Researchers
Specifically for NIH-funded researchers, *Molecular Microbiology* is launching a trial scheme for researchers who wish to have the final published version of the paper (rather than the accepted manuscript) deposited in PubMed Central (PMC). During the trial period, authors of NIH-funded work can choose one of three publication options:

1. OnlineOpen, which grants free and immediate availability of the article on publication and automatic, immediate deposition of the final pdf version with PMC. Each OnlineOpen article will be subject to a fee of US$3000, excluding colour charges, to be met by or on behalf of the Author in advance of publication.

2. Delayed OnlineOpen, an experimental service for NIH-funded authors that provides automatic deposit of the final, published version in PMC subject to a 12-month embargo. Each Delayed OnlineOpen article will be subject to a fee of US$1000, excluding colour charges, to be met by or on behalf of the Author in advance of publication.

3. Standard publication, under the terms of the Copyright Transfer Agreeement, will enable authors to meet the terms of their grant without paying a fee. Wiley-Blackwell will post the accepted version of manuscripts by NIH grantholders on PMC upon acceptance by the journal. This accepted version will be made publicly available in PMC 12 months after publication.

Proofs
The corresponding author will receive an email alert containing a link to a web site. A working email address must therefore be provided for the corresponding author. The proof can be dowloaded as a PDF (portable document format) file from this site. Acrobat Reader will be required in order to read this file. This software can be downloaded free of charge from the following web site: http://www.adobe.com/products/acrobat/readstep2.html

This will enable the file to be opened, read on screen and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no email address is available. Excessive changes made by the author, excluding typesetting errors, will be charged seperately.

Offprints
A PDF offprint of the online published article will be provided free of charge to the corresponding author, and may be distributed subject to the Publisher's terms and conditions. Paper offprints of the printed published article may be purchased if ordered via the method stipulated on the instructions that will accompany the proofs. Printed offprints are posted to the correspondence address given for the paper unless a different address is specified when ordered. Note that it is not uncommon for printed offprints to take up to eight weeks to arrive after publication of the journal.

Distribution of Strains and Materials
The publication of an article in *Molecular Microbiology* is subject to the understanding that authors will distribute freely any strains, clones or antibodies described therein for use in academic research. Authors might wish to make their plasmid constructs available free of charge through Addgene (www.addgene.org).

Supporting Information and Data Deposition
On-line Supporting Information can include additional explanatory notes, data sets, lists and sequences of oligonucleotides or additional figures or tables that will not be published in the print edition of the journal. Supporting Information must be submitted to and approved by the journal editor with the rest of the article. Any such material that is submitted after acceptance of the main article will be referred to the Editor for approval and may delay publication. If the editor accepts the accompanying paper, the supporting information must be supplied as a single pdf file headed by the title of the paper and the authors' names, addresses and contact information. Supporting Information will be published exactly as supplied and it is the author's responsibility to ensure that the material is logically laid out, adequately described and in a format that is likely to be accessible to readers. Figures and tables should be embedded in the text. Animations and other moving images or sound files in common Internet standard formats such as AVI, MPG, WAV, QuickTime, animated GIF or Flash must be supplied as separate files. Figures and tables in Supporting Information that are referred to in the main text should be labelled Fig. S1, Fig. S2 or Table S1, etc, in the order in which they are cited in the main text. An address for online access to Supporting Information will be added to the main text before it is published.

It is the policy of *Molecular Microbiology* that sequence data must be deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries, the accession number cross-referenced in the published manuscript, and the data made fully available at the time of publication. It is only necessary to submit to one database, without regard to where the sequence data will be published. Data are exchanged between DDBJ, eMBL and GenBank on a daily basis. The suggested wording for referring to accession-number information is: 'These sequence data have been submitted to the DDBJ/eMBL/ GenBank databases under accession number U12345'. Addresses for these databases are as follows:

DDBJ
DNA Data Bank of Japan
Center for Information Biology
National Institute of Genetics
Mishima, Shizuoka 411
Japan
Tel: + 81 559 81 6853
Fax: + 81 559 81 6849
e-mail: ddbjsub@ddbj.nig.ac.jp (for data submissions)
WWW URL: http://www.ddbj.nig.ac.jp

EMBL
EMBL Nucleotide Sequence Submissions
European Bioinformatics Institute
Wellcome Trust Genome Campus
Hinxton
Cambridge, CB10 1SD
UK
Tel: + 44 1223 494400
Fax: + 44 1223 494472
e-mail: datasubs@ebi.ac.uk
WWW URL: www.ebi.ac.uk

GenBank
National Center for Biotechnology Information
National Library of Medicine
Bldg. 38A, Rm 8N-803
Bethesda, MD 20894
USA
Tel: + 1 301 496 2475
Fax: + 1 301 480 9241
e-mail: gb-sub@ncbi.nlm.nih.gov
WWW URL: www.ncbi.nlm.nih.gov

MicroArray data will not be presented as Supporting Information in published manuscripts. Instead, they should be organized and presented in compliance with current practices (see for example (MGeD Society at http://www.mged.org/) and deposited in an accredited data base such as Array express (http://www.ebi.ac.uk/microarray-as/ae/) or NCIB GEO (http://www.ncbi.nlm.nih.gov/geo/). The corresponding reference to the database entry should be included in the text and will be hot-linked in the electronic version of the manuscript when it is published. Authors must ensure that the data are in the database immediately when the manuscript is accepted for publication. At earlier stages, authors should supply the data as supplementary material for referee access.

Author material archive policy
Please note that unless specifically requested Wiley-Blackwell will dispose of all hard copy or electronic material submitted 2 months after publication. If you require the return of any material submitted, please inform the editorial office or production editor as soon as possible if you have not already done so.