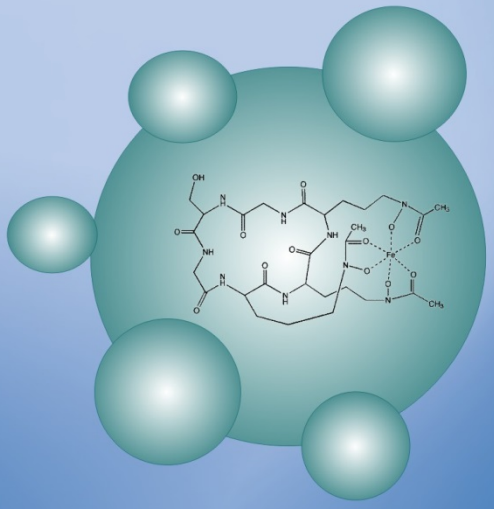
UNIVERSIDADE DE BRASÍLIA
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO
EM PATOLOGIA MOLECULAR

Captação de ferro mediada por sideróforos em *Paracoccidioides* spp.

TESE DE DOUTORADO

Candidata: Mirelle Garcia Silva
Orientadora: Dra. Célia Maria de Almeida Soares



Brasília - 2014

UNIVERSIDADE DE BRASÍLIA

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA MOLECULAR

Captação de ferro mediada por sideróforos em *Paracoccidioides* spp.

Tese apresentada ao Programa de Pós-Graduação em Patologia Molecular, da Faculdade de Medicina, Universidade de Brasília, como requisito para obtenção do título de Doutor em Patologia Molecular

Candidata: Mirelle Garcia Silva

Orientadora: Prof[a]. Dr[a]. Célia Maria de Almeida Soares

Brasília – DF

Fevereiro – 2014

Ficha catalográfica elaborada pela Biblioteca Central da Universidade de Brasília. Acervo 1014798.

Silva, Mirelle Garcia.
S586c Captação de ferro mediada por sideróforos em Paracoccidioides spp. / Mirelle Garcia Silva. -- 2014.
xiii, 130 f. : il. ; 30 cm.

Tese (doutorado) - Universidade de Brasília, Faculdade de Medicina, Programa de Pós-graduação em Patologia Molecular, 2014.
Inclui bibliografia.
Orientação: Maria de Almeida Soares.

1. Paracoccidioides brasiliensis. 2. Ferro. 3. Sideróforos. I. Soares, Célia Maria de Almeida. II. Título.

CDU 616.993.192.1(7/8=4)

TRABALHO REALIZADO NO LABORATÓRIO DE BIOLOGIA MOLECULAR, DO DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR, DO INSTITUTO DE CIÊNCIAS BIOLÓGICAS DA UNIVERSIDADE FEDERAL DE GOIÁS.

APOIO FINANCEIRO: CAPES/ CNPq/ FINEP/ FAPEG.

BANCA EXAMINADORA

TITULARES

Profa. Dra. Célia Maria de Almeida Soares – Presidente da banca
Instituto de Ciências Biológicas, Universidade Federal de Goiás

Profa. Dra. Nilce Maria Martinez-Rossi
Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo

Prof. Dr. Sébastien Olivier Charneau
Instituto de Ciências Biológicas, Universidade de Brasília

Profa. Dra. Izabela Marques Dourado Bastos
Instituto de Ciências Biológicas, Universidade de Brasília

Profa. Dra. Maristela Pereira
Instituto de Ciências Biológicas, Universidade Federal de Goiás

SUPLENTE

Profa. Dra. Anamélia Lorenzetii Bocca
Instituto de Ciências Biológicas, Universidade de Brasília

O que se multiplica em nós é a soma de tudo aquilo que dividimos.

Manoel Affonso de Mello

Às pessoas que dão sentido à minha vida...

...meus pais José e Lucélia.

Pai e mãe, serenidade e astúcia, estas duas qualidades, inerentes a cada um de vocês dois, se uniram e em um só espírito, me educaram. Deram-me bons conselhos, pautados em ótimos exemplos. Ensinaram-me que bons frutos são colhidos após árduo trabalho. Mostraram-me que a honestidade e o amor e respeito ao próximo são caminhos para uma vida harmônica e feliz. Agradeço pelos ensinamentos e amor incondicional.

...meu esposo Alexandre.

No início, sua postura no trabalho chamou minha atenção. Com o tempo, seus gestos de bondade e honestidade mereceram minha admiração. Agora espero que meu amor seja suficiente para agradecer pelo fato de você ter escolhido viver uma só vida junto a mim. Obrigada por ser paciente, carinhoso e meu maior incentivador. Sua presença torna meus dias mais serenos e minha vida cheia de esperança.

...minha irmã Marielle.

Agradeço pelo apoio e pela ajuda nunca negada. É muito bom crescer junto e ter com quem dividir as experiências e surpresas de cada dia.

AGRADECIMENTOS

A Deus, pela oportunidade da vida e pela capacidade de vivê-la.

À minha orientadora Célia, por me receber em seu grupo de trabalho. Agradeço pela orientação e por contribuir para meu amadurecimento profissional e pessoal. Obrigada pelas oportunidades concedidas e por empenhar-se no intuito de sempre oferecer aos seus alunos subsídios para realização de trabalhos de qualidade.

Ao meu co-orientador durante o estágio no exterior, Dr. Hubertus Haas, e toda sua equipe de trabalho (Beatrix, Fabio, Nicola, Mia, Michael e Lukas). Agradeço a hospitalidade e por sempre estarem dispostos a me auxiliar. Obrigada por compartilharem comigo seu conhecimento. Fazer parte do grupo por 4 meses foi uma experiência enriquecedora.

A CAPES, pela concessão da bolsa de doutorado no Brasil e de doutorado sanduíche no exterior. Aos demais órgãos financiadores de pesquisa CNPq, FINEP e FAPEG.

Aos professores do Laboratório de Biologia Molecular, da Universidade Federal de Goiás, Maristela Pereira, Alexandre Bailão, Clayton Borges, Juliana Parente e Sílvia Salém-Izaac, por trabalharem em conjunto com o objetivo de manter um laboratório de pesquisa funcional frente a condições tão adversas de trabalho.

Aos professores da Universidade de Brasília, Bergmann Ribeiro, Izabela Dourado e Sébastien Charneau pelas contribuições e sugestões durante o exame de qualificação. A todos os outros professores da mesma universidade que ministraram as disciplinas durante o doutorado e aos funcionários do programa de pós-graduação, Jaqueline e Dênis Bonfim, pela agilidade e disponibilidade em ajudar.

Aos professores m embros da banca, por disponibilizarem parte de seu tempo e por contribuírem para o aperfeiçoamento do trabalho.

A todos os colaboradores do trabalho pela contribuição e agregação de conhecimento.

Às minhas amigas e companheiras de trabalho, Elisa Flávia Bailão e Patrícia Lima. Obrigada por compartilharem comigo suas experiências e conquistas. Agradeço pelo carinho, força e pelas palavras e gestos de incentivo. Patrícia, obrigada por tornar meus dias de trabalho mais alegres e prazerosos. Elisa Flávia, agradeço por me ajudar a ser mais objetiva e, ao mesmo tempo, sensível. Espero conviver com vocês por tempo ainda...

Aos amigos e colegas de trabalho, Luciana Casaletti, Lilian Baeza, Juliana de Cúrcio, Mariana Tomazett e Lucas Nojosa, obrigada por contribuírem para o bom andamento laboratório e, consequentemente, para o bom andamento do trabalhado de cada aluno. Agradeço também pelos ótimos momentos de descontração, que ajudam a aliviar o cansaço de uma rotina intensa.

A todos os colegas do Laboratório de Biologia Molecular pela boa convivência.

À minha amiga Daniele Silva, por todo o apoio, principalmente durante o estágio sanduíche.

A todos os membros da minha família pelo apoio incondicional e palavras de incentivo. Suas orações e energia positiva me deram forças para nunca desistir.

A todos que, de alguma maneira, contribuíram para o desenvolvimento e a finalização deste trabalho.

SUMÁRIO

LISTA DE ABREVIATURAS

CoA – coenzima A

DA – ácido dimerúmico

DMT1 – transportador de metal divalente 1

FO – ferrioxamina

FPN – ferroportina

gp 43 – glicoproteína 43

H_2O_2 – peróxido de hidrogênio

IFN-γ – intérferon gama

IL-1 – interleucina-1

IL-6 – interleucina-6

Kb – kilo (quilo) pares bases

Lcn – lipocalina

Mb – mega pares bases

MFS – classe de proteínas transportadoras de membrana

Mr – massa molecular relativa

Nramp1 – proteína de macrófago 1 associada à resistência natural

NRPS – peptídeo sintetases não ribossômicas

*Pb*01 – isolado 01 de *Paracoccidioides lutzii*

*Pb*02 – isolado 2 de *Paracoccidioides brasiliensis*

*Pb*18 – isolado 18 de *Paracoccidioides brasiliensis*

*Pb*Epm83 – isolado Emp83 de *Paracoccidioides brasiliensis*

PCM – paracoccidioidomicose

PS2 – espécie filogenética 2

PS3 – espécie filogenética 3

PS4 – espécie filogenética 4

PTS – sinal de endereçamento peroxissomal

qRT-PCR – reação em cadeia da polimerase (PCR) quantitativa (q) utilizando a enzima transcritase reversa (RT)

RP-HPLC – cromotagrafia líquida de fase reversa

S1 – espécie 1

SIT – transportadores do complexo sideróforo-Fe^{3+}

TAFC – triacetilfusarinina C

TFR - receptor de transferrina

TNF-α – fator de necrose tumoral alfa

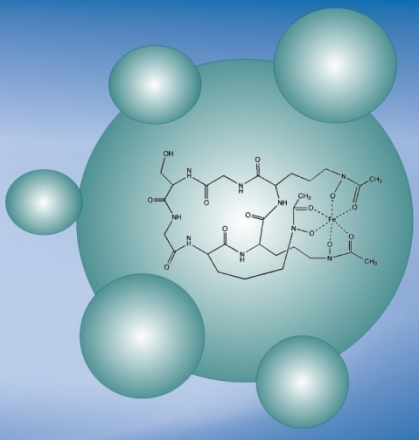
RESUMO

O gênero *Paracoccidioides* inclui espécies fúngicas termodimórficas, causadoras da paracoccidioidomicose, uma micose sistêmica endêmica da América Latina. A infecção ocorre quando propágulos micelianos ou conídios são inalados pelo hospedeiro. Após conversão para levedura nos alvéolos pulmonares o fungo pode disseminar-se para outros órgãos e tecidos. O ferro é um micronutriente essencial para todos os eucariotos, pois participa de vários processos biológicos essenciais. Entretanto, a biodisponibilidade deste metal dentro do hospedeiro é baixa. Como consequência, micro-organismos patogênicos desenvolveram mecanismos de aquisição de alta afinidade como forma de obter ferro durante a infecção. O presente trabalho descreve a aquisição de ferro mediada por sideróforos neste fungo. Análises *in silico* demonstraram que as espécies do gênero *Paracoccidioides* possuem todos os genes necessários para síntese e captação de sideróforos, os quais são produzidos em condições de depleção de ferro. Análises de cromatografia líquida de fase reversa e espectrometria de massas revelaram que *Paracoccidioides* spp. produz sideróforos do tipo hidroxamato. O fungo sintetiza e secreta coprogeno B, o qual gera ácido dimerúmico como produto de degradação, e também produz ferricrocina e ferricromo C como sideróforos intracelulares. Adicionalmente, *Paracoccidioides* spp. é capaz de crescer na presença de sideróforos como única fonte de ferro, demonstrando que além de produzir, o fungo também utiliza siderofóros para o crescimento, incluindo o xenosideróforo ferrioxamina. A exposição prévia a ferrioxamina aumentou a sobrevivência de *Paracoccidioides* spp. após fagocitose por macrófagos ativados. Além disso, o fungo provavelmente induz a síntese de sideróforos quando no interior destas células, demonstrando que estas moléculas provavelmente desempenham papel importante durante a interação patógeno-hospedeiro. Ademais, sideróforos produzidos por *Paracoccidioides* spp. podem ser utilizados como fontes de ferro por *Aspergillus nidulans*. Em conjunto, estes dados demonstraram que a síntese e a utilização de sideróforos são mecanismos empregados por *Paracoccidioides* spp. para superar a limitação de ferro. Como a escassez deste micronutriente é encontrada no hospedeiro, a produção de sideróforos está provavelmente relacionada à patogenicidade e virulência do fungo e representa um possível alvo para terapia com antifúngicos levando-se em consideração a ausência de tal via em humanos.

ABSTRACT

The genus *Paracoccidioides* includes termodimorphic fungal species which causes paracoccidioidomycosis, a systemic mycosis endemic in Latin America. The infection occurs when mycelium propagules or conidia are inhaled by the host. After conversion to yeast in the alveoli the fungus may disseminate to other organs and tissues. Iron is an essential micronutrient for all eukaryotes, since it participates in a variety of essential biological processes. However, the bioavailability of this metal is low inside the host. As a consequence, pathogenic microorganisms evolved high affinity acquisition mechanisms to obtain iron during infection. Here we describe the siderophore mediated iron acquisition in this fungus. *In silico* analysis demonstrated that species from *Paracoccidioides* genus possess all the necessary genes for synthesis and uptake of siderophores, which are produced under iron limiting conditions. Reversed phase liquid chromatography and mass spectrometry analysis revealed that *Paracoccidioides* spp. produce siderophores of hydroxamate type. The fungus synthesizes and secretes coprogen B, which generates dimerumic acid as a breakdown product, and also produces ferricrocin and ferrichrome C as intracellular siderophores. Moreover, *Paracoccidioides* spp. is able to grow in presence of siderophores as the only iron sources, demonstrating that beyond producing, the fungus also utilizes siderophores for growth, including the xenosiderophore ferrioxamine. Previous exposure to ferrioxamine increased *Paracoccidioides* spp. survival following phagocytosis by activated macrophages. Moreover, the fungus probably induces siderophore synthesis inside these cells, demonstrating that these iron chelators play an important role during host-pathogen interaction. Additionally, siderophores produced by *Paracoccidioides* spp. can be utilized as iron sources by *Aspergillus nidulans*. Altogether, these data demonstrated that synthesis and utilization of siderophores are mechanisms employed by *Paracoccidioides* spp. to surpass iron limitation. As iron paucity is found within the host, siderophore production may be related to fungus pathogenicity and virulence and represents a possible target for antifungal therapy since these pathway is absent in humans.

Capítulo 1



INTRODUÇÃO

1.1. O fungo *Paracoccidioides* spp.

O gênero *Paracoccidioides* inclui fungos patogênicos causadores da paracoccidioidomicose (PCM) pertencentes ao filo Ascomycota, classe Euromyceto, ordem Onygenales e família Ajellomycetaceae, a qual inclui também os patógenos *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immites* e *Coccidioides posadasii* (BAGAGLI et al., 2006; UNTEREINER et al., 2004). A capacidade de alternar entre duas formas distintas, cada uma associada a condições ambientais específicas, insere tais patógenos no grupo dos fungos dimórficos, responsáveis pela maioria das infeções sistêmicas em humanos e outros mamíferos. Esses micro-organismos são considerados patógenos primários, ou seja, podem causar doença em indivíduos imunocompetentes (RAPPLEYE e GOLDMAN, 2006). O estímulo mais evidente no dimorfismo de *Paracoccidioides* spp. é a temperatura, o que o classifica como termodimórfico. Quando cultivado em temperaturas inferiores a 28 ^{o}C, a forma de micélio se desenvolve. Já a 36 ^{o}C, temperatura do hospedeiro mamífero, *Paracoccidioides* spp. cresce como levedura (BAGAGLI et al., 2006). Assim, o sucesso da infecção depende da capacidade do fungo de transitar da forma filamentosa para leveduras unicelulares. Isolados incapazes de se diferenciar não são virulentos (DE MORAES BORBA e SCHAFFER, 2002; ROONEY e KLEIN, 2002).

Paracoccidioides brasiliensis foi considerada a única espécie representante do gênero até 2006. Análises filogenéticas baseadas em dados de polimorfismo de 65 isolados de *P. brasiliensis* permitiram a classificação do fungo em três espécies crípticas distintas: S1 (espécie 1) com 38 isolados, PS2 (espécie filogenética 2) com 6 isolados e PS3 (espécie filogenética 3) com 21 isolados. S1 inclui isolados do Brasil, Argentina, Paraguai, Uruguai, Peru e Venezuela. PS2 ocorre no Brasil e Venezuela, enquanto PS3 é restrita à Colômbia, sendo considerada evolucionariamente independente (MATUTE et al., 2006) (**Figura 1**). PS4 (espécie filogenética 4) foi descrita recentemente e inclui 5 isolados clínicos da Venezuela (BOCCA et al., 2013; SALGADO-SALAZAR et al., 2010). Estudos filogenéticos posteriores realizados com 21 isolados de *P. brasiliensis* demonstraram que todos eles foram agrupados nos grupos filogenéticos descritos anteriormente (S1 e PS3), com exceção do isolado 01 (*Pb*01), sugerindo a possibilidade da existência de mais de três espécies crípticas (CARRERO et al., 2008). Tal hipótese

foi investigada por meio da comparação entre o grupo formado pelas espécies S1, PS2 e PS3 e o grupo "semelhante a *Pb*01", compreendendo o isolado *Pb*01 e outros isolados da América Latina. A análise de 13 *loci* polimórficos indicou que o fluxo genético entre estes dois grupos foi bloqueado, sugerindo isolamento reprodutivo. Com base nos dados, foi proposta a descrição de uma nova espécie dentro do gênero *Paracoccidioides,* contendo o clado "semelhante a *Pb*01" (TEIXEIRA et al., 2009). Análises filogenéticas moleculares baseadas em marcadores mitocondriais ofereceram novas evidências de que *Pb*01 poderia realmente ser uma nova espécie (SALGADO-SALAZAR et al., 2010), a qual foi proposta recentemente como *Paracoccidioides lutzii*, em homenagem à Adolpho Lutz, o primeiro pesquisador a observar o fungo em 1908 (TEIXEIRA et al., 2013a; TEIXEIRA et al., 2009). Propõe-se que *P. lutzii* seja endêmico da região Centro-Oeste do Brasil. No entanto, isolados pertencentes à nova espécie já foram detectados em Rondônia e no Equador (TEIXEIRA et al., 2013a; TEIXEIRA et al., 2009) (**Figura 1**).

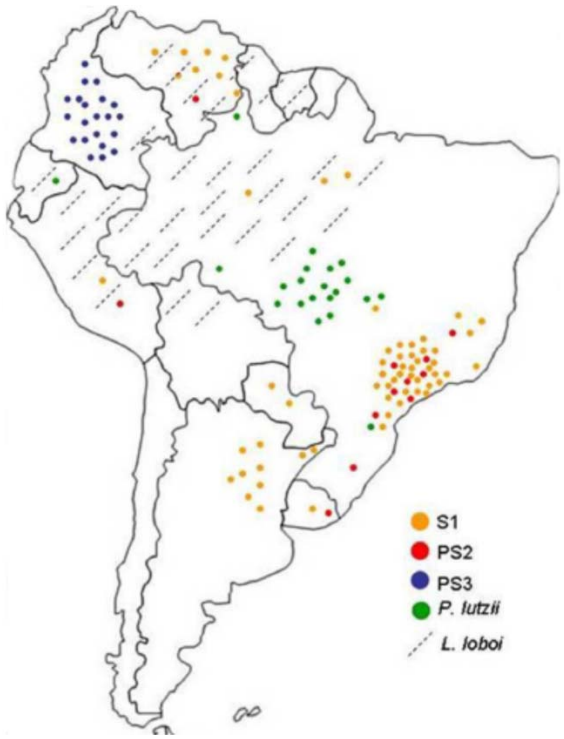


Figura 1. Distribuição geográfica do gênero *Paracoccidioides*. Distribuição geográfica atual das espécies do gênero: *P. brasiliensis* (S1, PS2, PS3) e *P. lutzii*. O fungo patogênico *Lacazia loboi* (*L. loboi*) foi incluído recentemente na família Ajellomycetaceae, sendo considerada espécie irmã de *Paracoccidioides* spp. Fonte: Theodoro et al., (2012).

Análises comparativas entre genomas demonstraram que isolados de *P. brasiliensis* são mais similares entre si do que quando comparados a *P. lutzii*. Os genomas de *Pb*18 (S1) e *Pb*03 (S2) compreendem, respectivamente, 30 e 29,1 Mb, enquanto o de *P. lutzii* (*Pb*01) possui 32,9 Mb. A porcentagem de similaridade de sequências é maior entre *Pb*18 e *Pb*03 (~96%) em comparação com *P. lutzii* (~90%). Diferenças também existem com relação ao genoma mitocondrial, que em *Pb*18 e *Pb*03 compreende cerca de 75 kb, o que é praticamente duas vezes o tamanho predito para *P. lutzii* (~31 kb). O número total de genes preditos codificantes para proteínas também vaira entre os três genomas, entre 7.875 (*Pb*03) e 9.132 (*P. lutzii*) (DESJARDINS et al., 2011).

Perfis metabólicos específicos entre membros do gênero *Paracoccidioides* foram evidenciados recentemente por meio de análises proteômicas. *Pb*01, representando *P. lutzii*, utiliza preferencialmente a via anaeróbia (glicólise e fermentação) para produzir energia a partir de glicose, o que foi corroborado pela detecção de maior quantidade de etanol nas células desta linhagem. Representantes de *P. brasiliensis* apresentam características metabólicas distintas, quando comparados entre si e com *P. lutzii*. A beta-oxidação, que permite a geração de acetil-CoA para o metabolismo central de carbono a partir de ácidos graxos, é marcante em *Pb*339 (S1). *Pb*2 (PS2) parece obter energia principalmente pela degradação de aminoácidos, que fornece substratos para o ciclo do ácido tricarboxílico. Já *Pb*Epm83 (PS3) utiliza a via aeróbia para obter energia a partir do catabolismo da glicose pela via glicolítica e ciclo do ácido tricarboxílico (PIGOSSO et al., 2013).

No que diz respeito à morfologia, levedura e micélio de ambas as espécies do gênero *Paracoccidioides* são similares. Ambas as formas são multinucleadas, enquanto os conídios produzidos pelo micélio apresentam somente um núcleo (CANO et al., 1998; MCEWEN et al., 1987b). Microscopicamente, as leveduras apresentam um aspecto de roda de leme de navio, onde uma célula central grande é circundada por células periféricas menores, as quais são brotamentos múltiplos originados por evaginações da célula-mãe (**Figura 2A**). Os micélios são septados com conídios terminais ou intercalares (**Figura 2C**) (QUEIROZ-TELLES, 1994; RESTREPO-MORENO, 2003). Macroscopicamente, as colônias de leveduras são rugosas e amareladas, apresentando aspecto cerebriforme (**Figura 2B**). Já as colônias de micélio são pequenas, irregulares e com aspecto algodonoso (**Figura 2D**) (BRUMMER et al.,

1993). A forma e tamanho dos conídeos, esporo assexuais, parecem ser as únicas características morfológicas distintas entre *P. lutzii* e *P. brasiliensis*. Conídios produzidos por isolados de *P. lutzii* são maiores e mais alongados (BOCCA et al., 2013; TEIXEIRA et al., 2013a; TEIXEIRA et al., 2009; THEODORO et al., 2012).

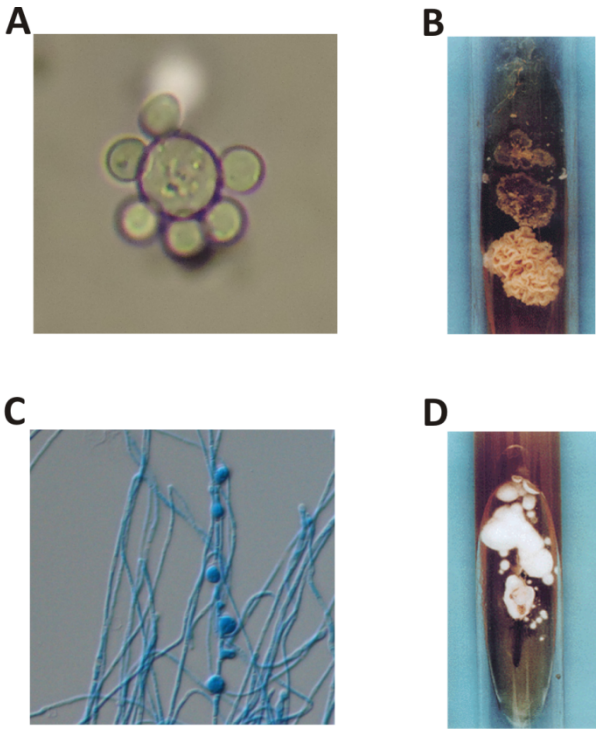


Figura 2. Morfologia de *Paracoccidioides* spp. Características microscópicas de levedura (**A**) e micélio (**C**). Aspecto de colônias crescidas a 37 ^{o}C (levedura, **B**) e 25 ^{o}C (micélio, **D**). Fontes: Laboratório de Biologia Molecular, Universidade Federal de Goiás; Lacaz et al., (1999) e Universidade de Chiba, Japão (http://www.pf.chiba-u.ac.jp/gallery/fungi/p/Paracoccidioides_brasiliensis_mycelial_form-2.htm).

Para a maioria das doenças infecciosas não transmissíveis, as áreas nas quais a infecção ocorre geralmente não coincidem com aquelas nas quais os casos são diagnosticados. Com base nos longos períodos de latência registrados para a PCM, o termo "reservárea" foi proposto para designar os lugares onde *Paracoccidioides* spp. encontra-se na natureza e nos quais o homem adquire a infecção (BORELLI, 1964). Em virtude desse período de latência prolongado no hospedeiro humano, o habitat e nicho ecológico do fungo permanecem imprecisos. Entretanto, estudos sugerem que a forma miceliana do fungo viva saprobioticamente na natureza, pois o mesmo já foi isolado de solo, água e plantas (RESTREPO et al., 2001). Supõe-se que o fungo ocorra

normalmente em ambientes úmidos, próximos a rios, onde possa ser protegido por representantes de espécies aquáticas heterotérmicas como moluscos, anfíbios, peixes e artrópodes. Estes organismos forneceriam nutrientes, umidade, competição biológica limitada e temperatura apropriada ao parasito para sobrevivência do mesmo no meio ambiente (CONTI-DIAZ, 2007). O cultivo de isolados de *Paracoccidioides* spp. em diferentes tipos de solo demonstrou que o fungo pode se desenvolver em solos arenosos e argilosos, com alta umidade. A produção de conídeos, estruturas que aumentam a capacidade de sobrevivência no meio ambiente, foi observada em alguns isolados. (TERÇARIOLI et al., 2007). Apesar da ausência de um estágio teleomórfico, dados moleculares e morfológicos evidenciaram a possibilidade de existência de um ciclo sexual no gênero *Paracoccidioides* (TEIXEIRA et al., 2013b).

Em decorrência do isolamento raro e não reprodutível do fungo do solo, acredita-se que este ambiente não seja o habitat permanente do patógeno, sugerindo que o mesmo reside por tempo variável neste local (CONTI-DIAZ, 2007). *Paracoccidioides* spp. é frequentemente isolado de tatus (*Dasypus novemcinctus* e *Cabassous centralis*) e já foi detectado em animais domésticos, como cachorros (RICCI et al., 2004). Estes organismos devem ser considerados hospedeiros acidentais e não reservas naturais do fungo, pois os mesmos podem ser acometidos com a doença (CONTI-DIAZ, 2007). Recentemente, infecção por *P. brasiliensis* foi reportada em porcos domésticos, apesar de o fungo não ter sido isolado dos animais (BELITARDO et al., 2014). Por estarem em contato constante com o solo e possuírem uma resposta imunológica celular fraca, tatus podem infectar-se repetidas vezes (RICHINI-PEREIRA et al., 2009). Dessa forma, sugere-se um possível papel desses animais no ciclo natural do fungo devido à sua habilidade de eliminar e/ou disseminar o patógeno no ambiente.

1.2. A paracoccidioidomicose (PCM)

A PCM é uma micose sistêmica causada pela inalação dos conídeos e/ou propágulos produzidos pela forma miceliana de *Paracoccidioides* spp. que, ao atingirem o epitélio pulmonar, transformam-se em levedura, a forma parasitária (MCEWEN et al., 1987a). A partir dos pulmões o fungo pode disseminar-se através das vias hematogênica e/ou linfática, chegando a outros órgãos e sistemas, podendo ser fatal (CAMARGO e FRANCO, 2000; VALERA et al., 2008).

Geograficamente, a doença é restrita às Américas Central e do Sul, com maior prevalência no Brasil, Colômbia, Venezuela e Argentina. Como não há notificação compulsória da doença, os dados sobre sua incidência são baseados em relatos de inquéritos epidemiológicos e de séries de casos. No Brasil, acredita-se que em zonas endêmicas a incidência varie de 3 a 4 novos casos/milhão até 1 a 3 casos por 100 mil habitantes ao ano (SHIKANAI-YASUDA et al., 2006). Entre os anos de 1980 a 1995, a PCM foi considerada como a oitava causa de mortalidade por doença infecciosa predominantemente crônica entre as doenças infecciosas e parasitárias (COUTINHO et al., 2002). A maioria das mortes causadas por micoses sistêmicas entre 1996 e 2006 ocorreu devido à PCM, com uma média anual de 148 mortes no período 2005-2006. Os maiores índices de mortalidade estão na região Sudeste, principalmente nos estados de São Paulo, Rio de Janeiro e Minas Gerais, e na região Sul, nos estados do Paraná e Rio Grande do Sul (PRADO et al., 2009). Casos de PCM em áreas não endêmicas já foram diagnosticados na Europa, nos Estados Unidos da América e na Ásia (AJELLO e POLONELLI, 1985; BUITRAGO et al., 2011; CHIKAMORI et al., 1984; JOSEPH et al., 1966). Isso pode ser explicado pela existência de um período assintomático da PCM, o qual é em média 15,3 anos, que ocorre após o contato do paciente com a área endêmica até a manifestação da doença (BRUMMER et al., 1993).

A classificação clínica da PCM inclui: PCM infecção (forma subclínica) e PCM doença, a qual inclui a forma aguda/subaguda (tipo juvenil) e a forma crônica (tipo adulto). Na PCM infecção o paciente não apresenta sinais ou sintomas da doença mas possui reatividade positiva ao antígeno paracoccidioidina. A forma juvenil é responsável por 3 a 5% dos casos da doença e é caracterizada pelo envolvimento do sistema fagocítico mononuclear (linfonodos, fígado, baço e medula óssea) (**Figura 3A**). Acomete crianças, adolescentes e adultos jovens (com menos de 35 anos) de ambos os sexos, sendo considerada de evolução rápida e severa. A forma crônica responde por mais de 90% dos casos e geralmente ocorre em adultos entre 30 e 60 anos de idade, sendo o sexo masculino o mais afetado. Pode ser unifocal quando um único órgão é afetado, geralmente os pulmões, ou multifocal, quando a doença dissemina-se para vários órgãos. Os pulmões são os órgãos mais afetados, seguidos pela pele e membranas mucosas, oral e nasal principalmente (**Figura 3B**). A forma crônica progride lentamente, de forma silenciosa, podendo levar anos até que seja diagnosticada (BOCCA et al., 2013; SHIKANAI-YASUDA et al., 2006).

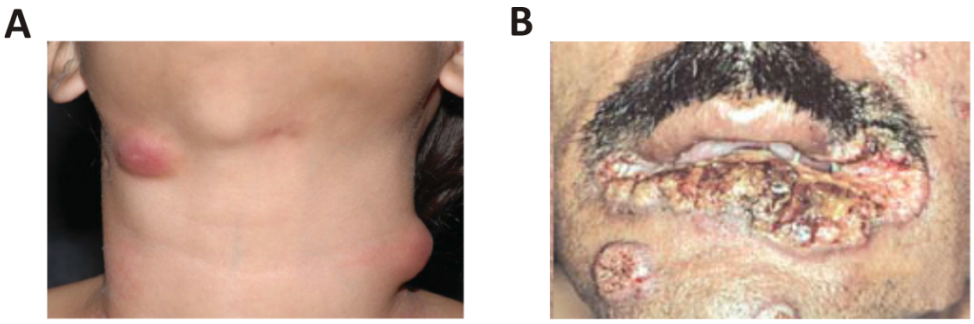


Figura 3. Manifestações clínicas da PCM. A. Forma aguda apresentando linfonodos aumentados, com aspecto inflamatório e formação de abcesso. **B**. Acometimento peri-oral e mentoniano na forma crônica. Fontes: Marques (2013) e Shikanai-Yasusa et al., 2006.

A menor incidência da PCM em mulheres pode ser explicada pela interação do fungo com o hormônio feminino β-estradiol. Análises da transição dimórfica de isolados de *Paracoccidioides* spp. na presença de 17β-estradiol demonstraram que o número de células que transitaram para a forma de levedura é menor (RESTREPO et al., 1984). Estudos adicionais evidenciaram a ligação do estradiol a uma proteína presente no citoplasma de micélio e levedura (LOOSE et al., 1983; STOVER et al., 1986). Além disso, o bloqueio da transição foi demonstrado em estudos com animais (ARISTIZABAL et al., 2002; ARISTIZABAL et al., 1998). Acredita-se que a proteína encontrada no citoplasma do fungo funcione como um receptor para o hormônio e que este iniba a transição micélio-levedura, impedindo, desta forma, o estabelecimento da infecção. Análises transcricionais indicaram que as ações inibitórias de 17β-estradiol são decorrentes da ação de genes sinalizadores que regulam o dimorfismo (SHANKAR et al., 2011). Estes dados, aliados ao menor contato de mulheres com o solo, corroboram dados epidemiológicos que demonstram a menor incidência da PCM no sexo feminino.

O estabelecimento e a severidade da PCM dependem tanto de fatores inerentes ao fungo, como virulência e composição antigênica, quanto da habilidade do hospedeiro em desenvolver uma resposta imunitária efetiva. O controle da infecção depende da resposta imune celular, geralmente associada ao padrão tipo 1, caracterizado pela síntese de citocinas que ativam macrófagos, os quais apresentam atividades fungistáticas e fungicidas. Como resultado, granulomas compactos se formam permitindo o controle da replicação do fungo. Entretanto, em pacientes infectados que apresentam depressão da resposta tipo 1, granulomas não são formados e há evolução

para doença. Neste contexto, formas mais graves evoluem com predomínio de resposta imunológica tipo 2, onde há maior ativação de linfócitos B, hipergamaglobulinemia e altos títulos de anticorpos específicos, cuja magnitude, em geral, correlaciona-se positivamente com a gravidade e disseminação da doença. Apesar de altos níveis séricos de anticorpos específicos serem detectados em pacientes com formas graves e disseminadas, a resposta imunitária humoral não é efetiva contra o fungo (FORTES et al., 2011; SHIKANAI-YASUDA et al., 2006).

O diagnóstico definitivo da PCM requer a demonstração direta do fungo em exame a fresco de escarro, raspado de lesão ou aspirado de linfonodos e/ou fragmento de biopsia de órgãos supostamente acometidos. Entretanto, a condição do paciente pode às vezes dificultar a coleta do material biológico para o exame direto. Assim, provas sorológicas específicas são realizadas e têm importância não apenas no auxílio do diagnóstico como, também, na avaliação da resposta do hospedeiro ao tratamento específico (SHIKANAI-YASUDA et al., 2006), o qual comumente inclui os antifúngicos trimetropim-sulfametoxazol, anfotericina B e itraconazol. O primeiro é utilizado no tratamento das formas clínicas brandas a moderadas enquanto as formas severas e disseminadas são tratadas com anfotericina B. De forma geral, o tratamento é de longa duração, para permitir o controle das manifestações clínicas da micose e evitar as recaídas (BOCCA et al., 2013; SHIKANAI-YASUDA et al., 2006).

Apesar de ambas as espécies de *Paracoccidioides* serem capazes de causar PCM, particularidades regionais, ainda não explicadas, já foram observadas no diagnóstico, manifestações clínicas e tratamento da doença. A presença frequente de uma forma linfática-abdominal da PCM foi observada em Goiás (BARBOSA et al., 1968), fato que pode ser relacionado à *P. lutzii* como agente causador, já que esta espécie é endêmica no estado (TEIXEIRA et al., 2013a). Adicionalmente, já foi reportado que isolados de *P. lutzii*, mas não de *P. brasiliensis*, causaram peritonite progressiva e intensa seguida de rápida disseminação para o baço, fígado, linfonodos e mesentério intestinal em camundongos (MOLINARI-MADLUM et al., 1999). Testes sorológicos com o antígeno gp43 são realizados frequentemente para auxiliar no diagnóstico e tratamento da PCM. O teste padrão utiliza antígenos produzidos pela linhagem 339 de *P. brasiliensis*, pertencente à espécie críptica S1. Foi observado que soros de pacientes da região Centro-Norte do Brasil apresentaram baixa reatividade na presença do antígeno padrão (BATISTA et al., 2010). Além disso, amostras sorológicas

da região Centro-Oeste do Brasil também exibiram baixa reatividade quando confrontadas com gp43 produzida por isolado da espécie críptica PS3 de *P. brasiliensis* (MACHADO et al., 2013). Tais fatos indicam que testes sorológicos desenvolvidos para *Paracoccidioides* spp não são igualmente eficientes para todas as espécies, o que pode ser explicado pelo alto nível de variação de aminoácidos na cadeia polipeptídica do antígeno gp43 de *P. lutzii* e *P. brasiliensis* (MORAIS et al., 2000; TEIXEIRA et al., 2009). No que diz respeito ao tratamento, paciente infectados com *P. lutzii* respondem melhor ao trimetropim-sulfametoxazol do que aqueles acometidos por *P. brasiliensis* (HAHN et al., 2003). Tais estudos denotam a necessidade de contínuas e posteriores investigações a respeito do papel de ambas as espécies do gênero *Paracoccidioides* na patogenicidade da PCM.

1.3. O micronutriente ferro e sua homeostase

O ferro é o segundo metal, depois do alumínio, e o quarto elemento mais abundante da crosta terrestre. As propriedades deste metal de transição permitem sua existência em dois principais estados de oxidação: a forma reduzida, ou íon ferroso (Fe^{2+}), e a forma oxidada, ou íon férrico (Fe^{3+}) (OUTTEN e THEIL, 2009). Esta capacidade de ganhar ou perder elétrons permite que o ferro seja sensível às flutuações no potencial óxido-redutor, o que é relevante do ponto de vista fisiológico. Sozinho, incorporado em grupos ferro-enxofre ou no grupo heme o ferro liga-se a várias proteínas envolvidas em processos celulares essenciais que incluem a respiração, o ciclo do ácido tricarboxílico, a regulação gênica, bem como a biossíntese de aminoácidos, desoxirribonucleotídeos, lipídeos e esteróis (CAIRO et al., 2006). Apesar de ser descrito que bactérias ácidas láticas não requerem ferro para o metabolismo geral (PANDEY et al., 1994), já foi demonstrado que algumas utilizam este metal para sobrevivência em longo prazo na fase estacionária (DUHUTREL et al., 2010).

Apesar de sua abundância e essencialidade, a biodisponibilidade do ferro é baixa. O íon Fe^{2+} é relativamente estável e solúvel em soluções aquosas e pH neutro mas, na presença de oxigênio, é espontaneamente auto oxidado. O Fe^{3+} resultante é essencialmente insolúvel em água e pH neutro, sendo a concentração de Fe^{3+} livre aproximadamente 10^{-18} M em ambientes aquosos (KOSMAN, 2003; RAYMOND et al., 2003). Quando em excesso, o ferro é tóxico. Na célula, uma quantidade significativa

deste íon é reduzida e pode participar da reação de Fenton, na qual Fe^{2+} reage com peróxido de hidrogênio (H_2O_2) gerando radicais hidroxil muito reativos. Estes radicais causam danos celulares ao reagirem com membranas lipídicas, proteínas e ácidos nucléicos (ONG et al., 2006).

Como o ferro é essencial, mas pode ser ao mesmo tempo deletério, a concentração deste metal deve ser cuidadosamente controlada em nível sistêmico e celular. O metabolismo do ferro em ambos os níveis envolve três processos principais: captação, utilização e armazenamento. Em virtude da ausência de um mecanismo ativo de exportação deste íon pelo organismo, a absorção de ferro é um processo estritamente controlado. Contrariamente, em nível celular há uma exportação regulada deste metal (HENTZE et al., 2004).

A manutenção da homeostase sistêmica de ferro em mamíferos depende de mecanismos que incluem a captação regulada de ferro proveniente da dieta, a mobilização de ferro armazenado nos hepatócitos e a reciclagem de eritrócitos. Tais mecanismos envolvem, por sua vez, o metabolismo celular deste íon.

O ferro é absorvido no duodeno após a redução de íons Fe^{3+} por redutases férricas presentes na borda dos enterócitos. O Fe^{2+} é então transportado para o interior celular por meio do transportador DMT1 (ou Nramp2). No citoplasma, os íons podem ser armazenados, utilizados em processos celulares ou, pela ação do transportador ferroportina (FPN), são liberados para o plasma onde circulam na forma oxidada ligados à transferrina (**Figura 4A**). FPN também é responsável pela liberação para o plasma do ferro armazenado em macrófagos e hepatócitos. A transferrina previne a formação de radicais livres catalisada pelo ferro e, ao mesmo tempo, facilita o transporte deste nutriente para células alvo. A liberação do ferro em tais células é mediada pelo receptor de transferrina (TFR), o qual é reciclado e volta para a superfície celular após a liberação do ferro no citoplasma (**Figura 4B**)(CASSAT e SKAAR, 2013; HENTZE et al., 2010)

A maior parte do ferro do corpo de um adulto humano está complexada, na forma reduzida Fe^{2+}, ao grupo heme da hemoglobina presente nos eritrócitos e mioglobina dos músculos. Grande parte do ferro necessário para as atividades diárias do organismo é provida por macrófagos que reciclam eritrócitos senescentes, pois a quantidade de íons absorvidos pela alimentação é pequena e ainda ocorrem perdas decorrentes da descamação da pele e células epiteliais do intestino (**Figura 5**) Cada eritrócito pode

conter mais de 1 bilhão de átomos de ferro. Estas células, senescentes ou danificadas, são permanentemente degradas por macrófagos principalmente no baço, fígado e medula óssea. A enzima heme oxigenase catalisa a degradação do grupo heme e o ferro liberado é posteriormente transportado para o citoplasma (**Figura 4B**) (CASSAT e SKAAR, 2013; MEYNARD et al., 2014).

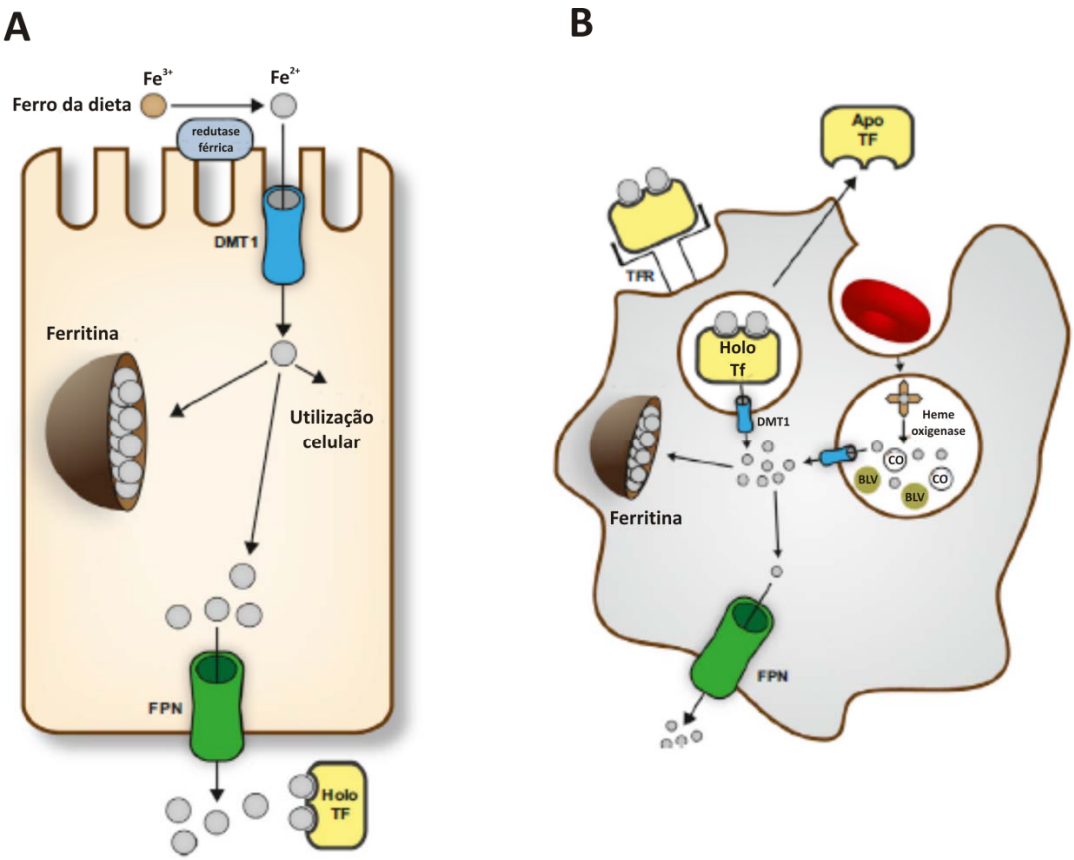


Figura 4. Homeostase de ferro em humanos. **A.** Ao chegar ao duodeno, o ferro proveniente da dieta é reduzido por redutases férricas presentes na borda apical dos enterócitos. Após ser transportado por DMT1, o íon Fe^{2+} é armazenado pela ferritina, utilizado no metabolismo celular ou liberado para o plasma pela ferroportina (FPN). No ambiente extracelular se liga à apo-transferrina, dando origem à holo-transferrina. **B.** Macrófagos, e outras células, adquirem ferro por meio da endocitose da holo-transferrina mediada por TFR. A acidificação do endossomo facilita a liberação do ferro e o complexo apo-transferrina-TFR é reciclado para a superfície celular. Os íons Fe^{3+} são reduzidos no endossomo e subsequentemente transportados para o citoplasma por DMT1. A heme oxigenasse catalisa a degradação do grupo heme a ferro, monóxido de carbono (CO) e biliverdina (BLV). O ferro é transportado por DMT1 para o citoplasma e pode ser armazenado ou liberado para o plasma pela FPN. Adaptado de Cassat e Skaar, 2013.

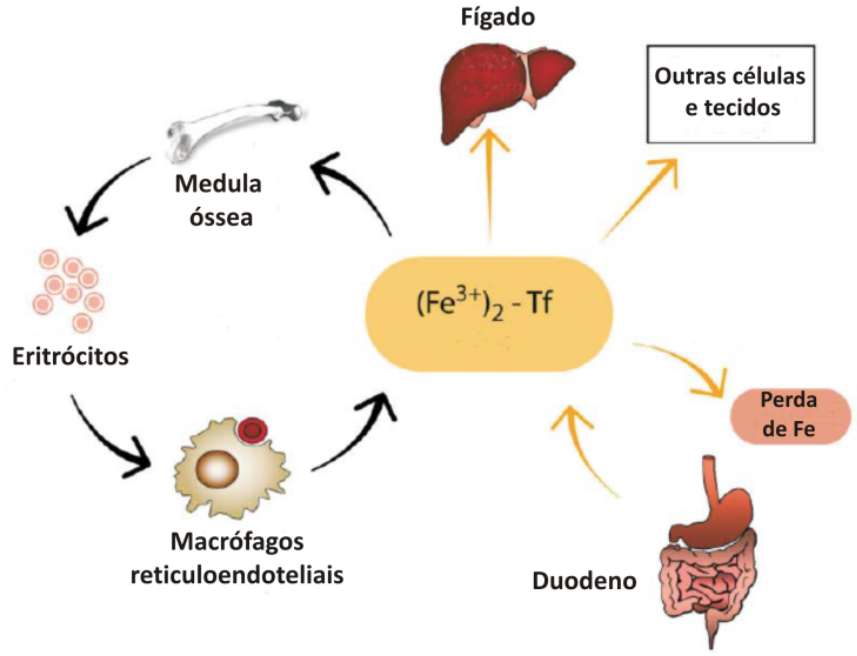


Figura 5. Distribuição sistêmica do ferro. O ferro é absorvido no duodeno e circula no plasma ligado à transferrina, que o distribui para o fígado, células eritróides e outras células e tecidos do organismo. Adaptado de Hentze et al., 2004.

O hormônio hepicidina e o transportador ferroportina desempenham papel importante na regulação da concentração de ferro no plasma. A hepicidina, hormônio peptídico produzido pelo fígado, regula sistemicamente o metabolismo de ferro bloqueando a liberação para o plasma dos íons provenientes da dieta, da reciclagem por macrófagos e dos hepatócitos. Ao ligar-se à ferroportina, o principal exportador de ferro na superfície celular, a hepicidina promove a internalização deste transportador o qual é subsequentemente degradado nos lisossomos. Desta forma, a quantidade de ferro liberada para o plasma pode ser regulada (NEMETH et al., 2004)

1.4. O ferro na interação patógeno-hospedeiro

O controle da homeostase de ferro é de grande importância na interação patógeno-hospedeiro, visto que ambos competem por este micronutriente essencial. Durante o processo infeccioso, vários mecanismos da imunidade inata do hospedeiro restringem o suprimento de ferro aos micro-organismos invasores, o que constitui a

chamada imunidade nutricional, uma estratégia para diminuir a taxa de proliferação microbiana (GANZ, 2009).

Durante a resposta de fase aguda que ocorre após o reconhecimento do patógeno, macrófagos ativados e neutrófilos secretam a citocina pró-inflamatória interleucina-6 (IL-6) a qual estimula a produção de hepicidina pelo fígado (**Figura 6A**). A indução deste hormônio promove uma diminuição dos níveis plasmáticos de ferro em consequência da internalização e degradação da ferroportina. Em contrapartida, há um acúmulo deste metal no citosol, o qual é armazenado pela ferritina. Além da hepicidina, outros mecanismos contribuem para a diminuição das concentrações de ferro no plasma em resposta à infecção. Citocinas como intérferon gama (IFN-γ), fator de necrose tumoral alfa (TNF-α) e interleucina 1 (IL-1) produzidas por células do sistema imunitário regulam o metabolismo de ferro para fortalecer o combate ao micro-organismo invasor (**Figura 6B**).

Os patógenos também se deparam com a baixa disponibilidade de ferro mesmo depois de fagocitados. Citocinas pró-inflamatórias diminuem a expressão do receptor de transferrina na superfície dos fagócitos e induzem a expressão de Nramp1 (APPELBERG, 2006). Nramp1 (*natural resistence-associated macrophage protein 1*) é um transportador de íons metálicos divalentes expresso unicamente na membrana de vesículas lisossomais de macrófagos e grânulos terciários de neutrófilos. Durante a fagocitose, Nramp1 é recrutada para a membrana do fagossomo e transporta o ferro deste compartimento para o citosol, onde este metal é armazenado pela ferritina (GRUENHEID et al., 1997). Desta forma, o patógeno fagocitado fica desprovido de ferro para seu crescimento e multiplicação (**Figura 6C**). Foi descrito recentemente que *Leishmania amazonensis*, um protozoário parasita intracelular, inibe a expressão de ferroportina nos macrófagos infectados. Esta estratégia impede a exportação de ferro dessas células resultando no crescimento e multiplicação do parasita no interior dos fagócitos (BEN-OTHMAN et al., 2014).

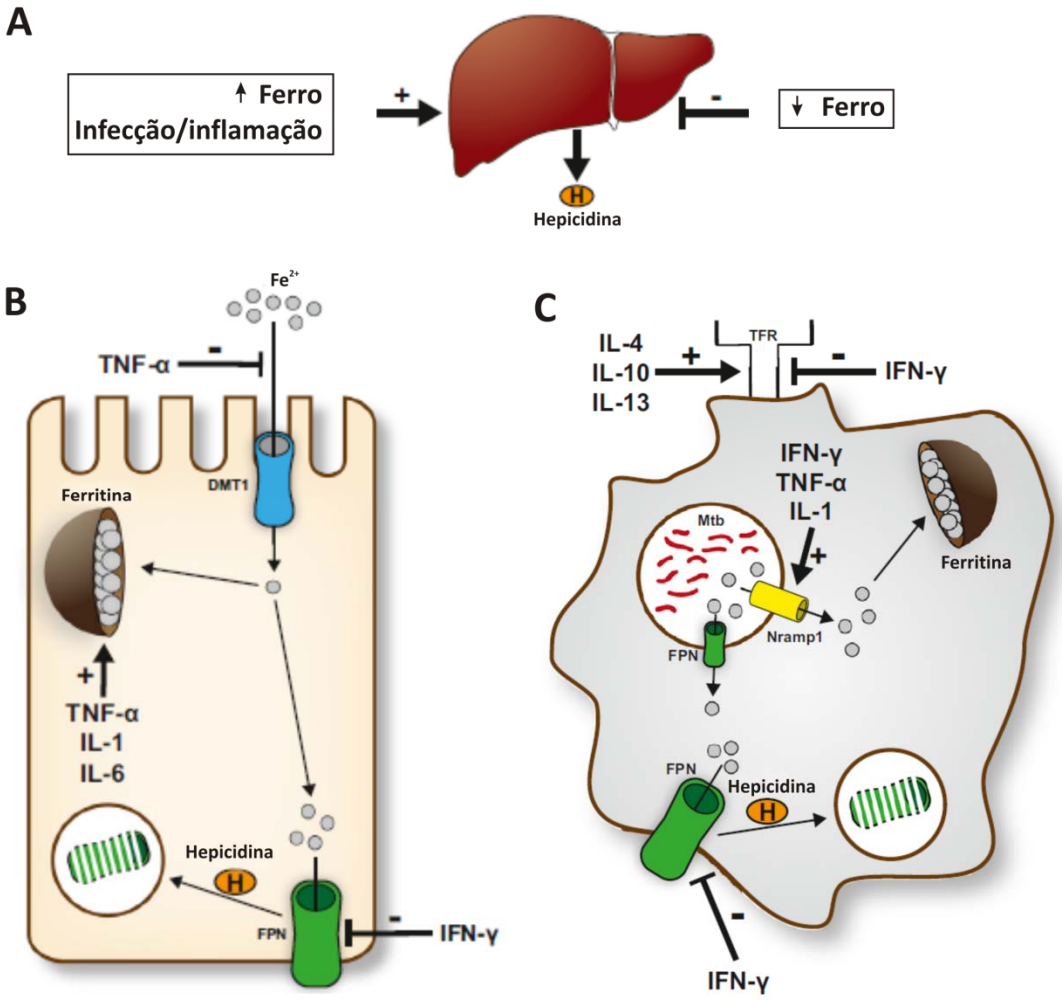


Figura 6. Limitação de ferro como uma defesa imunitária inata. A. Elevadas concentrações de ferro no plasma e/ou infecção induzem a produção de hepicidina (H), cuja síntese é inibida mediante baixas concentrações plasmáticas de ferro. **B.** Citocinas pró-inflamatórias reprimem a absorção de ferro mediada por DMT1 e induzem a síntese de ferritina. A hepicidina impede a liberação de ferro para o plasma por induzir a internalização e degradação da ferroportina (FPN). **C.** Em resposta a sinais inflamatórios, macrófagos diminuem a expressão do receptor de transferrina (TFR) e induzem a expressão de Nramp1, promovendo a remoção de ferro do fagossomo. Adaptado de Cassat e Skaar, 2013.

Além da hipoferremia induzida sistemicamente pela hepicidina, efetores da resposta imunitária também restringem o ferro ao micro-organismo invasor no local da infecção. A lactoferrina, uma glicoproteína da família das transferrinas, liga-se ao ferro livre com alta afinidade e está presente em superfícies mucosas onde restringe a disponibilidade de ferro a possíveis micro-organismos invasores (WARD e CONNEELY, 2004). Adicionalmente, os grânulos secundários de neutrófilos contêm

lactoferrina, a qual é liberada no sítio de infecção em resposta a citocinas (MASSON et al., 1969). Diferente da transferrina, a lactoferrina não libera o ferro ligado em pH ácido, que é prevalente em áreas infectadas.

Proteínas da família das lipocalinas (Lcn) também limitam a disponibilidade de ferro para os micro-organismos invasores durante a infecção. Tais proteínas se ligam a moléculas produzidas pelos patógenos para aquisição de ferro, os sideróforos, os quais serão discutidos a seguir. Humanos produzem Lcn1, que se liga a sideróforos produzidos por fungos e bactérias, e Lcn2, a qual reconhece sideróforos bacterianos somente (FLO et al., 2004; FLUCKINGER et al., 2004).

1.5. Captação de ferro por patógenos: o papel dos sideróforos

Diante da condição de privação de ferro imposta pelo hospedeiro, os patógenos, como os fungos, desenvolveram mecanismos de alta afinidade para a captação deste metal, já que o mesmo é essencial para o sucesso da infecção. Várias evidências demonstram que infecções aumentam em frequência e severidade em pessoas com sobrecarga de ferro no organismo (CASSAT e SKAAR, 2013). Em geral, a aquisição de ferro por patógenos fúngicos inclui três mecanismos, não necessariamente mutuamente exclusivos: a redução do íon férrico (Fe^{3+}) ao íon ferroso (Fe^{2+}), a aquisição do ferro ligado ao grupo heme e a solubilização do Fe^{3+} pela ligação à sideróforos.

Como os íons Fe^{3+} são insolúveis em pH fisiológico em presença de oxigênio, a redução destes a Fe^{2+} é necessária para que o ferro torne-se solúvel e seja captado mais facilmente. Neste processo, denominado via de captação redutiva, o Fe^{3+} é inicialmente reduzido por ação de uma redutase férrica presente na superfície celular. O Fe^{2+} gerado é então oxidado novamente por uma multicobre oxidase acoplada a uma permease, a qual transporta o íon Fe^{3+} diretamente para o interior celular. Este mecanismo de captação de ferro de alta afinidade, induzido em condições limitantes deste micronutriente, é utilizado por fungos patogênicos e não patogênicos como a levedura *Saccharomyces cerevisiae* (KOSMAN, 2003). Componentes da maquinaria redutiva já foram identificados nos patógenos *Candida albicans* (KNIGHT et al., 2002), *Cryptococcus neoformans* (JUNG e KRONSTAD, 2008), *Histoplasma capsulatum* (TIMMERMAN e WOODS, 2001) e *Aspergillus fumigatus* (SCHRETTL et al., 2004). Deleção de genes envolvidos na captação de ferro por esta via em *C. neoformans*

(JUNG et al., 2009) e *C. albicans* (KNIGHT et al., 2005) geraram linhagens com severa redução de virulência em modelos de infecção.

Devido ao fato de que a maior parte do ferro no corpo humano está complexada à hemoglobina nos eritrócitos, fungos patogênicos desenvolveram mecanismos para utilizar o grupo heme como fonte de ferro. Já foi demonstrado que *C. albicans* possui fatores hemolíticos e receptores de superfície para hemoglobina (MANNS et al., 1994; WEISSMAN e KORNITZER, 2004), os quais facilitam a internalização desta molécula em vesículas endocíticas (WEISSMAN et al., 2008). Dentro do vacúolo o grupo heme é extraído da hemoglobina e degradado por uma heme oxigenase, liberando o Fe^{2+} para ser utilizado pela célula (PENDRAK et al., 2004). Os patógenos *H. capsulatum* e *C. neoformans* também são capazes de utilizar o grupo heme, pois crescem em condições em que somente esta fonte de ferro está disponível (FOSTER, 2002; JUNG et al., 2008).

Outra estratégia utilizada por fungos patogênicos para a aquisição de ferro consiste na síntese e captação de sideróforos, um mecanismo não redutivo de alta afinidade. Sideróforos (do grego "portadores de ferro") são compostos de baixa massa molecular (Mr <1500) que apresentam alta afinidade por íons férricos (NEILANDS, 1993) e sua função é fornecer ferro para a célula através da solubilização de Fe^{3+} extracelular. Também funcionam como moléculas armazenadoras de ferro na maioria dos fungos (MATZANKE et al., 1987). Podem ser classificados em três grupos principais dependendo da natureza química dos motivos que doam os ligantes de oxigênio para o ferro: catecóis, carboxilatos e hidroxamatos (MIETHKE e MARAHIEL, 2007). Com exceção da rizoferrina, um carboxilato produzido por zigomicetos (VAN DER HELM e WINKELMANN, 1994), a maioria dos sideróforos produzidos por fungos pertencem à classe dos hidroxamatos, os quais são derivados do aminoácido não proteinogênico ornitina e podem ser agrupados em quatro famílias estruturais: ácido rodotorúlico, ferricromos, coprogenos e fusarininas (HAAS et al., 2008). Após a síntese, os sideróforos extracelulares são secretados. Depois da ligação ao ferro, o complexo sideróforo-Fe^{3+} é internalizado por meio de transportadores localizados na membrana celular (WINKELMANN, 2002).

A biossíntese de sideróforos (**Figura 7**) é bem descrita em *A. fumigatus* (BLATZER et al., 2011). Como mecanismo de aquisição de ferro de alta afinidade, tais moléculas são produzidas em condições de escassez deste micronutriente. A biossíntese de hidroxamatos inicia-se com a hidroxilação da ornitina, catalisada pela enzima

ornitina oxigenase (SidA). O primeiro gene codificante para esta enzima identificado em fungos foi *sid1*, do patógeno de plantas *Ustilago maydis* (MEI et al., 1993). Ortólogos foram posteriormente identificados em patógenos humanos, *A. fumigatus (sidA)* (HISSEN et al., 2005) e *H. capsulatum (sid1)* (HWANG et al., 2008), e fungos não patogênicos como *Aspergillus nidulans (sidA)* (EISENDLE et al., 2003). A segunda etapa da via de biossíntese consiste na formação do grupo hidroxamato caracterizada pela transferência de um grupo acil para a hidroxiornitina. Em *A. fumigatus* duas transacilases catalisam a adição de diferentes grupos acil à ornitina hidroxilada: SidF, que adiciona anidromevalonil-CoA, e SidL que catalisa a adição de acetil-CoA. Nesta etapa, duas vias para geração de diferentes sideróforos surgem devido à escolha do grupo acil, o qual define a natureza de cada molécula. Ácido rodotorúlico, ferricromo e ferricrocina (família dos ferricromos) possuem o grupo acetil, enquanto fusarininas e coprogenos apresentam anidromevalonil. No terceiro passo da via biossintética, peptídeo sintetases não ribossômicas (NRPSs; SidC e SidD) promovem a união dos grupos hidroxamatos por meio de ligações peptídicas (ferricrocina) e de ligações éster (fusarinina C-FSC). A acetil transferase SidG catalisa a formação de triacetilfusarinina C (TAFC) a partir de FSC (BLATZER et al., 2011; SCHRETTL et al., 2007). Além do sideróforo intracelular ferricrocina, envolvido no armazenamento e distribuição de ferro em hifas, *A. fumigatus* também produz hidroxiferricrocina (HFC), a qual participa do armazenamento deste metal em conídeos (WALLNER et al., 2009). Estudos revelaram que neste fungo a via de biossíntese de TAFC e a síntese de ergosterol são conectadas por meio da acil-CoA ligase SidI e da enoil-CoA hidratase SidH (YASMIN et al., 2012), como demonstrado na **Figura 7**. Recentemente foi demonstrado que SidI, SidH e SidF possuem sinal de endereçamento peroxissomal (PTS) e que a síntese de TAFC é parcialmente localizada nos peroxissomos. Em adição, análises da sequência de ortólogos destas proteínas em outros fungos produtores de sideróforos revelaram a presença de PTS, demonstrando uma função metabólica nova e evolutivamente conservada dos peroxissomos (GRUNDLINGER et al., 2013). As estruturas de alguns sideróforos do tipo hidroxamato produzidos por fungos são apresentadas na **Figura 8**.

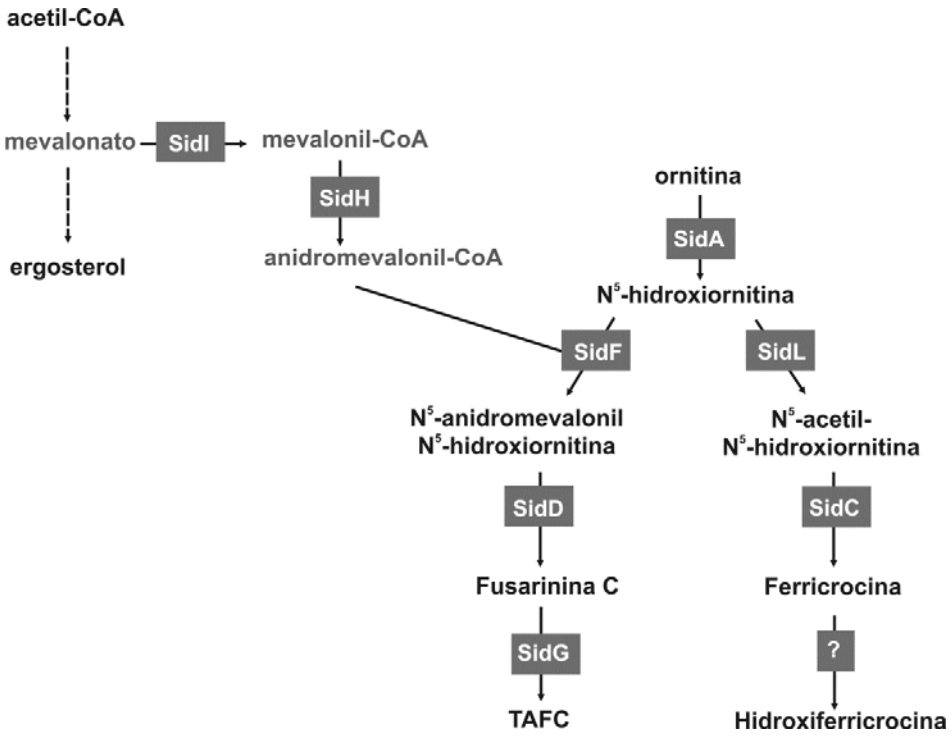


Figura 7. Biossíntese de sideróforos em *A. fumigatus*. Adaptado de Yasmin et al, 2012.

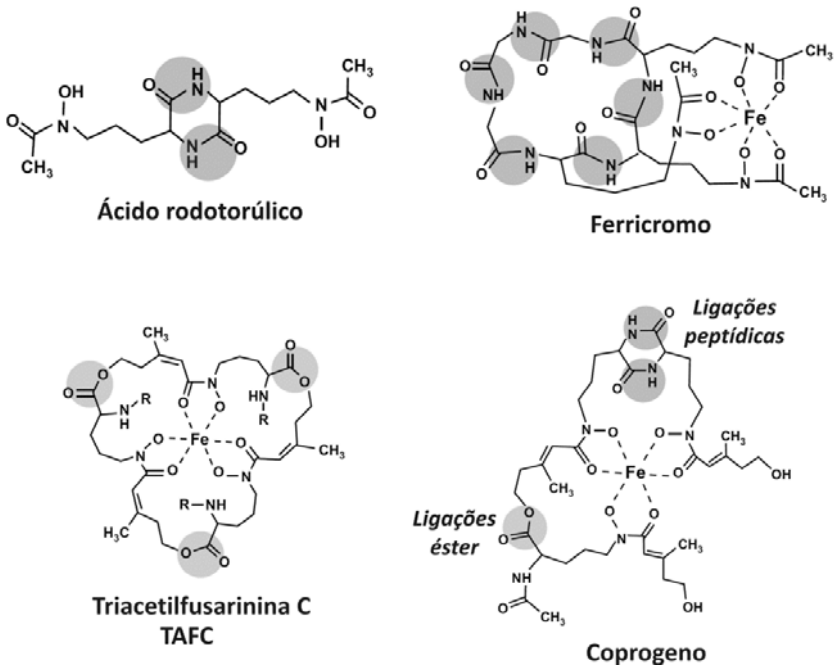


Figura 8. Estruturas de sideróforos do tipo hidroxamato. Ácido rodotorúlico e ferricromos apresentam somente ligações peptídicas entre os grupos hidroxamatos. TAFC é formada por ligações éster, enquanto coprogenos apresentam ambos os tipos de ligações. Adaptado de Haas et al., 2008.

Análises funcionais das proteínas envolvidas na biossíntese de sideróforos em *A. fumigatus* demonstraram que estas moléculas são essenciais durante a infecção, visto que a linhagem mutante Δ*sidA* demonstrou-se desprovida de virulência em modelo murino de aspergilose invasiva (HISSEN et al., 2005). Adicionalmente, as linhagens Δ*sidF,* Δ*sidC,* Δ*sidD,* Δ*sidG,* Δ*sidH* e Δ*sidI* apresentaram virulência atenuada em modelo murino de infeção (SCHRETTL et al., 2007; YASMIN et al., 2012). Coerente com papel dos sideróforos na virulência do fungo, *sidD* foi o gene codificante para NRPSs mais expresso após a incubação de *A. fumigatus* com macrófagos (CRAMER et al., 2006). Ademais, análises de expressão global demonstraram indução significativa dos genes *sidC*, *sidD*, *sidF* e *sidG* durante estágio inicial de infecção em camundongos neutropênicos (SCHRETTL et al., 2007). Assim como em *A. fumigatus*, a produção de sideróforos em *H. capsulatum* é de fundamental importância para a aquisição de ferro durante a infecção. A deleção de s*id1*, ortólogo de *sidA*, resultou na diminuição do crescimento de leveduras em macrófagos e no comprometimento da virulência em camundongos (HILTY et al., 2011; HWANG et al., 2008). Tais fatos denotam a importância da aquisição de ferro mediada por sideróforos durante o processo infeccioso.

A internalização do complexo ferro-sideróforo é geralmente mediada por transportadores da subfamília SIT (*Siderophore-iron transporter,* subfamília 16), pertencentes à superfamília MFS (*Major facilitator superfamily*) (PAO et al., 1998). Estes transportadores secundários contêm de 12 a 14 domínios transmembrana preditos. Estudos realizados com *Neurospora crassa* e *S. cerevisiae* demonstraram que o reconhecimento dos sideróforos é altamente estéreo específico (WINKELMANN, 2001), indicando que a ligação ao transportador não depende do tamanho ou hidrofobicidade da molécula, mas sim de sítios de ligação específicos.

A captação de ferro mediada por transportadores SIT parece ser conservada universalmente no reino Fungi, mesmo em espécies não produtoras de sideróforos, como *S. cerevisiae*, *Candida* spp. e *C. neorformans*, as quais utilizam sideróforos produzidos por outros organismos (xenosideróforos). O genoma de *Candida glabrata* codifica um único transportador de sideróforo, Sit1, o qual é essencial para a sobrevivência deste fungo em macrófagos e responsável pelo transporte de hidroxamato do tipo ferricromo (NEVITT e THIELE, 2011). Em *C. albicans* o transportador Sit1 também participa do transporte de ferricromos e é requerido durante a invasão epitelial

(HEYMANN et al., 2002). O requerimento de Sit1 no transporte de hidroxamato também já foi demonstrado em *C. neoformans*. Estudos com a linhagem mutante Δ*sit1* evidenciaram a necessidade deste transportador na utilização de ferrioxamina B como fonte de ferro e no crescimento do fungo em ambientes cuja disponibilidade de ferro é baixa (TANGEN et al., 2007). *A. fumigatus* e A. *nidulans* codificam 10 e 7 possíveis transportadores de sideróforos, respectivamente (HAAS et al., 2008). Estudos de expressão heteróloga em uma linhagem de *S. cerevisiae* incapaz de captar sideróforos indicaram que os transportadores MirA e MirB de *A. nidulans* transportam, respectivamente, o sideróforo bacteriano enterobactina e TAFC (HAAS et al., 2003). A presença de vários transportadores provavelmente reflete a habilidade dos micro-organismos em utilizar tipos diferentes de sideróforos, incluindo os xenosideróforos. De fato, *A. nidulans* é capaz de captar os xenosideróforos enterobactina e ferrioxamina B além dos sideróforos nativos ferricrocina e TAFC (OBEREGGER et al., 2001).

Após a internalização, o destino de sideróforos diferentes parece não ser o mesmo. Em *S. cerevisiae*, ferricromos acumulam no citoplasma (MOORE et al., 2003) enquanto ferrioxamina B é provavelmente compartimentalizada em vacúolos (FROISSARD et al., 2007). Já em *A. fumigatus* e *A. nidulans*, as ligações éster de TAFC são hidrolisadas após a captação e os produtos da hidrólise são secretados (KRAGL et al., 2007; OBEREGGER et al., 2001).

O ferro ligado ao sideróforo pode, alternativamente, ser utilizado pela via redutiva. Neste caso, não há internalização do complexo sideróforo-Fe^{3+}. Após a redução na superfície celular, os íons Fe^{2+} são transportados para o interior da célula (RENSHAW et al., 2002).

Em fungos, a homeostase de íons metálicos é mantida principalmente por meio da regulação transcricional da expressão gênica. Um grupo de fatores de transcrição do tipo GATA responsivos ao ferro medeiam a repressão de genes para a aquisição deste micronutriente quando o mesmo encontra-se disponível em concentrações suficientes (RUTHERFORD e BIRD, 2004). Tais fatores já foram descritos em *C. albicans* (Sfu1), *H. capsulatum* (Sre1), *Blastomyces dermatitidis* (SreB), *C. neoformans* (Cir1) e *A. fumigatus* (SreA) (GAUTHIER et al., 2010; JUNG e KRONSTAD, 2011; LAN et al., 2004; SCHRETTL et al., 2008). A resposta a baixas concentrações de ferro é em contrapartida mediada por reguladores do tipo bZIP. Em *A. fumigatus*, HapX reprime vias que dependem de ferro, como respiração, ciclo do ácido tricarboxílico e biossíntese

do grupo heme, durante a penúria deste nutriente. Ao mesmo tempo, a captação de ferro mediada por sideróforos é induzida (SCHRETTL et al., 2010a). Assim, fatores do tipo GATA e reguladores bZIP agem de maneira oposta dependendo da disponibilidade de ferro no ambiente em que a célula se encontra.

1.6. Metabolismo de ferro em *Paracoccidioides* spp.

Assim como em outros organismos, o ferro é essencial para o desenvolvimento de *Paracoccidioides* spp. Sobrenadantes de cultura de *Paracoccidioides* spp. cultivado em meio com baixas concentrações de ferro apresentaram maior capacidade de ligação à este metal quando comparados com sobrenadantes de meio de cultura ricos em ferro. A adição de quelante ao meio já desprovido de ferro inibe parcialmente o crescimento de leveduras nos primeiros dias de incubação. Entretanto, depois de alguns dias, a taxa de crescimento é a mesma daquela observada quando o fungo é cultivado na ausência do quelante (ARANGO e RESTREPO, 1988). Adicionalmente, o crescimento de *Paracoccidioides* spp. é melhorado através do cultivo em um meio suplementado com filtrados de cultura do próprio fungo (CASTANEDA et al., 1988). Estes dados sugerem que os fatores presentes no filtrado são moléculas capazes de se ligar ao ferro e que, por esta propriedade, potencializam o crescimento do fungo. Tais moléculas são, provavelmente, sideróforos. Tal fato sugere que *Paracoccidioides* spp. possui mecanismos de captação de ferro que são relevantes para o crescimento *in vitro* e durante a interação com o hospedeiro. De fato, o requerimento por ferro também já foi demonstrado na interação de *Paracoccidioides* spp. com células hospedeiras.

A sobrevivência de leveduras no interior de monócitos humanos é suprimida pelo tratamento dessas células com deferoxamina, um quelante de ferro. O efeito da deferoxamina é revertido pela holotransferrina, mas não pela apotransferrina, evidenciando que a sobrevivência do fungo em monócitos é dependente de ferro (DIAS-MELICIO et al., 2005). Esse micronutriente também é essencial para o dimorfismo em *Paracoccidioides* spp, pois o tratamento de macrófagos com deferoxamina inibe a transição conídeo-levedura (CANO et al., 1994). Adicionalmente, camundongos tratados com ferro exógeno são mais susceptíveis à infecção por *Paracoccidioides* sp. (PARENTE et al., 2011).

O estado metabólico de *Paracoccidioides* sp. é alterado de acordo com a disponibilidade de ferro, como revelado por análises proteômicas. A restrição de ferro

influencia principalmente os mecanismos relacionados à produção de energia. Em condições de baixa disponibilidade deste micronutriente a glicólise é a via metabólica mais favorecida em detrimento das vias oxidativas, que são dependentes de enzimas que possuem grupos ferro-enxofre. Assim, o fungo prioriza vias independentes de ferro. Este rearranjo metabólico é provavelmente um mecanismo de sobrevivência indispensável quando *Paracoccidioides* sp. encontra-se em condições de escassez de ferro, como no hospedeiro. De fato, as mudanças vistas em nível proteômico foram também observadas em nível transcricional após a análise de alguns genes em leveduras de *Paracoccidioides* sp. recuperadas de baços de camundongos (PARENTE et al., 2011).

2. JUSTIFICATIVA

A paracoccidioidomicose é uma micose sistêmica com alta incidência no Brasil e endêmica entre populações de áreas rurais, nas quais afeta indivíduos do sexo masculino, principalmente em sua idade economicamente ativa. Esta doença constitui um sério problema de saúde pública, pois é potencialmente incapacitante e causa mortes prematuras. A capacidade de *Paracoccidioides* spp. provocar esta micose com grande variedade de manifestações clínicas está relacionada tanto à biologia do próprio fungo quanto a de seu hospedeiro humano. Conhecer os mecanismos utilizados pelo agente etiológico para causar a doença é o primeiro passo para entender a relação patógeno-hospedeiro e, consequentemente, para dar subsídios a um tratamento e/ou profilaxia eficazes.

O sucesso da infecção depende, entre outras coisas, da capacidade do patógeno em adquirir micronutrientes, os quais se tornam escassos no hospedeiro humano como uma forma de defesa contra o micro-organismo invasor. Entre estes micronutrientes está o ferro que, por participar de vários processos biológicos essenciais, é vital para praticamente todos os organismos vivos. Estudos já evidenciaram a necessidade deste micronutriente para o desenvolvimento de *Paracoccidioides* spp. tanto *in vitro* quanto em condições de infecção. Análises transcricionais do fungo recuperado de camundongos e na presença de sangue e plasma humanos demonstraram que genes relacionados à captação de ferro são induzidos. Deste modo, o conhecimento das estratégias utilizadas pelo fungo para a aquisição deste nutriente é importante para o entendimento dos mecanismos de sobrevivência e multiplicação no hospedeiro.

A produção e captação de sideróforos são mecanismos utilizados por vários patógenos para a aquisição de ferro em seus hospedeiros. A produção e utilização destas moléculas por *Paracoccidioides* spp. já foi mencionada, mas nenhum dado sobre a natureza das mesmas e sua possível contribuição para a patogênese e virulência do fungo foi obtido até o momento. Portanto, o estudo sobre a captação de ferro mediada por sideróforos em *Paracoccidioides* spp. faz-se necessário para esclarecer tais questões e, consequentemente, gerar informações importantes que auxiliem na compreensão e no tratamento da doença.

3. OBJETIVOS

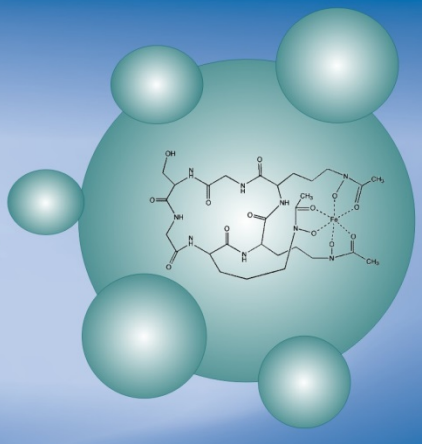
3.1. Objetivo geral

Analisar a aquisição de ferro mediada pela produção e captação de sideróforos em *Paracoccidioides* spp. e a função destas moléculas na interação com células hospedeiras.

3.2 . Objetivos específicos

- Buscar genes ortólogos relacionados à biossíntese e ao transporte de sideróforos no genoma de *Paracoccidioides* spp;
- Avaliar a expressão dos genes encontrados em condições de privação de ferro;
- Verificar a produção de sideróforos em *Paracoccidioides* spp;
- Identificar os possíveis sideróforos produzidos por *Paracoccidioides* spp.;
- Avaliar o crescimento de *Paracoccidioides* spp. na presença de sideróforos como única fonte de ferro;
- Avaliar a função de sideróforos em condições de infecção em macrófagos murinos;
- Verificar se os possíveis sideróforos produzidos por *Paracoccidioides* spp. podem ser utilizados como fonte de ferro por outros organismos.

Capítulo 2



frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 21 March 2011
doi: 10.3389/fmicb.2011.00049

The homeostasis of iron, copper, and zinc in *Paracoccidioides brasiliensis*, *Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii*: a comparative analysis

Mirelle Garcia Silva[1], Augusto Schrank[2], Elisa Flávia L.C. Bailão[1], Alexandre Melo Bailão[1], Clayton Luiz Borges[1], Charley Christian Staats[2], Juliana Alves Parente[1], Maristela Pereira[1], Silvia Maria Salem-Izacc[1], Maria José Soares Mendes-Giannini[3], Rosely Maria Zancopé Oliveira[4], Lívia Kmetzsch Rosa e Silva[2], Joshua D. Nosanchuk[5,6], Marilene Henning Vainstein[2] and Célia Maria de Almeida Soares[1]*

[1] *Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Goiás, Brazil*
[2] *Laboratório de Biologia Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil*
[3] *Faculdade de Ciências Farmacêuticas, Universidade Estadual Júlio de Mesquista Filho, Araraquara, São Paulo, Brazil*
[4] *Laboratório de Micologia, Instituto de Pesquisa Evandro Chagas, Fundação Oswaldo Cruz, Rio De Janeiro, Brazil*
[5] *Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA*
[6] *Department Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, USA*

Edited by:
James A. Fraser, University of Queensland, Australia

Reviewed by:
James A. Fraser, University of Queensland, Australia
Dennis J. Thiele, Duke University School of Medicine, USA

****Correspondence:***
Célia Maria de Almeida Soares, Laboratório de Biologia Molecular, Departamento de Bioquímica e Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, ICB II, Campus II, 74690-900 Goiânia, Goiás, Brazil.
e-mail: celia@icb.ufg.br

Iron, copper, and zinc are essential for all living organisms. Moreover, the homeostasis of these metals is vital to microorganisms during pathogenic interactions with a host. Most pathogens have developed specific mechanisms for the uptake of micronutrients from their hosts in order to counteract the low availability of essential ions in infected tissues. We report here an analysis of genes potentially involved in iron, copper, and zinc uptake and homeostasis in the fungal pathogens *Paracoccidioides brasiliensis*, *Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii*. Although prior studies have identified certain aspects of metal regulation in *Cryptococcus* species, little is known regarding the regulation of these elements in *P. brasiliensis*. We also present amino acid sequences analyses of deduced proteins in order to examine possible conserved domains. The genomic data reveals, for the first time, genes associated to iron, copper, and zinc assimilation and homeostasis in *P. brasiliensis*. Furthermore, analyses of the three fungal species identified homologs to genes associated with high-affinity uptake systems, vacuolar and mitochondrial iron storage, copper uptake and reduction, and zinc assimilation. However, homologs to genes involved in siderophore production were only found in *P. brasiliensis*. Interestingly, *in silico* analysis of the genomes of *P. brasiliensis Pb*01, *Pb*03, and *Pb*18 revealed significant differences in the presence and/or number of genes involved in metal homeostasis, such as in genes related to iron reduction and oxidation. The broad analyses of the genomes of *P. brasiliensis, C. neoformans* var. *grubii*, and *C. gattii* for genes involved in metal homeostasis provide important groundwork for numerous interesting future areas of investigation that are required in order to validate and explore the function of the identified genes and gene pathways.

Keywords: micronutrient homeostasis, pathogenic fungi, infection

INTRODUCTION

A sufficient supply of iron, copper and zinc is essential for all living and proliferating organisms. In infectious diseases, iron, copper and zinc metabolism are important for both the host and the pathogen, and complex responses in each occur to maintain adequate resources of these elements to preserve homeostasis. Iron, in the form of heme and iron–sulfur clusters, is essential as a cofactor of various enzymes, oxygen carriers, and electron-transfer systems involved in vital cellular functions ranging from respiration to DNA replication (Schaible and Kaufmann, 2004). Copper is a redox-active metal ion essential for most aerobic organisms, which also serves as a catalytic and structural cofactor for enzymes involved in energy generation, iron acquisition, oxygen transport, and cellular metabolism, among other processes (Kim et al., 2008). Zinc is also a crucial metal, since it is at the catalytic center of numerous enzymes and plays important roles in the functionality of a wide variety of proteins (Van Ho et al., 2002). Mammalian hosts and microbes have developed sophisticated strategies to acquire these metals, even under conditions in which their availability is limited. One of the strategies developed by mammalian hosts to prevent microbial infections is to limit the availability of iron (Weinberg, 2009). Recently, it has been demonstrated that zinc deprivation is a host defense mechanism utilized by macrophages during *Histoplasma capsulatum* infection (Winters et al., 2010). In addition, the binding of copper to calgranulin C in human neutrophils could be a mechanism of antimicrobial action (Moroz et al., 2003). In order to counteract these and other host responses, microorganisms employ a range of uptake mechanisms for the targeted acquisition of iron, copper and zinc.

Ferric iron is generally insoluble at physiological pH in the presence of oxygen. Thus, the common mechanisms of iron-assimilation include the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}),

and solubilization of Fe^{3+} by binding siderophores (Kornitzer, 2009). The reductive system in fungi is regulated by three different mechanisms. First, a low-affinity iron reductase that functions in iron-rich environments generates Fe^{2+}, which is transported into the cell by a non-specific low-affinity iron permease. Second, a regulated high-affinity ferric reductase operates in low iron conditions, such as those present in a mammalian host. The produced Fe^{2+} is further oxidized to Fe^{3+} by a membrane multi-copper-oxidase before being transported across the cell membrane by a high-affinity iron permease. The third mechanism is a non-enzymatic reduction, such as that promoted by 3-hydroxyanthranilic acid (3HAA), which is known to maintain a reduced environment to facilitate the release and sustain the presence of Fe^{2+} at the fungal membrane until transport occurs (Howard, 1999).

Ferric iron uptake mediated by siderophores is considered a non-reductive high-affinity mechanism by which microorganisms acquire iron. Siderophores are low-molecular weight (M_r < 1500), ferric iron-specific chelators (Neilands, 1993). Microorganisms produce siderophores as scavenging agents in low iron concentration environments in order to supply iron to the cell through the solubilization of extracellular ferric iron. Siderophores are also produced intracellularly for iron storage in most fungi (Matzanke et al., 1987). Siderophores can be classified into three main groups depending on the chemical nature of the moieties donating the oxygen ligands for Fe^{3+}: catechols, carboxylates and hydroxamates (Miethke and Marahiel, 2007). With the exception of the carboxylate rhizoferrin produced by zygomycetes, the other known fungal siderophores are all hydroxamates (Van der Helm and Winkelmann, 1994). Fungal hydroxamates are derived from the non-proteinogenic amino acid ornithine and can be grouped into four structural families: rhodotorulic acid, ferrichromes, coprogens and fusarinines. Siderophores are named based on their iron-charged forms, existing in the iron-free form of the ligand called desferri-siderophore. Not all fungi produce siderophores. For example, *Saccharomyces cerevisiae* is not a siderophore producer (Neilands et al., 1987). Similarly, *Cryptococcus* species and *Candida albicans* are also unable to produce siderophores. However, these pathogenic fungi can utilize iron bound to siderophores secreted by other species (bacteria and fungi), the xenosiderophores (Howard, 1999). After siderophores are synthesized, they can be utilized intracellularly or secreted to the extracellular medium to solubilize ferric iron. For secreted siderophores, the captured metal of the siderophore–iron complex may be utilized either by reductive iron assimilatory systems or by internalization of the whole complex by specific transporters. In fungi, the uptake of siderophore–iron chelates is accomplished by transporters of the siderophore–iron transporter (SIT) subfamily, previously designated as family 16 of the major facilitator superfamily (MFS; Pao et al., 1998). These transporters are integral membrane proteins, with 12–14 predicted transmembrane domains, that mediate the import of siderophores in a highly regulated process (Philpott, 2006).

Several homeostatic mechanisms that ensure the maintenance of copper at a sufficient concentration for cell growth have been identified. Copper homeostasis in fungi is maintained by the transcriptional regulation of genes involved in copper acquisition, mobilization and sequestration and also at the posttranslational level (Gross et al., 2000). In *S. cerevisiae* copper is reduced from Cu (II) to Cu (I) by cell surface metalloreductases (Hassett and Kosman, 1995; Georgatsou et al., 1997) and uptake is mediated by Ctr1p and Ctr3p, two high-affinity transporters. Both *ctr1* and *ctr3* genes are regulated at the transcriptional level in response to copper availability, being induced by copper deprivation (Dancis et al., 1994a; Pena et al., 2000). The vacuolar copper transporter Ctr2p is also involved in the intracellular copper homeostasis, since it provides copper via mobilization of intracellular copper stores (Rees et al., 2004).

Zinc homeostasis is maintained by posttranslational and transcriptional homeostatic regulatory mechanisms (Lyons et al., 2000; Eide, 2003). Unlike iron and copper, zinc is taken up as divalent cation. Once inside the cell, zinc is neither oxidized nor reduced (Berg and Shi, 1996). In *S. cerevisiae* the uptake of zinc is mediated by two separate systems. One system has a high-affinity for this metal and is active in zinc-limited conditions (Zhao and Eide, 1996a). The second system has a lower affinity for zinc and is not highly regulated by zinc concentrations (Zhao and Eide, 1996b). The expression of the high-affinity zinc transporter Zrt1p and the low-affinity zinc transporter Zrt2p is regulated by the transcription factor Zap1p, which plays a central role in zinc homeostasis (Zhao and Eide, 1997). The zinc transporter activity is also posttranslationally regulated. High levels of extracellular zinc trigger the inactivation of Zrt1p through endocytosis of the protein and its subsequent degradation in the vacuole (Gitan et al., 1998).

This paper focuses on the metabolism of iron, copper and zinc in the fungal pathogens *Paracoccidioides brasiliensis, Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii*. Low iron conditions have been associated with the susceptibility of *P. brasiliensis*, the etiological agent of paracoccidioidomycosis (PCM), to the antimicrobial action of monocytes (Dias-Melicio et al., 2005). Major phenotypic changes in *C. neoformans*, the etiological agent of cryptococcosis, are regulated by iron availability. For example, low iron concentrations result in the induction of capsule enlargement and the repression of laccase (Jung and Kronstad, 2008). Although iron regulation is well described in *Cryptococcus* species (Jung et al., 2008), iron associated processes are poorly understood in *P. brasiliensis*. Further, there is limited information on the impact of copper and zinc in *P. brasiliensis*, as well as the impact of zinc in *Cryptococcus* species. In this paper we performed *in silico* analyses of genes related to iron, copper and zinc metabolism in *P. brasiliensis, C. neoformans* var. *grubii* and *C. gattii*. We also compared the obtained information with data available from *S. cerevisiae*, which represents the most deeply studied model fungus, and other fungi.

MATERIALS AND METHODS

Sequences of genes related to iron, copper and zinc uptake, as well as to siderophore biosynthesis and uptake were used in the search of orthologs of *P. brasiliensis* and *Cryptococcus* species genomes. The *P. brasiliensis* database[1] includes the genomes of three isolates (*Pb*01, *Pb*03, and *Pb*18) and the cryptococcal database includes genomes of *C. neoformans* var. *grubii*[2] and *C. gattii*[3]. The sequences used in

[1]http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html

[2]http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html

[3]http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans_b/MultiHome.html

the *in silico* analysis were obtained from the NCBI databank[4], and they are primarily from *S. cerevisiae*, but also include genes from other fungi, such as *Aspergillus fumigatus*, *Aspergillus nidulans*, *C. albicans* and *H. capsulatum*. The search by orthologs was based on sequence similarity by using the BLAST tool. The expectation value adopted in the databases search was *E*-value $\leq 10^{-5}$.

The deduced amino acid sequences of the orthologs found in *P. brasiliensis* isolates and *Cryptococcus* species were analyzed. Searches for conserved domains and signal peptides in the orthologs proteins were performed using the Conserved Domain Database at NCBI[5] and the online software SMART[6]. Predictions of putative transmembrane segments were made using the TopPred[7] server and SMART software. Amino acid sequences alignment were performed using the ClustalX2 (Larkin et al., 2007).

RESULTS AND DISCUSSION

IRON

Uptake of iron at the cell surface by the reductive system

To better understand how *P. brasiliensis* could acquire iron by the reductive system, *in silico* analyses were performed utilizing *S. cerevisiae*[8] and *C. albicans*[9] sequences. The data showed that *Pb*01 contains four metalloreductase (Frep) homologs, *Pb*03 five homologs, and *Pb*18 three homologs (**Table 1**). The genes encoding metalloreductases were *fre1*, *fre3*, *fre5*, *fre7* and *frp1*. Also, *Pb*01 and *Pb*03 have two homologs each of the ferroxidase Fetp and *Pb*18 has one. The reductive uptake system was first described in *S. cerevisiae* (Lesuisse et al., 1987). The enzymatic reduction step in *S. cerevisiae* is catalyzed by members of the FRE family of metalloreductases. The products of the *fre* genes are not specific for iron reduction, since they can also promote copper reduction. *S. cerevisiae* Fre1p and Fre2p are required for growth on media with low concentrations of ferric iron salts. Fre3p and Fre4p catalyze uptake of iron from siderophores and Fre7p is under the control of the copper-dependent transcription factor Mac1p (Philpott and Protchenko, 2008). The expression of *C. albicans* ferric reductase Frp1p is upregulated by alkaline pH and iron-limited conditions (Liang et al., 2009). Future studies are required to dissect the roles of the different *P. brasiliensis* reductases, especially in *in vivo* conditions.

Homologs for iron permeases (Ftrp and Fthp) were not found in *P. brasiliensis* genomes, corroborating the hypothesis that iron is transported by the zinc permeases, as previously suggested by transcriptional analyses (Bailão et al., 2006, 2007; Costa et al., 2007). However, in the present *in silico* analysis, we identified five zinc transporters (**Table 1**). These permeases could be coupled with one or more of the ferroxidases homologs (Fet5p, Fet31p and Fet33p) identified in the *P. brasiliensis* genome database. In *S. cerevisiae*, reduced iron is taken up through a high-affinity transport complex that consists of Fet3p, a multi-copper ferroxidase, and Ftr1p, a permease. Independent studies have demonstrated that Fet3p produced by *S. cerevisiae* Δ*ftr1* mutant cells is retained in a cytoplasmic compartment in a copper-free, inactive form. Correspondingly, Ftr1p produced by *S. cerevisiae* Δ*fet3* mutant cells fails to reach the plasma membrane (Stearman et al., 1996). These observations are in agreement with a model in which the two proteins form a heterodimer or higher order structure for correct maturation and trafficking to the plasma membrane (Kosman, 2003).

The *P. brasiliensis* genomes analysis revealed the presence of a *ggt1* homolog. This gene is presumably responsible for the glutathione (GSH)-dependent iron reduction activity previously identified in functional studies (Zarnowski and Woods, 2005). The proposed mechanism comprises secretion of a glutathione-dependent ferric reductase (GSH–FeR), named Ggt1p, that purportedly utilizes siderophores and Fe^{3+}-binding proteins as substrates, enhancing the enzymatic activity under iron-limiting conditions, which is consistent with the function of a high-affinity uptake system, as described in *H. capsulatum* (Timmerman and Woods, 2001).

Homologs of permease genes involved in low-affinity iron reductive systems, such as *smf*, were not detected in our analysis. Hence, the low-affinity permease utilized by *P. brasiliensis* to acquire iron could be one of the zinc permeases, as suggested (**Table 1**). Despite the absence of iron permease *fth1* gene homologs, *P. brasiliensis* has one *ccc1* gene homolog that could drive iron vacuolar transport. *P. brasiliensis* also has homologs of the mitochondrial iron transporters genes *mrs3* and *mrs4* and the mitochondrial iron chaperone Yfh1p, suggesting mitochondrial iron homeostasis in this pathogen (**Table 1**). Since mitochondria are major users of iron, it follows that they should contain machinery required for its transport. Mrs3p and Mrs4p are homologous and functionally redundant proteins found in the inner mitochondrial membrane of *S. cerevisiae*, which are involved in transport under iron-limiting conditions (Foury and Roganti, 2002). Yfh1p, a homolog of human frataxin, is also involved in mitochondrial iron homeostasis (Babcock et al., 1997). While Mrs3p and Mrs4p mediate iron delivery from the outside to the inside of mitochondria, the frataxin homolog facilitates the use of iron within this organelle, functioning as a mitochondrial matrix iron chaperone (Zhang et al., 2006; Froschauer et al., 2009).

Cryptococcal genomic databases analysis revealed both *S. cerevisiae* and *C. albicans* homologs for proteins related to iron metabolism (**Table 1**). Remarkably, the *C. neoformans* var. *grubii* database contains four metalloreductase homologs, while the *C. gattii* genome has three similar homologs. The reason for the multiplicity of metalloreductases isoenzymes is not clear, although it is speculated that some sets of genes are expressed under specific conditions for iron acquisition (Kornitzer, 2009). Concerning the ferroxidases, *C. neoformans* var. *grubii* has three homologs and *C. gatti* contains one. Both genomes possess two iron permeases homologs, whose presence is supported by prior functional analyses (Jung et al., 2008). Two iron permeases, gene orthologs of *S. cerevisiae ftr1*, have been identified in *C. neoformans,* namely Cft1p and Cft2p (Jung et al., 2008). The expression of the *cft1* gene is down-regulated at high iron concentrations, suggesting that its product functions as a high-affinity iron permease. The role of *cft2* is still unclear, although it supposedly encodes a low-affinity iron permease or a vacuolar permease that could transport stored iron to the cytoplasm, similar to what occurs in *S. cerevisiae* with the iron permease Fth1p. One of the iron permeases here identified is probably a Fth1p homolog, which

[4]http://www.ncbi.nlm.nih.gov/guide/

[5]http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

[6]http://smart.embl-heidelberg.de/

[7]http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred

[8]http://www.yeastgenome.org/

[9]http://www.candidagenome.org/

Table 1 | Orthologs to genes related to iron, copper and zinc uptake by reductive systems in *P. brasiliensis* and *Cryptococcus* species.

Gene	Organism/accession number	Predicted function	Orthologs in *Pb* 01, 03 and 18 (accession numbers)†	*E*-value*	Orthologs in *Cryptococcus* species (accession numbers)†	*E*-value*
fre1	*S. cerevisiae* NP_013315	Metalloreductase	PAAG_05370.1 PABG_06003.1	e-22 e-19	Not identified	–
fre3	*S. cerevisiae* NP_015026	Metalloreductase	PAAG_02079.1 PABG_02329.1 PADG_00813.1	e-35 e-35 e-35	Not identified	–
fre5	*S. cerevisiae* NP_015029	Metalloreductase	PABG_07812.1	e-26	Not identified	–
fre7	*S. cerevisiae* NP_014489	Metalloreductase	PAAG_06164.1 PABG_06497.1 PADG_07957.1	0.0 0.0 0.0	CNAG_00876.2 CNBG_6082.2	e-37 e-37
fre8	*S. cerevisiae* NP_013148	Metalloreductase	Not identified	–	CNAG_07334.2 CNBG_2116.2	e-10 e-07
fre10	*C. albicans* XP_711543	Metalloreductase	Not identified	–	CNAG_06821.2 CNBG_5888.2	e-34 e-27
cfl4	*C. albicans* XP_715639	Metalloreductase	Not identified	–	CNAG_06524.2	e-32
frp1	*C. albicans* XP_713315	Metalloreductase	PAAG_04493.1 PABG_04278.1 PADG_04652.1	e-26 e-26 e-26	Not identified	–
fet3	*S. cerevisiae* NP_013774	Ferroxidase	Not identified	–	CNAG_06241.2	0.0
fet5	*S. cerevisiae* NP_116612	Ferroxidase	PABG_05667.1 PADG_05994.1	e-40 e-37	CNAG_07865.2 CNBG_4942.2	0.0 0.0
fet31	*C. albicans* XP_711263	Ferroxidase	PAAG_06004.1	e-39	CNAG_02958.2	0.0
fet33	*C. albicans* XP_711265	Ferroxidase	PAAG_00163.1 PABG_05183.1	e-33 e-33	Not identified	–
ftr1/ftr2	*C. albicans* XP_715020/ XP_715031	Iron permease	Not identified	–	CNAG_06242.2 CNBG_3602.2	0.0 0.0
smf1	*S. cerevisiae* NP_014519	Low-affinity permease	Not identified	–	CNAG_05640.2 CNBG_6162.2	0.0 0.0
fth1	*C. albicans* XP_723298	Vacuolar transporter	Not identified	–	CNAG_02959.2 CNBG_4943.2	0.0 0.0
ccc1	*S. cerevisiae* NP_013321	Vacuolar transporter	PAAG_07762.1 PABG_00362.1 PADG_02775.1	e-31 e-31 e-31	CNAG_05154.2 CNBG_4540.2	e-23 e-18
mrs3/ mrs4	*S. cerevisiae* NP_012402/ NP_012978	Mitochondrial iron transporter	PAAG_05053.1 PABG_04509.1 PADG_04903.1	0.0 0.0 0.0	CNAG_02522.2 CNBG_4218.2	0.0 0.0
yfh1	*S. cerevisiae* NP_010163	Mitochondrial matrix iron chaperone	PAAG_02608.1 PABG_03095.1 PADG_01626.1	e-15 e-09 e-16	CNAG_05011.2 CNBG_4670.2	e-18 e-18

(Continued)

Table 1 | Continued

Gene	Organism/accession number	Predicted function	Orthologs in *Pb* 01, 03 and 18 (accession numbers)†	*E*-value*	Orthologs in *Cryptococcus* species (accession numbers)†	*E*-value*
ggt1	*H. capsulatum* EGC49121	Secreted glutathione-dependent ferric reductase	PAAG_06130.1 PABG_06527.1 PADG_07986.1	0.0 0.0 0.0	CNAG_02888.2 CNBG_3537.2	0.0 0.0
mac1	*S. cerevisiae* NP_013734	Copper metalloregulatory transcription factor	PAAG_08210.1 PABG_07429.1	e-5 e-5	CNAG_07724.2 CNBG_2252.2	e-7 e-7
ctr3	*S. cerevisiae* NP_013515	High-affinity copper transporter of the plasma membrane	PAAG_05251.1 PABG_07607.1 PADG_05084.1	e-22 e-21 e-21	CNAG_00979.2 CNBG_0560.2	e-14 e-14
ctr1	*S. cerevisiae* NP_015449	High-affinity copper transporter of the plasma membrane	Not identified	–	Not identified	–
ctr2	*S. cerevisiae* NP_012045	Putative low-affinity copper transporter of the vacuolar membrane	PABG_01536.1 PADG_04146.1	e-14 e-14	CNAG_01872.2	e-13
atx1	*S. cerevisiae* NP_14140	Cytosolic copper metallochaperone	PAAG_00326.1 PABG_06615.1 PADG_02352.1	e-12 e-12 e-12	CNAG_02434.2 CNBG_4136.2	e-10 e-11
ccc2	*S. cerevisiae* NP_010556	Cu^{2+} transporting P-type ATPase	PAAG_07053.1 PABG_03057.1 PADG_01582.1	0.0 0.0 0.0	CNAG_06415.2 CNBG_5045.2	0.0 0.0
cup1	*S. cerevisiae* NP_011920	Metallothionein	Not identified	–	Not identified	–
cup2	*S. cerevisiae* NP_011922	Metallothionein	Not identified	–	Not identified	–
sod1	*S. cerevisiae* NP_012638	Cytosolic superoxide dismutase	PAAG_04164.1 PABG_03954.1 PADG_07418.1	0.0 0.0 0.0	CNAG_01019.2 CNBG_0599.2	0.0 0.0
sod2	*S. cerevisiae* NP_011872	Mitochondrial superoxide dismutase	PAAG_02725.1 PABG_03204.1 PADG_01755.1	0.0 0.0 0.0	CNAG_04388.2 CNBG_2661.2	0.0 0.0
*zrt*1	*S. cerevisiae* NP_011259	High-affinity zinc transporter of the plasma membrane	PAAG_08727.1 PABG_07725.1 PADG_08567.1	0.0 0.0	CNAG_03398.2 CNBG_2209.2	e-40 e-41
zrt2	*S. cerevisiae* NP_013231	Low-affinity zinc transporter of the plasma membrane	PAAG_03419.1 PABG_05498.1 PADG_06417.1	e-27 e-26 e-28	CNAG_00895.2	0.0

(Continued)

Table 1 | Continued

Gene	Organism/accession number	Predicted function	Orthologs in *Pb* 01, 03 and 18 (accession numbers)†	*E*-value*	Orthologs in *Cryptococcus* species (accession numbers)†	*E*-value*
*zrc*1	*S. cerevisiae* NP_013970	Vacuolar membrane zinc transporter	PAAG_00702.1	e-41	Not identified	–
*cot*1	*S. cerevisiae* NP_014961	Vacuolar membrane zinc transporter	PAAG_07885.1 PABG_07467.1 PADG_08196.1	e-44 0.0 0.0	CNAG_02806.2 CNBG_3460.2	e-40 e-37
*zrt*3	*S. cerevisiae* NP_012746	Vacuolar membrane zinc transporter	PAAG_09074.1 PABG_04697.1 PADG_05322.1	e-23 e-22 e-23	Not identified	–
*msc*2	*S. cerevisiae* NP_010491	Cation diffusion facilitator protein of the endoplasmic reticulum and nucleus	PABG_07115.1 PADG_06381.1	e-40 e-40	CNAG_05394.2 CNBG_4458.2	e-23 e-24
*zap*1	*S. cerevisiae* NP_012479	Zinc-regulated transcription factor	PAAG_03645.1 PABG_03305.1 PADG_01870.1	e-20 e-18 e-24	CNAG_05392.2 CNBG_4460.2	e-40 e-28

**Similarities with E-values < 10^{-5} were considered significant.*
†Accession numbers: PAAG refers to Pb01; PABG refers to Pb03; PADG refers to Pb18; CNAG refers to C. neoformans *var.* grubii *and CNBG refers to* C. gattii.

is likely involved in vacuolar iron uptake. Moreover, we could identify iron transporter *ccc1* gene homologs in the genome, suggesting that a vacuolar iron homeostasis system exists in *Cryptococcus*. Data mining revealed one homolog of the low-affinity gene *smf* family, confirming the presence of both high and low-affinity iron reductase systems, as described (Jacobson et al., 1998). The presence of mitochondrial *mrs3, mrs4* and *yfh1* gene homologs in *C. neoformans* var. *grubii* supports a mechanism for iron homeostasis (Nyhus and Jacobson, 1999; Jacobson et al., 2005). Additionally, our *in silico* analyses demonstrated that cryptococcal reductive systems are closely related to that of *S. cerevisiae* (**Table 1**). Although no activity for the enzyme glutathione-dependent ferric reductase had been reported in *Cryptococcus*, both genomes contain *ggt1* homologs suggesting the presence of a GSH–FeR system. A comparative analysis of iron uptake by reductive systems in *P. brasiliensis*, *C. neoformans* var. *grubii* and *C. gattii* is depicted in **Figure 1**.

Conserved domains in proteins related to the reductive iron metabolism

Amino acid sequence analyses of orthologs proteins found in the *P. brasiliensis* isolates and *Cryptococcus* species may support the assumption of conserved functions. Searching for conserved domains in all the analyzed sequences (**Table A1** in Appendix) revealed that most of the *P. brasiliensis* and *Cryptococcus* deduced proteins codified by the genes related to reductive iron metabolism contain conserved domains related to specific functions. Regarding to metalloreductases, the presence of a ferric reductase domain and a FAD- and/or a NAD-binding domain can be essential for functional enzymatic activity, since they are responsible for electron donation, as described in other organisms (De Luca and Wood, 2000). A schematic diagram presenting the cited motifs in a metalloreductase Frep is shown in **Figure 2**. An HPFT*XX*S motif is believed to be a site for FAD-binding and a glycine-rich motif and a cysteine–glycine couple are thought to be involved in NADPH binding (Shatwell et al., 1996). As well, copper-oxidase domains are required for ferroxidase activity. *S. cerevisiae* Fet3p is a multi-copper-oxidase and, like other copper proteins, possesses three distinct types of Cu^{2+}-binding sites. Oxidation of Fe^{2+} occurs at the type 1 copper site followed by the reduction of molecular oxygen to $2H_2O$ at the other two copper sites (Hassett et al., 1998; Kosman, 2003). The ferroxidases in the *P. brasiliensis* isolates and *Cryptococcus* species present such domain, suggesting they are functional proteins.

Siderophore production

Culture supernatants of *P. brasiliensis* grown in media with low iron concentrations display higher iron binding capacity when compared with culture supernatants from iron-rich media (Arango and Restrepo, 1988), which has suggested that siderophores are involved in iron acquisition in this fungus. Furthermore, *in silico* analysis of *P. brasiliensis* structural genomes indicates that this fungus can potentially produce siderophores. The three sequenced *P. brasiliensis* genomes show sequences that potentially encode all the necessary enzymes for siderophore synthesis: *sidA*, *sidF*, *sidC* and *sidD* (*A. fumigatus* orthologs), as shown in **Table 2** and **Figure 1**. This biosynthetic pathway may lead to the production of hydroxamate-type siderophores. The first committed step in siderophore biosynthesis is the N^5-hydroxylation of ornithine catalyzed by ornithine-N^5-oxygenase. The *sid1* gene of *Ustilago maydis,* the etiologic agent of corn smut, was the first characterized fungal ornithine-N^5-oxygenase-encoding gene (Mei et al., 1993).

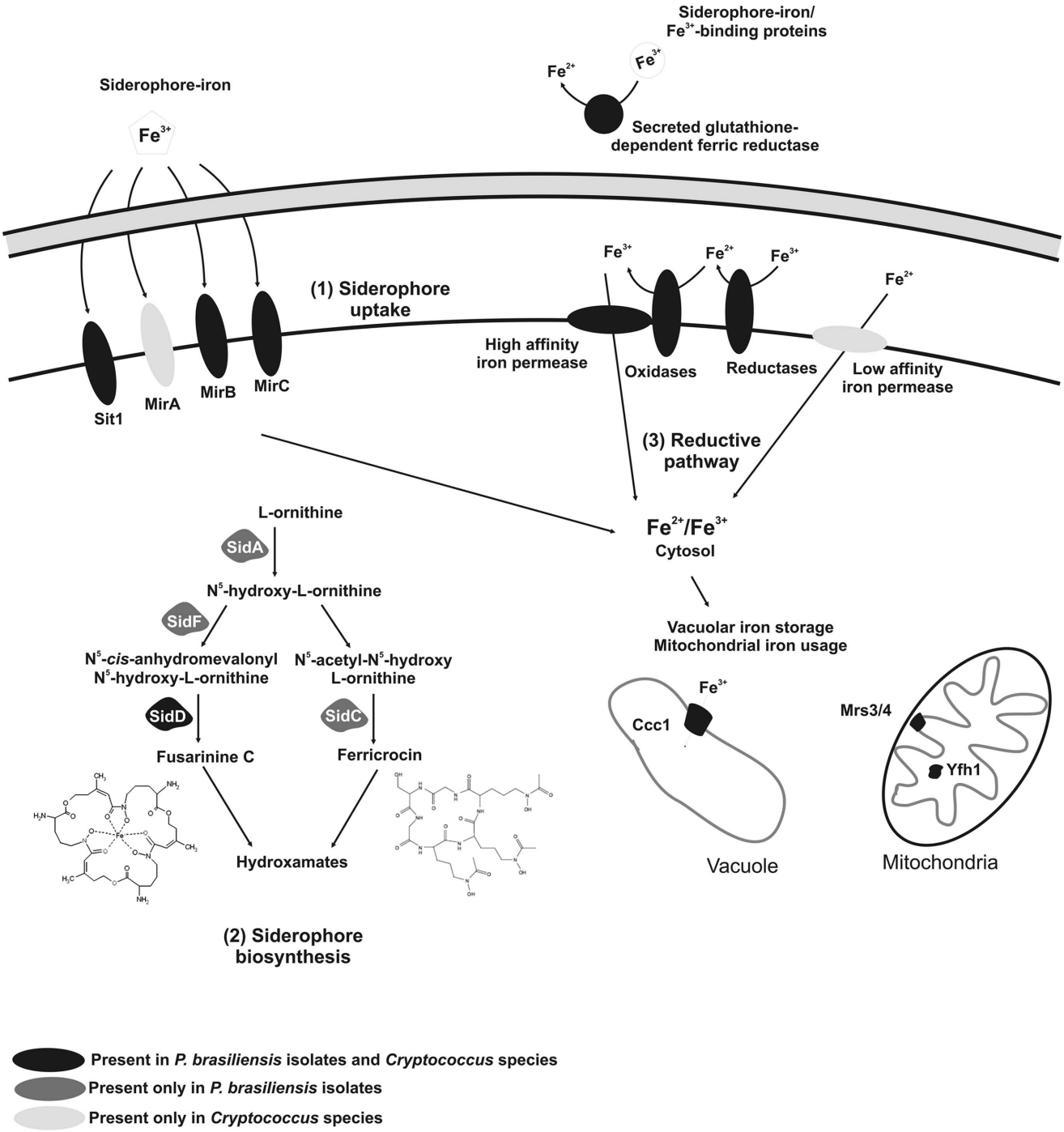


FIGURE 1 | Schematic comparison of iron metabolism in *P. brasiliensis* isolates and *Cryptococcus* species. Sit1p, MirAp, MirBp and MirCp are membrane transporters that traffic siderophores bound to ferric iron into the intracellular environment. SidAp, SidFp, SidCp and SidDp are enzymes from the biosynthetic pathway of hydroxamate-type siderophores. Ccc1p is a vacuolar membrane iron transporter. Mrs3/4p are iron transporters found in the inner mitochondrial membrane and Yfh1p is a mitochondrial matrix iron chaperone.

Orthologs of *sid1* have been identified in *A. fumigatus* (*sidA*) and *H. capsulatum* (*sid1*). In the latter, disruption of *sid1* causes poor growth under low iron conditions and loss of siderophore production, suggesting an important role of siderophore production in iron-limiting conditions (Schrettl et al., 2004; Hwang et al., 2008). The formation of the hydroxamate group consists of the transfer of an acyl group from acyl-coenzyme A to N^5-hydroxyornithine. Different acyl group usage results in the production of distinct

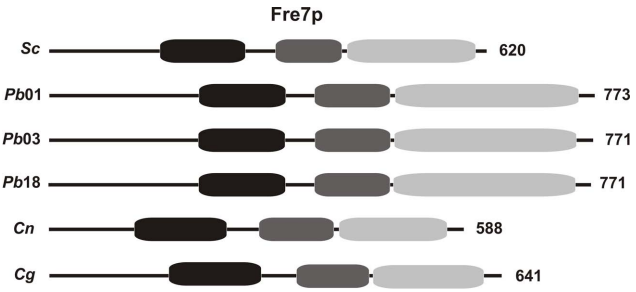


FIGURE 2 | Domains found in a Frep related to iron reductive uptake in *P. brasiliensis* isolates and *Cryptococcus* species. The found domains are: ferric reductase domain (black boxes), FAD-binding domain (dark gray boxes) and NAD-binding domain (light gray boxes). The length of each protein, in amino acids, is shown on the right. Accession numbers: *Sc* (NP_014489), *Pb*01 (PAAG_06164.1), *Pb*03 (PABG_06497.1), *Pb*18 (PADG_07957.1), *Cn* (CNAG_00876.2) and *Cg* (CNBG_6082.2).

siderophores. Acetyl is used for rhodotorulic acid and ferrichrome synthesis, while anhydromevalonyl is utilized in the fusarinines and coprogens pathway (Haas et al., 2008). *A. fumigatus sidF* encodes an N^5-hydroxyornithine:*cis* anhydromevalonyl coenzyme A-N^5-transacylase involved in the synthesis of fusarinine and triacetylfusarinine (Schrettl et al., 2007). The *sidF* ortholog of *H. capsulatum, sid3* gene, is transcriptionally induced under iron restricted conditions (Hwang et al., 2008). Hydroxamates are covalently linked via peptide (rhodotorulic acid, ferrichromes, coprogens) or ester bonds (fusarinines, coprogens) carried out by non-ribosomal peptide synthetases (NRPSs; Finking and Marahiel, 2004). In *A. fumigatus*, *sidC* and *sidD* encode two NRPSs involved in ferricrocin (intracellular siderophore) and triacetylfusarinine C (TAFC) biosynthesis, respectively. Some siderophores additionally require acetylation at the N^2-amino group, such as coprogen and TAFC. For example, *sidG* deletion in *A. fumigatus* results in the abrogation of the TAFC siderophore production (Schrettl et al., 2007). Given that our *in silico* analysis of *P. brasiliensis* identified sequences capable of coding for SidAp, SidFp, SidCp and SidDp, it is reasonable to hypothesize that *P. brasiliensis* may be able to synthesize both extracellular and intracellular siderophores.

Although *Cryptococcus* species have been described as unable to produce siderophores (Jacobson and Petro, 1987), *in silico* analysis of *C. neoformans* var. *grubii* and *C. gattii* structural genomes indicates the presence of *sidD* and *sidG* genes, which are also involved in other metabolic pathways in fungi. However, *sidA* and *sidF* genes were not found, and these genes are essential, especially since they act early in the pathway for siderophores production (**Table 2**; **Figure 1**). It will be interesting to examine if *sidA* and *sidF* have other functions and how siderophore-associated iron uptake was replaced to account for this loss.

Conserved domains in proteins related to siderophore biosynthesis

As described above, the third siderophore biosynthetic step is performed by NRPSs. These enzymes have a modular structure where one module, the catalytic unit, is composed of an adenylation domain (A) for substrate specificity and activation, a peptidyl carrier (PCP) domain that binds a 4′phosphopantetheine cofactor for attachment of the activated substrate, and a condensation (C) domain for bond formation (Finking and Marahiel, 2004). As *Cryptococcus* species are not siderophore producers, NRPSs domains analysis was performed only with SidCp ortholog found in *P. brasiliensis* genomes. These analyses revealed that, as in *A. fumigatus*, the three domains essential for NRPS function are present in SidCp from the three *P. brasiliensis* isolates examined (**Figure 3A**). Domains found in other siderophore biosynthesis related proteins are shown in **Table A2** in Appendix.

Siderophore uptake

The presence of orthologs for appropriate siderophore genes and the fact that the iron binding capacity of medium from low iron cultures of *P. brasiliensis* is greater than that of iron-replete medium (Arango and Restrepo, 1988) supports our hypothesis that *P. brasiliensis* produces and captures siderophores from the extracellular environment. Therefore, we have categorized putative *P. brasiliensis* siderophore transporters by sequence homology analysis (**Table 2**; **Figure 1**). Searches of the *P. brasiliensis* genomes revealed that all three isolates contain the *S. cerevisiae* gene homolog SIT *sit1*. *S. cerevisiae* can utilize siderophore-bound iron either by the reductive iron-assimilation system or by membrane transporters. In the latter case, the uptake is mediated by four transporters that differ in substrate specificity: Sit1p/Arn3p, Arn1p, Taf1p/Arn2p, Enb1p/Arn4p (Lesuisse et al., 1998; Heymann et al., 1999, 2000; Yun et al., 2000a,b). Sit1p/Arn3p recognizes ferrioxamines, coprogen, and ferrichromes lacking anhydromevalonic acid. Additionally, *P. brasiliensis* isolates possess the *A. nidulans* SIT gene homologs, *mirB*, and *mirC* (**Table 2**; **Figure 1**). Heterologs expression assays of *A. nidulans mir* genes in a *S. cerevisiae* mutant strain unable to uptake siderophores have demonstrated that MirBp transports native TAFC, a hydroxamate siderophore. The growth of *P. brasiliensis* is stimulated by coprogen B and dimerum acid (DA), a derivative of rhodotorulic acid from *Blastomyces dermatitidis,* suggesting that *P. brasiliensis* can use hydroxamate compounds as iron sources (Castaneda et al., 1988).

The siderophore transporter Sit1p/Arn3p and the transporters of the SIT-family (*mirA*, *mirB* and *mirC*) were found in *C. neoformans* var. *grubii* and *C. gattii* (**Table 2**; **Figure 1**). The homolog gene *sit1/arn3* was previously identified in *C. neoformans* var. *neoformans* using SAGE employed to examine the transcriptome under iron-limiting and iron-replete conditions (Lian et al., 2005). Mutants defective in *sit1* had increased melanin production and elevated transcript levels for the laccase gene, *lac1*. The melanin phenotype may be caused by changes in iron homeostasis or membrane trafficking, perhaps leading to altered copper loading of laccase in the cell wall. Studies with mutants lacking *sit1/arn3* in *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* have demonstrated that the gene *sit1* is required for siderophore utilization (ferrioxamine B) and growth in low iron-environments (Tangen et al., 2007). An overview of the siderophore biosynthesis and uptake in *P. brasiliensis* and *Cryptococcus* species is shown in **Figure 1**.

Analysis of transmembrane domains in siderophore–iron transporters

Amino acid sequences of siderophore transporter orthologs found in *P. brasiliensis* isolates and *Cryptococcus* species were analyzed in the TopPred server to predict their transmembrane domain topologies. **Figure 3B** presents the transmembrane segments of Sit1p in *S. cerevisiae*, *P. brasiliensis* isolates, *C. neoformans* var. *grubii* and *C. gattii*.

Table 2 | Orthologs to genes related to siderophore biosynthesis and to iron uptake by the non-reductive siderophore transport system in *P. brasiliensis* and *Cryptococcus* species.

Gene	Organism/ accession number	Predicted function	Orthologs in *Pb* 01, 03 and 18 (accession numbers)†	*E*-value*	Orthologs in *Cryptococcus* species (accession numbers)†	*E*-value*
sidA	*A. fumigatus*	Ornithine-N^5-	PAAG_01682.1	0.0	Not identified	–
	XP_755103	monooxygenase	PABG_03730.1	0.0		
			PADG_00097.1	0.0		
sidF	*A. fumigatus*	N^5-transacylases	PAAG_01680.1	0.0	Not identified	–
	XM_743567		PABG_03728.1	0.0		
			PADG_00100.1	0.0		
sidC	*A. fumigatus*	Non-ribosomal	PAAG_08527.1	0.0	Not identified	–
	XP_753088	peptide synthetase	PABG_04670.1	0.0		
			PADG_05295.1	0.0		
sidD	*A. fumigatus*	Non-ribosomal	PAAG_01679.1	0.0	CNAG_03588.2	e-40
	XP_748662	peptide synthetase	PABG_03726.1	0.0	CNBG_2041.2	e-41
			PADG_00102.1	0.0		
sidG	*A. fumigatus*	N^2-transacetylase	Not identified	–	CNAG_04355.2	2e-5
	XP_748685				CNBG_2703.2	e-6
sit1/arn3	*S. cerevisiae*	Siderophore	PAAG_06516.1	0.0	CNAG_00815.2	0.0
	NP_010849	transporter	PABG_02063.1	0.0	CNBG_1123.2	0.0
			PADG_00462.1	0.0		
mirA	*A. nidulans*	Siderophore	Not identified	–	CNAG_02083.2	0.0
	AY027565	transporter			CNBG_5232.2	0.0
mirB	*A. nidulans*	Siderophore	PAAG_01685.1	0.0	CNAG_07751.2	0.0
	XP_681809	transporter	PABG_03732.1	0.0	CNBG_2036.2	0.0
			PADG_00095.1	0.0		
mirC	*A. nidulans*	Siderophore	PAAG_02233.1	0.0	CNAG_07519.2	0.0
	AY135152	transporter	PABG_04747.1	0.0	CNBG_1087.2	e-44
			PADG_05373.1	0.0		

**Similarities with E-values < 10^{-5} were considered significant.*
†Accession numbers: PAAG refers to Pb01; PABG refers to Pb03; PADG refers to Pb18; CNAG refers to C. neoformans *var.* grubii *and CNBG refers to* C. gattii.

The number of segments varies between 12 and 15. Identical topology was found in Sit1p from *P. brasiliensis* isolates *Pb*03 and *Pb*18, whereas *Pb*01 has a different topology. Transmembrane domains were also identified in all the other siderophore transporters, as shown in **Table A2** in Appendix. These transporters also contain a MFS1 domain, which indicates that they belong to the MFS of transporters.

Iron source preferences

Several fungal pathogens utilize heme or hemoglobin as sources of iron (Foster, 2002; Jung et al., 2008). *C. albicans* expresses surface receptors for hemoglobin and hemolytic factors (Manns et al., 1994). Interestingly, heme–iron utilization in *C. albicans* is facilitated by Rbt5p, an extracellular glycosylphophatidylinositol (GPI)-anchored hemoglobin-binding protein (Weissman et al., 2008). Although there is no experimental evidence regarding the utilization of iron from the heme group by *P. brasiliensis*, there are genes that show similarity with Hmx-1p (Pendrak et al., 2004), and exhibit a heme oxygenase domain (PAAG_06626.1 in *Pb*01; PABG_02644.1 in *Pb*03; PADG_01082.1 in *Pb*18) in each of the *P. brasiliensis* isolates. These genes are annotated as conserved hypothetical or as predicted proteins. *C. neoformans* var. *grubii* is also able to utilize heme and hemoglobin as iron sources, but the mechanism(s) of heme utilization by this fungus are still unclear (Jung et al., 2008).

Transferrin has also been shown to be an iron source for both *C. albicans* and *C. neoformans* var. *grubii*. These fungi employ high-affinity permeases to acquire iron from transferrin in mammalian

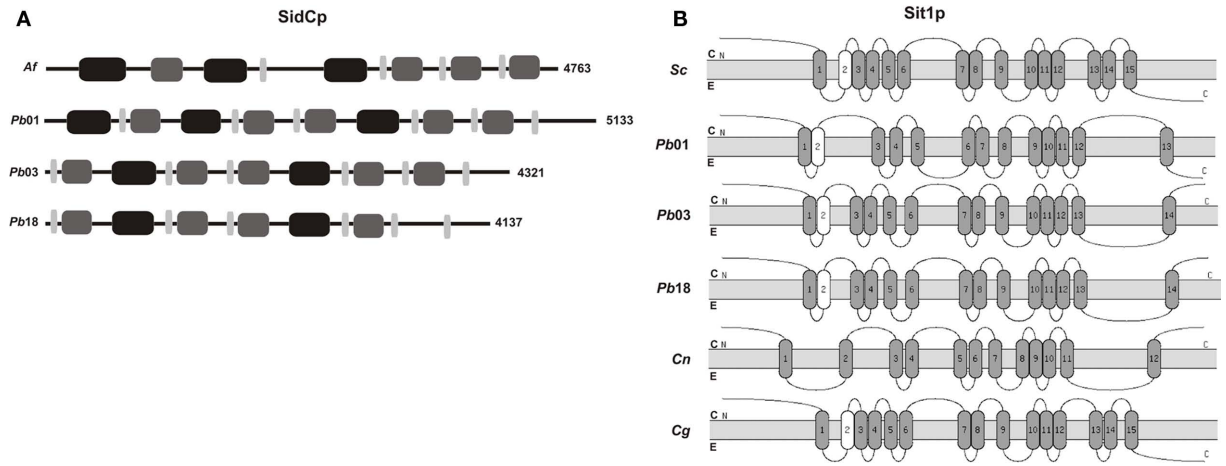


FIGURE 3 | Conserved features in proteins involved in biosynthesis and uptake of siderophores. (A) Modular organization of SidCp in *P. brasiliensis* isolates. Black boxes represent adenylation (A) domain, dark gray boxes illustrate the condensation (C) domain and light gray boxes represent the peptidyl carrier (PCP) domain. The length of each protein, in amino acids, is shown on the right. **(B)** Topology of transmembrane domains of Sit1p from *S. cerevisiae*, *P. brasiliensis* isolates and *Cryptococcus* species. White boxes represent putative segments, according to cutoff parameters (cutoff for certain transmembrane segments 1.00; cutoff for putative transmembrane segments 0.60). E: extracellular environment; C: cytosol. The topology prediction was performed using the TopPred server. Accession numbers in A: *Af* (XP_753088), *Pb*01 (PAAG_08527.1), *Pb*03 (PABG_04670.1), *Pb*18 (PADG_05295.1). Accession numbers in B: *Sc* (NP_010849), *Pb*01 (PAAG_06516.1), *Pb*03 (PABG_02063.1), *Pb*18 (PADG_00462.1), *Cn* (CNAG_00815.2), and *Cg* (CNBG_1123.2).

hosts through the reductive system (Knight et al., 2005; Jung et al., 2008). In the *P. brasiliensis* genome databases, genes were found (PAAG_04670.1; PABG_00038.1; PADG02428.1, respectively for isolates *Pb*01, *Pb*03 and *Pb*18) with high similarity to Cft1p, a permease from *C. neoformans* var. *grubii* required for iron utilization from transferrin (Jung et al., 2008).

COPPER

Copper uptake by the reductive system

Little is known about copper metabolism in *P. brasiliensis*. However, our *in silico* analyses of the *S. cerevisiae* copper metabolism-related genes in comparison to *P. brasiliensis* genomic databases revealed genes related to the copper reduction metalloreductase, *fre*. Copper transport is well described in *S. cerevisiae* where it is reduced from Cu (II) to Cu (I) by several cell surface metalloreductases encoded by several *fre* genes. These metalloreductases are regulated by iron and copper availability, mediated by the transcriptional factor Mac1p (Jungmann et al., 1993). Homologs of the copper metalloregulatory transcription factor gene (*mac*1) are present in both *Pb*01 and *Pb*03 genomes, but not in *Pb*18. Additionally, the high-affinity copper transport (Ctr3p) was found in all three isolate genomes. In *S. cerevisiae*, after reduction, copper is transported by the high-affinity copper transporter comprised by Ctr3p and Ctr1p, which are functionally redundant, although they have distinct amino acid sequences. Ctr3p is an integral membrane protein that assembles as a trimer to form a competent copper uptake permease at the plasma membrane. *S. cerevisiae* Ctr1p is localized at the plasma membrane and exists as an oligomer *in vivo*. These two high-affinity copper transport proteins are induced by copper deprivation and repressed by copper excess (Dancis et al., 1994a; Pena et al., 2000). In our *in silico* analyses, genes for the high-affinity copper transporter of the plasma membrane (*ctr1*) were not found, suggesting that high-affinity copper transport is performed only by the Ctr3p protein.

Genes related to metallochaperone (*atx*1), Cu^{2+} transporting P-type ATPase (*ccc2*) and superoxide dismutases (*sod1* and *sod2*; **Table 1**) were also found in *P. brasiliensis* genomes. In the cell, copper is transported by Atx1p, a cytosolic copper metallochaperone protein, that transports Cu (I) to Ccc2p, a transporting P-type ATPase containing a cytoplasmic region containing two distinct soluble metal-binding domains that interact with Atx1p (Banci et al., 2007). Ccc2p mediates the export of copper from the cytosol and distributes it to cupric proteins (Yuan et al., 1997). *S. cerevisiae* also has a detoxification pathway formed by Cup1p and Cup2p, metallothioneins (**Table 1**), that protect against copper poisoning (Hamer et al., 1985). An alternative copper transport system is mediated by Ctr2p, a vacuolar membrane protein of *S. cerevisiae*, that mobilizes vacuolar copper stores to cytosolic copper chaperones (Rees et al., 2004). Homologs of the low-affinity copper transporter of the vacuolar membrane (Ctr2p) are in *Pb*03 and *Pb*18, but not in *Pb*01. Additionally, the metallothioneins (encoded by *cup1* and *cup2* genes) were not identified in *P. brasiliensis* isolates *Pb*01, *Pb*03 and *Pb*18.

In silico analysis (**Table 1**) revealed that *Cryptococcus* species have orthologs encoding ferric/cupric reductases, suggesting that the copper reduction process is similar to that described for *S. cerevisiae*. Homologs of the high-affinity copper transporter *ctr3* gene and copper metalloregulatory transcription factor gene (*mac1*) have previously been identified (Waterman et al., 2007). Also, proteins with similarity to the cytosolic copper metallochaperone (*atx1* gene), the Cu^{2+} transporting P-type ATPase (*ccc2* gene) and the cytosolic and mitochondrial superoxide dismutases (*sod1* and *sod2* genes) have also identified, suggesting that copper distribution in *Cryptococcus* species occurs as described in *S. cerevisiae*. A homolog of the *ctr2* gene was identified only in *C. neoformans* var. *grubii*. Recently it was demonstrated that Ctr2p links copper homeostasis to polysaccharide capsule production in *C. neoformans*. The lack of this protein resulted in increased phagocytosis by murine macro-

phage, sensitivity to copper starvation and defects in polysaccharide capsule formation and melanization (Chun and Madhani, 2010). The gene *ctr1* for the high-affinity copper transporter of the plasma membrane and the genes *cup1* and *cup2* for metallothioneins were not found in *Cryptococcus* species. These analyses suggest that the high-affinity copper transport in cryptococcal cells is primarily performed by the protein encoded by *ctr*3.

Analysis of conserved motifs present in copper transporters

Searches for conserved domains revealed the presence of Mets and M*XXX*M motifs in the Ctr3p of the *P. brasiliensis* isolates and the *Cryptococcus* species (**Figure 4**). Studies in yeast and mammalian cells have revealed that proteins of the CTR family are integral membrane proteins containing three membrane-spanning domains, with high protein sequence homology (Dancis et al., 1994a; Lee et al., 2002). With the exception of *S. cerevisiae* Ctr3p, all CTR family members are rich in methionine residues within the amino-terminal portion (Labbe et al., 1999). These residues are arranged as M*XX*M and/or M*X*M, called Mets motifs, and it has been suggested that they could be involved in extracellular copper binding (Dancis et al., 1994b). It has been demonstrated that these clustered methionine residues together with an M*XXX*M motif in the transmembrane domain of CTR family members are important for copper uptake (Puig et al., 2002). In *P. brasiliensis* the M*XXX*M motif is found within the third transmembrane segment. The Ctr3p of *Cryptococcus* species contains only two predicted transmembrane domains instead of the three transmembrane segments described for other fungi. In *C. neoformans* var. *grubii* and *C. gattii*, the M*XXX*M motif is within the second transmembrane domain. Conserved domains were also found in amino acid sequences of other proteins involved in copper metabolism (**Table A1** in Appendix), suggesting that the orthologs found in *P. brasiliensis* and *Cryptococcus* may have activities that are similar to genes with established functions in other fungi.

ZINC

Zinc uptake

Comparisons to the *S. cerevisiae* genes related to zinc metabolism performed in *P. brasiliensis* genomes are presented in **Table 1**. Analyses demonstrate that *P. brasiliensis* has homologs to zinc transporters described in *S. cerevisiae* that are localized in the plasmatic, vacuolar and endoplasmic reticulum membranes. Importantly, five genes encoding to transporters of the ZIP family, with homology to *S. cerevisiae* Zrt1p or Zrt2p, are in the *P. brasiliensis* genomic database. In *S. cerevisiae,* zinc is transported by proteins belonging to the ZIP family, which is composed by a zinc high-affinity transporter protein encoded by the *zrt1* gene and a low-affinity transporter encoded by the *zrt2* gene (Gaither and Eide, 2001). We have previously identified homologs of zinc transporters by transcriptional analysis of *P. brasiliensis* yeast cells after incubation in human blood and plasma (Bailão et al., 2006, 2007). Interestingly, *P. brasiliensis* isolate *Pb*01 has two vacuolar membrane zinc transporters, encoded by the *zrc1* and *cot1* genes, whereas isolates *Pb*03 and *Pb*18 contain only the *cot1* homolog. Intracellularly, zinc is in vacuoles in association with the vacuolar membrane proteins Zrc1p and Cot1p, members of the cation diffusion facilitator (CDF) family (MacDiarmid et al., 2002). A homolog of the transcription factor Zap1p is also present in the three *P. brasiliensis* isolates. The expression of the genes associated with zinc homeostasis is positively regulated in *S. cerevisiae* by the transcription factor Zap1p, which regulates the expression of *zrt1*, *zrt2*, *zrt3*, *fet4*, and *zcr1* under zinc limiting conditions (Wu et al., 2008). Therefore, zinc assimilation in *P. brasiliensis* may be similar to that of *S. cerevisiae*.

Similarly, zinc homeostasis in *Cryptococcus* species is poorly studied. *In silico* analysis was performed by comparing *S. cerevisiae* genes related to zinc metabolism in genomic cryptococcal databases (**Table 1**). The results show that *C. neoformans* var. *grubii* and *C. gattii* have Zrt1p and Zrt2p zinc transporters homologs. These proteins putatively internalize zinc into the cell. Further, homologs of the vacuolar transporter Cot1p and the CDF Msc2p are present. Cot1p is presumably in the vacuolar membrane and should be related to zinc storage in this compartment. Msc2p, an endoplasmic reticulum membrane zinc transporter, could be related to zinc transport to this organelle. The protein encoded by *msc2* (CDF) is responsible for zinc homeostasis in the endoplasmic reticulum in *S. cerevisiae* (Ellis et al., 2004). A homolog of the transcription factor Zap1p is also present in *Cryptococcus*. Since homologs to the vacuolar membrane zinc transporter gene *zrt3* were not identified, the *zrc1* and *cot1* genes, encoding vacuolar membrane zinc transporters

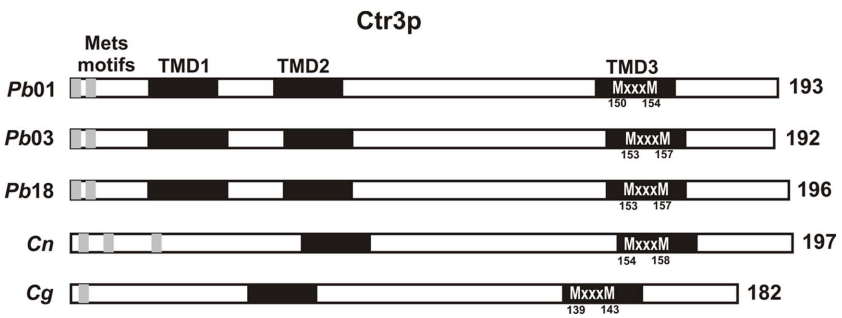


FIGURE 4 | Conserved features found in the primary structure of Ctr3p of *P. brasiliensis* isolates and *Cryptococcus* species. Ctr3p from *P. brasiliensis* isolates contains three putative transmembrane domains (TMD1-3, shown in black) while Ctr3p from *Cryptococcus* species presents only two TMDs. All species contain putative copper binding motifs (Mets motifs) arranged as M*XX*M and/or M*X*M. M*XXX*M motif in TMD3 in *P. brasiliensis* isolates and TMD2 in *Cryptococcus* species are represented in white characters. The length of each protein, in amino acids, is shown on the right. Accession numbers: *Pb*01 (PAAG_05251.1), *Pb*03 (PABG_07607.1), *Pb*18 (PADG_05084.1), *Cn* (CNAG_00979.2) and *Cg* (CNBG_0560.2).

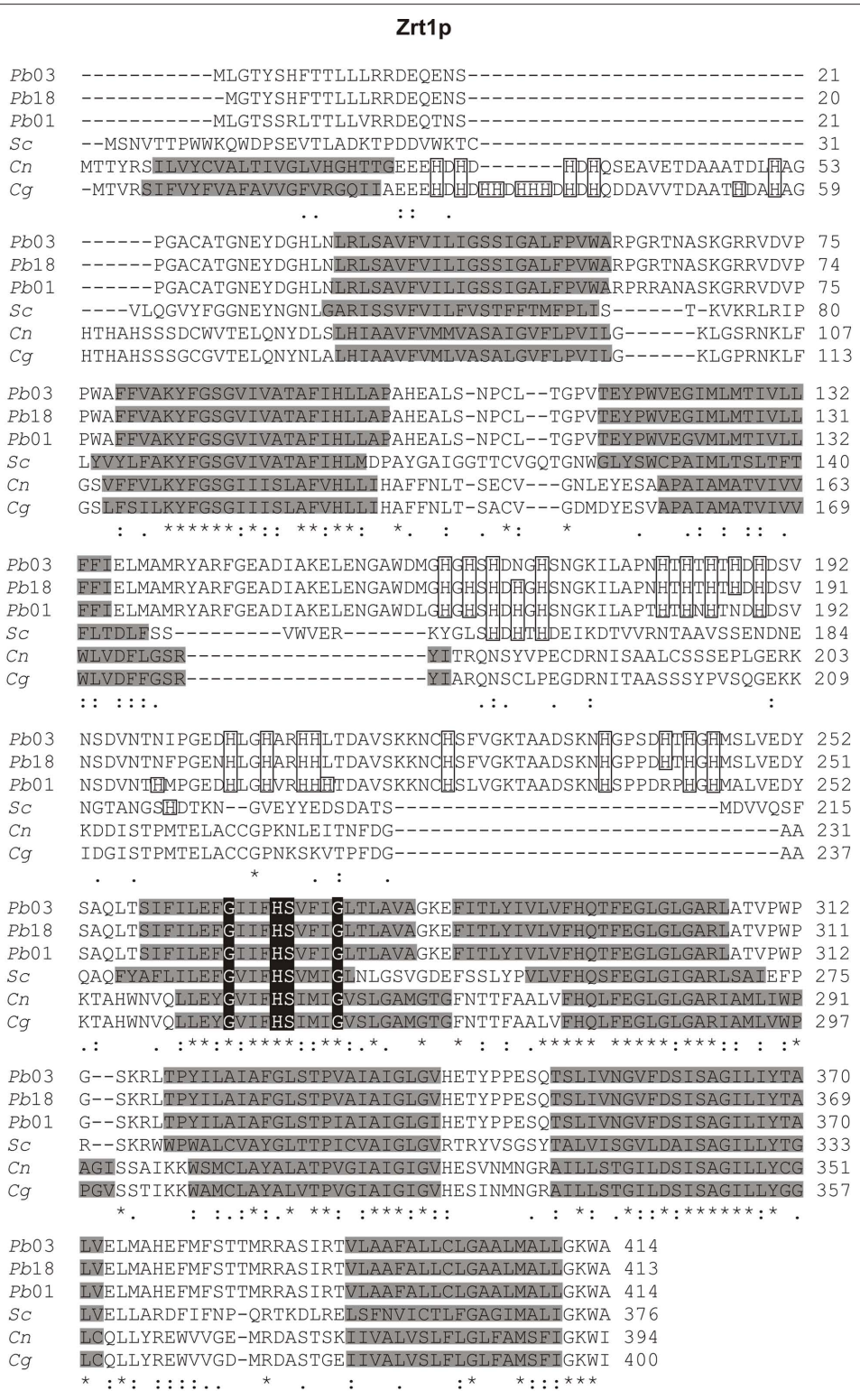


FIGURE 5 | Alignment of amino acid sequences of Zrt1p from *S. cerevisiae*, *P. brasiliensis* isolates and *Cryptococcus* species. The predicted transmembrane domains are shown in gray boxes. The black boxes inside the transmembrane segment contain conserved histidine-serine and glycine residues. The histidines found in the amino-terminal region of Zrt1p from *Cryptococcus* species and in the loop between transmembrane domains III and IV in *P. brasiliensis* and *S. cerevisiae* are boxed. Asterisks indicate amino acid identity and dots represent conserved substitutions. Accession numbers: *Pb*03 (PABG_07725.1), *Pb*18 (PADG_08567.1), *Pb*01 (PAAG_08727.1), *Sc* (NP_011259), *Cn* (CNAG_03398.2) and *Cg* (CNBG_2209.2).

could be responsible for the zinc transport to this organelle. This analysis suggests that *C. neoformans* var. *grubii* and *C. gattii* could obtain zinc via routes similar to that described for *S. cerevisiae*.

Analysis of conserved regions in the high-affinity zinc transporter (Zrt1p) in P. brasiliensis *isolates and* Cryptococcus *species*

Alignment of Zrt1p amino acid sequence from *S. cerevisiae*, *P. brasiliensis* isolates and *Cryptococcus* species revealed some conserved features (**Figure 5**). Concerning the predicted transmembrane domain number, all *P. brasiliensis* isolates contain eight predicted domains, while both *C. neoformans* var. *grubii* and *C. gattii* have nine. Proteins belonging to the ZIP family are predicted to have from five to eight transmembrane domains and they vary in size from 233 to 477 amino acid residues. The variations in the amino-terminal portion are usually responsible for the differences in size. The transmembrane domain IV has the most conserved portions of ZIP family proteins, with conserved histidine and glycine residues. The histidine residue and the adjacent polar residue, usually a serine, within the transmembrane domain are predicted to comprise part of a heavy metal-binding site in the center of the membrane (Eng et al., 1998). The amino acid sequence of *S. cerevisiae* Zrt1p presents a number of histidine residues in a large loop between the transmembrane segments III and IV, which is a putative metal ion binding site (Zhao and Eide, 1996a). The histidine-serine and glycine residues are conserved within the fourth transmembrane region in *P. brasiliensis* and within the fifth transmembrane region in *Cryptococcus*. Regarding the histidine rich region, it is conserved between transmembrane domains III and IV in *P. brasiliensis* isolates, whereas are conserved at the amino-terminal portion in *Cryptococcus* species, as occurs in other members of the ZIP family (Eng et al., 1998). Conserved domains are also found in amino acid sequences of other proteins involved in zinc metabolism that were identified in the search for orthologs (**Table A1** in Appendix).

CONCLUSION

As we have described, microorganisms are extremely well equipped to exploit host metal sources during growth and infection. *Cryptococcus* species demonstrate remarkable flexibility in gaining access to and utilizing iron, the most investigated micronutrient in this organism. Our laboratories have begun to elucidate the mechanisms for the uptake and metabolism of micronutrients such as iron, copper and zinc in *P. brasiliensis*. Studies on individual genes and pathways are revealing unique features of micronutrients metabolism in this fungus. The application of systems biology approaches that incorporates genomic and proteomic data will further generate hypotheses about the common and specific responses to micronutrient deprivation in both pathogenic fungi and potentially lead to the development of novel therapeutics exploiting their metal requirements.

ACKNOWLEDGMENT

This work at laboratories was supported by grants from MCT/FINEP/Rede GENOPROT Grant number 01.07.0552.00.

REFERENCES

Arango, R., and Restrepo, A. (1988). Growth and production of iron chelants by *Paracoccidioides brasiliensis* mycelial and yeast forms. *J. Med. Vet. Mycol.* 26, 113–118.

Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfo, M., and Kaplan, J. (1997). Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. *Science* 276, 1709–1712.

Bailão, A. M., Schrank, A., Borges, C. L., Dutra, V., Molinari-Madlum, E. E. W. I., Felipe, M. S. S., Mendes-Giannini, M. J. S., Martins, W. S., Pereira, M., and Soares, C. M. A. (2006). Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. *Microbes Infect.* 8, 2686–2697.

Bailão, A. M., Shrank, A., Borges, C. L., Parente, J. A., Dutra, V., Felipe, M. S., Fiuza, R. B., Pereira, M., and Soares, C. M. A. (2007). The transcriptional profile of *Paracoccidioides brasiliensis* yeast cells is influenced by human plasma. *FEMS Immunol. Med. Microbiol.* 51, 43–57.

Banci, L., Bertini, I., Chasapis, C. T., Rosato, A., and Tenori, L. (2007). Interaction of the two soluble metal-binding domains of yeast Ccc2 with copper(I)-Atx1. *Biochem. Biophys. Res. Commun.* 364, 645–649.

Berg, J. M., and Shi, Y. (1996). The galvanization of biology: a growing appreciation for the roles of zinc. *Science* 271, 1081–1085.

Castaneda, E., Brummer, E., Perlman, A. M., McEwen, J. G., and Stevens, D. A. (1988). A culture medium for *Paracoccidioides brasiliensis* with high plating efficiency, and the effect of siderophores. *J. Med. Vet. Mycol.* 26, 351–358.

Chun, C. D., and Madhani, H. D. (2010). Ctr2 links copper homeostasis to polysaccharide capsule formation and phagocytosis inhibition in the human fungal pathogen *Cryptococcus neoformans*. *PLoS ONE* 5, e12503. doi: 10.1371/journal.pone.0012503

Costa, M., Borges, C. L., Bailao, A. M., Meirelles, G. V., Mendonca, Y. A., Dantas, S. F., de Faria, F. P., Felipe, M. S., Molinari-Madlum, E. E., Mendes-Giannini, M. J., Fiuza, R. B., Martins, W. S., Pereira, M., and Soares, C. M. (2007). Transcriptome profiling of *Paracoccidioides brasiliensis* yeast-phase cells recovered from infected mice brings new insights into fungal response upon host interaction. *Microbiology* 153, 4194–4207.

Dancis, A., Haile, D., Yuan, D. S., and Klausner, R. D. (1994a). The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. *J. Biol. Chem.* 269, 25660–25667.

Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J., and Klausner, R. D. (1994b). Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. *Cell* 76, 393–402.

De Luca, N. G., and Wood, P. M. (2000). Iron uptake by fungi: contrasted mechanisms with internal or external reduction. *Adv. Microb. Physiol.* 43, 39–74.

Dias-Melicio, L. A., Calvi, S. A., Peracoli, M. T., and Soares, A. M. (2005). Inhibitory effect of deferoxamine on *Paracoccidioides brasiliensis* survival in human monocytes: reversal by holotransferrin not by apotransferrin. *Rev. Inst. Med. Trop. Sao Paulo* 47, 263–266.

Eide, D. J. (2003). Multiple regulatory mechanisms maintain zinc homeostasis in *Saccharomyces cerevisiae*. *J. Nutr.* 133, 1532S–1535S.

Ellis, C. D., Wang, F., MacDiarmid, C. W., Clark, S., Lyons, T., and Eide, D. J. (2004). Zinc and the Msc2 zinc transporter protein are required for endoplasmic reticulum function. *J. Cell Biol.* 166, 325–335.

Eng, B. H., Guerinot, M. L., Eide, D., and Saier, M. H. Jr. (1998). Sequence analyses and phylogenetic characterization of the ZIP family of metal ion transport proteins. *J. Membr. Biol.* 166, 1–7.

Finking, R., and Marahiel, M. A. (2004). Biosynthesis of nonribosomal peptides. *Annu. Rev. Microbiol.* 58, 453–488.

Foster, L. A. (2002). Utilization and cell-surface binding of hemin by *Histoplasma capsulatum*. *Can. J. Microbiol.* 48, 437–442.

Foury, F., and Roganti, T. (2002). Deletion of the mitochondrial carrier genes MRS3 and MRS4 suppresses mitochondrial iron accumulation in a yeast frataxin-deficient strain. *J. Biol. Chem.* 277, 24475–24483.

Froschauer, E. M., Schweyen, R. J., and Wiesenberger, G. (2009). The yeast mitochondrial carrier proteins Mrs3p/Mrs4p mediate iron transport across the inner mitochondrial membrane. *Biochim. Biophys. Acta* 1788, 1044–1050.

Gaither, L. A., and Eide, D. J. (2001). Eukaryotic zinc transporters and their regulation. *Biometals* 14, 251–270.

Georgatsou, E., Mavrogiannis, L. A., Fragiadakis, G. S., and Alexandraki, D. (1997). The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *J. Biol. Chem.* 272, 13786–13792.

Gitan, R. S., Luo, H., Rodgers, J., Broderius, M., and Eide, D. (1998). Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation. *J. Biol. Chem.* 273, 28617–28624.

Gross, C., Kelleher, M., Iyer, V. R., Brown, P. O., and Winge, D. R. (2000). Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays. *J. Biol. Chem.* 275, 32310–32316.

Haas, H., Eisendle, M., and Turgeon, B. G. (2008). Siderophores in fungal physiology and virulence. *Annu. Rev. Phytopathol.* 46, 149–187.

Hamer, D. H., Thiele, D. J., and Lemontt, J. E. (1985). Function and autoregulation of yeast copperthionein. *Science* 228, 685–690.

Hassett, R., and Kosman, D. J. (1995). Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. *J. Biol. Chem.* 270, 128–134.

Hassett, R. F., Yuan, D. S., and Kosman, D. J. (1998). Spectral and kinetic properties of the Fet3 protein from *Saccharomyces cerevisiae*, a multinuclear copper ferroxidase enzyme. *J. Biol. Chem.* 273, 23274–23282.

Heymann, P., Ernst, J. F., and Winkelmann, G. (1999). Identification of a fungal triacetylfusarinine C siderophore transport gene (TAF1) in *Saccharomyces cerevisiae* as a member of the major facilitator superfamily. *Biometals* 12, 301–306.

Heymann, P., Ernst, J. F., and Winkelmann, G. (2000). A gene of the major facilitator superfamily encodes a transporter for enterobactin (Enb1p) in *Saccharomyces cerevisiae*. *Biometals* 13, 65–72.

Howard, D. H. (1999). Acquisition, transport, and storage of iron by pathogenic fungi. *Clin. Microbiol. Rev.* 12, 394–404.

Hwang, L. H., Mayfield, J. A., Rine, J., and Sil, A. (2008). *Histoplasma* requires SID1, a member of an iron-regulated siderophore gene cluster, for host colonization. *PLoS Pathog.* 4, e1000044. doi: 10.1371/journal.ppat.1000044

Jacobson, E. S., Goodner, A. P., and Nyhus, K. J. (1998). Ferrous iron uptake in *Cryptococcus neoformans*. *Infect. Immun.* 66, 4169–4175.

Jacobson, E. S., and Petro, M. J. (1987). Extracellular iron chelation in *Cryptococcus neoformans*. *J. Med. Vet. Mycol.* 25, 415–418.

Jacobson, E. S., Troy, A. J., and Nyhus, K. J. (2005). Mitochondrial functioning of constitutive iron uptake mutations in *Cryptococcus neoformans*. *Mycopathologia* 159, 1–6.

Jung, W. H., and Kronstad, J. W. (2008). Iron and fungal pathogenesis: a case study with *Cryptococcus neoformans*. *Cell. Microbiol.* 10, 277–284.

Jung, W. H., Sham, A., Lian, T., Singh, A., Kosman, D. J., and Kronstad, J. W. (2008). Iron source preference and regulation of iron uptake in *Cryptococcus neoformans*. *PLoS Pathog.* 4, e45. doi: 10.1371/journal.ppat.0040045

Jungmann, J., Reins, H. A., Lee, J., Romeo, A., Hassett, R., Kosman, D., and Jentsch, S. (1993). MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. *EMBO J.* 12, 5051–5056.

Kim, B. E., Nevitt, T., and Thiele, D. J. (2008). Mechanisms for copper acquisition, distribution and regulation. *Nat. Chem. Biol.* 4, 176–185.

Knight, S. A., Vilaire, G., Lesuisse, E., and Dancis, A. (2005). Iron acquisition from transferrin by *Candida albicans* depends on the reductive pathway. *Infect. Immun.* 73, 5482–5492.

Kornitzer, D. (2009). Fungal mechanisms for host iron acquisition. *Curr. Opin. Microbiol.* 12, 377–383.

Kosman, D. J. (2003). Molecular mechanisms of iron uptake in fungi. *Mol. Microbiol.* 47, 1185–1197.

Labbe, S., Pena, M. M., Fernandes, A. R., and Thiele, D. J. (1999). A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe*. *J. Biol. Chem.* 274, 36252–36260.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948.

Lee, J., Pena, M. M., Nose, Y., and Thiele, D. J. (2002). Biochemical characterization of the human copper transporter Ctr1. *J. Biol. Chem.* 277, 4380–4387.

Lesuisse, E., Raguzzi, F., and Crichton, R. R. (1987). Iron uptake by the yeast *Saccharomyces cerevisiae*: involvement of a reduction step. *J. Gen. Microbiol.* 133, 3229–3236.

Lesuisse, E., Simon-Casteras, M., and Labbe, P. (1998). Siderophore-mediated iron uptake in *Saccharomyces cerevisiae*: the SIT1 gene encodes a ferrioxamine B permease that belongs to the major facilitator superfamily. *Microbiology* 144(Pt 12), 3455–3462.

Lian, T., Simmer, M. I., D'Souza, C. A., Steen, B. R., Zuyderduyn, S. D., Jones, S. J., Marra, M. A., and Kronstad, J. W. (2005). Iron-regulated transcription and capsule formation in the fungal pathogen *Cryptococcus neoformans*. *Mol. Microbiol.* 55, 1452–1472.

Liang, Y., Gui, L., Wei, D. S., Zheng, W., Xing, L. J., and Li, M. C. (2009). *Candida albicans* ferric reductase FRP1 is regulated by direct interaction with Rim101p transcription factor. *FEMS Yeast Res.* 9, 270–277.

Lyons, T. J., Gasch, A. P., Gaither, L. A., Botstein, D., Brown, P. O., and Eide, D. J. (2000). Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 97, 7957–7962.

MacDiarmid, C. W., Milanick, M. A., and Eide, D. J. (2002). Biochemical properties of vacuolar zinc transport systems of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 277, 39187–39194.

Manns, J. M., Mosser, D. M., and Buckley, H. R. (1994). Production of a hemolytic factor by *Candida albicans*. *Infect. Immun.* 62, 5154–5156.

Matzanke, B. F., Bill, E., Trautwein, A. X., and Winkelmann, G. (1987). Role of siderophores in iron storage in spores of *Neurospora crassa* and *Aspergillus ochraceus*. *J. Bacteriol.* 169, 5873–5876.

Mei, B., Budde, A. D., and Leong, S. A. (1993). sid1, a gene initiating siderophore biosynthesis in *Ustilago maydis*: molecular characterization, regulation by iron, and role in phytopathogenicity. *Proc. Natl. Acad. Sci. U.S.A.* 90, 903–907.

Miethke, M., and Marahiel, M. A. (2007). Siderophore-based iron acquisition and pathogen control. *Microbiol. Mol. Biol. Rev.* 71, 413–451.

Moroz, O. V., Antson, A. A., Grist, S. J., Maitland, N. J., Dodson, G. G., Wilson, K. S., Lukanidin, E., and Bronstein, I. B. (2003). Structure of the human S100A12-copper complex: implications for host-parasite defence. *Acta Crystallogr. D Biol. Crystallogr.* 59, 859–867.

Neilands, J. B. (1993). Siderophores. *Arch. Biochem. Biophys.* 302, 1–3.

Neilands, J. B., Konopka, K., Schwyn, B., Coy, M., Francis, R. T., Paw, B. H., and Bagg, A. (1987). "Comparative biochemistry of microbial iron assimilation," in *Iron Transport in Microbes, Plants and Animals*, eds G. Winkelmann, D. Van der Helm, and J. B. Neilands (New York: VCH Publishers), 3–34.

Nyhus, K. J., and Jacobson, E. S. (1999). Genetic and physiologic characterization of ferric/cupric reductase constitutive mutants of *Cryptococcus neoformans*. *Infect. Immun.* 67, 2357–2365.

Pao, S. S., Paulsen, I. T., and Saier, M. H. Jr. (1998). Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* 62, 1–34.

Pena, M. M., Puig, S., and Thiele, D. J. (2000). Characterization of the *Saccharomyces cerevisiae* high affinity copper transporter Ctr3. *J. Biol. Chem.* 275, 33244–33251.

Pendrak, M. L., Chao, M. P., Yan, S. S., and Roberts, D. D. (2004). Heme oxygenase in *Candida albicans* is regulated by hemoglobin and is necessary for metabolism of exogenous heme and hemoglobin to alpha-biliverdin. *J. Biol. Chem.* 279, 3426–3433.

Philpott, C. C. (2006). Iron uptake in fungi: a system for every source. *Biochim. Biophys. Acta* 1763, 636–645.

Philpott, C. C., and Protchenko, O. (2008). Response to iron deprivation in *Saccharomyces cerevisiae*. *Eukaryot. Cell* 7, 20–27.

Puig, S., Lee, J., Lau, M., and Thiele, D. J. (2002). Biochemical and genetic analyses of yeast and human high affinity copper transporters suggest a conserved mechanism for copper uptake. *J. Biol. Chem.* 277, 26021–26030.

Rees, E. M., Lee, J., and Thiele, D. J. (2004). Mobilization of intracellular copper stores by the ctr2 vacuolar copper transporter. *J. Biol. Chem.* 279, 54221–54229.

Schaible, U. E., and Kaufmann, S. H. (2004). Iron and microbial infection. *Nat. Rev. Microbiol.* 2, 946–953.

Schrettl, M., Bignell, E., Kragl, C., Joechl, C., Rogers, T., Arst, H. N. Jr., Haynes, K., and Haas, H. (2004). Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. *J. Exp. Med.* 200, 1213–1219.

Schrettl, M., Bignell, E., Kragl, C., Sabiha, Y., Loss, O., Eisendle, M., Wallner, A., Arst, H. N. Jr., Haynes, K., and Haas, H. (2007). Distinct roles for intra- and extracellular siderophores during *Aspergillus fumigatus* infection. *PLoS Pathog.* 3, 1195–1207. doi: 10.1371/journal.ppat.0030128

Shatwell, K. P., Dancis, A., Cross, A. R., Klausner, R. D., and Segal, A. W. (1996). The FRE1 ferric reductase of *Saccharomyces cerevisiae* is a cytochrome b similar to that of NADPH oxidase. *J. Biol. Chem.* 271, 14240–14244.

Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D., and Dancis, A. (1996). A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science* 271, 1552–1557.

Tangen, K. L., Jung, W. H., Sham, A. P., Lian, T., and Kronstad, J. W. (2007). The iron- and cAMP-regulated gene SIT1 influences ferrioxamine B utilization, melanization and cell wall structure in *Cryptococcus neoformans*. *Microbiology* 153, 29–41.

Timmerman, M. M., and Woods, J. P. (2001). Potential role for extracellular glutathione-dependent ferric

reductase in utilization of environmental and host ferric compounds by *Histoplasma capsulatum*. *Infect. Immun.* 69, 7671–7678.

Van der Helm, D., and Winkelmann, G. (1994). "Hydroxamates and polycarbonates as iron transport agents (siderophores) in fungi," in *Metal Ions in Fungi*, eds G. Winkelmann and D. R. Winge (New York: Marcel Dekker), 39–148.

Van Ho, A., Ward, D. M., and Kaplan, J. (2002). Transition metal transport in yeast. *Annu. Rev. Microbiol.* 56, 237–261.

Waterman, S. R., Hacham, M., Hu, G., Zhu, X., Park, Y. D., Shin, S., Panepinto, J., Valyi-Nagy, T., Beam, C., Husain, S., Singh, N., and Williamson, P. R. (2007). Role of a CUF1/CTR4 copper regulatory axis in the virulence of *Cryptococcus neoformans*. *J. Clin. Invest.* 117, 794–802.

Weinberg, E. D. (2009). Iron availability and infection. *Biochim. Biophys. Acta* 1790, 600–605.

Weissman, Z., Shemer, R., Conibear, E., and Kornitzer, D. (2008). An endocytic mechanism for haemoglobin-iron acquisition in *Candida albicans*. *Mol. Microbiol.* 69, 201–217.

Winters, M. S., Chan, Q., Caruso, J. A., and Deepe, G. S. Jr. (2010). Metallomic analysis of macrophages infected with *Histoplasma capsulatum* reveals a fundamental role for zinc in host defenses. *J. Infect. Dis.* 202, 1136–1145.

Wu, C. Y., Bird, A. J., Chung, L. M., Newton, M. A., Winge, D. R., and Eide, D. J. (2008). Differential control of Zap1-regulated genes in response to zinc deficiency in *Saccharomyces cerevisiae*. *BMC Genomics* 9, 370. doi: 10.1186/1471-2164-9-370

Yuan, D. S., Dancis, A., and Klausner, R. D. (1997). Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. *J. Biol. Chem.* 272, 25787–25793.

Yun, C. W., Ferea, T., Rashford, J., Ardon, O., Brown, P. O., Botstein, D., Kaplan, J., and Philpott, C. C. (2000a). Desferrioxamine-mediated iron uptake in *Saccharomyces cerevisiae*. Evidence for two pathways of iron uptake. *J. Biol. Chem.* 275, 10709–10715.

Yun, C. W., Tiedeman, J. S., Moore, R. E., and Philpott, C. C. (2000b). Siderophore-iron uptake in *Saccharomyces cerevisiae*. Identification of ferrichrome and fusarinine transporters. *J. Biol. Chem.* 275, 16354–16359.

Zarnowski, R., and Woods, J. P. (2005). Glutathione-dependent extracellular ferric reductase activities in dimorphic zoopathogenic fungi. *Microbiology* 151, 2233–2240.

Zhang, Y., Lyver, E. R., Knight, S. A., Pain, D., Lesuisse, E., and Dancis, A. (2006). Mrs3p, Mrs4p, and frataxin provide iron for Fe-S cluster synthesis in mitochondria. *J. Biol. Chem.* 281, 22493–22502.

Zhao, H., and Eide, D. (1996a). The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. *Proc. Natl. Acad. Sci. U.S.A.* 93, 2454–2458.

Zhao, H., and Eide, D. (1996b). The ZRT2 gene encodes the low affinity zinc transporter in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 271, 23203–23210.

Zhao, H., and Eide, D. J. (1997). Zap1p, a metalloregulatory protein involved in zinc-responsive transcriptional regulation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 17, 5044–5052.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 August 2010; accepted: 03 March 2011; published online: 21 March 2011.

Citation: Silva MG, Schrank A, Bailão EFLC, Bailão AM, Borges CL, Staats CC, Parente JA, Pereira M, Salem-Izacc SM, Mendes-Giannini MJS, Oliveira RMZ, Rosa e Silva LK, Nosanchuk JD, Vainstein MH and Soares CMA (2011) The homeostasis of iron, copper, and zinc in Paracoccidioides brasiliensis, Cryptococcus neoformans var. grubii, and Cryptococcus gattii: a comparative analysis. Front. Microbio. ***2****:49. doi: 10.3389/fmicb.2011.00049*

This article was submitted to Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology.

Copyright © 2011 Silva, Schrank, Bailão, Bailão, Borges, Staats, Parente, Pereira, Salem-Izacc, Mendes-Giannini, Oliveira, Rosa e Silva, Nosanchuk, Vainstein and Soares. This is an open-access article subject to an exclusive license agreement between the authors and Frontiers Media SA, which permits unrestricted use, distribution, and reproduction in any medium, provided the original authors and source are credited.

APPENDIX

Table A1 | Conserved domains in proteins involved in iron, copper and zinc uptake by reductive systems in *P. brasiliensis* isolates and *Cryptococcus* species.

Gene product	Predicted function	Organism/accession number†	Conserved domains*	Transmembrane domains*	Signal peptide*
Fre1	Metalloreductase	*P. brasiliensis* 01/PAAG_05370.1	Ferric reductase domain	7	Yes
		P. brasiliensis 03/PABG_06003.1	FAD-binding domain	6	No
			NAD-binding domain		
Fre3	Metalloreductase	*P. brasiliensis* 01/PAAG_02079.1	Ferric reductase domain	6	Yes
		P. brasiliensis 03/PABG_02329.1	FAD-binding domain	6	Yes
		P. brasiliensis 18/PADG_00813.1	NAD-binding domain	6	Yes
Fre5	Metalloreductase	*P. brasiliensis* 03/PABG_07812.1	Ferric reductase domain	6	No
			FAD-binding domain		
			NAD-binding domain		
Fre7	Metalloreductase	*P. brasiliensis* 01/PAAG_06164.1		8	No
		P. brasiliensis 03/PABG_06497.1	Ferric reductase domain	8	No
		P. brasiliensis 18/PADG_07957.1	FAD-binding domain	8	No
		C. neoformans/CNAG_00876.2	NAD-binding domain	7	No
		C. gattii/CNBG_6082.2		8	No
Fre8	Metalloreductase	*C. neoformans*/CNAG_07334.2	Ferric reductase domain	6	No
			FAD-binding domain		
		C. gattii/CNBG_2116.2	NAD-binding domain	6	No
Fre10	Metalloreductase	*C. neoformans*/CNAG_06821.2	Ferric reductase domain	4	No
			FAD-binding domain		
		C. gattii/CNBG_5888.2	NAD-binding domain	4	No
Cfl4	Metalloreductase	*C. neoformans*/CNAG_06524.2	Ferric reductase domain	5	No
			FAD-binding domain		
			NAD-binding domain		
Frp1	Metalloreductase	*P. brasiliensis* 01/PAAG_04493.1	Ferric reductase domain	5	No
		P. brasiliensis 03/PABG_04278.1	FAD-binding domain	6	No
		P. brasiliensis 18/PADG_04652.1	NAD-binding domain	5	No
Fet3	Ferroxidase	*C. neoformans* CNAG_06241.2	Copper-oxidase domain	1	Yes
Fet5	Ferroxidase	*P. brasiliensis* 03/PABG_05667.1	Copper-oxidase domain	–	No
		P. brasiliensis 18/PADG_05994.1		–	No
		C. neoformans/CNAG_07865.2		1	Yes
		C. gattii/CNBG_4942.2		1	Yes
Fet31	Ferroxidase	*P. brasiliensis* 01/PAAG_06004.1	Copper-oxidase domain	1	No
		C. neoformans/CNAG_02958.2		–	Yes
Fet33	Ferroxidase	*P. brasiliensis* 01/PAAG_00163.1	Copper-oxidase domain	–	No
		P. brasiliensis 03/PABG_05183.1		–	Yes
Ftr1/Ftr2	Iron permease	*C. neoformans*/CNAG_06242.2	FTR1 domain	7	Yes
		C. gattii/CNBG_3602.2		6	Yes
Fth1	Iron permease	*C. neoformans*/CNAG_02959.2	FTR1 domain	7	Yes
		C. gattii/CNBG_4943.2		7	Yes

(Continued)

Table A1 | Continued

Gene product	Predicted function	Organism/accession number†	Conserved domains*	Transmembrane domains*	Signal peptide*
Smf1	Low-affinity Permease	*C. neoformans*/CNAG_05640.2	Nramp domain	11	No
		C. gattii/CNBG_6162.2		11	No
Ccc1	Vacuolar transporter	*P. brasiliensis* 01/PAAG_07762.1	DUF125 domain	4	No
		P. brasiliensis 03/PABG_00362.1		4	No
		P. brasiliensis 18/PADG_02775.1		4	No
		C. neoformans/CNAG_05154.2		4	No
		C. gattii/CNBG_4540.2		4	No
Mrs3/Mrs4	Mitochondrial iron transporter	*P. brasiliensis* 01/PAAG_05053.1	Mitochodrial carrier domain	–	No
		P. brasiliensis 03/PABG_04509.1		–	No
		P. brasiliensis 18/PADG_04903.1		–	No
		C. neoformans/CNAG_02522.2		–	No
		C. gattii/CNBG_4218.2		–	No
Yfh1	Mitochondrial matrix iron chaperone	*P. brasiliensis* 01/PAAG_02608.1	Frataxin domain	–	No
		P. brasiliensis 03/PABG_03095.1		–	No
		P. brasiliensis 18/PADG_01626.1		–	No
		C. neoformans/CNAG_05011.2		–	No
		C. gattii/CNBG_4670.2		–	No
Ggt1	Secreted glutathione-dependent ferric reductase	*P. brasiliensis* 01/PAAG_06130.1	Gamma-glutamyltranspeptidase domain	1	Yes
		P. brasiliensis 03/PABG_06527.1		1	Yes
		P. brasiliensis 18/PADG_07986.1		1	Yes
		C. neoformans/CNAG_02888.2		–	No
		C. gattii/CNBG_3537.2		–	No
Mac1	Copper metalloregulatory transcription factor	*P. brasiliensis* 01/PAAG_08210.1	Copper fist domain	–	No
		P. brasiliensis 03/PABG_07429.1		–	No
		C. neoformans/CNAG_07724.2		–	No
		C. gattii/CNBG_2252.2		–	No
Ctr3	High-affinity copper transporter of the plasma membrane	*P. brasiliensis* 01/PAAG_05251.1	Ctr domain	3	No
		P. brasiliensis 03/PABG_07607.1		3	No
		P. brasiliensis 18/PADG_05084.1		3	No
		C. neoformans/CNAG_00979.2		2	No
		C. gattii/CNBG_0560.2		2	No
Ctr2	Putative low-affinity copper transporter of the vacuolar membrane	*P. brasiliensis* 03/PABG_01536.1	Ctr domain	3	No
		P. brasiliensis 18/PADG_04146.1		3	No
		C. neoformans/CNAG_01872.2		3	No
Atx1	Cytosolic copper metallochaperone	*P. brasiliensis* 01/PAAG_00326.1	HMA domain	–	No
		P. brasiliensis 03/PABG_06615.1		–	No
		P. brasiliensis 18/PADG_02352.1		–	No
		C. neoformans/CNAG_02434.2		–	No
		C. gattii/CNBG_4136.2		–	No
Ccc2	Cu^{2+} transporting P-type ATPase	*P. brasiliensis* 01/PAAG_07053.1		7	No
		P. brasiliensis 03/PABG_03057.1	HMA domain	8	No

(Continued)

Table A1 | Continued

Gene product	Predicted function	Organism/accession number†	Conserved domains*	Transmembrane domains*	Signal peptide*
		P. brasiliensis 18/PADG_01582.1	Hydrolase domain	8	No
		C. neoformans/CNAG_06415.2	E1-E2 ATPase domain	8	No
		C. gattii/CNBG_5045.2		8	No
Sod1	Cytosolic superoxide dismutase	*P. brasiliensis* 01/PAAG_04164.1	SOD domain	–	No
		P. brasiliensis 03/PABG_03954.1		–	No
		P. brasiliensis 18/PADG_07418.1		–	No
		C. neoformans/CNAG_01019.2		–	No
		C. gattii/CNBG_0599.2		–	No
Sod2	Mitochondrial superoxide dismutase	*P. brasiliensis* 01/PAAG_02725.1	SOD N-terminal domain	–	No
		P. brasiliensis 03/PABG_03204.1	SOD C-terminal domain	–	No
		P. brasiliensis 18/PADG_01755.1		–	No
		C. neoformans/CNAG_04388.2		–	No
		C. gattii/CNBG_2661.2		–	No
Zrt1	High-affinity zinc transporter of the plasma membrane	*P. brasiliensis* 01/PAAG_08727.1	Zip domain	8	No
		P. brasiliensis 03/PABG_07725.1		8	No
		P. brasiliensis 18/PADG_08567.1		8	No
		C. neoformans/CNAG_03398.2		9	Yes
		C. gattii/CNBG_2209.2		9	Yes
Zrt2	Low-affinity zinc transporter of the plasma membrane	*P. brasiliensis* 01/PAAG_03419.1	Zip domain	8	Yes
		P. brasiliensis 03/PABG_05498.1		7	No
		P. brasiliensis 18/PADG_06417.1		8	Yes
		C. neoformans/CNAG_00895.2		8	Yes
Zrc1	Vacuolar membrane zinc transporter	*P. brasiliensis* 01/PAAG_00702.1	Cation efflux domain	6	Yes
Cot1	Vacuolar membrane zinc transporter	*P. brasiliensis* 01/PAAG_07885.1	Cation efflux domain	5	Yes
		P. brasiliensis 03/PABG_07467.1		4	No
		P. brasiliensis 18/PADG_08196.1		5	Yes
		C. neoformans/CNAG_02806.2		6	Yes
		C. gattii/CNBG_3460.2		4	Yes
Zrt3	Vacuolar membrane zinc transporter	*P. brasiliensis* 01/PAAG_09074.1	Zip domain	6	No
		P. brasiliensis 03/PABG_04697.1		6	No
		P. brasiliensis 18/PADG_05322.1		6	No
Msc2	Cation diffusion facilitator protein of the endoplasmic reticulum and nucleus	*P. brasiliensis* 03/PABG_07115.1	Cation efflux domain	10	No
		P. brasiliensis 18/PADG_06381.1		10	No
		C. neoformans/CNAG_05394.2		11	No
		C. gattii/CNBG_4458.2		10	No
Zap1	Zinc-regulated transcription factor	*P. brasiliensis* 01/PAAG_03645.1	Zinc finger C_2H_2 domain	–	No
		P. brasiliensis 03/PABG_03305.1		–	No
		P. brasiliensis 18/PADG_01870.1		–	No
		C. neoformans/CNAG_05392.2		–	No
		C. gattii/CNBG_4460.2		–	No

**Amino acid sequence analysis was performed using the online software SMART.*

†Accession numbers: PAAG refers to Pb01; PABG refers to Pb03; PADG refers to Pb18; CNAG refers to C. neoformans *var.* grubii *and CNBG refers to* C. gattii.

Table A2 | Conserved domains in proteins related to siderophore biosynthesis and to iron uptake by the non-reductive siderophore transport system in *P. brasiliensis* isolates and *Cryptococcus* species.

Gene product	Predicted function	Organism/accession number†	Conserved domains*	Transmembrane domains*	Signal peptide*
SidA	Ornithine-N^5-monooxygenase	*P. brasiliensis* 01/PAAG_01682.1	Pyr_redox_2 domain	–	No
		P. brasiliensis 03/PABG_03730.1		–	No
		P. brasiliensis 18/PADG_00097.1		–	No
SidF	N^5-transacylases	*P. brasiliensis* 01/PAAG_01680.1	AlcB domain	–	No
		P. brasiliensis 03/PABG_03728.1		–	No
		P. brasiliensis 18/PADG_00100.1		–	No
SidC	Non-ribosomal peptide synthetase	*P. brasiliensis* 01/PAAG_08527.1	Adenylation domain	–	No
		P. brasiliensis 03/PABG_04670.1	Peptidyl carrier domain	–	No
		P. brasiliensis 18/PADG_05295.1	Condensation domain	–	No
SidD	Non-ribosomal peptide synthetase	*P. brasiliensis* 01/PAAG_01679.1	Adenylation domain	–	Yes
		P. brasiliensis 03/PABG_03726.1	Peptidyl carrier domain	–	No
		P. brasiliensis 18/PADG_00102.1		–	No
		C. neoformans/CNAG_03588.2	Condensation domain	–	No
		C. gattii/CNBG_2041.2		–	No
SidG	N^2-transacetylase	*C. neoformans*/CNAG_04355.2	MYND-type zinc finger domains	–	No
		C. gattii/CNBG_2703.2	Acetyltransferase domain	–	No
Sit1/Arn3	Siderophore transporter	*P. brasiliensis* 01/PAAG_06516.1	MFS1 domain	12	No
		P. brasiliensis 03/PABG_02063.1		14	No
		P. brasiliensis 18/PADG_00462.1		14	No
		C. neoformans/CNAG_00815.2		13	No
		C. gattii/CNBG_1123.2		13	No
MirA	Siderophore transporter	*C. neoformans*/CNAG_02083.2	MFS1 domain	12	No
		C. gattii/CNBG_5232.2		11	No
MirB	Siderophore transporter	*P. brasiliensis* 01/PAAG_01685.1	MFS1 domain	14	No
		P. brasiliensis 03/PABG_03732.1		14	No
		P. brasiliensis 18/PADG_00095.1		14	No
		C. neoformans/CNAG_07751.2		14	No
		C. gattii/CNBG_2036.2		14	No
MirC	Siderophore transporter	*P. brasiliensis* 01/PAAG_02233.1	MFS1 domain	8	No
		P. brasiliensis 03/PABG_04747.1		12	No
		P. brasiliensis 18/PADG_05373.1		12	No
		C. neoformans/CNAG_07519.2		10	No
		C. gattii/CNBG_1087.2		14	Yes

**Amino acid sequence analysis was performed using the online software SMART.*

†Accession numbers: PAAG refers to Pb01; PABG refers to Pb03; PADG refers to Pb18; CNAG refers to C. neoformans *var.* grubii *and CNBG refers to* C. gattii.

Hydroxamate production as a high affinity iron acquisition mechanism in *Paracoccidioides* spp.

Mirelle Garcia Silva-Bailão[1,2], Elisa Flávia Luiz Cardoso Bailão[1,3], Beatrix Elisabeth Lechner[4], Gregory M. Gauthier[6], Herbert Lindner[5], Alexandre Melo Bailão[1], Hubertus Haas[4] and Célia Maria de Almeida Soares[1#]

[1]Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Goiás, Brazil

[2]Programa de Pós-Graduação em Patologia Molecular, Universidade de Brasília, Brasília, Brazil

[3]Unidade Universitária de Iporá, Universidade Estadual de Goiás, Iporá, Goiás, Brazil

Divisions of [4]Molecular Biology/Biocenter and [5]Clinical Biochemistry/Biocenter, Innsbruck Medical University, Innrain 80, 6020 Innsbruck, Austria

[6]Department of Medicine, Section of Infectious Diseases, University of Wisconsin, Madison, USA

[#]Corresponding author:
Célia Maria de Almeida Soares
Laboratório de Biologia Molecular
Instituto de Ciências Biológicas II
Campus Samambaia
Universidade Federal de Goiás
74690-900
Goiânia, GO - Brazil
e-mail: cmasoares@gmail.com/ Tel./fax: +55 62 3521 1110

Keywords: iron, siderophores, reversed-phase HPLC, coprogen B, ferricrocin, infection

Abstract

Iron is a micronutrient required by almost all living organisms, including fungi. Although this metal is abundant, its bioavailability is low either in aerobic environments or within mammalian hosts. As a consequence, pathogenic microorganisms evolved high affinity iron acquisition mechanisms which include the production and uptake of siderophores. Here we investigated the utilization of these molecules by species of the *Paracoccidioides* genus, the causative agents of a systemic mycosis. It was demonstrated that iron starvation induces the expression of *Paracoccidioides* orthologs genes for siderophore biosynthesis and transport. Reversed-phase HPLC analysis revealed that the fungus produces and secretes coprogen B, which generates dimerumic acid as a breakdown product. Ferricrocin and ferrichrome C were detected in *Paracoccidioides* as the intracellular produced siderophores. Moreover, the fungus is also able to grow in presence of siderophores as the only iron sources, demonstrating that beyond producing, *Paracoccidioides* is also able to utilize siderophores for growth, including the xenosiderophore ferrioxamine. Exposure to exogenous ferrioxamine increased fungus survival during co-cultivation with macrophages indicating that these molecules play a role during host-pathogen interaction. Furthermore, cross-feeding experiments revealed that *Paracoccidioides* siderophores promotes growth of *Aspergillus nidulans* strain unable to produce these iron chelators. Together, these data denote that synthesis and utilization of siderophores is a mechanism used by *Paracoccidioides* to surpass iron limitation. As iron paucity is found within the host, siderophore production may be related to fungus pathogenicity.

Introduction

The requirement of iron for growth and proliferation is a feature of virtually all organisms, with the exception of a few bacteria. The biological significance of iron lies on its ability to cycle between two oxidation states: the reduced ferrous (Fe^{2+}) and oxidized ferric (Fe^{3+}). The capacity to accept and donate electrons gives iron a redox versatility to function as a cofactor for various cellular enzymes involved in several essential biological processes including respiration, the tricarboxylic acid cycle, synthesis of amino acids, desoxyribonucleotides, lipids and sterols as well as oxidative stress detoxification. Although essential, iron can also be toxic in high concentrations since Fe^{2+} has the potential to generate cell damaging reactive oxygen species (ROS) via

the Fenton/Haber Weiss reaction [1,2]. Thereby, cellular iron homeostasis depends on the precise regulation of iron acquisition, utilization and storage.

Under aerobic conditions, iron is oxidized and Fe^{3+} is essentially insoluble in water at neutral pH [3]. Beyond the environmental low iron availability, pathogenic microorganisms are also confronted by iron scarcity during interaction with the host. In mammalian hosts, the assimilated iron is bound to proteins, such as hemoglobin, transferrin, ferritin and lactoferrin [4]. Following infection, iron concentrations in extracellular fluid and plasma decrease. Macrophages play an important role in the iron withholding. These defense cells limit the release of iron obtained from old erythrocytes and, under the influence of cytokines, they inhibit multiplication of phagocytosed microorganisms by moving iron from the phagosome to cytoplasmic ferritin [5,6]. Since both host and pathogen require iron for metabolism, the control over access to this nutrient can dictate the fate of an infection.

Microorganisms, including fungi, have evolved high affinity uptake strategies for iron acquisition in order to overcome the low bioavailability of this ion in aqueous environments (concentration of free Fe^{3+} approximately 10^{-18} M at pH 7) and within mammalian hosts (concentration of free iron in serum in the order of 10^{-24} M) [7]. One of these strategies consists on the synthesis and secretion of siderophores, defined as low molecular weight organic chelators with high affinity for Fe^{3+}. Such molecules are produced under iron limiting conditions and make insoluble Fe^{3+} available for consumption [8]. The high affinity for iron allows siderophores to compete with host proteins transferrin and lactoferrin. Indeed, the pathogen *Aspergillus fumigatus* overcomes the iron limitation of serum by secreting siderophores which remove iron from serum transferrin [9,10].

With the exception of carboxylates produced by zygomycetes [11], virtually all fungal siderophores are hydroxamates, derived from the non proteinogenic amino acid ornithine. In the proposed biosynthetic pathway for fungal hydroxamates, ornithine-N^5-monooxygenase (SidA) catalyzes N^5-hydroxylation of ornithine. The hydroxamate group is formed next by N^5-acylation of N^5-hydroxyornithine catalyzed by N^5-transacylases. In *A. fumigatus* two transacylases, which add different acyl groups to hydroxyornithine, were identified thus far: SidF, which adds anhydromevalonyl-CoA, and SidL, which catalyzes the addition of acetyl-CoA. In this step, the pathway for distinct siderophores splits for the first time since the choice of the acyl group defines the nature of the molecules. Ferricrocin and ferrichrome, for example, are linked to

acetyl whilst fusarinines and coprogens possess anhydromevalonyl. The latter moiety is derived of mevalonate, from the ergosterol biosynthetic pathway, by the subsequent action of the acyl-CoA ligase SidI and the enoyl-CoA hydratase SidH. The following step is the covalent linkage of hydroxamates via peptide (ferrichromes, coprogens) or ester bonds (fusarinines, coprogens), performed by nonribosomal peptide synthetases (NRPSs). The NRPSs SidD and SidC are involved, respectively, in the synthesis of extra- and intracellular siderophores in *A. fumigatus* [12,13,14]. Given the role of siderophores as iron scavengers, the ability to produce these molecules is advantageous for pathogenic microorganisms and has been considered a virulence attribute for either human or plant fungal pathogens [12,15,16,17,18,19].

The dimorphic fungal pathogens of *Paracoccidioides* genus are the causative agents of paracoccidioidomycosis, a systemic mycosis endemic in Latin America. Once inhaled by the host, fungal propagules are converted into yeasts in the lungs, from where they can disseminate throughout the body. It was already demonstrated that both mycelial and yeasts forms of *Paracoccidioides* have a metabolic requirement for iron [20] and that iron availability increases the susceptibility of mice to fungus infection [21]. A former report described that *Paracoccidioides* infected patients who have restricted pulmonary disease exhibit no alterations in transferrin saturation or in levels of serum iron. On the other hand, low iron concentrations and reduced saturation of transferrin were found in patients with disseminated disease [22,23]. Differential gene expression analysis revealed that genes involved in high-affinity iron uptake were induced by *Paracoccidioides* upon infection of mice and during the incubation with human blood and plasma [24,25]. It was also demonstrated recently that the human plasma protein hemopexin, which tightly binds to heme group, associates with *Paracoccidioides* cell wall [26].

Taken together, these data demonstrate that the fungus faces iron deprivation within the host and have to overcome the scarcity of this micronutrient. Even though the production of iron chelants by *Paracoccidioides* was already reported, the details about this iron acquisition pathway as well as the nature of the produced molecules were unknown. In a previous study, we demonstrated that *Paracoccidioides* genomes encode orthologs for siderophore biosynthesis (*sidA*, *sidF*, *sidC*, *sidD*) as well as siderophore uptake genes (*sit1*, *mirB*, *mirC*) [27]. In the current study we show that iron limiting conditions trigger synthesis and secretion of hydroxamates coprogen B and dimerumic acid by *Paracoccidioides*. The fungus also produces ferricrocin and ferrichrome C as

intracellular siderophores. Additionally, *Paracoccidioides* is able to grow in presence of siderophores as irons sources, including the xenosiderophore ferrioxamine. Siderophore utilization is also important during fungal infection as demonstrated by interaction with macrophages. The findings point to a possible role of siderophores in fungal pathogenicity.

Materials and Methods

Strains and growth conditions

Fungal strains used in this study are listed in **Table 1**. *Paracoccidioides* yeasts cells were maintained in brain heart infusion (BHI) medium supplemented with 4 % glucose at 36 ^{o}C. Except for expression analysis under infectious conditions, all the experiments were performed with strains cultivated in chemically defined medium MMcM [28] after growth to exponential phase in liquid BHI and two washes with phosphate buffered saline solution 1X (PBS 1X; 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl; pH 7.4). For growth on iron sources, *P. lutzii* and *Pb*18 were incubated in MMcM with no iron addition and containing 50 µM of bathophenanthroline-disulfonic acid (BPS) (B-1375 Sigma-Aldrich, St. Louis, MO), a ferrous iron-specific chelator, for 24 h under rotation. Cells were collected by centrifugation and washed twice with PBS 1X. Serial 10-fold dilutions of cellular suspensions were then spotted on MMcM agar plates containing 50 µM BPS supplemented or not with 10 µM of the iron sources: ammonium ferrous sulfate, ammonium ferric citrate, dimerumic acid (DA), ferricrocin (FC) and ferrioxamine (FO). DA and FC were purchased from EMC Microcollections, Tuebingen, Germany. FO was prepared by incubating equal molar amounts of $FeCl_3$ and deferoxamine mesylate (D9533 Sigma-Aldrich, St. Louis, MO) together in 1 M Tris pH 7.4 for 30 minutes at room temperature.

A. nidulans Δ*sidA* strain [29] was grown at 37 ^{o}C in *Aspergillus* minimal medium (AMM), as described [30], containing 1% glucose as carbon source, 20 mM glutamine as the nitrogen source, 10 µM $FeSO_4$, 20 µg l^{-1} biotin and 10 µM triacetylfusarinine C (TAFC).

RNA isolation and quantitative real time PCR (qRT-PCR)

P. lutzii yeast cells were incubated in MMcM supplemented with 50 μM BPS or in MMcM containing 3.5 μM ammonium ferrous sulfate. Cells were collected after 30 min, 1, 3 and 24 h and total RNA was isolated using trizol (TRI Reagent®, Sigma-Aldrich, St. Louis, MO) and mechanical cell rupture (Mini-Beadbeater™ - Biospec Products Inc., Bartlesville, OK). RNAs were reverse-transcribed using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen™, Life Technologies) and cDNAs were submitted to qRT-PCR in the StepOnePlus™ real-time PCR system (Applied Biosystems Inc.). SYBR green PCR master mix (Applied Biosystems, Foster City, CA) was used in the reaction mixture and the PCR thermal cycling was 40 cycles of 95 ^{o}C for 15 s and 60 ^{o}C for 1 min. The sequences of forward and reverse oligonucleotides used are listed in **Table S1**. One primer in each pair spanned an intron, preventing amplification from genomic DNA. The qRT-PCR reaction was performed in triplicate for each cDNA sample and a melting curve analysis was accomplished to confirm a single PCR product. The data were normalized with the transcript for α-tubulin (GenBank accession number XM_002796593) amplified in each set of qRT-PCR experiments. A non-template control was included. A relative standard curve was generated by pooling an aliquot from each cDNA sample which was serially diluted 1:5 to 1:125. Relative expression levels of transcripts of interest were calculated using the standard curve method for relative quantification [31]. Student's *t*-test was applied in the statistical analyses and *P* values of 0.05 or less were considered statistically significant.

In silico analysis of putative *Paracoccidioides sidH* and *sidI* orthologs

The amino acid sequences of putative *Paracoccidioides sidH* and *sidI* orthologs were obtained at the Dimorphic Fungal Database of the Broad Institute site at (http://www.broadinstitute.org/annotation/genome/dimorph_collab//MultiHome.html) based on homology search. The sequences have been submitted to GenBank with the following accession numbers SidH: *P. lutzii* (XP_002791730), *Pb*18 (EEH45393) and *Pb*03 (EEH20631); SidI: *P. lutzii* (XP_002796673), *Pb*18 (EEH43810) and *Pb*03 (EEH21513). The amino acid sequences of *Paracoccidioides* and *A. fumigatus* (SidH XP_748661; SidI XP_753087) orthologs were aligned using CLUSTALX2 [32]. The peroxisomal targeting signal-PTS1 scores of proteins were obtained using the PTS1-

predictor program http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp [33]. Positive scores indicate high probability of peroxisomal targeting. PTS2 motifs were identified using the PTS2 finder http://www.peroxisomedb.org/diy_PTS2.html.

Upstream sequences analysis

Upstream regions of siderophore biosynthesis and *mirB* genes were inspected for the presence of conserved sequences related to iron regulated transcription of siderophore genes. For all genes (*sidD*, *sidF*, *sidA*, *sidI* and *mirB*) the upstream region comprehends the entire intergenic region from the 5´open reading frame.

Chrome azurol S (CAS) assays

Siderophore production by *P. lutzii* and *P. brasiliensis* was qualitatively analyzed with an overlay-CAS (O-CAS) as described [34]. *P. lutzii* and *P. brasiliensis Pb*18, *Pb*02 and *Pb*Epm83 yeasts were grown for 13 days at 36 ^{o}C on MMcM agar plates, without iron addition. For iron sufficiency (control), ammonium ferrous sulfate was used in a final concentration of 30 µM. CAS medium was prepared according to [35] with minor modifications. Briefly, 100 ml of O-CAS was prepared with 6.05 mg CAS dissolved in 5 ml water and mixed with 83.2 µl of ferric chloride solution (30 mM $FeCl_3 \cdot 6\ H_2O$ in HCl 10 mM). Under stirring this solution was slowly added to 7.29 mg hexadecyltrimetyl ammonium bromide (HDTMA) dissolved in 4 ml water. The resultant dark blue liquid was autoclaved at 121^{o}C for 15 min. A mixture of 3.024 g piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) dissolved in 75 ml water (pH 6.8) was also autoclaved with agarose (0.9%, w/v) as the gelling agent. After cooling to 50 ^{o}C, both PIPES and dye solutions were mixed with enough care to avoid foaming. After that, 15 ml of O-CAS were applied over the plates in order to detect secreted siderophores. The ternary complex chrome azurol S/Fe^{3+}/HDTMA serves as an indicator. When a strong chelator, as siderophores, removes the iron from the dye its color turns from blue to orange.

The percentage of siderophores in *P. lutzii* and *Pb*18 supernatants was determined as described [36]. Yeast cells were cultured at 36 ^{o}C in MMcM liquid medium with no iron addition and MMcM containing 30 µM ammonium ferrous sulfate. Supernatants were collected after 6, 10 and 15 days of incubation. After sterile filtration with 0.22 µM pore filter, 500 µl of supernatants as well as a reference prepared with non-

inoculated MMcM were added to 500 µl of CAS liquid medium also prepared according to Schwyn and Neilands [35]. Briefly, 6 ml of 10 mM HDTMA solution was placed in 100 ml volumetric flask. A mixture of 1.5 ml of ferric chloride solution (3 mM $FeCl_3 \cdot 6 H_2O$ in HCl 10 mM) and 2 mM aqueous CAS solution was slowly added to the HDTMA flask under stirring. An aqueous solution containing 4.307 g PIPES (pH 5.6) was added to the volumetric flask which was then filled with water to afford 100 ml of CAS assay solution. The mixture of CAS-supernatants (s) and CAS-reference (r) was incubated at room temperature and absorbance at 630 nm was measured after 1 h (Ultraspec 2000 UV/Visible Spectrophotometer, Pharmacia Biotech). The percentage of siderophores were calculated by subtracting the sample absorbance values from the reference according to the formula $[(A_r - A_s/A_r)] \times 100$.

Glassware was acid treated to remove residual traces of iron [37]. All the reagents used for CAS medium preparation were purchased from Sigma-Aldrich, St. Louis, MO.

Isolation and identification of siderophores

For isolation and characterization of *P. lutzii* and *Pb*18 secreted siderophores, yeasts cells were cultivated for 4, 10 and 18 days in MMcM medium with no iron addition. Culture supernatants were filtered (0.22 µM) and lyophilized. Samples were then dissolved and concentrated to one tenth of the original volume with MilliQ-water. 250 µl of 100 mM $FeSO_4$ was added to the samples in order to convert desferri-siderophores in ferri-ones. An aliquot of 2.5 ml was applied to an Amberlite XAD-16 column (Rohm and Haas, Philadelphia, PA, USA). Siderophore-iron complexes were eluted with 2 ml of methanol and collected. Methanol was discarded by speed vacuum centrifugation overnight. The dried pellet was solubilized in 100 µl of water and 10 µl were applied to reversed phase HPLC (RP-HPLC). Samples were separated using a Nucleosil 100-5 C_{18} column (250mm x 4mm I.D.; 5 µm particle pore size; Macherey-Nagel, Düren, Germany). Chromatography was performed within 40 min at a constant flow of 0.5 ml min^{-1} with a two-step acetonitrile gradient starting at solvent A - solvent B (94:6) (solvent A: water containing 0.1% TFA; solvent B: 85% acetonitrile and 0.1% TFA). The concentration of solvent B was increased linearly from 6% to 15% during 10 min, from 15% to 60% during 25 min and held at 60% for 5 min. Fractions obtained in this way were collected, lyophilized and stored at -20°C.

Determination of the molecular mass of the samples obtained by RP-HPLC was carried out using an LTQ Velos ion trap mass spectrometer (Thermo Fisher Scientific)

equipped with an electrospray source (ESI-MS, Electrospray Ionization Mass Spectrometry). Samples were dissolved in 50% aqueous methanol containing 0.1% formic acid, and infused directly into the ion source using the syringe pump. The electrospray voltage was set at 4.0 kV and the heated capillary was held at 270°C.

For analysis of cellular siderophores, equal number of *P. lutzii* and *Pb*18 yeasts cells were cultivated for 8 days in MMcM medium with no iron addition. Cells were harvested by centrifugation and washed five times with PBS 1X in order to get rid of extracellular siderophores. Subsequently, cellular extracts were prepared by grinding yeast cells into a fine powder using a mortar and pestle under liquid nitrogen. The powder was resuspended in water (1 ml sterile water/4 ml culture) and the suspension was centrifuged. Cellular debris were discarded, the supernatants were filtered (0.22 μM) and lyophilized. Samples were dissolved and concentrated to one tenth of the original volume with MilliQ-water and analyzed as described for the extracellular siderophores.

Macrophage infection experiments

Murine macrophage cell line J774 A.1 (BCRJ Cell Bank, Rio de Janeiro, accession number 0121) maintained in RPMI medium (RPMI 1640, Vitrocell, Brazil) supplemented with non-essential amino acids (M7145; Sigma-Aldrich, St. Louis, MO), 10% (v/v) fetal bovine serum (FBS), at 37 ^{o}C in 5% CO_2, were used in the assays. $1x10^6$ macrophages were seeded into each well of a 24-well tissue culture plate and 100 U ml^{-1} of murine gamma interferon (IFN-γ; PeproTech, Rocky Hill, New Jersey, USA) was added for 24 h at 37 °C in 5% CO_2 for macrophage activation as described [38].

Paracoccidioides yeast cells exposed to siderophores were co-cultivated with activated macrophages and the number of viable fungal cells after phagocytosis was assessed by colony forming unit (CFU) counts. Briefly, *P. lutzii* and *Pb*18 were incubated in MMcM with no iron addition and containing 50 μM of BPS for 24 h under rotation. Equal number of fungal cells was next exposed to 10 μM of each ammonium ferrous sulfate, DA and FO for 3 h. $2x10^6$ *Paracoccidioides* viable yeasts cells were then added to the wells containing $1x10^6$ macrophages (yeast-to-macrophage ratio 2:1). The cells were co-cultivated for 24 h at 37 °C in 5% CO_2 to allow fungal internalization. Each well was washed twice with 1 ml PBS 1X in order to get rid of non-internalized yeasts. Infected macrophages were lysed with water and dilutions of the lysates containing the phagocytized yeasts were plated on BHI medium supplemented with 4%

(v/v) sheep blood and 4% glucose. After incubation at 36 ^{o}C for 9 days, the number of CFU was determined to check the ability of yeast cells exposed to siderophores to survive in macrophages. CFU were expressed as the mean value ± the standard error from triplicates. Student's *t*-test was applied in the statistical analyses and *P* values of 0.05 or less were considered statistically significant.

For gene expression analysis, *P. lutzii* was grown in BHI and, after three washes with PBS1X, $2x10^6$ viable yeasts cells were incubated with $1x10^6$ activated macrophages in presence of 50 µM BPS (added to the RPMI immediately before addition of yeast cells). Cells were co-cultivated for 24 h at 37 °C in 5% CO_2 to allow fungal internalization. Each well was washed twice with 1 ml PBS 1X in order to get rid of non-internalized yeasts. Trizol was added to each well and total RNA of internalized yeasts was isolated. RNAs from uninfected macrophages and from *P. lutzii* yeast cells cultured in RPMI 1640 medium, also in presence of BPS, were obtained as control. After reverse transcription, cDNAs were submitted to qRT-PCR, as described above.

Cross-feeding experiments

P. lutzii and *Pb*18 yeasts cells were incubated in MMcM with no iron addition and containing 50 µM BPS. After 24 h, $1x10^7$ yeasts cells were spotted on MMcM supplemented with 200 µM BPS and incubated at 36 ^{o}C for 7 days. Next, $1x10^7$ *A. nidulans* Δ*sidA* spores were point-inoculated 2 cm distant from the borders of *Paracoccidioides* colonies and plates were incubated for 48 h. As control, *A. nidulans* Δ*sidA* spores were also spotted on MMcM 200 µM BPS plates in the absence of *Paracoccidioides* yeasts.

Results

Transcriptional analysis of siderophore orthologs genes

Analysis performed by Parente et al. [21] showed an induction of *Paracoccidioides* genes putatively related to siderophore biosyntheis (*sidA*) and uptake (*sit1*) under iron limiting conditions, indicating that *Paracoccidioides* may produce and capture siderophores to overcome iron starvation. To verify that the other orthologs genes related to siderophore biosynthesis and uptake are also transcriptionally regulated by iron availability, quantitative RT-PCRs (qRT-PCRs) were carried out. All analyzed transcripts, *sidF*, *sidH*, *sidI*, *sidC*, *sidD*, *mirB* and *mirC*, were induced under iron

limiting conditions (50 μM BPS), especially after 24 h of incubation, as depicted in **Figure 1**. Transcripts of orthologs genes, which encode the acyl-CoA ligase SidI and the enoyl-CoA hydratase SidH were also induced. Both enzymes link ergosterol and siderophore biosynthesis in *A. fumigatus* [13] and are found in most fungi that produce siderophores. BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches retrieved *P. lutzii*, *Pb*18 and *Pb*03 sequences presenting, respectively, 68, 65 and 65 % identity with *A. fumigatus* SidI (**Figure S1**). The SidH orthologs from *A. fumigatus* and *P. lutzii*, *Pb*18, *Pb*03 share, respectively 52, 47 and 48% identity at the amino acid sequence level (**Figure S2**). As in other *Ascomycetes* [39], SidI and SidH from *Paracoccidioides* also carry putative PTS motifs. PTS2 was found in all *Paracoccidioides* SidI whereas PTS1 is present in *P. lutzii* and *Pb*18 SidH (**Figure S1 and S2**). The latter indicates that siderophore biosynthesis is partially localized in peroxisomes as previously shown in *A. nidulans* and *A. fumigatus* [39]. Taken together, the presence of orthologs involved in siderophore biosynthesis and uptake and their induction during iron-deficient conditions indicates that *Paracoccidioides* is a siderophore producer.

Genomic organization and identification of putative regulatory sites of *Paracoccidioides* siderophore genes

Genes involved in the siderophore biosynthesis pathway tend to be genomically clustered [39] and a similar pattern of organization was found in *Paracoccidioides*. Four out of the six iron regulated biosynthetic genes (including *sidA*) are located next to each other in a region of approximately 22 kb of *P. lutzii* genome (**Figure 2A**), which interestingly also includes the putative siderophore transporter-encoding gene *mirB*. The gene cluster organization of these iron regulated genes was also found in *Pb*18 and *Pb*03 genomes.

Since all genes in the siderophore biosynthetic cluster were similarly iron-regulated at the transcriptional level, the 5′ upstream regions of these genes were examined in order to identify conserved sequences. As depicted in **Figure 2B**, the upstream regions of all iron regulated genes presented at least one HGATAR motif, the consensus recognition sequence of fungal GATA factors [40]. An extended version of the HGATAR motif, 5′-(G/A)ATC(T/A)GATAA-3′, formerly identified in the 5′regions of the siderophore biosynthetic cluster in *Histoplasma capsulatum* [15] and *A. fumigatus* [41], was also found in the upstream regions of the siderophore gene cluster in *Paracoccidioides*, excepting *sidD*.

Detection of secreted siderophores in *Paracoccidioides* cultures

Since orthologs genes for siderophore production were induced under iron limiting conditions, *Paracoccidioides* cultures were examined for the presence of these molecules by using the CAS detection medium [35]. *P. lutzii* and *P. brasiliensis Pb*18, *Pb*02 and *Pb*Epm83 yeasts were incubated at 36 °C on MMcM agar plates with no iron addition or with 30 μM ammonium ferrous sulfate. After 13 days, an overlay-CAS (O-CAS) was added to the plates and a change in color from blue to orange was observed after a few hours in the iron depleted plates (**Figure 3A**), which indicates the secretion of hydroxamate siderophores [34].

The percentage of these molecules was then determined in *P. lutzii* and *Pb*18 supernatants, by mixing it with CAS, after 6, 10 and 15 days of incubation in MMcM. As shown in **Figure 3B**, siderophores were produced in higher amounts in 10 and 15 days of growth under iron limiting conditions. Changes in the blue color of CAS solutions were also observed. An increase in biomass was perceived during incubation in both solid and liquid iron poor media (data not show), demonstrating that *Paracoccidioides* possesses a functional high affinity iron uptake mechanism.

Identification and characterization of *Paracoccidioides* siderophores

In order to identify the siderophores detected by the CAS solution, *P. lutzii* and *Pb*18 yeasts were grown under iron limiting conditions and supernatants were analyzed by RP-HPLC and mass spectrometry. Following addition of $FeSO_4$, supernatants turned orange colored (**Figure 4A**), an indicative of siderophore-iron chelates [42]. Reversed-phased HPLC showed that compounds exhibiting absorption at 435 nm were secreted by both *P. lutzii* and *Pb*18 under iron depleted conditions. **Figure 4B** shows HPLC analysis of *P. lutzii* culture supernatants after 4 days of cultivation. High-resolution mass spectrometry of RP-HPLC peaks displaying absorption at 435 nm, which is typically for iron-saturated siderophores, yielded the molecular masses m/z $(M-2H+Fe)^+$=538.1724 matching $C_{22}H_{36}N_4O_8Fe$ (dimerumic acid, calculated molecular mass 538.1717) and $(M-2H+Fe)^+$=780.2992 matching $C_{33}H_{54}N_6O_{12}Fe$ (coprogen B, calculated molecular mass 780.2979). Notably, dimerumic acid is most likely a breakdown product of coprogen B. In agreement, tandem mass spectrometry (MS/MS) of coprogen B generated dimerumic acid (**Figure S3A**). Further analysis of *P. lutzii* and *Pb*18 supernatants from 10 days of growth under iron limiting conditions reinforced that dimerumic acid is a breakdown product of coprogen B, since the amount of coprogen B

401 decreased while dimerumic acid increased compared to 4 days supernatants (**Figure**
402 **S3B**). Also, after 18 days, only dimerumic acid was found (data not shown).
403 Some ascomycetes are known to produce intracellular siderophores for iron
404 storage [43,44]. To further investigate the production of these molecules in the
405 *Paracoccidioides* genus, cell extracts of *P. lutzii* and *Pb*18 were prepared as described
406 in *Materials and Methods* and submitted to reversed-phased HPLC. RP-HPLC peaks
407 displaying absorption at 435 nm were seen in both *P. lutzii* and *Pb*18 cell extracts.
408 **Figure 4C** depicts HPLC analysis of *Pb*18 exemplary. High-resolution mass
409 spectrometry of RP-HPLC peaks yielded the molecular masses m/z (M-
410 2H+Fe)+=771.2497 matching $C_{28}H_{47}N_9O_{13}Fe$ (ferricrocin, calculated molecular mass
411 771.2475) and (M-2H+Fe)+=755.2549 matching $C_{28}H_{47}N_9O_{12}Fe$ (ferrichrome C,
412 calculated molecular mass 755.2526), suggesting that the main intracellular
413 siderophores of *P. lutzii* and *Pb*18 are ferricrocin and ferrichrome C (**Figure S4**).
414
415 **Growth of *Paracoccidioides* in presence of siderophores as iron sources**
416 The presence of putative siderophore transporters in the *Paracoccidioides*
417 genomes and its induction under iron limiting conditions indicates that the fungus
418 besides producing is able to utilize siderophores as well. To test this hypothesis, serial
419 dilutions of *P. lutzii* and *Pb*18 yeasts cells were spotted on MMcM agar plates
420 containing BPS and supplemented or not (iron limiting conditions) with hydroxamate
421 siderophores as well as organic and inorganic compounds as iron sources. Although
422 minor differences were seen in the growth profile, both strains grew better in presence
423 of siderophores than in other iron sources, as show in **Figure 5**. This suggests that
424 *Paracoccidioides* may be able to uptake siderophore-iron complexes from the
425 extracellular environment and, subsequently, utilize the iron for metabolism and growth.
426 Residual growth in presence of BPS is not surprising, since this ferrous iron-specific
427 chelator does not affect siderophore-iron utilization.
428
429 **Infection of murine macrophages cell line with *Paracoccidioides* yeasts cells**
430 **exposed to siderophores**
431 Lung resident macrophages are the first defense cells which interact with
432 *Paracoccidioides* following host invasion. It was demonstrated that fungal survival
433 within human monocytes is iron dependent since the inhibitory effect of the iron
434 chelator deferoxamine is reversed by holotransferrin [45]. IFN-γ and other cytokines

435 modulate cellular iron metabolism to strengthen host iron withholding defenses,
436 culminating in reduced iron availability to pathogens inside macrophages [46]. As iron
437 is critical for *Paracoccidioides* yeasts survival in monocytes, the susceptibility of yeast
438 cells exposed to siderophores to killing by IFN-γ activated macrophages was evaluated.
439 Following growth on iron limiting conditions (50 μM BPS), *P. lutzii* and *Pb*18 yeasts
440 cells were incubated in the presence of DA, FO and ammonium ferrous sulfate prior to
441 co-cultivation with macrophages (**Figure 6A**). As shown by CFU counting, *P. lutzii* and
442 *Pb*18 yeasts cells survival in infected macrophages increased following exposure to DA
443 and FO (**Figure 6B**). The increased ability to survive to macrophage killing is probably
444 a result of siderophore-iron utilization.
445
446 **Transcriptional analysis of putative siderophore biosynthesis *sidA* gene during**
447 **infection of murine macrophages cell line**
448 Taking into account the putative role of *Paracoccidioides sidA* gene in
449 siderophore production and its induction under iron limiting conditions *in vitro* [21], the
450 expression of this gene was examined in *P. lutzii* yeasts cells after co-cultivation with
451 murine macrophages. Following growth to exponential phase, viable yeasts were co-
452 cultivated with IFN-γ activated macrophages and, after 24 h, RNAs from phagocytosed
453 yeasts were obtained. As shown **Figure 7**, the abundance of *sidA* transcripts increased
454 2.3 times during co-cultivation with macrophages when compared to the non-infectious
455 condition. This indicates that *sidA* may play a relevant role during fungus-macrophage
456 interaction.
457
458 **Cross-feeding between *Paracoccidioides* and *A. nidulans* Δ*sidA* mutant**
459 Siderophore utilization is not restricted to the producing microorganisms. Several
460 bacteria and fungi can take up and utilize iron bound to siderophores produced by other
461 microbial species (xenosiderophores). *In vitro* growth and CFU recovery from co-
462 cultivation with murine macrophages suggest that *Paracoccidioides* may utilize the
463 xenosiderophore ferrioxamine and DA as an iron source. To check if *Paraccoccidioides*
464 siderophores could be utilized as iron sources by other fungal species, growth of *A.*
465 *nidulans* Δ*sidA* strain, which is unable to produce these molecules, was tested. Spores of
466 *A. nidulans* Δ*sidA* mutant were point-inoculated in vicinity to 7 days-old colonies of *P.*
467 *lutzii* and *Pb*18 on plates (MMcM + 200 μM BPS). As shown in **Figure 8A**, in 24 h
468 *Aspergillus* hyphae started growing toward *Paracoccidioides* colonies and the radial

growth was sustained for the next 24 h. This suggests that siderophores secreted by *Paracoccidioides* promoted the growth Δ*sidA* mutant, since no growth was observed in absence of *Paracoccidioides* (**Figure 8B**).

Discussion

As for other organisms, iron is also essential for growth of *Paracoccidioides* [20]. Since this fungus is the causative agent of a systemic mycosis, it faces host iron-withholding and must be able to overcome this condition in order to establish the infection. The knowledge of the strategies used by pathogenic microorganisms to acquire iron is extremely important to understand the host-pathogen interaction and, consequently, for the treatment of the disease. Nevertheless, information about the mechanisms developed by *Paracoccidioides* for iron uptake is still scarce.

In an effort to start deciphering the molecular mechanisms employed by this fungus for iron acquisition, we firstly screened its genomic sequence for genes putatively involved in iron homeostasis. Based on homology analysis and precedent from other fungi, orthologs of genes related to high affinity iron uptake systems, including those for siderophore biosynthesis and uptake, were found [27]. Here we show that these genes putatively involved in siderophore production and utilization were transcriptionally induced under iron limiting conditions, which is in agreement with their possible role in a high affinity uptake system.

Genes encoding proteins, which are involved in a common metabolic pathway, tend to be clustered in the genome. Indeed, most *Paracoccidioides* siderophore biosynthetic genes were co-localized in a genome region that also included the transport gene *mirB*. We have previously shown that MirB amino acid sequence present a major facilitator superfamily (MFS) domain (MFS1) [27]. Interestingly, a MFS transporter (*MFS2*) was also found within an iron regulated siderophore biosynthetic gene cluster in the closely related pathogen *H. capsulatum* [15]. Taking into account the organization in cluster and the co-regulation of these genes in response to iron levels, their upstream regions were inspected for the presence of conserved sequences. The HGATAR motif and the RATCWGATAA consensus sequence were found. In many pathogenic and non-pathogenic fungi the GATA sequences localized in the promoter regions of siderophore metabolism genes are recognized by GATA-type transcription factors that promote repression of siderophore synthesis under iron sufficiency [41,47,48,49]. We

hypothesize that *Paracoccidioides* siderophore genes could also be target of regulation by GATA factors.

Taken together, the presence and putative regulatory elements of siderophore biosynthetic genes strongly suggested that *Paracoccidioides* could be a siderophore producer. The presence of these molecules was firstly detected in iron deprived *Paracoccidioides* agar plates with an overlay of CAS solution, which indicated the presence of hydroxamates, siderophores typically produced by fungi [11]. Even when iron was omitted from the culture medium, the production and secretion of desferri-hydroxamates, which extracellularly chelated traces of iron, and the subsequent uptake of the iron-siderophore complex allowed fungal growth. Siderophore production by *Paracoccidioides* was more affected by the iron addition in solid than in liquid medium. Iron at 30 μM completely abrogated siderophore production in agar plates, but the same concentration did not impaired siderophore secretion in culture supernatants. Growth rate on liquid medium is higher than in solid. As the utilization of iron increases according to the incubation time, due the consumption by the growing cells, the availability of this nutrient decreases and induces the production and secretion of siderophores, even if iron was added initially. The same differences in siderophore production were observed in *Aspergillus* species [36].

Fungal hydroxamate siderophores can be classified into four structural families: coprogens, ferrichromes, fusarinines and rhodotorulic acid. Reversed-phase HPLC and mass spectrometry analysis allowed the confirmation of hydroxamate production by *Paracoccidioides*. Coprogen B and dimerumic acid were identified as extracellular siderophores while ferrichrome C and ferricrocin as the intracellular ones. Coprogen-type siderophores contains anhydromevalonyl residues linked to the hydroxylated ornithine by the action of the transacylase SidF. This acyl group derives from mevalonate by CoA ligation and dehydration catalyzed, respectively, by SidI and SidH [13]. Accordingly, the presence of orthologs to *sidI*, *sidH* and *sidF* in *Paracoccidioides* genome is not surprising. Coprogen B consists of a fusarinine molecule linked to the dihydroxamate dimerumic acid. In *Paracoccidioides* young cultures the amount of coprogen B in the supernatants is higher than the amount of dimerumic acid while in older cultures this proportion is reversed. This strongly indicates that the dimerumic acid found in *Paracoccidioides* supernatants is a byproduct of coprogen B. Similarly, dimerumic acid was identified in *H. capsulatum* cultures as a breakdown product of coprogen B [50,51]. Both siderophores were also recognized in supernatants of

Blastomyces dermatitidis grown under iron-poor conditions [52]. Ferrichrome C and ferricrocin are cyclic hexapeptides in which the acyl group bound to the hydroxyornithine is an acetyl [53]. Ferricrocin is produced intracellularly by *A. fumigatus* and *A. nidulans* for hyphal iron storage and distribution [12,44,54]. The basidiomycete *Ustilago maydis* produces ferrichrome and ferrichrome A for iron acquisition [55], while ferrichrome C is produced by the dermatophyte *Trichophyton rubrum*, which also synthesizes ferricrocin [56]. Although both ferrichrome C and ferrricrocin were identified in *Paracoccidioides* cellular extracts, their role in iron storage requires further investigation. Interestingly, the extra- and intracellular siderophores discussed above are not only produced by human pathogens. The plant pathogen *Magnaporthe grisea* and the non-pathogenic model organism *Neurospora crassa* secrete siderophores of the coprogen-type for iron acquisition and use ferricrocin for intracellular iron storage [57,58]. This demonstrates the broad utilization of siderophores as a strategy for iron acquisition in fungi.

A previous study demonstrated that *Paracoccidioides* plating efficiency is enhanced in presence of coprogen B, dimerumic acid (both isolated from *B. dermatitidis*) and ferrichrome, the latter being the most effective growth factor [59]. Here we showed that ferricrocin and ferrioxamine can also be used by *Paracoccidioides* as iron sources. In fungi, the uptake of siderophore-iron complex is usually mediated by transporters belonging to the UMF/SIT subfamily of the major facilitator superfamily (MFS). The three genes encoding putative siderophore transporter orthologs found at *Paracoccidioides* genome present a MFS1 domain indicating that they belong to the MFS [27]. The presence of more than one putative siderophore transporter in *Paracoccidioides* may reflect its ability to utilize a variety of siderophore as iron sources, including the xenosiderophore ferrioxamine.

Cross-feeding experiments demonstrated that siderophores secreted by *Paracoccidioides* restored the growth of the non-producer *A. nidulans* Δ*sidA* mutant. This strain is unable to grow in standard growth media unless siderophores are supplied [29]. *A. nidulans* encodes 10 putative siderophore transporters [60,61]. This fact, associated with the lack of a reductive iron assimilation system, is in accordance with the ability of this fungus to utilize xenosiderophores. Beyond the native siderophores ferricrocin and TAFC, *A. nidulans* is also able to utilize iron from enterobactin, a catecholate-type siderophore produced by bacteria, and from ferrioxamine B, a less

569 effective iron source [62]. Thereby, utilization of *Paracoccidioides* siderophores by the
570 *A. nidulans* Δ*sidA* mutant is in agreement.

571 Since siderophore production and uptake has been described as an important
572 virulence attribute for pathogens [10,15], we started checking the influence of these
573 molecules during fungus interaction with murine macrophages. Following inhalation by
574 the host, *Paracoccidioides* propagules bind to macrophages in the lung. Once
575 phagocytosed, fungal cells are able to survive and multiply in non-activated
576 macrophages. However, IFN-γ activated macrophages prevent multiplication of
577 ingested fungus and, consequently, its survival [63]. Here we demonstrated that
578 exposure of *Paracoccidioides* yeasts cells to ferrioxamine before co-cultivation with
579 IFN-γ activated macrophages, resulted in an increase in survival when compared to
580 yeasts exposed to $FeSO_4$ only. We hypothesize that the siderophore utilization before
581 infection provided the iron requirements for fungal metabolism and for defense against
582 oxidative stress generated by macrophages. The demand for iron for *Paracoccidioides*
583 survival during interaction with phagocytes was already investigated. Results
584 demonstrated that iron is essential for intracellular transformation of ingested conidia to
585 yeast in murine macrophages [64] and for survival of yeast cells inside human
586 monocytes [45]. In agreement with the growth assay in presence of siderophores, we
587 demonstrated that these molecules play a more effective role as iron sources for
588 *Paracoccidioides* when compared to organic or inorganic compounds. It was formerly
589 demonstrated that addition of $FeCl_3$ to *Paracoccidioides* minimal medium is not as
590 effective in the increase of fungus plating efficiency, as supplementation with
591 siderophores [59]. The exposure to ferrichrome also enhanced the survival of the
592 opportunistic fungal pathogen *Candida glabrata* to macrophage killing [65].

593 Quantification of transcripts level of the putative siderophore biosynthetic gene
594 *sidA* revealed that this gene was induced during co-cultivation of *Paracoccidioides* with
595 macrophages. This suggests that the fungus probably produce siderophores to overcome
596 low iron availability imposed by these activated phagocytic cells. Such strategy is
597 employed by other fugal pathogens. Extra- and intracellular siderophores were shown to
598 be crucial for intracellular growth of *A. fumigatus* within alveolar murine macrophages
599 [66] and expression of siderophore biosynthetic genes was detected during murine
600 infection with conidia [67]. In *H. capsulatum* the expression of the *sidA* ortholog *sid1*
601 was also induced after phagocytosis and required for adequate cellular growth in human
602 macrophages [68].

Altogether, our results revealed the ability of *Paracoccidioides* to synthesize and utilize siderophore as iron sources. Although the production of these iron chelators had been formerly reported, we demonstrated here, for the first time, the identity of the produced siderophores including the intracellular ones, whose production was not mentioned before. Additionally, infection experiments carried out with a murine macrophage cell line revealed that siderophore utilization plays an important role during the interaction of *Paracoccidioides* with mammalian cells. Despite some studies had demonstrated the importance of iron in the scenario of *Paracoccidioides* infection, evaluation of the impact of iron metabolism on fungus pathogenicity was not deeply investigated. This study was the first step of upcoming molecular and functional analysis of siderophore biosynthetic and uptake genes in *Paracoccidioides*. Indeed, studies are being carried out in order to investigate the role of these genes as possible virulence factors in this pathogenic fungus. The added knowledge is clinically important since siderophore biosynthesis and uptake represent possible targets for an antifungal chemotherapy due the absence of these pathways in human cells.

Acknowledgments

Work at Universidade Federal de Goiás was supported by grants from Financiadora de Estudos e Projetos (FINEP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG), Coordenação de Pessoal do Ensino Superior (CAPES) and Pronex. Work at Innsbruck Medical University was supported by the Austrian Science Foundation (FWF P21643-B11 to HH). MGSB was supported by a fellowship from CAPES.

626 **References**

627

628 1. Haber F, Weiss J (1934) The catalytic decomposition of hydrogen peroxide by iron
629 salts. Proc R Soc Lond 147: 332-351.

630 2. Halliwell B, Gutteridge JM (1984) Oxygen toxicity, oxygen radicals, transition
631 metals and disease. Biochem J 219: 1-14.

632 3. Kosman DJ (2003) Molecular mechanisms of iron uptake in fungi. Mol Microbiol 47:
633 1185-1197.

634 4. Ganz T (2009) Iron in innate immunity: starve the invaders. Curr Opin Immunol 21:
635 63-67.

636 5. Cassat JE, Skaar EP (2013) Iron in infection and immunity. Cell Host Microbe 13:
637 509-519.

638 6. Recalcati S, Locati M, Cairo G (2012) Systemic and cellular consequences of
639 macrophage control of iron metabolism. Semin Immunol 24: 393-398.

640 7. Raymond KN, Dertz EA, Kim SS (2003) Enterobactin: an archetype for microbial
641 iron transport. Proc Natl Acad Sci U S A 100: 3584-3588.

642 8. Neilands JB (1993) Siderophores. Arch Biochem Biophys 302: 1-3.

643 9. Hissen AH, Chow JM, Pinto LJ, Moore MM (2004) Survival of *Aspergillus*
644 *fumigatus* in serum involves removal of iron from transferrin: the role of
645 siderophores. Infect Immun 72: 1402-1408.

646 10. Schrettl M, Bignell E, Kragl C, Joechl C, Rogers T, et al. (2004) Siderophore
647 biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus*
648 virulence. J Exp Med 200: 1213-1219.

649 11. Van der Helm D, Winkelmann G (1994) Hydroxamates and polycarbonates as iron
650 transport agents (siderophores) in fungi. In: Winkelmann G, Winge DR, editors.
651 Metal ions in fungi. New York. pp. 39-148.

652 12. Schrettl M, Bignell E, Kragl C, Sabiha Y, Loss O, et al. (2007) Distinct roles for
653 intra- and extracellular siderophores during *Aspergillus fumigatus* infection. PLoS
654 Pathog 3: 1195-1207.

655 13. Yasmin S, Alcazar-Fuoli L, Grundlinger M, Puempel T, Cairns T, et al. (2012)
656 Mevalonate governs interdependency of ergosterol and siderophore biosyntheses in
657 the fungal pathogen *Aspergillus fumigatus*. Proc Natl Acad Sci U S A 109: E497-
658 504.

659 14. Blatzer M, Schrettl M, Sarg B, Lindner HH, Pfaller K, et al. (2011) SidL, an
660 *Aspergillus fumigatus* transacetylase involved in biosynthesis of the siderophores
661 ferricrocin and hydroxyferricrocin. Appl Environ Microbiol 77: 4959-4966.

662 15. Hwang LH, Mayfield JA, Rine J, Sil A (2008) *Histoplasma* requires *SID1*, a
663 member of an iron-regulated siderophore gene cluster, for host colonization. PLoS
664 Pathog 4: e1000044.

665 16. Greenshields DL, Liu G, Feng J, Selvaraj G, Wei Y (2007) The siderophore
666 biosynthetic gene SID1, but not the ferroxidase gene FET3, is required for full
667 *Fusarium graminearum* virulence. Mol Plant Pathol 8: 411-421.

668 17. Hof C, Eisfeld K, Welzel K, Antelo L, Foster AJ, et al. (2007) Ferricrocin synthesis
669 in *Magnaporthe grisea* and its role in pathogenicity in rice. Mol Plant Pathol 8: 163-
670 172.

671 18. Hissen AH, Wan AN, Warwas ML, Pinto LJ, Moore MM (2005) The *Aspergillus*
672 *fumigatus* siderophore biosynthetic gene *sidA*, encoding L-ornithine N5-oxygenase,
673 is required for virulence. Infect Immun 73: 5493-5503.

674 19. Oide S, Moeder W, Krasnoff S, Gibson D, Haas H, et al. (2006) NPS6, encoding a
675 nonribosomal peptide synthetase involved in siderophore-mediated iron metabolism,
676 is a conserved virulence determinant of plant pathogenic ascomycetes. Plant Cell 18:
677 2836-2853.

678 20. Arango R, Restrepo A (1988) Growth and production of iron chelants by
679 *Paracoccidioides brasiliensis* mycelial and yeast forms. J Med Vet Mycol 26: 113-
680 118.

681 21. Parente AF, Bailao AM, Borges CL, Parente JA, Magalhaes AD, et al. (2011)
682 Proteomic analysis reveals that iron availability alters the metabolic status of the
683 pathogenic fungus *Paracoccidioides brasiliensis*. PLoS One 6: e22810.

684 22. Martinez R, Fiorillo AM (1978) Iron metabolism in patients with South American
685 blastomycosis (paracoccidioidomycosis). Rev Inst Med Trop Sao Paulo 20: 195-
686 201.

687 23. Martinez R, Fiorillo AM (1978) Effect of treatment of South American
688 blastomycosis (paracoccidioidomycosis) on parameters of iron metabolism. Rev Inst
689 Med Trop Sao Paulo 20: 190-194.

690 24. Bailão AM, Schrank A, Borges CL, Dutra V, Molinari-Madlum EEWI, et al. (2006)
691 Differential gene expression by *Paracoccidioides brasiliensis* in host interaction
692 conditions: representational difference analysis identifies candidate genes associated
693 with fungal pathogenesis. Microbes Infect 8: 2686-2697.

694 25. Bailão AM, Shrank A, Borges CL, Parente JA, Dutra V, et al. (2007) The
695 transcriptional profile of *Paracoccidioides brasiliensis* yeast cells is influenced by
696 human plasma. FEMS Immunol Med Microbiol 51: 43-57.

697 26. Longo LV, Nakayasu ES, Matsuo AL, Peres da Silva R, Sobreira TJ, et al. (2013)
698 Identification of human plasma proteins associated with the cell wall of the
699 pathogenic fungus *Paracoccidioides brasiliensis*. FEMS Microbiol Lett 341: 87-95.

700 27. Silva MG, Schrank A, Bailao EF, Bailao AM, Borges CL, et al. (2011) The
701 homeostasis of iron, copper, and zinc in *Paracoccidioides brasiliensis*,
702 *Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii*: a comparative
703 analysis. Front Microbiol 2: 49.

704 28. Restrepo A, Jimenez BE (1980) Growth of *Paracoccidioides brasiliensis* yeast
705 phase in a chemically defined culture medium. J Clin Microbiol 12: 279-281.

706 29. Eisendle M, Oberegger H, Zadra I, Haas H (2003) The siderophore system is
707 essential for viability of *Aspergillus nidulans*: functional analysis of two genes
708 encoding l-ornithine N 5-monooxygenase (*sidA*) and a non-ribosomal peptide
709 synthetase (*sidC*). Mol Microbiol 49: 359-375.

710 30. Pontecorvo G, Roper JA, Hemmons LM, Macdonald KD, Bufton AW (1953) The
711 genetics of *Aspergillus nidulans*. Adv Genet 5: 141-238.

712 31. Bookout AL, Cummins CL, Mangelsdorf DJ, Pesola JM, Kramer MF (2006) High-
713 throughput real-time quantitative reverse transcription PCR. Curr Protoc Mol Biol
714 Chapter 15: Unit 15 18.

715 32. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, et al. (2007)
716 Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948.

717 33. Neuberger G, Maurer-Stroh S, Eisenhaber B, Hartig A, Eisenhaber F (2003)
718 Prediction of peroxisomal targeting signal 1 containing proteins from amino acid
719 sequence. J Mol Biol 328: 581-592.

720 34. Perez-Miranda S, Cabirol N, George-Tellez R, Zamudio-Rivera LS, Fernandez FJ
721 (2007) O-CAS, a fast and universal method for siderophore detection. J Microbiol
722 Methods 70: 127-131.

723 35. Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and
724 determination of siderophores. Anal Biochem 160: 47-56.

725 36. Machuca A, Milagres AM (2003) Use of CAS-agar plate modified to study the
726 effect of different variables on the siderophore production by *Aspergillus*. Lett Appl
727 Microbiol 36: 177-181.

728 37. Cox CD (1994) Deferration of laboratory media and assays for ferric and ferrous
729 ions. Methods Enzymol 235: 315-329.

730 38. Youseff BH, Holbrook ED, Smolnycki KA, Rappleye CA (2012) Extracellular
731 superoxide dismutase protects *Histoplasma* yeast cells from host-derived oxidative
732 stress. PLoS Pathog 8: e1002713.

733 39. Grundlinger M, Yasmin S, Lechner BE, Geley S, Schrettl M, et al. (2013) Fungal
734 siderophore biosynthesis is partially localized in peroxisomes. Mol Microbiol 88:
735 862-875.

736 40. Scazzocchio C (2000) The fungal GATA factors. Curr Opin Microbiol 3: 126-131.

737 41. Schrettl M, Kim HS, Eisendle M, Kragl C, Nierman WC, et al. (2008) SreA-
738 mediated iron regulation in *Aspergillus fumigatus*. Mol Microbiol 70: 27-43.

739 42. Gsaller F, Blatzer M, Abt B, Schrettl M, Lindner H, et al. (2013) The first promoter
740 for conditional gene expression in *Acremonium chrysogenum*: iron starvation-
741 inducible *mir1*^{p}. J Biotechnol 163: 77-80.

742 43. Charlang G, Ng B, Horowitz NH, Horowitz RM (1981) Cellular and extracellular
743 siderophores of *Aspergillus nidulans* and *Penicillium chrysogenum*. Mol Cell Biol 1:
744 94-100.

745 44. Wallner A, Blatzer M, Schrettl M, Sarg B, Lindner H, et al. (2009) Ferricrocin, a
746 siderophore involved in intra- and transcellular iron distribution in *Aspergillus*
747 *fumigatus*. Appl Environ Microbiol 75: 4194-4196.

748 45. Dias-Melicio LA, Calvi SA, Peracoli MT, Soares AM (2005) Inhibitory effect of
749 deferoxamine on *Paracoccidioides brasiliensis* survival in human monocytes:
750 reversal by holotransferrin not by apotransferrin. Rev Inst Med Trop Sao Paulo 47:
751 263-266.

752 46. Weiss G (2005) Modification of iron regulation by the inflammatory response. Best
753 Pract Res Clin Haematol 18: 183-201.

754 47. Haas H, Zadra I, Stoffler G, Angermayr K (1999) The *Aspergillus nidulans* GATA
755 factor SREA is involved in regulation of siderophore biosynthesis and control of
756 iron uptake. J Biol Chem 274: 4613-4619.

757 48. Chao LY, Marletta MA, Rine J (2008) Sre1, an iron-modulated GATA DNA-
758 binding protein of iron-uptake genes in the fungal pathogen *Histoplasma*
759 *capsulatum*. Biochemistry 47: 7274-7283.

760 49. Harrison KA, Marzluf GA (2002) Characterization of DNA binding and the cysteine
761 rich region of SRE, a GATA factor in *Neurospora crassa* involved in siderophore
762 synthesis. Biochemistry 41: 15288-15295.

763 50. Burt WR (1982) Identification of coprogen B and its breakdown products from
764 *Histoplasma capsulatum*. Infect Immun 35: 990-996.

765 51. Howard DH, Rafie R, Tiwari A, Faull KF (2000) Hydroxamate siderophores of
766 *Histoplasma capsulatum*. Infect Immun 68: 2338-2343.

767 52. Gauthier GM, Sullivan TD, Gallardo SS, Brandhorst TT, Vanden Wymelenberg AJ,
768 et al. (2010) SREB, a GATA transcription factor that directs disparate fates in
769 *Blastomyces dermatitidis* including morphogenesis and siderophore biosynthesis.
770 PLoS Pathog 6: e1000846.

771 53. Renshaw JC, Robson GD, Trinci APJ, Wiebe MG, Livens FR, et al. (2002) Fungal
772 siderophores: structures, functions and applications. Mycol Res 106: 1123-1142.

773 54. Eisendle M, Schrettl M, Kragl C, Muller D, Illmer P, et al. (2006) The intracellular
774 siderophore ferricrocin is involved in iron storage, oxidative-stress resistance,
775 germination, and sexual development in *Aspergillus nidulans*. Eukaryot Cell 5:
776 1596-1603.

777 55. Winterberg B, Uhlmann S, Linne U, Lessing F, Marahiel MA, et al. (2010)
778 Elucidation of the complete ferrichrome A biosynthetic pathway in *Ustilago maydis*.
779 Mol Microbiol 75: 1260-1271.

780 56. Mor H, Kashman Y, Winkelmann G, Barash I (1992) Characterization of
781 siderophores produced by different species of the dermatophytic fungi *Microsporum*
782 and *Trichophyton*. Biometals 5: 213-216.

783 57. Antelo L, Hof C, Welzel K, Eisfeld K, Sterner O, et al. (2006) Siderophores
784 produced by *Magnaporthe grisea* in the presence and absence of iron. Z Naturforsch
785 C 61: 461-464.

786 58. Matzanke BF, Bill E, Trautwein AX, Winkelmann G (1987) Role of siderophores in
787 iron storage in spores of *Neurospora crassa* and *Aspergillus ochraceus*. J Bacteriol
788 169: 5873-5876.

789 59. Castaneda E, Brummer E, Perlman AM, McEwen JG, Stevens DA (1988) A culture
790 medium for *Paracoccidioides brasiliensis* with high plating efficiency, and the
791 effect of siderophores. J Med Vet Mycol 26: 351-358.

792 60. Haas H, Eisendle M, Turgeon BG (2008) Siderophores in fungal physiology and
793 virulence. Annu Rev Phytopathol 46: 149-187.

794 61. Haas H, Schoeser M, Lesuisse E, Ernst JF, Parson W, et al. (2003) Characterization
795 of the *Aspergillus nidulans* transporters for the siderophores enterobactin and
796 triacetylfusarinine C. Biochem J 371: 505-513.

797 62. Oberegger H, Schoeser M, Zadra I, Abt B, Haas H (2001) SREA is involved in
798 regulation of siderophore biosynthesis, utilization and uptake in *Aspergillus*
799 *nidulans*. Mol Microbiol 41: 1077-1089.

800 63. Brummer E, Hanson LH, Restrepo A, Stevens DA (1989) Intracellular
801 multiplication of *Paracoccidioides brasiliensis* in macrophages: killing and
802 restriction of multiplication by activated macrophages. Infect Immun 57: 2289-2294.

803 64. Cano LE, Gomez B, Brummer E, Restrepo A, Stevens DA (1994) Inhibitory effect
804 of deferoxamine or macrophage activation on transformation of *Paracoccidioides*
805 *brasiliensis* conidia ingested by macrophages: reversal by holotransferrin. Infect
806 Immun 62: 1494-1496.

807 65. Nevitt T, Thiele DJ (2011) Host iron withholding demands siderophore utilization
808 for *Candida glabrata* to survive macrophage killing. PLoS Pathog 7: e1001322.

809 66. Schrettl M, Ibrahim-Granet O, Droin S, Huerre M, Latge JP, et al. (2010) The
810 crucial role of the *Aspergillus fumigatus* siderophore system in interaction with
811 alveolar macrophages. Microbes Infect 12: 1035-1041.

812 67. McDonagh A, Fedorova ND, Crabtree J, Yu Y, Kim S, et al. (2008) Sub-telomere
813 directed gene expression during initiation of invasive aspergillosis. PLoS Pathog 4:
814 e1000154.

815 68. Hilty J, George Smulian A, Newman SL (2011) *Histoplasma capsulatum* utilizes
816 siderophores for intracellular iron acquisition in macrophages. Med Mycol 49: 633-
817 642.

818 69. Teixeira MD, Theodoro RC, Oliveira FF, Machado GC, Hahn RC, et al. (2013)
819 *Paracoccidioides lutzii* sp. nov.: biological and clinical implications. Med Mycol.

820 70. Matute DR, McEwen JG, Puccia R, Montes BA, San-Blas G, et al. (2006) Cryptic
821 speciation and recombination in the fungus *Paracoccidioides brasiliensis* as
822 revealed by gene genealogies. Mol Biol Evol 23: 65-73.

823 71. Theodoro RC, Bagagli E, Oliveira C (2008) Phylogenetic analysis of PRP8 intein in
824 *Paracoccidioides brasiliensis* species complex. Fungal Genet Biol 45: 1284-1291.

825

826

Figures

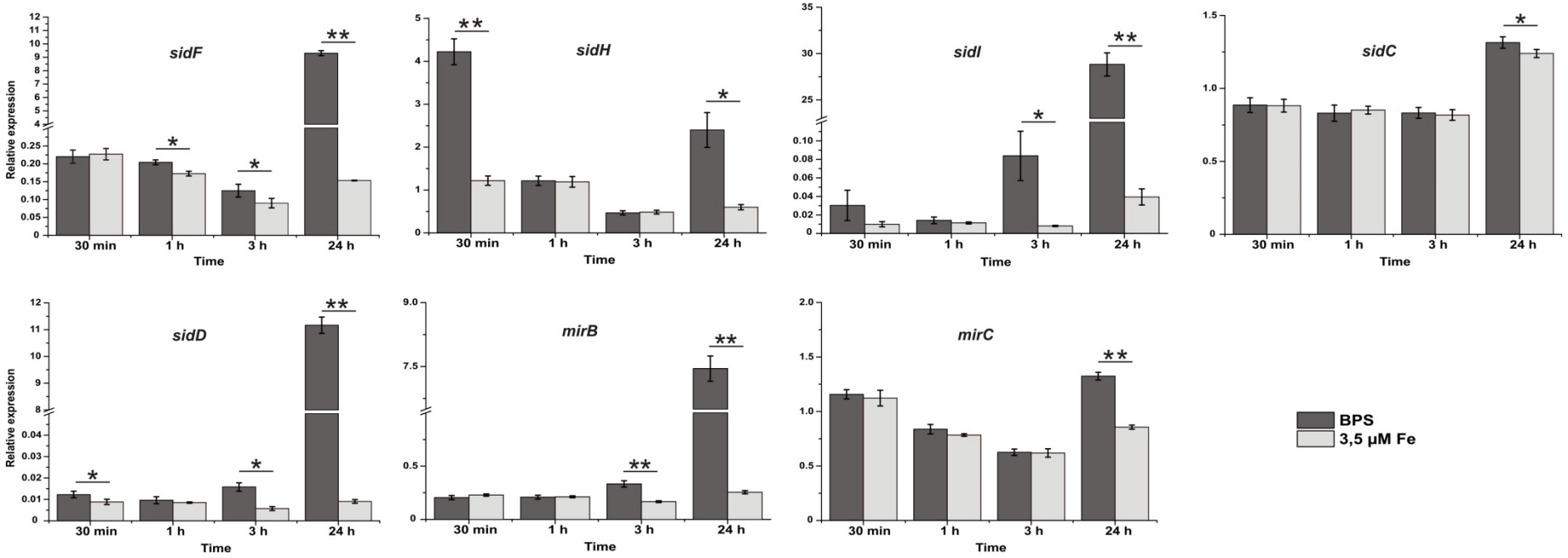


Figure 1. Low iron availability induces expression of putative siderophore biosynthesis and uptake genes. Quantitative RT-PCR was performed with transcripts of *P. lutzii* yeast cells under iron-limited conditions (50 µM BPS) and incubated with 3.5 µM ammonium ferrous sulfate. Expression values of siderophores biosynthesis (*sidF*, *sidI*, *sidH*, *mirC*, *sidC*) and transport genes (*mirB*, *mirC*) were calculated using α-tubulin as endogenous control. Data are expressed as mean ± standard deviation from triplicates. Statistically significant difference was determined by Student's *t*-test (*p < 0.05/ ** p < 0.001).

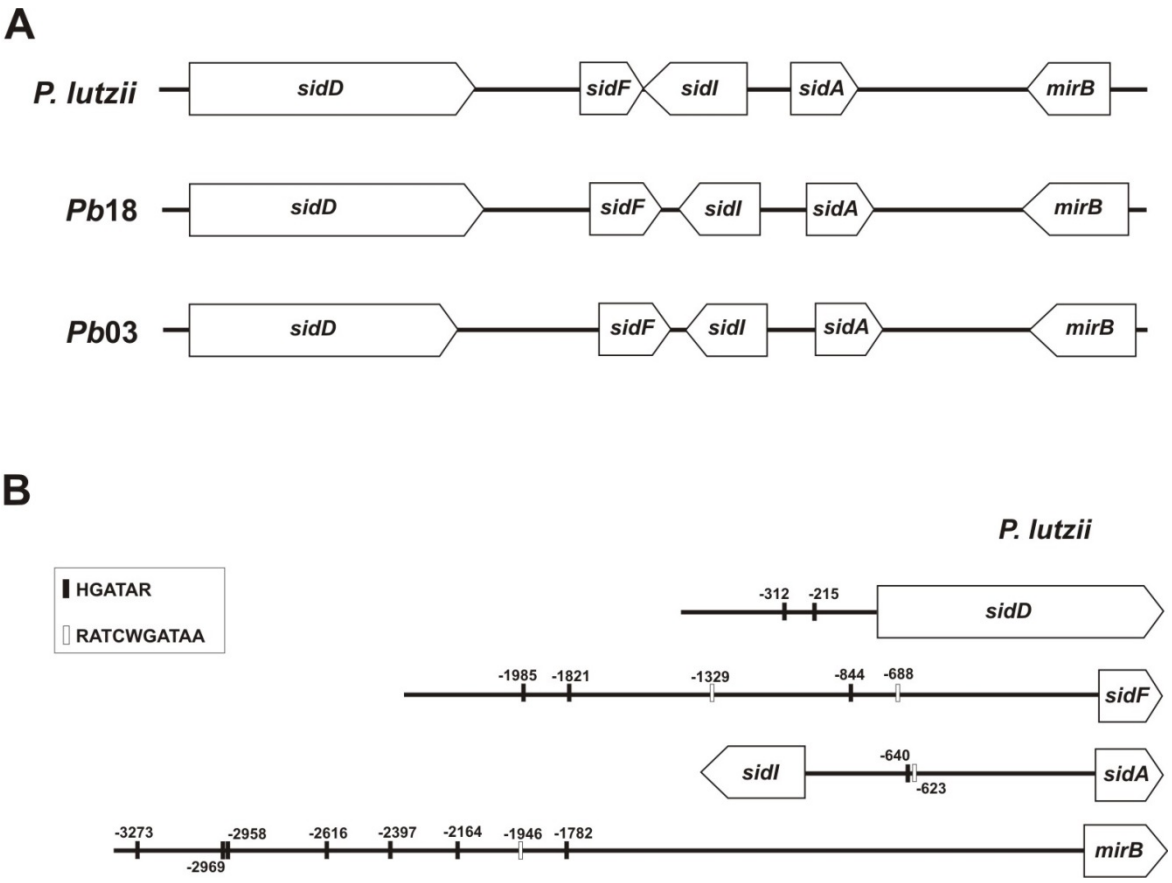


Figure 2. Genomic organization and regulatory sites in upstream regions of siderophore genes. A. Representation of siderophore biosynthesis and uptake genes localization in *P. lutzii*, *Pb*18 and *Pb*013 genomes. **B.** Schematic figure showing the position and sequence of putative regulatory sites in upstream regions of siderophore genes in *P. lutzii*. A black line represents nucleotides (nt) located 5´ to the open reading frame of each gene: *sidD* 679 nt, *sidF* 2409 nt, *mirB* 3376 nt. The intergenic region between *sidI* and *sidA* includes 1005 nt. Numbers represent the number of nucleotides upstream the start codon of each gene where the regulatory sequence was found (vertical line). In case of divergent genes, the nucleotide position is relative to *sidA*. H: A/T/C; R: A/G; W: A/T. Accession numbers are available in **Table S2**.

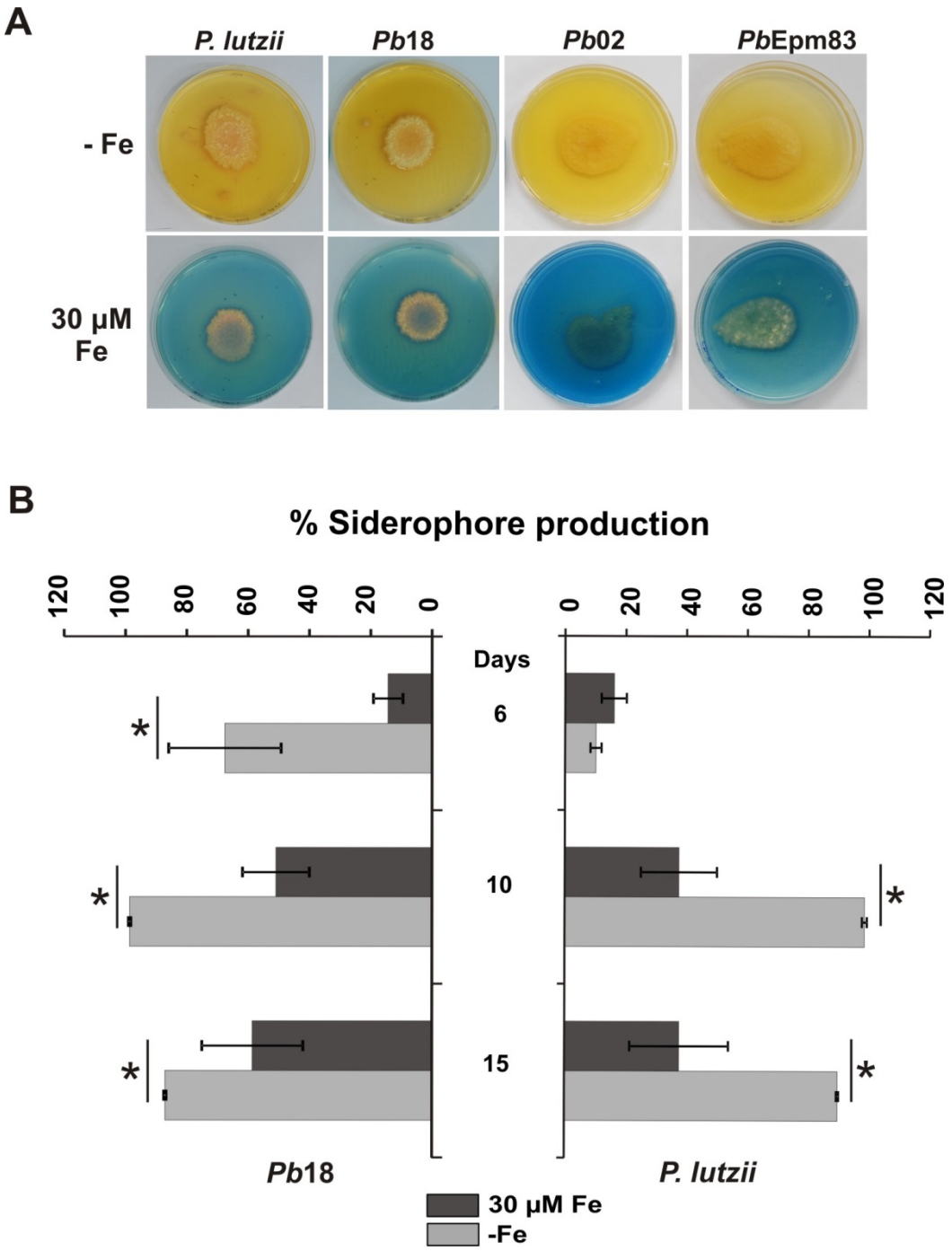


Figure 3. Iron limiting conditions induce biosynthesis and secretion of siderophores. A. Detection of hydroxamate siderophores in MMcM agar plates. Under iron sufficiency (30 µM Fe), the production of these chelators was repressed in solid medium. **B.** Percentage of siderophores was determined in *P. lutzii* and *Pb*18 supernatants. Values are expressed as mean ± standard deviation of three experiments. *statistically significant difference determined by Student's *t*-test ($p < 0.05$).

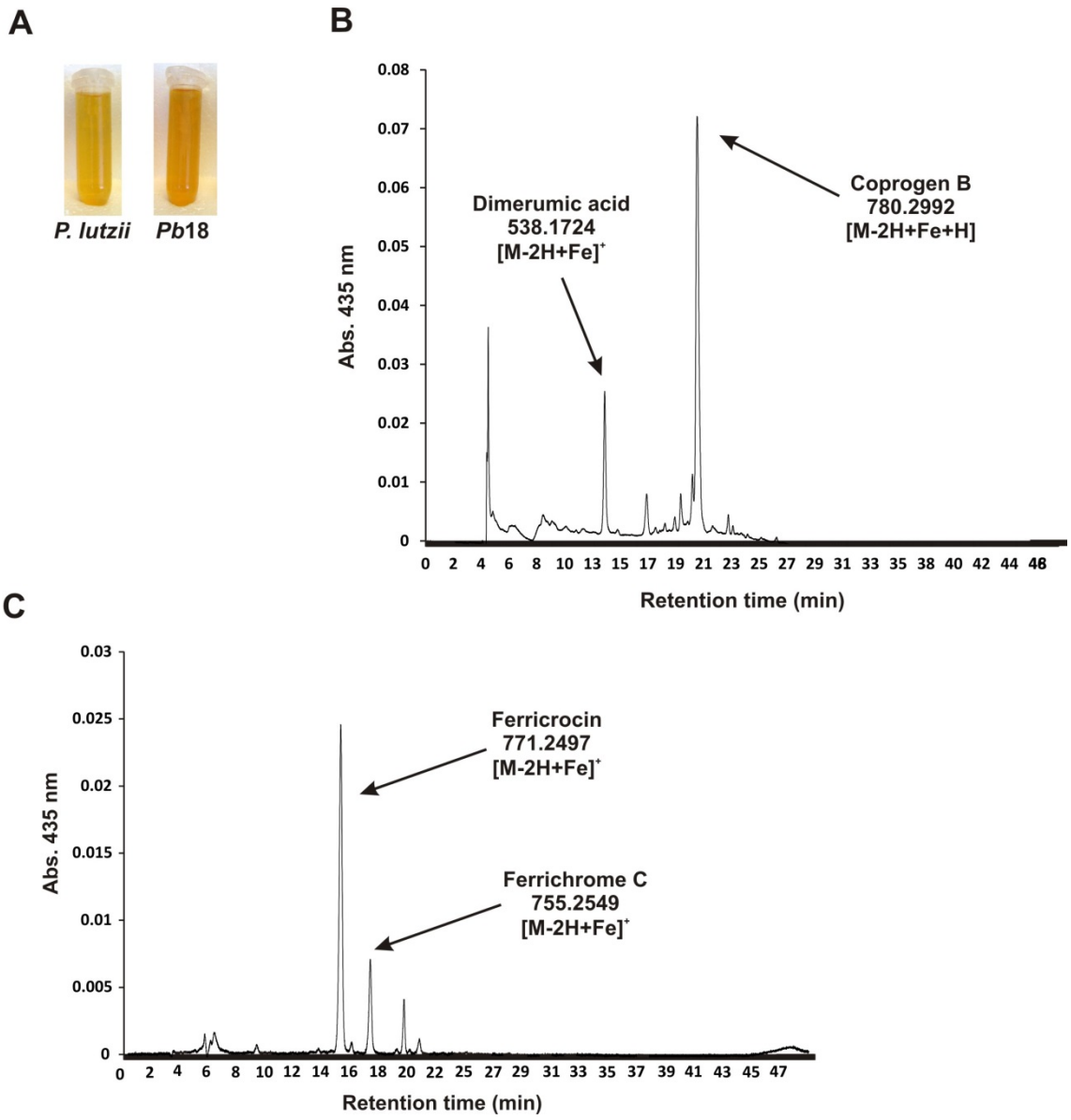


Figure 4. Identification of extra- and intracellular siderophores produced by *Paracoccidioides*. A. Culture supernatants after saturation with iron. **B.** Reversed-phase HPLC of *P. lutzii* culture supernatants after 4 days of incubation under iron limiting conditions. Coprogen B and dimerumic acid were identified as extracellular siderophores. **C.** RP-HPLC of *Pb*18 cell extracts obtained after cultivation for 8 days in iron depleted medium. The intracellular siderophores identified were ferricrocin and ferrichrome C. Molecular masses of iron bound compounds are indicated.

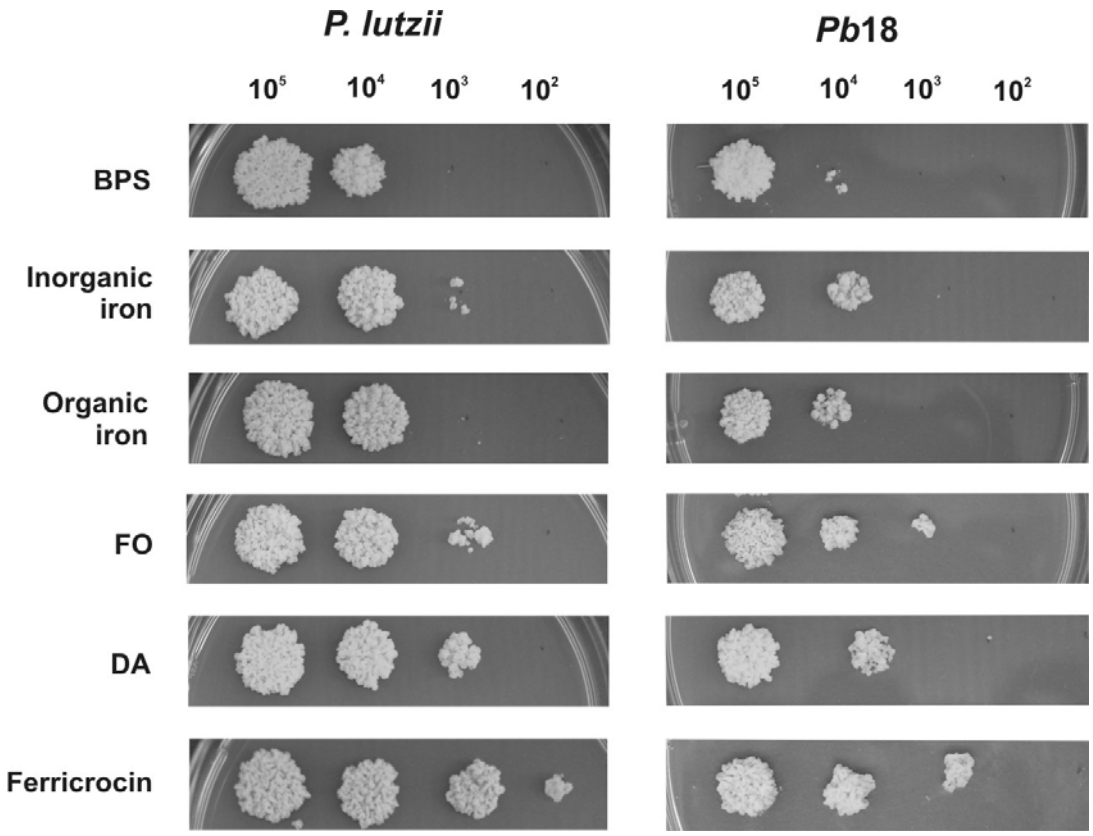


Figure 5. Effect of siderophores on *Paracoccidioides* growth. After growth on MMcM + BPS for 24 h, *P. lutzii* and *Pb*18 yeast cells were washed and serially diluted. 10^5 to 10^2 cells were spotted on MMcM agar plates containing 50 μM BPS. Iron sources were added, or not, in the final concentration of 10 μM. Inorganic iron: ammonium ferrous sulfate; organic iron: ammonium ferric citrate; DA: dimerumic acid; FO: ferrioxamine.

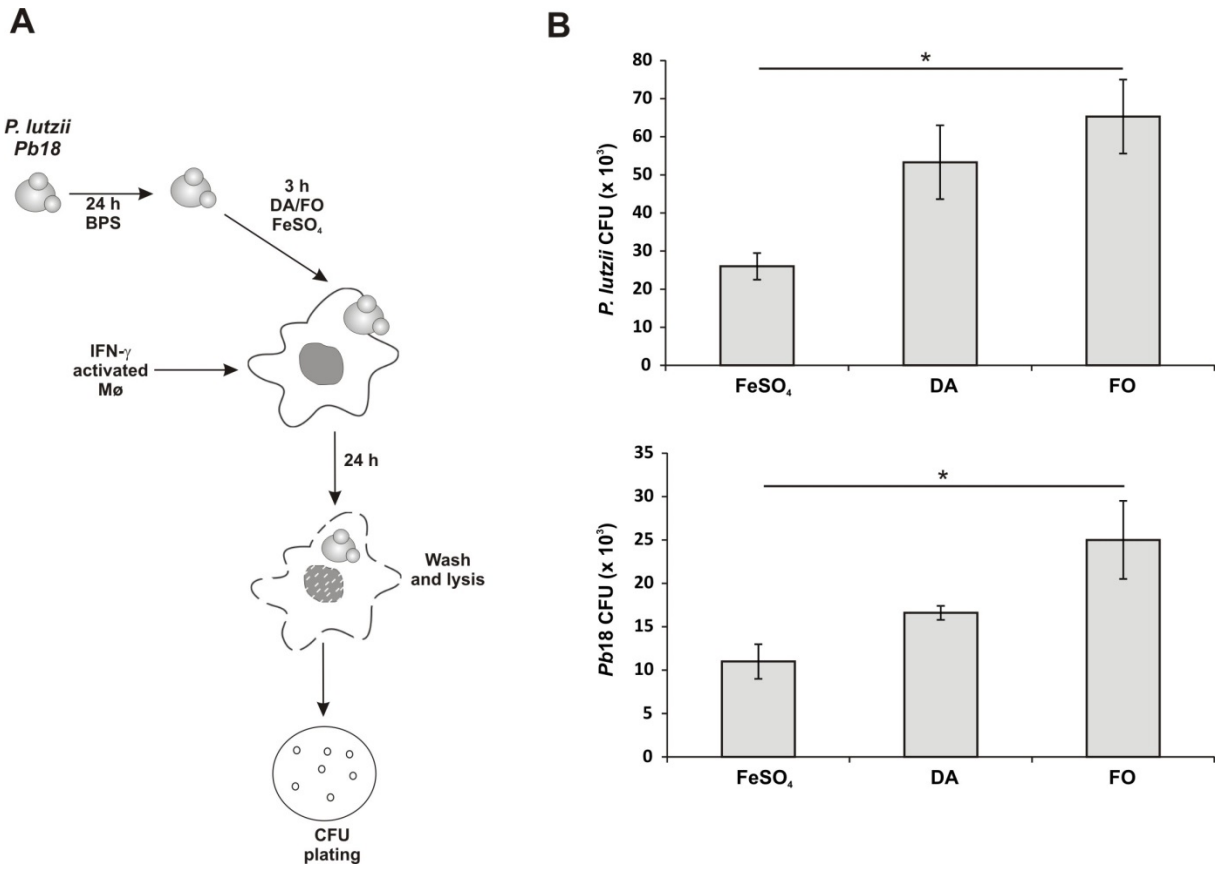


Figure 6. Exposure to siderophores increase *Paracoccidioides* ability to survive macrophage killing. A. Schematic flowchart of murine macrophage infection by *Paracoccidioides* yeasts. **B**. Prior exposure to 10 μM FO and DA enhanced *P. lutzii* and *Pb*18 survival to macrophage killing. CFU counts are expressed as mean ± standard error from triplicates, representative of two independent experiments. *statistically significant difference determined by Student's *t*-test ($p < 0.05$).

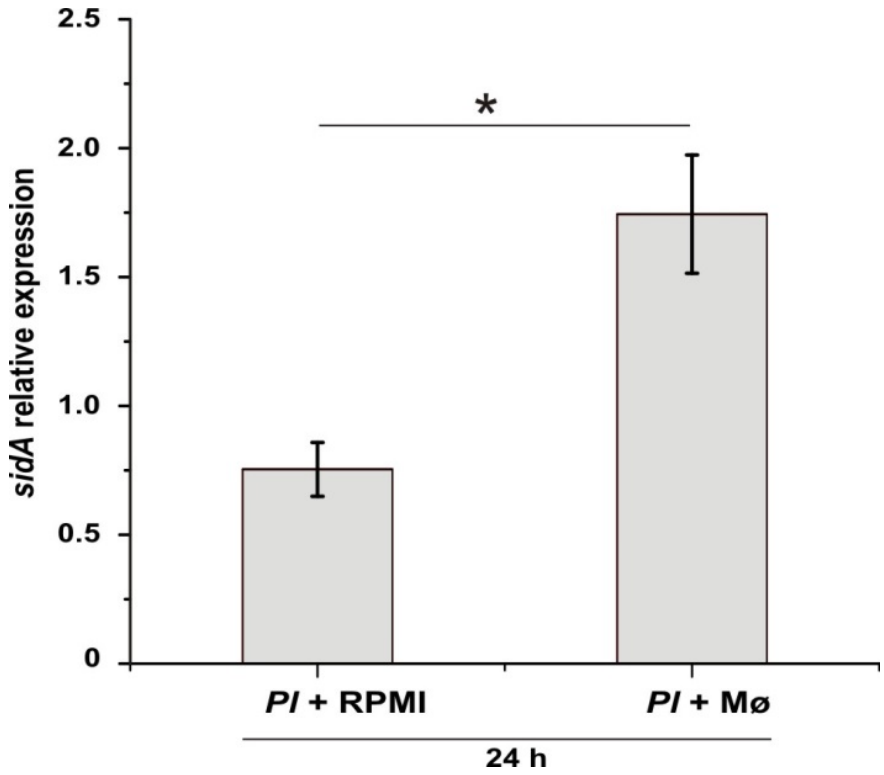


Figure 7. *Paracoccidioides sidA* expression is induced during murine macrophage infection. Quantitative RT-PCR was performed with transcripts of *P. lutzii* yeast cells phagocytosed by murine macrophage after 24 h of co-incubation. As control, yeast cells were incubated for 24 h in RPMI medium. 50 μM BPS was added to the RPMI medium in both conditions. Expression values were calculated using α-tubulin as endogenous control. Data are expressed as mean ± standard deviation from triplicates. *statistically significant difference determined by Student's *t*-test ($p < 0.001$).

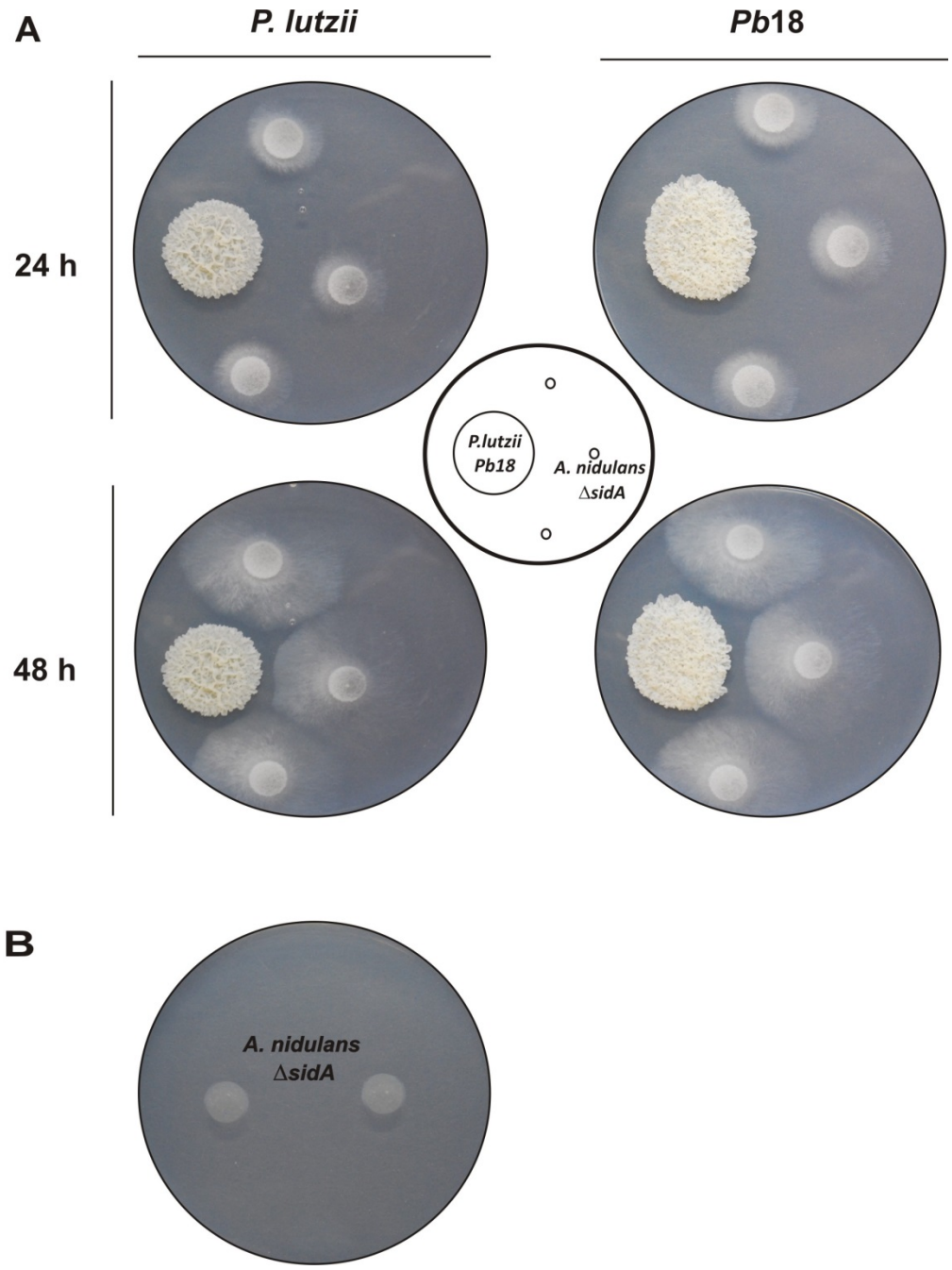


Figure 8. *Paracoccidioides* siderophores promote growth of *A. nidulans* Δ*sidA* mutant. A. *Aspergillus* hyphae grew on plates containing *P. lutzii* and *Pb*18 yeasts cells grown under iron limiting conditions. **B.** No growth was observed in absence of *Paracoccidioides*.

Supporting Figures

```
PlsidI      MATTLNDSSLWRLQQTLNHIQPQHIRENEERLSIVHGPTQPALWEMTLGGLLEFQCLQYR 60
AfsidI      MAT------IRRLQQTLSHLRPP---KAPQLLSIVEGPTQPELLDITLGELLTLQSLQYG 51
Pb03sidI    MATTLNDSSLWRLQQTLNHIQPQCIRENEQRLSIVHGPTHPALWEMTLGGLLEFQCLRYR 60
Pb18sidI    MATTLNDSSLWRLQQTLNHIQPQCIRENEQRLSIVHGPTHPALWEMTLGGLLEFQCLRYR 60
            ***      : ******.*::*    :  : ****.***:* * ::*** ** :*.*:*

PlsidI      DLECVVIPWTGARWTYGHLEHESGRLARGLLAKGIQRGDRIGVMAGNCEEYVSLFFAAAR 120
AfsidI      DYECLVFPWTGARWTYTDLKDEADRVARGLLAMGIQKGDRIGIMAGNCEQYISVFFAAAR 111
Pb03sidI    DLECVVVPWTGARWTYGRLEHESGRLARGLLAKGIQRGDRIGVMAGNCEEYVSLFFAAAR 120
Pb18sidI    DLECVVVPWTGARWTYGRLEHESGRLARGLLAKGIQRGDRIGVMAGNCEEYVSLFFAAAR 120
            * **:*.*********  *:.*:.*:****** ***:*****:******:*:*:******

PlsidI      VGAILVVINNTYTQTELIYALHHT-------ACKLLFIVPRIGRRNLENALDDLAS-PDI 172
AfsidI      VGAILVVLNNTYTPSELYYALGHT-------DCRLLFLTPRIGRHSLEEVLAKLGPRPKE 164
Pb03sidI    VGAILVVINNTYTQTELIYALNHTGMFVPRYSMLLLFIVPRIGRRNLENTLDDLAS-PDI 179
Pb18sidI    VGAILVVINNTYTQTELIYALNHTGMIVPRYSMLLLFIVPRIGRRNLENTLDDLAS-PDI 179
            *******:***** :** *** **          ***:.*****:.**:.* .*.. *.

PlsidI      SERVPNLEETILIRGNYKKFRTYESVALEGNSVSMKAVQRRQDTLSPFDVCNLQFTSGST 232
AfsidI      QGTSSALEEIIILRGQYSGFSTYEHVIQRGLPLPSHALQDREAELHSTDVCNLQFTSGST 224
Pb03sidI    SKRVPNLEETILIRGNYQKFGTYESVALEGNSVSMKAIQRRQDTLSPFDVCNLQFTSGST 239
Pb18sidI    SKRVPNLEETILIRGNYQKFGTYESVALEGNSVSMKAIQRRQDTLSPFDVCNLQFTSGST 239
            .   . *** *:**:*. * *** *  .* .:. :*:* *:  * . ************

PlsidI      GNPKASMLTHHNLINNSRFIGDRMDFTEYDILCCPPPLFHCFGLVLGLLACITHGAKVVY 292
AfsidI      GNPKAAMLTHHNLVNNSRFIGDRMNLTSFDILCCPPPLFHCFGLVLGMLAVVTHGSKIIF 284
Pb03sidI    GNPKASMLTHHNLINNSRFIGDRMDFTEYDILCCPPPLFHCFGLVLGLLACITHGAKVVY 299
Pb18sidI    GNPKASMLTHHNLINNSRFIGDRMDFTEYDILCCPPPLFHCFGLVLGLLACITHGAKVVY 299
            *****:*******:**********::*.:******************:** :***:*:::

PlsidI      PAETFEPGAVLKALSDERCTALHGVPTMFEAILALPRPDTFDCSQLRTGIIAGAPVPRPL 352
AfsidI      PSETFDPTAVLHAISDEKCTALHGVPTMFEAILSLPKPPNFDCSNLRTGIIAGAPVPRPL 344
Pb03sidI    PAETFEPGAVLKALSDERCTALHGVPTMFEAILALPRPDTFDCSQLRTGIIAGAPVPRPL 359
Pb18sidI    PAETFEPGAVLKALSDERCTALHGVPTMFEAILALPRPDTFDCSQLRTGIIAGAPVPRPL 359
            *:***:* ***:*:***:****************:**:* .****:***************

PlsidI      MKRLWNELNMTEFTSSYGLTEASPTCFNAFTSDPIDTRLTTVGTVLPHASAKIINPNTGE 412
AfsidI      MKRLLEELNMTEYTSSYGLTEASPTCFNALTTDSIERRLTTVGKVMPHAKAKIIDT-QGH 403
Pb03sidI    MKRLWNELNMTEFTSSYGLTEASPTCFNAFTSDSIDTRLSTVGTVLPHASAKIINPNTGE 419
Pb18sidI    MKRLWNELNMTEFTSSYGLTEASPTCFNAFTSDSIDTRLTTVGTVLPHASAKIINPNTGE 419
            **** :******:****************:*:*.*: **:***.*:***.****:.  *.

PlsidI      TVKVGERGELCMAGYQIHKGYWENPEKTAETLIEDEDGTIWLRTGDEAMFNSEGYCSITG 472
AfsidI      IVPIGQRGELCIAGYQLTKGYWNNPEKTAEALITDSDGVTWLKTGDEAIFDEEGYCSITG 463
Pb03sidI    TVKVGERGELCMSGYQIHKGYWENTEKTAEALIEDEDGTIWLRTGDEAVFNSEGYCSITG 479
Pb18sidI    TVKVGERGELCMAGYQIHKGYWENTEKTAEALIEDEDGTIWLRTGDEAVFNSKGYCSITG 479
             * :*:*****::***: ****:*.*****:** *.**. **:*****:*:.:*******

PlsidI      RFKDIIIRGGENIYPLEIEERLTAHPAISRAAVVGLPDKHYGEVVCAFLTLEESHDC-PS 531
AfsidI      RFKDIIIRGGENIYPLEIEERLAAHPAIEVASVIGIPDQKYGEVVGAFLALAADVSARPS 523
Pb03sidI    RFKDIIIRGGENIYPLEIEERLTAHPAISRAAVVGLPDKYYGEVVCAFLTLEESHDC-PS 538
Pb18sidI    RFKDIIIRGGENIYPLEIEERLTAHPAISRAAVVGLPDKYYGEVVCAFLTLEESHDC-PS 538
            **********************:*****. *:*:*:**: ***** ***:*  . .. **

PlsidI      DDEIRDWTRKKLGRHKAPKHVFVFGSDPRLPGDIPQTGSGKPLGRSTSSPRVSMANRYPN 591
AfsidI      DEELRAWTRETLGRHKAPQYFFVFGEE-GVDRTIPVTGSGKVRKVDLRKIAASVLERRLA 582
Pb03sidI    DDEIRDWTRKKLGGHKAPKHVFVFGSDPRLPGDIPQTGSGKVQKQILRDLGRKLI----- 593
Pb18sidI    DDEIRDWTRKKLGGHKAPKHVFVFGSDPRLPGDIPQTGSGKVQKQILRDLGRKLI----- 593
            *:*:* ***:.** ****::.****.:  :   ** *****       .   .:

PlsidI      SG------ 593
AfsidI      KTAAIKEK 590
Pb03sidI    --------
Pb18sidI    --------
```

Figure S1. Similarity of *A. fumigatus* SidI with putative acyl-CoA ligase from *P. lutzii*, *Pb*18 and *Pb*03. The amino acid sequences of the orthologs were aligned using the software ClustalX2. Asterisks: amino acid identity. Dots: conserved substitutions. Grey box: PTS2 motif.

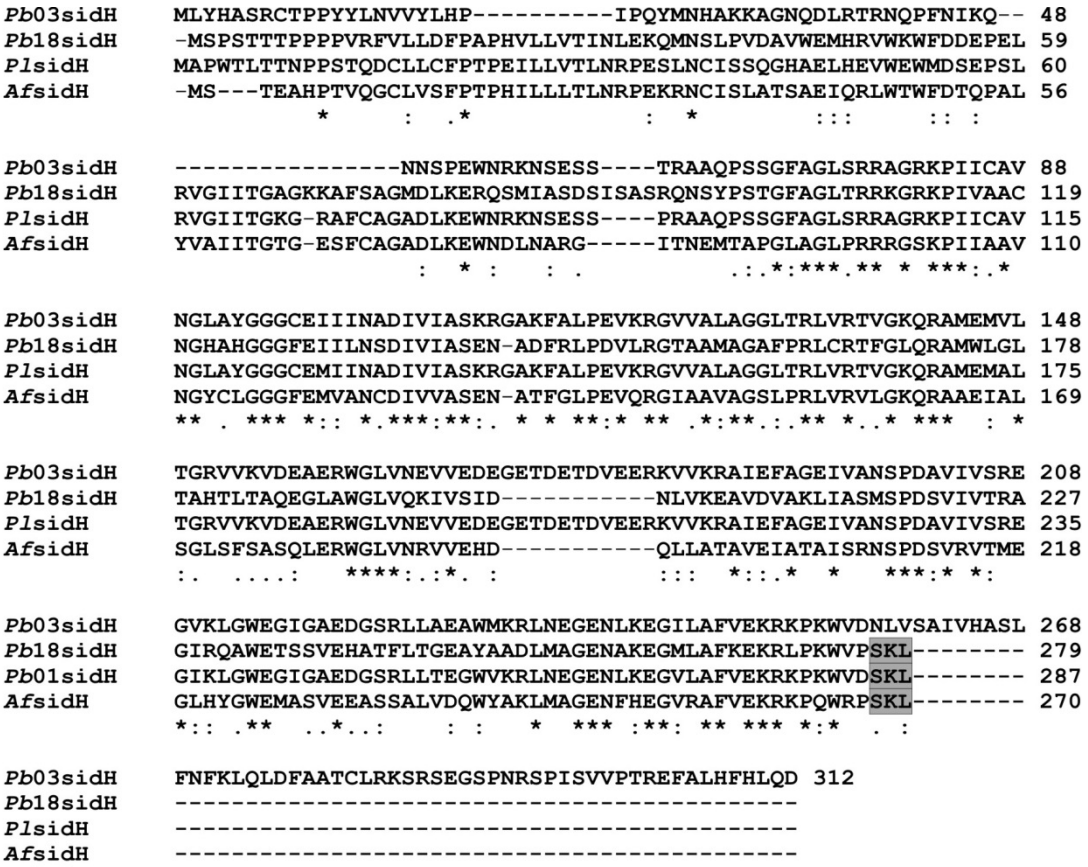


Figure S2. Similarity of *A. fumigatus* SidH with putative enoyl-CoA hydratase from *P. lutzii*, *Pb*18 and *Pb*03. The amino acid sequences of the orthologs were aligned using the software ClustalX2. Asterisks: amino acid identity. Dots: conserved substitutions. Grey box: PTS1 motif. PTS1 scores: *Pl* (8.8) and *Pb*18 (10.4).

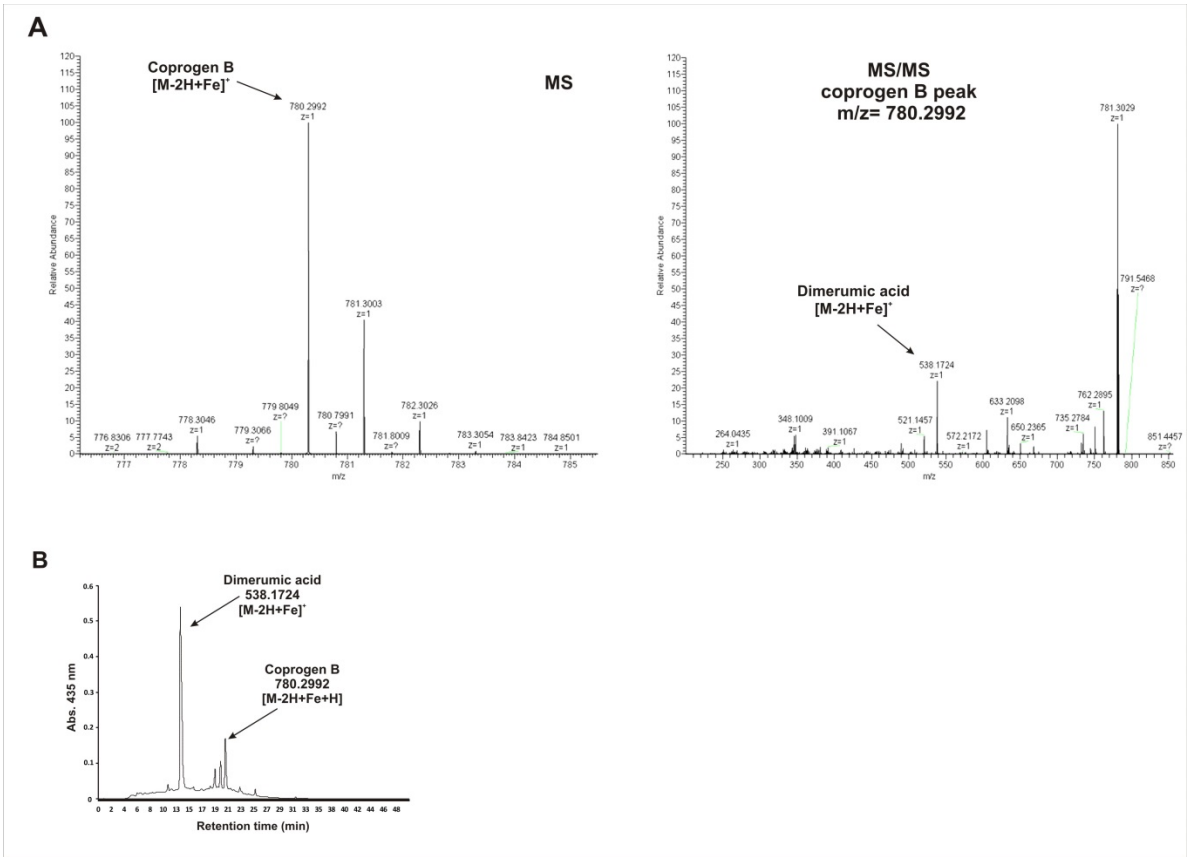


Figure S3. High-resolution mass spectrometry of *Paracoccidioides* extracellular siderophores. A. RP-HPLC peak corresponding to coprogen B in Figure 4B was submitted to MS and MS/MS analysis, demonstrating that dimerumic acid is as a breakdown product of coprogen B. **B.** Longer periods of cultivation result in an increase in the amount of dimerumic acid over coprogen B, as demonstrated by RP-HPLC peaks from *Pb*18 supernatants obtained after 10 days of incubation.

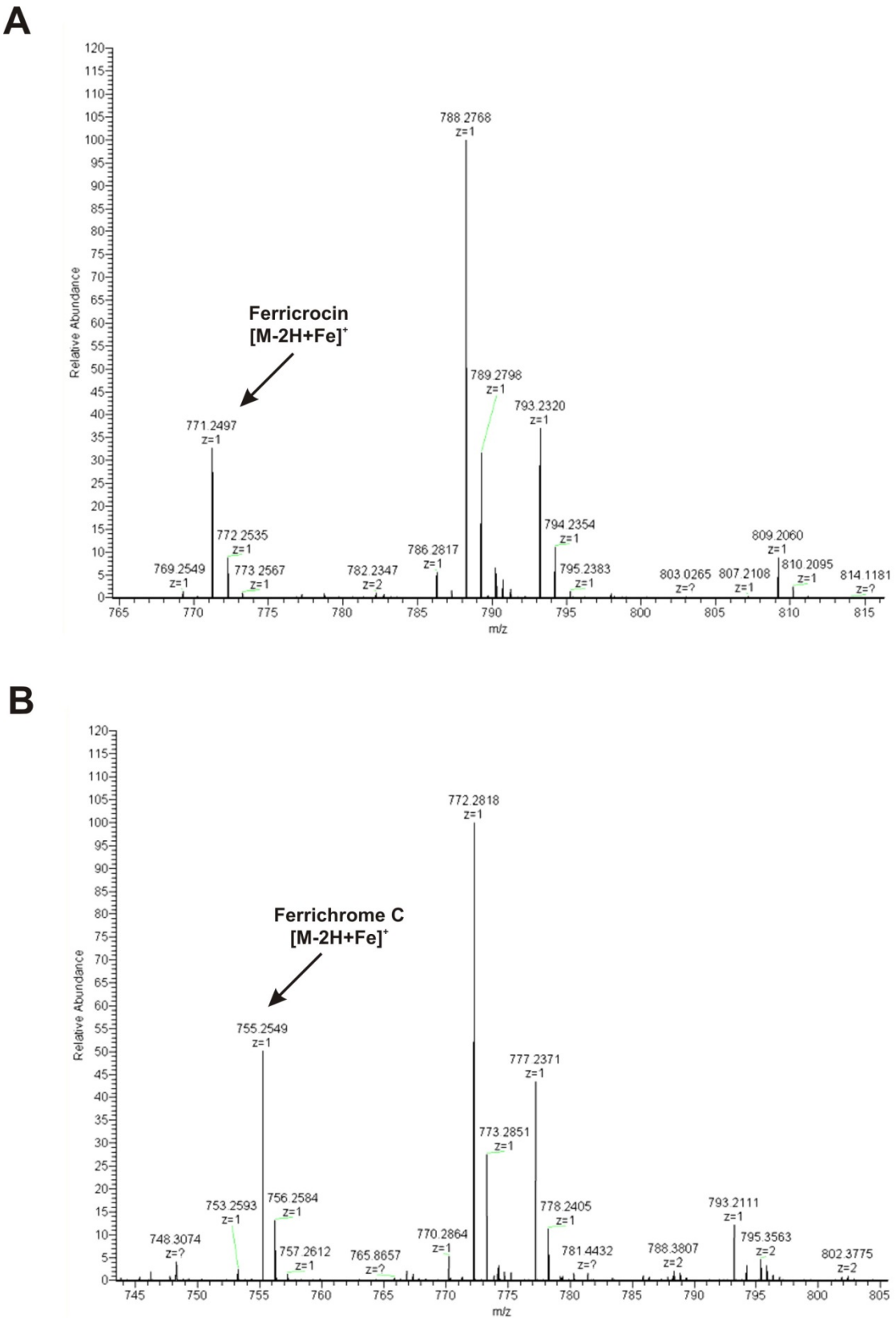


Figure S4. High-resolution mass spectrometry of *Paracoccidioides* intracellular siderophores. RP-HPLC peaks displayed at Figure 4C were submitted to mass spectrometry analysis for molecular masses definition of ferricrocin (**A**) and ferrichrome C (**B**).

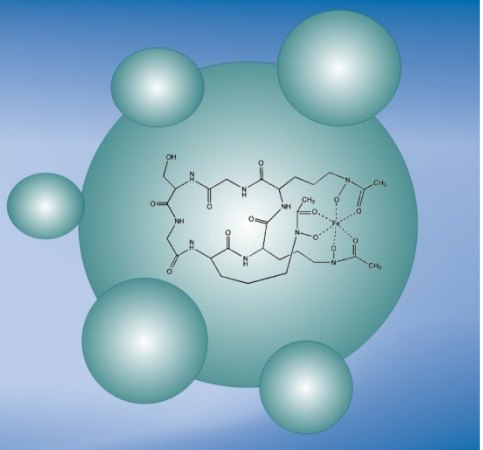
Table 1. *Paracoccidioides* strains used in this study.

Strain	Cryptic species	Reference
*Paracoccidioides lutzii Pb*01	"*Pb*01-like"	[69]
*Paracoccidioides brasiliensis Pb*18	S1	[70]
*Paracoccidioides brasiliensis Pb*02	PS2	[70]
*Paracoccidioides brasiliensis Pb*Epm83	PS3	[71]

Supp. Table 2. Accession numbers of *Paracoccidioides* siderophore genes available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

P. lutzii		***Pb*18**	***Pb*03**
L-ornithine 5-monooxygenase (*sidA*)	PAAG_01682	PADG_00097	PABG_03730
Acetylase (*sidF*)	PAAG_01680	PADG_00100	PABG_03728
Succinylbenzoate-CoA ligase (*sidI*)	PAAG_01681	PADG_00099	PABG_03729
Carnitinyl-CoA dehydratase (*sidH*)	PAAG_06469	PADG_01543	PABG_02862
D-alanine-poly(phosphoribitol) ligase subunit 1 (*sidC*)	PAAG_08527	PADG_05295	PABG_04670
D-alanine-poly(phosphoribitol) ligase subunit 1 (*sidD*)	PAAG_01679	PADG_00102	PABG_03726
Siderophore iron transporter (*mirB*)	PAAG_01685	PADG_00095	PABG_03732
Siderophore iron transporter (*mirC*)	PAAG_02233	PADG_00462	PABG_04747

Capítulo 3



1. DISCUSSÃO

O desenvolvimento de uma enfermidade depende de características que se expressam em ambos, patógeno e hospedeiro, no momento infecção. As reações desencadeadas em cada um dos organismos após o primeiro contato determinam ou o sucesso do patógeno, quando a infecção evolui e se instala a doença, ou a habilidade do hospedeiro em evitar a propagação do invasor. Neste último caso, as estratégias consistem em tornar o local invadido pelo micro-organismo, no hospedeiro, o mais hostil possível. A restrição de micronutrientes imposta por mamíferos frente à infecção constitui um dos artifícios empregados para minimizar e/ou evitar o desenvolvimento de patógenos e pode ser designada imunidade nutricional.

Devido sua participação em vários processos biológicos vitais, o ferro é essencial para sobrevivência de praticamente todos os organismos vivos. Assim, a disponibilidade deste metal durante o processo infeccioso está relacionada à capacidade do patógeno em causar doença e, ao mesmo tempo, à capacidade do hospedeiro de combater a infecção. Inúmeras evidências demonstram que infecções por diversos micro-organismos, incluindo fungos, bactérias, protozoários e até mesmo vírus, aumentam em frequência e severidade em humanos com sobrecarga de ferro no organismo (DRAKESMITH e PRENTICE, 2008; KHAN et al., 2007; PRENTICE, 2008).

Sabe-se que durante a infecção por *Paracoccidioides* spp., principalmente na forma aguda da PCM, ocorre a chamada anemia da inflamação, caracterizada por baixos níveis séricos de ferro e transferrina. Esta hipoferremia constitui provavelmente um mecanismo de defesa contra a forma parasitária do fungo, a qual, por sua vez, deve ser capaz de adquirir ferro. Apesar de importante, pouco se conhece sobre as estratégias utilizadas por espécies do gênero *Paracoccidioides* para aquisição de ferro. Neste trabalho, tais mecanismos foram investigados já que são elementares para o entendimento da relação patógeno-hospedeiro.

As análises iniciais foram facilitadas pela disponibilidade do genoma de isolados de *P. brasiliensis* (*Pb*18 e *Pb*03) e *P. lutzii* (*Pb*01). Genes relacionados à aquisição de ferro em outros fungos patogênicos (*A. fumigatus*, *C. albicans* e *H. capsulatum*) e não patogênicos (*S. cerevisiae* e *A. nidulans*) foram utilizados na busca por ortólogos no genoma de *Paracoccidioides* spp. Os resultados evidenciaram a presença de genes cujos produtos proteicos estão possivelmente envolvidos em dois

mecanismos de captação de ferro de alta afinidade: a via redutiva e a não redutiva, caracterizada pela produção e captação de sideróforos.

Genes ortólogos àqueles codificantes para metaloredutases e ferroxidases de membrana foram identificados nos três isolados. A ausência de permeases férricas adiciona evidências à hipótese de que o ferro possa ser transportado por permeases de zinco, como sugerido em análises transcrionais anteriores (BAILÃO et al., 2006; BAILÃO et al., 2007). Adicionalmente, a presença de ortólogos de uma redutase férrica secretada sugere que *Paracoccidioides* spp. pode potencialmente captar ferro pela via redutiva. De fato, análises de expressão demonstraram a indução dos genes codificantes para as redutases férricas de membrana e secretada em condições de baixa disponibilidade de ferro (BAILÃO et al., 2012). Análises das sequências de aminoácidos revelaram ainda que as metaloredutases e ferroxidases preditas nos genomas possuem domínios conservados relacionados a funções enzimáticas específicas. A presença de uma via redutiva funcional em *Paracoccidioides* spp. está sendo averiguada e resultados iniciais apontam a presença de metaloredutases ativas neste patógeno.

Concernente à via de captação de ferro não redutiva, a presença de genes ortólogos àqueles do sistema de produção e captação de sideróforos adicionou evidências a estudos anteriores que sugerem a utilização destas moléculas por *Paracoccidioides* spp. (ARANGO e RESTREPO, 1988; CASTANEDA et al., 1988). De acordo com as análises *in silico*, *Paracoccidioides* spp. é potencialmente capaz de produzir sideróforos do tipo hidroxamato por apresentar ortólogos a todas as enzimas descritas como necessárias para tal (SidA, SidF, SidC, SidD, SidI, SidH). Ademais, a presença de transportadores de membrana preditos (Sit1, MirB, MirC) sugere que o fungo, além de produzir, também seja apto a utilizar sideróforos como fonte de ferro. Estes transportadores possuem um domínio MFS1 que indica que os mesmos pertencem à superfamília MFS de transportadores, descritos como mediadores da internalização do complexo sideróforo-Fe^{3+} em outros fungos. Adicionalmente, o transportador predito Sit1 de ambas as espécies de *Paracoccidioides* apresenta 13-14 domínios transmembrana, o que também é característico da família MFS.

A função dos sideróforos como mecanismo de captação de ferro de alta afinidade em *Paraccocidioides* spp. foi inicialmente investigada por meio da análise da expressão dos genes relacionados à síntese e utilização destas moléculas. Transcritos de

todos os genes de *P. lutzii* avaliados neste trabalho (*sidF, sidC, sidD, sidI, sidH, mirB, mirC*) foram induzidos em condições de privação de ferro, assim como os transcritos de *sidA* e *sit1*, analisados previamente (PARENTE et al., 2011). Como os genes foram regulados da mesma forma pela disponibilidade de ferro, a organização genômica dos mesmos foi verificada. Genes que codificam proteínas que fazem parte de uma mesma via metabólica são geralmente localizados adjacentes um ao outro no genoma e este padrão de organização foi encontrado em *P. lutzii* (*Pb*01) e *P. brasiliensis* (*Pb*18 e *Pb*03) nos quais *sidD*, *sidF*, *sidI*, *sidA* e *mirB* são contíguos. Esta organização gênomica em grupo também é descrita em várias espécies de fungos produtores de sideróforos, incluindo ascomicetos e basidiomicetos (GRUNDLINGER et al., 2013). Em adição, um transportador da família MFS também foi identificado próximo a genes de biossíntese de sideróforos em *H. capsulatum* (HWANG et al., 2008), o que provavelmente está relacionado à sintenia existente entre *Paracoccidioides* spp. e este fungo (DESJARDINS et al., 2011). A indução em resposta à limitação de ferro e a organização genômica sugeriram que os genes poderiam ser igualmente regulados em nível transcricional. Análises da região à montante revelaram a presença do motivo (A/T/C)GATA(A/G) e da sequência consenso (A/G)ATC(A/T)GATAA. Em fungos patogênicos, como *A. fumigatus*, *H. capsulatum* e *B. dermatitidis* (CHAO et al., 2008; GAUTHIER et al., 2010; SCHRETTL et al., 2008), e não patogênicos, como *N. crassa* e *A. nidulans* (HAAS et al., 1999; HARRISON e MARZLUF, 2002), as sequências GATA localizadas nas regiões promotoras dos genes relacionados à síntese e captação de sideróforos são reconhecidas por fatores de transcrição do tipo GATA. Em condições em que o ferro é abundante, estes fatores inibem a produção e captação de sideróforos, os quais são utilizados somente em condições de privação de ferro, como mecanismo de aquisição de alta afinidade. Dessa maneira, sugere-se que em *Paracoccidioides* spp. o mesmo tipo de regulação possa acontecer.

A síntese de sideróforos por *Paracoccidioides* spp. foi investigada utilizando-se uma solução do indicador cromo azurol S (CAS), a qual é normalmente azul e muda de cor em presença de quelantes que possuem alta afinidade por Fe^{3+}, como os sideróforos. Ensaios em placa demonstraram que, em condições limitantes de ferro, isolados de *P. lutzii* (*Pb*01) e de todas as espécies crípticas de *P. brasiliensis, Pb*18 (S1), *Pb*02 (PS2) e *Pb*Epm83 (PS3), produziram e secretaram hidroxamatos, sideróforos tipicamente produzidos por fungos. A maior porcentagem destas moléculas nos

sobrenadantes de *P. lutzii* e *Pb*18 foi detectada após 10 dias de cultivo em meio sem ferro. A secreção de sideróforos nos sobrenadantes de cultura nos quais ferro foi adicionado incialmente deve-se provavelmente à maior taxa de crescimento do fungo em meio líquido. O requerimento por ferro aumenta com o tempo de incubação, o que diminui a disponibilidade deste nutriente e induz a síntese de sideróforos. É importante destacar que mesmo em condições limitantes de ferro, houve aumento da biomassa de todos os isolados em ambos os meios sólido e líquido, permitindo constatar que o fungo possui um sistema de captação de ferro funcional.

Análises de cromatografia líquida de fase reversa (RP-HPLC) e espectrometria de massas confirmaram a produção de hidroxamatos por *Paracoccidioides* spp. Coprogeno B e ácido dimerúmico foram identificados nos sobrenadantes de cultura enquanto ferricromo C e ferricrocina foram detectados nos extratos celulares do fungo. Na segunda etapa da via de biossíntese de sideróforos, um grupo acil é adicionado à ornitina hidroxilada pela ação de uma transacilase (SidF). No caso de coprogenos, este grupo é o anidromevalonil Co-A, o qual é derivado do mevalonato pela ação de uma acil-CoA ligase (SidI) e uma enoil-CoA hidratase (SidH) (YASMIN et al., 2012). O fato de *Paracoccidioides* spp. possuir ortólogos para todas estas enzimas, os quais foram induzidos na ausência de ferro, corrobora a produção de coprogeno B. O ácido dimerúmico identificado é provavelmente produto de degradação do coprogeno B, como ocorre em *H. capsulatum* (BURT, 1982; HOWARD et al., 2000). As evidências para tal hipótese são: (i) coprogeno B consiste de uma molécula de fusarinina ligada à uma de ácido dimerúmico e (ii) a quantidade de coprogeno B em sobrenadantes de cultura recentes é maior que a de ácido dimerúmico, enquanto esta proporção é inversa em culturas mais antigas. Ferricrocina é produzida por *A. fumigatus* e *A. nidulans* e funciona como molécula armazenadora e distribuidora de ferro em hifas (EISENDLE et al., 2006; SCHRETTL et al., 2007; WALLNER et al., 2009). Ferricromos também já foram identificados no basidiomiceto *Ustilago maydis* (WINTERBERG et al., 2010) e no dermatófito *Trichophyton rubrum* (MOR et al., 1992). O papel de ferricrocina e ferricromo C como armazenadores de ferro, ou não, em *Paracoccidioides* spp. requer análises adicionais.

Já foi demonstrado em um estudo anterior que o crescimento de *Paracoccidioides* spp. é melhorado na presença de três dos quatro sideróforos identificados: coprogeno B, ácido dimerúmico e ferricromo (CASTANEDA et al.,

1988). No presente estudo evidenciou-se que ferricrocina e o xenosideróforo ferrioxamina também podem ser utilizados como fonte de ferro pelo fungo. Provavelmente, a utilização desta variedade de sideróforos é possível em virtude da presença de 3 transportadores preditos da família MFS (Sit1, MirB e MirC), já que o reconhecimento do complexo sideróforo-Fe^{3+} é altamente estéreo específico.

A utilização de sideróforos não é restrita aos micro-organismos produtores. Vários fungos e bactérias são capazes de utilizar sideróforos sintetizados por outras espécies como fonte de ferro. Como demonstrado, *Paracoccidioides* spp. também exibe esta propriedade e, além disso, produz sideróforos que podem ser utilizados por outros micro-organismos, como evidenciado em experimentos com a linhagem de *A. nidulans* Δ*sidA*, a qual é incapaz de produzir estas moléculas (EISENDLE et al., 2003). *A. nidulans* possui 10 transportadores de sideróforos preditos (HAAS et al., 2008) e não apresenta via redutiva de captação de ferro. Tais fatos justificam a habilidade deste fungo em utilizar sideróforos produzidos por outras espécies. Além do coprogeno produzido por *Paracoccidioides* spp., *A. nidulans* também é capaz de utilizar enterobactina, um sideróforo bacteriano, e ferrioxamina B (HAAS et al., 2003; OBEREGGER et al., 2001). A capacidade de captar mais de um tipo de sideróforo, principalmente aqueles produzidos por outras espécies, é vantajosa para micro-organismos pois é uma forma de poupar energia. Além disso, representa a possibilidade de adquirir ferro a partir de várias fontes.

Como a utilização de sideróforos por organismos patogênicos está geralmente relacionada à virulência (HWANG et al., 2008; SCHRETTL et al., 2004), a influência destas moléculas durante a interação de *Paracoccidioides* spp. com uma linhagem de macrófagos murinos foi investigada. Macrófagos alveolares são as primeiras células de defesa com as quais o fungo se depara. Células fúngicas fagocitadas são capazes de sobreviver e multiplicar-se em macrófagos não ativados. Sugere-se que após o crescimento e multiplicação, os macrófagos sejam destruídos e várias leveduras sejam liberadas. Contudo, macrófagos ativados por citocinas, como IFN-γ, são capazes não só de prevenir a multiplicação, mas também de eliminar o fungo (BRUMMER et al., 1989). A restrição de ferro é um dos mecanismos utilizados pelos fagócitos para impedir o desenvolvimento do patógeno. Já foi descrito que em *Paracoccidioides* spp. tanto a transição conídeo-levedura quanto a sobrevivência de leveduras em fagócitos são processos dependentes de ferro (CANO et al., 1994; DIAS-MELICIO et al., 2005).

Demonstrou-se neste trabalho que a exposição de leveduras de *Paracoccocidioides* spp. à ferrioxamina antes do co-cultivo com macrófagos ativados por IFN-γ resultou em um aumento da sobrevivência, quando comparada à leveduras expostas somente à $FeSO_4$. Provavelmente, a utilização do sideróforo antes da infecção forneceu o ferro necessário para o metabolismo fúngico e para a defesa contra o estresse oxidativo gerado pelos fagócitos. Adicionalmente, a indução de *sidA* durante o co-cultivo com macrófagos ativados sugere que o fungo pode produzir sideróforos para superar a baixa disponibilidade de ferro no interior no fagossomo. Tal estratégia é utilizada pelos patógenos *A. fumigatus* (SCHRETTL et al., 2010b) e *H. capsulatum* (HILTY et al., 2011) para sobrevivência e crescimento no interior de fagócitos. Estes dados, aliados à capacidade de *Paracoccidioides* spp. em utilizar vários sideróforos como fonte de ferro *in vitro*, demonstram que o fungo efetivamente possui um sistema de captação de ferro de alta afinidade baseado na produção e captação de sideróforos.

2. CONCLUSÕES

Uma das estratégias utilizadas por micro-organismos patogênicos para sobreviver e perpetuar no hospedeiro é a expressão de mecanismos de captação de ferro de alta afinidade, os quais incluem: a produção e captação de sideróforos, a via redutiva e a captação do grupo heme.

Os dados obtidos neste trabalho demonstraram que *P. lutzii* e *P. brasiliensis*, fungos patogênicos dimórficos, são capazes de sintetizar e utilizar sideróforos como fonte de ferro. Em condições de baixa disponibilidade deste micronutriente, genes relacionados à produção e captação destas moléculas foram induzidos e sideróforos do tipo hidroxamato foram detectados em sobrenadantes de cultura e extratos celulares de *Paracoccidioides* spp. A produção destas moléculas já havia sido reportada anteriormente, mas só agora a identidade das mesmas foi revelada. *Paracoccidioides* spp. sintetiza e secreta coprogeno B e produz ferricrocina e ferricromo C como sideróforos intracelulares. O fungo é capaz de crescer na presença de ácido dimerúmico, ferricrocina e ferrioxamina, um xenosideróforo, como únicas fontes de ferro. Adicionalmente, experimentos realizados com macrófagos murinos ativados revelaram que (i) a utilização de ferrioxamina promove o aumento da sobrevivência de *Paracoccidioides* spp. no interior dos fagócitos e (ii) que o fungo provavelmente induz a síntese de sideróforos quando no interior destas células. Além disso, sideróforos produzidos por *Paracoccidioides* podem ser utilizados como fonte de ferro por *A. nidulans*.

Análises *in silico* demonstraram que genes relacionados à via de captação redutiva estão presentes no genoma de *Paracoccidioides* spp. A presença de um sistema redutivo funcional em *Paracoccidioides* spp. está sendo averiguada e resultados iniciais apontam a presença de metaloredutases ativas neste patógeno. Além disso, *Paracoccidioides* spp. também é capaz de adquirir ferro do grupo heme por meio da captação de hemoglobina (BAILÃO et al., submetido).

Apesar de alguns estudos terem demonstrado a importância do ferro no cenário da infecção por *Paracoccidioides* spp., o impacto do metabolismo deste micronutriente na patogenicidade e virulência do fungo ainda não foi investigado extensivamente. Este estudo foi o primeiro passo para as próximas análises moleculares e funcionais dos genes relacionados à captação e biossíntese de sideróforos no gênero *Paracoccidioides*.

O conhecimento adquirido é clinicamente importante, pois a produção e captação destes ligantes de ferro representam possíveis alvos para terapia com antifúngicos levando-se em consideração a ausência de tais vias em humanos.

3. PERSPECTIVAS

As perspectivas que surgiram a partir dos resultados deste trabalho são:

- Obter linhagens silenciadas para os genes *sidA* (biossíntese de sideróforos), *sit1* (transporte de sideróforos) e *sidI* (conexão entre a via de biossíntese de sideróforos e a via de síntese de ergosterol) e verificar o fenótipo dos transformantes;

- Realizar ensaios de virulência em modelo murino de infecção com as linhagens silenciadas;

- Analisar o perfil proteômico da linhagem silenciada para *sidA*;

- Investigar a localização peroxissomal de *sidI*, *sidH* e *sidF* em linhagens que expressem essas proteínas fusionadas à GFP;

- Purificar sideróforos produzidos por *Paracoccidioides* spp.

4. REFÊNCIAS BIBLIOGRÁFICAS

AJELLO, L.; POLONELLI, L. Imported paracoccidioidomycosis: a public health problem in non-endemic areas. **Eur J Epidemiol** 1(3): 160-165, 1985.

APPELBERG, R. Macrophage nutriprive antimicrobial mechanisms. **J Leukoc Biol** 79(6): 1117-1128, 2006.

ARANGO, R.; RESTREPO, A. Growth and production of iron chelants by *Paracoccidioides brasiliensis* mycelial and yeast forms. **J Med Vet Mycol** 26(2): 113-118, 1988.

ARISTIZABAL, B. H.; CLEMONS, K. V.; STEVENS, D. A.; RESTREPO, A. Morphological transition of *Paracoccidioides brasiliensis* conidia to yeast cells: *in vivo* inhibition in females. **Infect Immun** 66(11): 5587-5591, 1998.

ARISTIZABAL, B. H.; CLEMONS, K. V.; COCK, A. M.; RESTREPO, A.; STEVENS, D. A. Experimental *Paracoccidioides brasiliensis* infection in mice: influence of the hormonal status of the host on tissue responses. **Med Mycol** 40(2): 169-178, 2002.

BAGAGLI, E.; BOSCO, S. M.; THEODORO, R. C.; FRANCO, M. 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(5): 344-351, 2006.

BAILÃO, A. M.; SCHRANK, A.; BORGES, C. L.; DUTRA, V.; MOLINARI-MADLUM, E. E. W. I.; FELIPE, M. S. S.; MENDES-GIANNINI, M. J. S.; MARTINS, W. S.; PEREIRA, M.; SOARES, C. M. A. Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. **Microbes Infect** 8(12-13): 2686-2697, 2006.

BAILÃO, A. M.; SHRANK, A.; BORGES, C. L.; PARENTE, J. A.; DUTRA, V.; FELIPE, M. S.; FIUZA, R. B.; PEREIRA, M.; SOARES, C. M. A. The transcriptional profile of *Paracoccidioides brasiliensis* yeast cells is influenced by human plasma. **FEMS Immunol Med Microbiol** 51(1): 43-57, 2007.

BAILÃO, E. F. L. C.; PARENTE, A. F. A.; PARENTE, J. A.; SILVA-BAILÃO, M. G. S.; CASTRO, K. P.; KMETZSCH, L.; STAATS, C. C.; SCHRANK, A.; VAINSTEIN, M. H.; BORGES, C. L.; BAILO, A. M.; SOARES, C. M. A. Metal acquisition and homeostasis in fungi. **Curr Fungal Infect Rep** 6: 257-266, 2012.

BARBOSA, W.; DAHER, R.; DE OLIVEIRA, A. R. Lymphatic abdominal form of South American blastomycosis. **Rev Inst Med Trop Sao Paulo** 10(1): 16-27, 1968.

BATISTA, J., JR.; DE CAMARGO, Z. P.; FERNANDES, G. F.; VICENTINI, A. P.; FONTES, C. J.; HAHN, R. C. Is the geographical origin of a *Paracoccidioides*

brasiliensis isolate important for antigen production for regional diagnosis of paracoccidioidomycosis? **Mycoses** 53(2): 176-180, 2010.

BELITARDO, D. R.; CALEFI, A. S.; BORGES, I. K.; DE OLIVEIRA, G. G.; SBEGHEN, M. R.; ITANO, E. N.; DE CAMARGO, Z. P.; ONO, M. A. Detection of Antibodies Against *Paracoccidioides brasiliensis* in Free-Range Domestic Pigs (*Sus scrofa*). **Mycopathologia** 177(1-2): 91-95, 2014.

BEN-OTHMAN, R.; FLANNERY, A. R.; MIGUEL, D. C.; WARD, D. M.; KAPLAN, J.; ANDREWS, N. W. *Leishmania*-mediated inhibition of iron export promotes parasite replication in macrophages. **PLoS Pathog** 10(1): e1003901, 2014.

BLATZER, M.; SCHRETTL, M.; SARG, B.; LINDNER, H. H.; PFALLER, K.; HAAS, H. SidL, an *Aspergillus fumigatus* transacetylase involved in biosynthesis of the siderophores ferricrocin and hydroxyferricrocin. **Appl Environ Microbiol** 77(14): 4959-4966, 2011.

BOCCA, A. L.; AMARAL, A. C.; TEIXEIRA, M. M.; SATO, P. K.; SHIKANAI-YASUDA, M. A.; SOARES FELIPE, M. S. Paracoccidioidomycosis: eco-epidemiology, taxonomy and clinical and therapeutic issues. **Future Microbiol** 8(9): 1177-1191, 2013.

BORELLI, D. Concepto de Reservárea. La reducida reservárea de la paracoccidioidosis. **Dermat Venez** 4: 71-74, 1964.

BRUMMER, E.; HANSON, L. H.; RESTREPO, A.; STEVENS, D. A. Intracellular multiplication of *Paracoccidioides brasiliensis* in macrophages: killing and restriction of multiplication by activated macrophages. **Infect Immun** 57(8): 2289-2294, 1989.

BRUMMER, E.; CASTANEDA, E.; RESTREPO, A. Paracoccidioidomycosis: an update. **Clin Microbiol Rev** 6(2): 89-117, 1993.

BUITRAGO, M. J.; BERNAL-MARTINEZ, L.; CASTELLI, M. V.; RODRIGUEZ-TUDELA, J. L.; CUENCA-ESTRELLA, M. Histoplasmosis and paracoccidioidomycosis in a non-endemic area: a review of cases and diagnosis. **J Travel Med** 18(1): 26-33, 2011.

BURT, W. R. Identification of coprogen B and its breakdown products from *Histoplasma capsulatum*. **Infect Immun** 35(3): 990-996, 1982.

CAIRO, G.; BERNUZZI, F.; RECALCATI, S. A precious metal: Iron, an essential nutrient for all cells. **Genes Nutr** 1(1): 25-39, 2006.

CAMARGO, Z. P.; FRANCO, M. F. Current knowledge on pathogenesis and immunodiagnosis of paracoccidioidomycosis. **Rev Iberoam Micol** 17: 41-48 2000.

CANO, L. E.; GOMEZ, B.; BRUMMER, E.; RESTREPO, A.; STEVENS, D. A. Inhibitory effect of deferoxamine or macrophage activation on transformation of *Paracoccidioides brasiliensis* conidia ingested by macrophages: reversal by holotransferrin. **Infect Immun** 62(4): 1494-1496, 1994.

CANO, M. I.; CISALPINO, P. S.; GALINDO, I.; RAMIREZ, J. L.; MORTARA, R. A.; DA SILVEIRA, J. F. Electrophoretic karyotypes and genome sizing of the pathogenic fungus *Paracoccidioides brasiliensis*. **J Clin Microbiol** 36(3): 742-747, 1998.

CARRERO, L. L.; NINO-VEGA, G.; TEIXEIRA, M. M.; CARVALHO, M. J.; SOARES, C. M.; PEREIRA, M.; JESUINO, R. S.; MCEWEN, J. G.; MENDOZA, L.; TAYLOR, J. W.; FELIPE, M. S.; SAN-BLAS, G. New *Paracoccidioides brasiliensis* isolate reveals unexpected genomic variability in this human pathogen. **Fungal Genet Biol** 45(5): 605-612, 2008.

CASSAT, J. E.; SKAAR, E. P. Iron in infection and immunity. **Cell Host Microbe** 13(5): 509-519, 2013.

CASTANEDA, E.; BRUMMER, E.; PERLMAN, A. M.; MCEWEN, J. G.; STEVENS, D. A. A culture medium for *Paracoccidioides brasiliensis* with high plating efficiency, and the effect of siderophores. **J Med Vet Mycol** 26(6): 351-358, 1988.

CHAO, L. Y.; MARLETTA, M. A.; RINE, J. Sre1, an iron-modulated GATA DNA-binding protein of iron-uptake genes in the fungal pathogen *Histoplasma capsulatum*. **Biochemistry** 47(27): 7274-7283, 2008.

CHIKAMORI, T.; SAKA, S.; NAGANO, H.; SAEKI, S.; LACAZ CDA, S.; RODRIGUES, M. C.; CASSAGUERRA, C. M.; BRACCIALLI, M. L. Paracoccidioidomycosis in Japan. Report of a case. **Rev Inst Med Trop Sao Paulo** 26(5): 267-271, 1984.

CONTI-DIAZ, I. A. On the unknown ecological niche of *Paracoccidioides brasiliensis*: our hypothesis of 1989: present status and perspectives. **Rev Inst Med Trop Sao Paulo** 49(2): 131-134, 2007.

COUTINHO, Z. F.; SILVA, D.; LAZERA, M.; PETRI, V.; OLIVEIRA, R. M.; SABROZA, P. C.; WANKE, B. Paracoccidioidomycosis mortality in Brazil (1980-1995). **Cad Saude Publica** 18(5): 1441-1454, 2002.

CRAMER, R. A., JR.; STAJICH, J. E.; YAMANAKA, Y.; DIETRICH, F. S.; STEINBACH, W. J.; PERFECT, J. R. Phylogenomic analysis of non-ribosomal peptide synthetases in the genus *Aspergillus*. **Gene** 383: 24-32, 2006.

DE MORAES BORBA, C.; SCHAFFER, G. M. *Paracoccidioides brasiliensis*: virulence and an attempt to induce the dimorphic process with fetal calf serum. **Mycoses** 45(5-6): 174-179, 2002.

DESJARDINS, C. A.; CHAMPION, M. D.; HOLDER, J. W.; MUSZEWSKA, A.; GOLDBERG, J.; BAILAO, A. M.; BRIGIDO, M. M.; FERREIRA, M. E.; GARCIA, A. M.; GRYNBERG, M.; GUJJA, S.; HEIMAN, D. I.; HENN, M. R.; KODIRA, C. D.; LEON-NARVAEZ, H.; LONGO, L. V.; MA, L. J.; MALAVAZI, I.; MATSUO, A. L.; MORAIS, F. V.; PEREIRA, M.; RODRIGUEZ-BRITO, S.; SAKTHIKUMAR, S.; SALEM-IZACC, S. M.; SYKES, S. M.; TEIXEIRA, M. M.; VALLEJO, M. C.; WALTER, M. E.; YANDAVA, C.; YOUNG, S.; ZENG, Q.; ZUCKER, J.; FELIPE, M. S.; GOLDMAN, G. H.; HAAS, B. J.; MCEWEN, J. G.; NINO-VEGA, G.; PUCCIA,

R.; SAN-BLAS, G.; SOARES, C. M.; BIRREN, B. W.; CUOMO, C. A. Comparative genomic analysis of human fungal pathogens causing paracoccidioidomycosis. **PLoS Genet** 7(10): e1002345, 2011.

DIAS-MELICIO, L. A.; CALVI, S. A.; PERACOLI, M. T.; SOARES, A. M. Inhibitory effect of deferoxamine on *Paracoccidioides brasiliensis* survival in human monocytes: reversal by holotransferrin not by apotransferrin. **Rev Inst Med Trop Sao Paulo** 47(5): 263-266, 2005.

DRAKESMITH, H.; PRENTICE, A. Viral infection and iron metabolism. **Nat Rev Microbiol** 6(7): 541-552, 2008.

DUHUTREL, P.; BORDAT, C.; WU, T. D.; ZAGOREC, M.; GUERQUIN-KERN, J. L.; CHAMPOMIER-VERGES, M. C. Iron sources used by the nonpathogenic lactic acid bacterium *Lactobacillus sakei* as revealed by electron energy loss spectroscopy and secondary-ion mass spectrometry. **Appl Environ Microbiol** 76(2): 560-565, 2010.

EISENDLE, M.; OBEREGGER, H.; ZADRA, I.; HAAS, H. The siderophore system is essential for viability of *Aspergillus nidulans*: functional analysis of two genes encoding l-ornithine N 5-monooxygenase (*sidA*) and a non-ribosomal peptide synthetase (*sidC*). **Mol Microbiol** 49(2): 359-375, 2003.

EISENDLE, M.; SCHRETTL, M.; KRAGL, C.; MULLER, D.; ILLMER, P.; HAAS, H. The intracellular siderophore ferricrocin is involved in iron storage, oxidative-stress resistance, germination, and sexual development in *Aspergillus nidulans*. **Eukaryot Cell** 5(10): 1596-1603, 2006.

FLO, T. H.; SMITH, K. D.; SATO, S.; RODRIGUEZ, D. J.; HOLMES, M. A.; STRONG, R. K.; AKIRA, S.; ADEREM, A. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. **Nature** 432(7019): 917-921, 2004.

FLUCKINGER, M.; HAAS, H.; MERSCHAK, P.; GLASGOW, B. J.; REDL, B. Human tear lipocalin exhibits antimicrobial activity by scavenging microbial siderophores. **Antimicrob Agents Chemother** 48(9): 3367-3372, 2004.

FORTES, M. R.; MIOT, H. A.; KUROKAWA, C. S.; MARQUES, M. E.; MARQUES, S. A. Immunology of paracoccidioidomycosis. **An Bras Dermatol** 86(3): 516-524, 2011.

FOSTER, L. A. Utilization and cell-surface binding of hemin by *Histoplasma capsulatum*. **Can J Microbiol** 48(5): 437-442, 2002.

FROISSARD, M.; BELGAREH-TOUZE, N.; DIAS, M.; BUISSON, N.; CAMADRO, J. M.; HAGUENAUER-TSAPIS, R.; LESUISSE, E. Trafficking of siderophore transporters in *Saccharomyces cerevisiae* and intracellular fate of ferrioxamine B conjugates. **Traffic** 8(11): 1601-1616, 2007.

GANZ, T. Iron in innate immunity: starve the invaders. **Curr Opin Immunol** 21(1): 63-67, 2009.

GAUTHIER, G. M.; SULLIVAN, T. D.; GALLARDO, S. S.; BRANDHORST, T. T.; VANDEN WYMELENBERG, A. J.; CUOMO, C. A.; SUEN, G.; CURRIE, C. R.; KLEIN, B. S. SREB, a GATA transcription factor that directs disparate fates in *Blastomyces dermatitidis* including morphogenesis and siderophore biosynthesis. **PLoS Pathog** 6(4): e1000846, 2010.

GRUENHEID, S.; PINNER, E.; DESJARDINS, M.; GROS, P. Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. **J Exp Med** 185(4): 717-730, 1997.

GRUNDLINGER, M.; YASMIN, S.; LECHNER, B. E.; GELEY, S.; SCHRETTL, M.; HYNES, M.; HAAS, H. Fungal siderophore biosynthesis is partially localized in peroxisomes. **Mol Microbiol** 88(5): 862-875, 2013.

HAAS, H.; ZADRA, I.; STOFFLER, G.; ANGERMAYR, K. The *Aspergillus nidulans* GATA factor SREA is involved in regulation of siderophore biosynthesis and control of iron uptake. **J Biol Chem** 274(8): 4613-4619, 1999.

HAAS, H.; SCHOESER, M.; LESUISSE, E.; ERNST, J. F.; PARSON, W.; ABT, B.; WINKELMANN, G.; OBEREGGER, H. Characterization of the *Aspergillus nidulans* transporters for the siderophores enterobactin and triacetylfusarinine C. **Biochem J** 371(Pt 2): 505-513, 2003.

HAAS, H.; EISENDLE, M.; TURGEON, B. G. Siderophores in fungal physiology and virulence. **Annu Rev Phytopathol** 46: 149-187, 2008.

HAHN, R. C.; MACEDO, A. M.; FONTES, C. J.; BATISTA, R. D.; SANTOS, N. L.; HAMDAN, J. S. Randomly amplified polymorphic DNA as a valuable tool for epidemiological studies of *Paracoccidioides brasiliensis*. **J Clin Microbiol** 41(7): 2849-2854, 2003.

HARRISON, K. A.; MARZLUF, G. A. Characterization of DNA binding and the cysteine rich region of SRE, a GATA factor in *Neurospora crassa* involved in siderophore synthesis. **Biochemistry** 41(51): 15288-15295, 2002.

HENTZE, M. W.; MUCKENTHALER, M. U.; ANDREWS, N. C. Balancing acts: molecular control of mammalian iron metabolism. **Cell** 117(3): 285-297, 2004.

HENTZE, M. W.; MUCKENTHALER, M. U.; GALY, B.; CAMASCHELLA, C. Two to tango: regulation of mammalian iron metabolism. **Cell** 142(1): 24-38, 2010.

HEYMANN, P.; GERADS, M.; SCHALLER, M.; DROMER, F.; WINKELMANN, G.; ERNST, J. F. The siderophore iron transporter of *Candida albicans* (Sit1p/Arn1p) mediates uptake of ferrichrome-type siderophores and is required for epithelial invasion. **Infect Immun** 70(9): 5246-5255, 2002.

HILTY, J.; GEORGE SMULIAN, A.; NEWMAN, S. L. *Histoplasma capsulatum* utilizes siderophores for intracellular iron acquisition in macrophages. **Med Mycol** 49(6): 633-642, 2011.

HISSEN, A. H.; WAN, A. N.; WARWAS, M. L.; PINTO, L. J.; MOORE, M. M. The *Aspergillus fumigatus* siderophore biosynthetic gene *sidA*, encoding L-ornithine N5-oxygenase, is required for virulence. **Infect Immun** 73(9): 5493-5503, 2005.

HOWARD, D. H.; RAFIE, R.; TIWARI, A.; FAULL, K. F. Hydroxamate siderophores of *Histoplasma capsulatum*. **Infect Immun** 68(4): 2338-2343, 2000.

HWANG, L. H.; MAYFIELD, J. A.; RINE, J.; SIL, A. *Histoplasma* requires *SID1*, a member of an iron-regulated siderophore gene cluster, for host colonization. **PLoS Pathog** 4(4): e1000044, 2008.

JOSEPH, E. A.; MARE, A.; IRVING, W. R., JR. Oral South American blastomycosis in the United States of America. Report of a case. **Oral Surg Oral Med Oral Pathol** 21(6): 732-737, 1966.

JUNG, W. H.; KRONSTAD, J. W. Iron and fungal pathogenesis: a case study with *Cryptococcus neoformans*. **Cell Microbiol** 10(2): 277-284, 2008.

JUNG, W. H.; SHAM, A.; LIAN, T.; SINGH, A.; KOSMAN, D. J.; KRONSTAD, J. W. Iron source preference and regulation of iron uptake in *Cryptococcus neoformans*. **PLoS Pathog** 4(2): e45, 2008.

JUNG, W. H.; HU, G.; KUO, W.; KRONSTAD, J. W. Role of ferroxidases in iron uptake and virulence of *Cryptococcus neoformans*. **Eukaryot Cell** 8(10): 1511-1520, 2009.

JUNG, W. H.; KRONSTAD, J. W. Iron influences the abundance of the iron regulatory protein Cir1 in the fungal pathogen *Cryptococcus neoformans*. **FEBS Lett** 585(20): 3342-3347, 2011.

KHAN, F. A.; FISHER, M. A.; KHAKOO, R. A. Association of hemochromatosis with infectious diseases: expanding spectrum. **Int J Infect Dis** 11(6): 482-487, 2007.

KNIGHT, S. A.; LESUISSE, E.; STEARMAN, R.; KLAUSNER, R. D.; DANCIS, A. Reductive iron uptake by *Candida albicans*: role of copper, iron and the TUP1 regulator. **Microbiology** 148(Pt 1): 29-40, 2002.

KNIGHT, S. A.; VILAIRE, G.; LESUISSE, E.; DANCIS, A. Iron acquisition from transferrin by *Candida albicans* depends on the reductive pathway. **Infect Immun** 73(9): 5482-5492, 2005.

KOSMAN, D. J. Molecular mechanisms of iron uptake in fungi. **Mol Microbiol** 47(5): 1185-1197, 2003.

KRAGL, C.; SCHRETTL, M.; ABT, B.; SARG, B.; LINDNER, H. H.; HAAS, H. EstB-mediated hydrolysis of the siderophore triacetylfusarinine C optimizes iron uptake of *Aspergillus fumigatus*. **Eukaryot Cell** 6(8): 1278-1285, 2007.

LACAZ, C. S.; VIDAL, M. S.; HEINS-VACCARI, E. M.; DE MELO, N. T.; DEL NEGRO, G. M.; ARRIAGADA, G. L.; FREITAS RDOS, S. *Paracoccidioides*

brasiliensis. A mycologic and immunochemical study of two strains. **Rev Inst Med Trop Sao Paulo** 41(2): 79-86, 1999.

LAN, C. Y.; RODARTE, G.; MURILLO, L. A.; JONES, T.; DAVIS, R. W.; DUNGAN, J.; NEWPORT, G.; AGABIAN, N. Regulatory networks affected by iron availability in *Candida albicans*. **Mol Microbiol** 53(5): 1451-1469, 2004.

LOOSE, D. S.; STOVER, E. P.; RESTREPO, A.; STEVENS, D. A.; FELDMAN, D. Estradiol binds to a receptor-like cytosol binding protein and initiates a biological response in *Paracoccidioides brasiliensis*. **Proc Natl Acad Sci U S A** 80(24): 7659-7663, 1983.

MACHADO, G. C.; MORIS, D. V.; ARANTES, T. D.; SILVA, L. R.; THEODORO, R. C.; MENDES, R. P.; VICENTINI, A. P.; BAGAGLI, E. Cryptic species of *Paracoccidioides brasiliensis*: impact on paracoccidioidomycosis immunodiagnosis. **Mem Inst Oswaldo Cruz** 108(5): 637-643, 2013.

MANNS, J. M.; MOSSER, D. M.; BUCKLEY, H. R. Production of a hemolytic factor by *Candida albicans*. **Infect Immun** 62(11): 5154-5156, 1994.

MARQUES, S. A. Paracoccidioidomycosis: epidemiological, clinical, diagnostic and treatment up-dating. **An Bras Dermatol** 88(5): 700-711, 2013.

MASSON, P. L.; HEREMANS, J. F.; SCHONNE, E. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. **J Exp Med** 130(3): 643-658, 1969.

MATUTE, D. R.; MCEWEN, J. G.; PUCCIA, R.; MONTES, B. A.; SAN-BLAS, G.; BAGAGLI, E.; RAUSCHER, J. T.; RESTREPO, A.; MORAIS, F.; NINO-VEGA, G.; TAYLOR, J. W. Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. **Mol Biol Evol** 23(1): 65-73, 2006.

MATZANKE, B. F.; BILL, E.; TRAUTWEIN, A. X.; WINKELMANN, G. Role of siderophores in iron storage in spores of *Neurospora crassa* and *Aspergillus ochraceus*. **J Bacteriol** 169(12): 5873-5876, 1987.

MCEWEN, J. G.; BEDOYA, V.; PATINO, M. M.; SALAZAR, M. E.; RESTREPO, A. Experimental murine paracoccidiodomycosis induced by the inhalation of conidia. **J Med Vet Mycol** 25(3): 165-175, 1987a.

MCEWEN, J. G.; RESTREPO, B. I.; SALAZAR, M. E.; RESTREPO, A. Nuclear staining of *Paracoccidioides brasiliensis* conidia. **J Med Vet Mycol** 25(5): 343-345, 1987b.

MEI, B.; BUDDE, A. D.; LEONG, S. A. *sid1*, a gene initiating siderophore biosynthesis in *Ustilago maydis*: molecular characterization, regulation by iron, and role in phytopathogenicity. **Proc Natl Acad Sci U S A** 90(3): 903-907, 1993.

MEYNARD, D.; BABITT, J. L.; LIN, H. Y. The liver: conductor of systemic iron balance. **Blood** 123(2): 168-176, 2014.

MIETHKE, M.; MARAHIEL, M. A. Siderophore-based iron acquisition and pathogen control. **Microbiol Mol Biol Rev** 71(3): 413-451, 2007.

MOLINARI-MADLUM, E. E.; FELIPE, M. S.; SOARES, C. M. Virulence of *Paracoccidioides brasiliensis* isolates can be correlated to groups defined by random amplified polymorphic DNA analysis. **Med Mycol** 37(4): 269-276, 1999.

MOORE, R. E.; KIM, Y.; PHILPOTT, C. C. The mechanism of ferrichrome transport through Arn1p and its metabolism in *Saccharomyces cerevisiae*. **Proc Natl Acad Sci U S A** 100(10): 5664-5669, 2003.

MOR, H.; KASHMAN, Y.; WINKELMANN, G.; BARASH, I. Characterization of siderophores produced by different species of the dermatophytic fungi *Microsporum* and *Trichophyton*. **Biometals** 5: 213-216, 1992.

MORAIS, F. V.; BARROS, T. F.; FUKADA, M. K.; CISALPINO, P. S.; PUCCIA, R. Polymorphism in the gene coding for the immunodominant antigen gp43 from the pathogenic fungus *Paracoccidioides brasiliensis*. **J Clin Microbiol** 38(11): 3960-3966, 2000.

NEILANDS, J. B. Siderophores. **Arch Biochem Biophys** 302(1): 1-3, 1993.

NEMETH, E.; TUTTLE, M. S.; POWELSON, J.; VAUGHN, M. B.; DONOVAN, A.; WARD, D. M.; GANZ, T.; KAPLAN, J. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. **Science** 306(5704): 2090-2093, 2004.

NEVITT, T.; THIELE, D. J. Host iron withholding demands siderophore utilization for *Candida glabrata* to survive macrophage killing. **PLoS Pathog** 7(3): e1001322, 2011.

OBEREGGER, H.; SCHOESER, M.; ZADRA, I.; ABT, B.; HAAS, H. SREA is involved in regulation of siderophore biosynthesis, utilization and uptake in *Aspergillus nidulans*. **Mol Microbiol** 41(5): 1077-1089, 2001.

ONG, S. T.; HO, J. Z.; HO, B.; DING, J. L. Iron-withholding strategy in innate immunity. **Immunobiology** 211(4): 295-314, 2006.

OUTTEN, F. W.; THEIL, E. C. Iron-based redox switches in biology. **Antioxid Redox Signal** 11(5): 1029-1046, 2009.

PANDEY, A.; BRINGEL, F.; MEYER, J. M. Iron requirement and search for siderophores in lacti acid bacteria. **Appl Microbiol Biotechnol** 40: 735-739, 1994.

PAO, S. S.; PAULSEN, I. T.; SAIER, M. H., JR. Major facilitator superfamily. **Microbiol Mol Biol Rev** 62(1): 1-34, 1998.

PARENTE, A. F.; BAILAO, A. M.; BORGES, C. L.; PARENTE, J. A.; MAGALHAES, A. D.; RICART, C. A.; SOARES, C. M. Proteomic analysis reveals that iron availability alters the metabolic status of the pathogenic fungus *Paracoccidioides brasiliensis*. **PLoS One** 6(7): e22810, 2011.

PENDRAK, M. L.; CHAO, M. P.; YAN, S. S.; ROBERTS, D. D. Heme oxygenase in *Candida albicans* is regulated by hemoglobin and is necessary for metabolism of exogenous heme and hemoglobin to alpha-biliverdin. **J Biol Chem** 279(5): 3426-3433, 2004.

PIGOSSO, L. L.; PARENTE, A. F.; COELHO, A. S.; SILVA, L. P.; BORGES, C. L.; BAILAO, A. M.; SOARES, C. M. Comparative proteomics in the genus *Paracoccidioides*. **Fungal Genet Biol** 60: 87-100, 2013.

PRADO, M.; SILVA, M. B.; LAURENTI, R.; TRAVASSOS, L. R.; TABORDA, C. P. Mortality due to systemic mycoses as a primary cause of death or in association with AIDS in Brazil: a review from 1996 to 2006. **Mem Inst Oswaldo Cruz** 104(3): 513-521, 2009.

PRENTICE, A. M. Iron metabolism, malaria, and other infections: what is all the fuss about? **J Nutr** 138(12): 2537-2541, 2008.

QUEIROZ-TELLES, F. *Paracoccidioides brasiliensis* ultrastructural findings. In: _____. **Paracoccidioidomycosis**. M. Franco, C. S. Lacaz, A. Restrepo-Moreno and G. Del Negro. London: CRC Press, 1994. 27–44.

RAPPLEYE, C. A.; GOLDMAN, W. E. Defining virulence genes in the dimorphic fungi. **Annu Rev Microbiol** 60: 281-303, 2006.

RAYMOND, K. N.; DERTZ, E. A.; KIM, S. S. Enterobactin: an archetype for microbial iron transport. **Proc Natl Acad Sci U S A** 100(7): 3584-3588, 2003.

RENSHAW, J. C.; ROBSON, G. D.; TRINCI, A. P. J.; WIEBE, M. G.; LIVENS, F. R.; COLLISON, D.; TAYLOR, R. J. Fungal siderophores: structures, functions and applications. **Mycol Res** 106(10): 1123-1142, 2002.

RESTREPO-MORENO, A. Paracoccidioidomycosis. In: _____. **Clinical Mycology**. W. E. Dismukes, P. G. Pappas and J. Sobel. New York: Oxford University Press, 2003. 328–345.

RESTREPO, A.; SALAZAR, M. E.; CANO, L. E.; STOVER, E. P.; FELDMAN, D.; STEVENS, D. A. Estrogens inhibit mycelium-to-yeast transformation in the fungus *Paracoccidioides brasiliensis*: implications for resistance of females to paracoccidioidomycosis. **Infect Immun** 46(2): 346-353, 1984.

RESTREPO, A.; MCEWEN, J. G.; CASTANEDA, E. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? **Med Mycol** 39(3): 233-241, 2001.

RICCI, G.; MOTA, F. T.; WAKAMATSU, A.; SERAFIM, R. C.; BORRA, R. C.; FRANCO, M. Canine paracoccidioidomycosis. **Med Mycol** 42(4): 379-383, 2004.

RICHINI-PEREIRA, V. B.; BOSCO, S. M.; THEODORO, R. C.; BARROZO, L.; PEDRINI, S. C.; ROSA, P. S.; BAGAGLI, E. Importance of xenarthrans in the eco-epidemiology of *Paracoccidioides brasiliensis*. **BMC Res Notes** 2: 228, 2009.

ROONEY, P. J.; KLEIN, B. S. Linking fungal morphogenesis with virulence. **Cell Microbiol** 4(3): 127-137, 2002.

RUTHERFORD, J. C.; BIRD, A. J. Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. **Eukaryot Cell** 3(1): 1-13, 2004.

SALGADO-SALAZAR, C.; JONES, L. R.; RESTREPO, A.; MCEWEN, J. G. The human fungal pathogen *Paracoccidioides brasiliensis* (Onygenales: Ajellomycetaceae) is a complex of two species: phylogenetic evidence from five mitochondrial markers. **Cladistics** 26: 613-624, 2010.

SCHRETTL, M.; BIGNELL, E.; KRAGL, C.; JOECHL, C.; ROGERS, T.; ARST, H. N., JR.; HAYNES, K.; HAAS, H. Siderophore biosynthesis but not reductive iron assimilation is essential for *Aspergillus fumigatus* virulence. **J Exp Med** 200(9): 1213-1219, 2004.

SCHRETTL, M.; BIGNELL, E.; KRAGL, C.; SABIHA, Y.; LOSS, O.; EISENDLE, M.; WALLNER, A.; ARST, H. N., JR.; HAYNES, K.; HAAS, H. Distinct roles for intra- and extracellular siderophores during *Aspergillus fumigatus* infection. **PLoS Pathog** 3(9): 1195-1207, 2007.

SCHRETTL, M.; KIM, H. S.; EISENDLE, M.; KRAGL, C.; NIERMAN, W. C.; HEINEKAMP, T.; WERNER, E. R.; JACOBSEN, I.; ILLMER, P.; YI, H.; BRAKHAGE, A. A.; HAAS, H. SreA-mediated iron regulation in *Aspergillus fumigatus*. **Mol Microbiol** 70(1): 27-43, 2008.

SCHRETTL, M.; BECKMANN, N.; VARGA, J.; HEINEKAMP, T.; JACOBSEN, I. D.; JOCHL, C.; MOUSSA, T. A.; WANG, S.; GSALLER, F.; BLATZER, M.; WERNER, E. R.; NIERMANN, W. C.; BRAKHAGE, A. A.; HAAS, H. HapX-mediated adaption to iron starvation is crucial for virulence of *Aspergillus fumigatus*. **PLoS Pathog** 6(9): e1001124, 2010a.

SCHRETTL, M.; IBRAHIM-GRANET, O.; DROIN, S.; HUERRE, M.; LATGE, J. P.; HAAS, H. The crucial role of the *Aspergillus fumigatus* siderophore system in interaction with alveolar macrophages. **Microbes Infect** 12(12-13): 1035-1041, 2010b.

SHANKAR, J.; WU, T. D.; CLEMONS, K. V.; MONTEIRO, J. P.; MIRELS, L. F.; STEVENS, D. A. Influence of 17beta-estradiol on gene expression of *Paracoccidioides* during mycelia-to-yeast transition. **PLoS One** 6(12): e28402, 2011.

SHIKANAI-YASUDA, M. A.; TELLES FILHO FDE, Q.; MENDES, R. P.; COLOMBO, A. L.; MORETTI, M. L. Guidelines in paracoccidioidomycosis. **Rev Soc Bras Med Trop** 39(3): 297-310, 2006.

STOVER, E. P.; SCHAR, G.; CLEMONS, K. V.; STEVENS, D. A.; FELDMAN, D. Estradiol-binding proteins from mycelial and yeast-form cultures of *Paracoccidioides brasiliensis*. **Infect Immun** 51(1): 199-203, 1986.

TANGEN, K. L.; JUNG, W. H.; SHAM, A. P.; LIAN, T.; KRONSTAD, J. W. The iron- and cAMP-regulated gene SIT1 influences ferrioxamine B utilization,

melanization and cell wall structure in *Cryptococcus neoformans*. **Microbiology** 153(Pt 1): 29-41, 2007.

TEIXEIRA, M. D.; THEODORO, R. C.; OLIVEIRA, F. F.; MACHADO, G. C.; HAHN, R. C.; BAGAGLI, E.; SAN-BLAS, G.; FELIPE, M. S. *Paracoccidioides lutzii* sp. nov.: biological and clinical implications. **Med Mycol**2013a.

TEIXEIRA, M. M.; THEODORO, R. C.; DE CARVALHO, M. J.; FERNANDES, L.; PAES, H. C.; HAHN, R. C.; MENDOZA, L.; BAGAGLI, E.; SAN-BLAS, G.; FELIPE, M. S. Phylogenetic analysis reveals a high level of speciation in the *Paracoccidioides* genus. **Mol Phylogenet Evol** 52(2): 273-283, 2009.

TEIXEIRA, M. M.; THEODORO, R. C.; DERENGOWSKI LDA, S.; NICOLA, A. M.; BAGAGLI, E.; FELIPE, M. S. Molecular and morphological data support the existence of a sexual cycle in species of the genus *Paracoccidioides*. **Eukaryot Cell** 12(3): 380-389, 2013b.

TERÇARIOLI, G. R.; BAGAGLI, E.; REIS, G. M.; THEODORO, R. C.; BOSCO SDE, M.; MACORIS, S. A.; RICHINI-PEREIRA, V. B. Ecological study of *Paracoccidioides brasiliensis* in soil: growth ability, conidia production and molecular detection. **BMC Microbiol** 7: 92, 2007.

THEODORO, R. C.; TEIXEIRA MDE, M.; FELIPE, M. S.; PADUAN KDOS, S.; RIBOLLA, P. M.; SAN-BLAS, G.; BAGAGLI, E. Genus *Paracoccidioides*: Species recognition and biogeographic aspects. **PLoS One** 7(5): e37694, 2012.

TIMMERMAN, M. M.; WOODS, J. P. Potential role for extracellular glutathione-dependent ferric reductase in utilization of environmental and host ferric compounds by *Histoplasma capsulatum*. **Infect Immun** 69(12): 7671-7678, 2001.

UNTEREINER, W. A.; SCOTT, J. A.; NAVEAU, F. A.; SIGLER, L.; BACHEWICH, J.; ANGUS, A. The Ajellomycetaceae, a new family of vertebrate-associated Onygenales. **Mycologia** 96(4): 812-821, 2004.

VALERA, E. T.; MORI, B. M.; ENGEL, E. E.; COSTA, I. S.; BRANDAO, D. F.; NOGUEIRA-BARBOSA, M. H.; QUEIROZ, R. G.; SILVEIRA VDA, S.; SCRIDELI, C. A.; TONE, L. G. Fungal infection by *Paracoccidioides brasiliensis* mimicking bone tumor. **Pediatr Blood Cancer** 50(6): 1284-1286, 2008.

VAN DER HELM, D.; WINKELMANN, G. Hydroxamates and polycarbonates as iron transport agents (siderophores) in fungi. In: _____. **Metal ions in fungi**. G. Winkelmann and D. R. Winge. New York, 1994. 39-148.

WALLNER, A.; BLATZER, M.; SCHRETTL, M.; SARG, B.; LINDNER, H.; HAAS, H. Ferricrocin, a siderophore involved in intra- and transcellular iron distribution in *Aspergillus fumigatus*. **Appl Environ Microbiol** 75(12): 4194-4196, 2009.

WARD, P. P.; CONNEELY, O. M. Lactoferrin: role in iron homeostasis and host defense against microbial infection. **Biometals** 17(3): 203-208, 2004.

WEISSMAN, Z.; KORNITZER, D. A family of *Candida* cell surface haem-binding proteins involved in haemin and haemoglobin-iron utilization. **Mol Microbiol** 53(4): 1209-1220, 2004.

WEISSMAN, Z.; SHEMER, R.; CONIBEAR, E.; KORNITZER, D. An endocytic mechanism for haemoglobin-iron acquisition in *Candida albicans*. **Mol Microbiol** 69(1): 201-217, 2008.

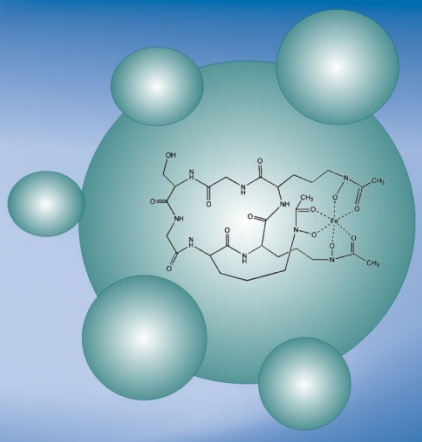
WINKELMANN, G. Siderophore transport in Fungi. In: _____. **Microbial Transport Systems**. G. Winkelmann. Weinheim: Wiley-VCH, 2001. 463-479.

WINKELMANN, G. Microbial siderophore-mediated transport. **Biochem Soc Trans** 30(4): 691-696, 2002.

WINTERBERG, B.; UHLMANN, S.; LINNE, U.; LESSING, F.; MARAHIEL, M. A.; EICHHORN, H.; KAHMANN, R.; SCHIRAWSKI, J. Elucidation of the complete ferrichrome A biosynthetic pathway in *Ustilago maydis*. **Mol Microbiol** 75(5): 1260-1271, 2010.

YASMIN, S.; ALCAZAR-FUOLI, L.; GRUNDLINGER, M.; PUEMPEL, T.; CAIRNS, T.; BLATZER, M.; LOPEZ, J. F.; GRIMALT, J. O.; BIGNELL, E.; HAAS, H. Mevalonate governs interdependency of ergosterol and siderophore biosyntheses in the fungal pathogen *Aspergillus fumigatus*. **Proc Natl Acad Sci U S A** 109(8): E497-504, 2012.

Anexos



Curr Fungal Infect Rep
DOI 10.1007/s12281-012-0108-8

GENOMICS AND PATHOGENESIS (S SHOHAM, SECTION EDITOR)

Metal Acquisition and Homeostasis in Fungi

Elisa Flávia Luiz Cardoso Bailão · Ana Flávia Alves Parente · Juliana Alves Parente · Mirelle Garcia Silva-Bailão · Kelly Pacheco de Castro · Lívia Kmetzsch · Charley Christian Staats · Augusto Schrank · Marilene Henning Vainstein · Clayton Luiz Borges · Alexandre Melo Bailão · Célia Maria de Almeida Soares

© Springer Science+Business Media, LLC 2012

Abstract Transition metals, particularly iron, zinc and copper, have multiple biological roles and are essential elements in biological processes. Among other micronutrients, these metals are frequently available to cells in only limited amounts, thus organisms have evolved highly regulated mechanisms to cope and to compete with their scarcity. The homeostasis of such metals within the animal hosts requires the integration of multiple signals producing depleted environments that restrict the growth of microorganisms, acting as a barrier to infection. As the hosts sequester the necessary transition metals from invading pathogens, some, as is the case of fungi, have evolved elaborate mechanisms to allow their survival and development to establish infection. Metalloregulatory factors allow fungal cells to sense and to adapt to the scarce metal availability in the environment, such as in host tissues. Here we review recent advances in the identification and function of molecules that drive the acquisition and homeostasis of iron, copper and zinc in pathogenic fungi.

E. F. L. C. Bailão · A. F. A. Parente · J. A. Parente · M. G. Silva-Bailão · K. P. de Castro · C. L. Borges · A. M. Bailão · C. M. de Almeida Soares (✉)
Laboratório de Biologia Molecular, Instituto de Ciências Biológicas II, Campus Samambaia,
Universidade Federal de Goiás,
74690-900, Goiânia, Goiás, Brazil
e-mail: cmasoares@gmail.com

E. F. L. C. Bailão
Unidade Universitária de Iporá, Universidade Estadual de Goiás,
Iporá, Goiás, Brazil

L. Kmetzsch · C. C. Staats · A. Schrank · M. H. Vainstein
Laboratório de Biologia Molecular, Centro de Biotecnologia,
Universidade Federal do Rio Grande do Sul,
Porto Alegre, Rio Grande do Sul, Brazil

Published online: 22 September 2012

Keywords Iron · Copper · Zinc · Fungal pathogens

Introduction

Metals such as iron, copper and zinc have numerous biological roles and play a central role at the host–pathogen interface. Mammalian and microbial cells have an essential demand for these metals, which act as both structural and catalytic cofactors for proteins, and are therefore required for biological processes. During infection, the competing demands for these nutrients culminate in a struggle for metal acquisition/utilization at the microbe–host interface [1•, 2•]. In the complex interactions between pathogens and their mammalian hosts, metal homeostasis plays an essential role in both virulence and host defense [3, 4].

Iron and copper participate in several oxidation–reduction reactions because of their ability to lose and gain electrons. This same property permits iron and copper to generate reactive oxygen species (ROS) [5, 6]. Zinc is also an essential cofactor of many enzymes, but in excess, may be toxic to cells [7]. For metal balance, cells usually regulate uptake, storage and consumption. Our understanding of the mechanisms involved in metal excretion is incomplete. This review summarizes the current knowledge regarding the most studied metals that contribute to virulence of fungal pathogens: iron, copper and zinc. We focus on the fungal pathogens *Candida albicans*, *Histoplasma capsulatum*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Paracoccidioides*. Specifically we discuss the struggle for control of transition metals during infection, the molecular mechanisms involved in iron, copper and zinc uptake and the regulation of metal homeostasis in those pathogens. Additionally we review the preferential host iron sources

and fungal genes related to iron acquisition/homeostasis directly involved in infection.

Host Metal Homeostasis During Infectious Processes

Among metals involved in fungal infection, the functions of iron are well characterized. Hosts have evolved mechanisms to efficiently acquire iron and at the same time decrease its availability to pathogens [2•]. Physiological conditions that lead to metal overload contribute to increased infections. For example, administration of exogenous iron results in exacerbation of cryptococcosis [8] and increases in free iron also results in higher fungal load in mouse tissues infected with *Paracoccidioides* [9]. At the interface between iron and immunity, macrophages appear as a cellular factory that manage metal homeostasis [3]. Upon infection, the iron efflux from macrophages is suppressed resulting in 70 % reduction in plasma iron, thus restricting the amount of the metal available to extracellular pathogens. Infected macrophages, conversely, restrict the amount of iron available to intracellular microbes by pumping out iron via the ferroportin transporter route. Mutation-impaired ferroportin function compromises the ability of macrophages to clear pathogens [10].

Lactoferrin is produced by neutrophils and epithelial cells to chelate iron in extracellular compartments resulting in impairment of proliferation of fungal invaders [11]. Induction of ferritin production to facilitate withholding of intracellular iron diminishes the amount of the metal available to intracellular pathogens [2•, 12]. Iron also influences immune functions mediated by macrophages, and cytokines affect systemic iron homeostasis and cellular iron efflux [13]. Reduced iron levels have been found in macrophages activated by exposure to interferon gamma (IFN-γ) or granulocyte macrophage colony-stimulating factor (GM-CSF) [14]. Transferrin can be used by pathogens as an iron source in host tissues. To counteract this process IFN-γ decreases the expression of transferrin receptor in macrophages. Moreover, the production of the cellular iron storage molecule ferritin can be regulated by proinflammatory signals [15]. So, in the complex host–pathogen interaction, the control of iron homeostasis is a battlefield where the host must withdraw the micronutrient from microbes and at the same time uses iron to elaborate an efficient oxidative burst, since this metal is required for generation of ROS.

Since copper is essential, it is not unexpected that both humans and pathogens share the requirement for acquiring sufficient levels of copper [6]. In response to fungal infection, macrophages phagocytose the fungal cells and initiate cellular events that culminate in the oxidative burst [6]. Studies suggest that fungal pathogens must obtain copper to develop an efficient survival mechanism in host tissues, since genes related to copper acquisition/homeostasis are upregulated during infection [16, 17]. *C. neoformans* fights the host defenses to acquire copper, which promotes melanin synthesis, a virulence factor for this fungus [18]. The dependence of fungi upon copper for survival under the host conditions can be related to their response to ROS generation by the host since superoxide dismutase is a copper-dependent enzyme.

Zinc levels are modulated during infectious processes. During inflammation, the liver sequesters zinc, likely limiting zinc bioavailability to pathogenic microbes [19]. Neutrophils display an antimicrobial mechanism based on competition for zinc. This zinc-chelating system, found in neutrophil cytoplasm and abscess fluid, exerts fungistatic activity based on the calcium- and zinc-binding protein calprotectin [20]. Abscess fluid inhibits the growth of several fungi and the addition of zinc results in fungal growth in this fluid [7], reinforcing the view that zinc sequestration is a strategy used by the host to combat fungal infections. A metallomic study has demonstrated that GM-CSF-activated macrophages reduce intracellular zinc concentrations upon *H. capsulatum* infection in order to kill the pathogen [14].

Molecular Mechanisms of Iron, Copper and Zinc Uptake

Iron uptake mechanisms are highly regulated in fungi since excess iron is toxic and iron excretion systems have not yet been described in fungi [21]. Fungi have evolved different mechanisms for iron acquisition [21]. A low-affinity iron uptake system characterized only in *Saccharomyces cerevisiae* involves permeases that transport not only iron, but also other metals. In the reductive high-affinity ferrous uptake, ferrireductases reduce ferric iron (Fe^{3+}) to its soluble ferrous form (Fe^{2+}). Fe^{2+} is then reoxidized by plasma membrane ferroxidases and Fe^{3+} is promptly internalized by a high-affinity permease [5]. Another high-affinity mechanism for iron uptake is mediated by siderophores, small molecules with high affinity for Fe^{3+}, that allow specific recognition and uptake of iron at the cell surface [22]. Most fungi produce and secrete hydroxamate-type siderophores under low-iron growth conditions [23]. Some fungi, such as *C. neoformans*, do not produce siderophores, but can transport molecules produced by other organisms (xenosiderophores) [24].

The *C. albicans* genome contains genes that encode 18 putative ferrireductases and five ferroxidase homologues [1•, 25]. The ferroxidase Fet34 localizes to the plasma membrane and possibly associates with the permease Ftr1 early in the secretory pathway, promoting the high-affinity iron uptake [26•]. *C. albicans* produces a siderophore transporter [27] that displays broad substrate specificity, transporting various hydroxamate-type siderophores [28].

Under iron-limiting conditions, *H. capsulatum* produces three different reductants: a secreted glutathione dependent γ-glutamyltransferase (Ggt1) [29], non-enzymatic reductants with low molecular weight, and cell surface ferric reducing agents [30]. The *H. capsulatum* genome contains genes that encode seven putative ferrireductases [31]. Although a high-affinity acquisition mechanism has not been described for *H. capsulatum*, genomic analysis of the strain G186AR revealed genes coding iron permease (ftr1) and ferroxidase (Fet3) homologues [32]. *H. capsulatum* is also able to produce multiple hydroxamate siderophores under conditions of low iron availability [33]. In addition, *H. capsulatum* can utilize xenosiderophores [34].

In *A. fumigatus*, the ferrireductase FreB has been characterized. After reduction, iron is internalized by the ferroxidase-permease complex FetC–FtrA [35]. *A. fumigatus* synthesizes three types of siderophores, two of which are responsible for iron storage [36–38]. The iron-loaded siderophore is internalized by specific transporters [39] and the ester bonds of triacetylfusarinine C are then hydrolyzed by an esterase [40]. The cleavage products (fusarinines) are excreted, and the free iron can either be used in cell metabolism or bind to intracellular siderophore desferri-ferricrocin for storage [37, 38, 41•].

Uptake of iron is probably mediated by two large groups of transporters in *C. neoformans*: high- and low-affinity systems [42]. Cft1 is a high-affinity iron permease associated with the reductive system. On the other hand *cft2* possibly encodes for a low-affinity uptake system, since no clear iron-related phenotypes could be detected in *cft2* null mutants [43]. Cfo1 and Cfo2 ferroxidases have also been described in *C. neoformans* [44]. Cfo1 is required for high-affinity and reductive iron transport, since mutants lacking the coding gene show reduced growth under low iron conditions and cannot use ferric iron for growth. Moreover, under low iron conditions, Cfo1 expression is increased and localized mainly in the cell surface [44]. Studies have shown the inability of *Cryptococcus* species to produce siderophores. This is supported by genomic analysis, which has revealed the absence of genes involved in steps of siderophore biosynthesis [24, 45]. Despite the inability to synthesize siderophores, *Cryptococcus* species are presumably able to transport xenosiderophores [43].

Molecular mechanisms for reductive iron uptake in the genus *Paracoccidioides* are coming to light. In silico analysis has revealed that the genome of this fungus contains genes that encode redundant ferrireductase homologues [45]. Experiments have demonstrated a significant increase in the expression of genes coding the ferrireductases *fre3*, *fre7*, *frp1* and *ggt1* upon iron restriction (Fig. 1a). *Paracoccidioides* has glutathione-dependent ferrireductase activity [46], an aspect that is corroborated by the presence of a *ggt1* homologue in the fungus genome [45]. Since iron permease homologues were not detected in the *Paracoccidioides* genome, it has been proposed that a zinc permease could function additionally as an iron permease to acquire this metal [45]. The importance of iron acquisition by siderophores in the *Paracoccidioides* genus have been noted and studies on siderophore production and uptake are in

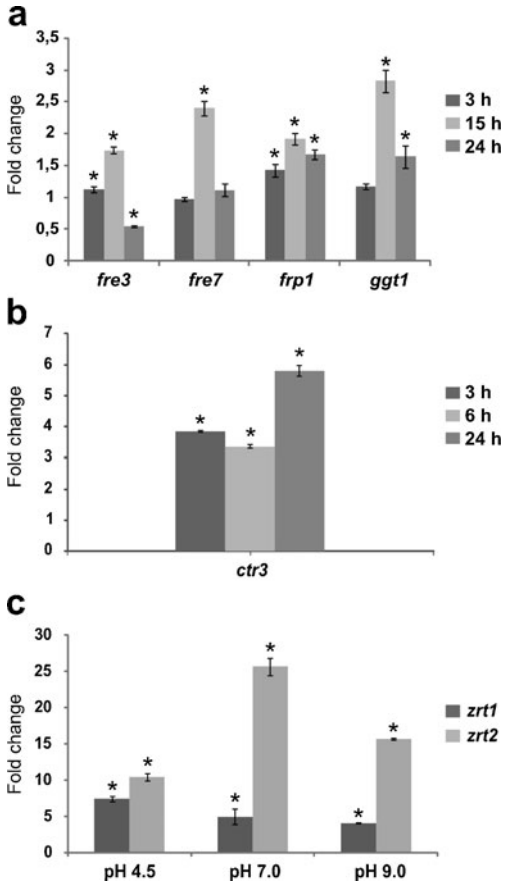


Fig. 1 Expression profile of *Paracoccidioides* (*Pb*01) genes during iron, copper and zinc starvation. *Pb*01 yeast cells were incubated in chemically defined medium containing different concentrations of iron, copper or zinc. Cells were harvested and total RNA was extracted using Trizol and mechanical cell rupture. After in vitro reverse transcription, the cDNAs were submitted to quantitative RT-PCR. The expression values were calculated using the transcripts alpha tubulin or l34 as endogenous controls [9, 82]. Data are presented as fold change relative to experimental controls. **a** Expression of ferrireductases encoding transcripts *fre3*, *fre7*, *frp1*, and *ggt1* evaluated in yeast cells in medium containing 3.5 μM iron (experimental control) or no iron for 3 h, 15 h and 24 h. **b** Expression of copper transporter encoding transcript *ctr3* evaluated in yeast cells in medium containing 50 μM copper (experimental control) or under conditions of copper starvation produced by adding the copper chelator bathocuproine disulphonate (BCS) for 3 h, 6 h and 24 h. **c** Expression level of zinc transporters encoding transcripts *zrt1* and *zrt2* evaluated in yeast cells in medium containing 30 μM zinc (experimental control) or under conditions of zinc depletion produced by adding the zinc chelator *N*,*N*,*N*,*N*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) at different pH values (4.5, 7.0 and 9.0) for 6 h. Data are presented as means ± SD from triplicate determinations. *$p \leq 0.05$, *t* test, in relation to the data obtained from the experimental controls

progress. The major evidence of iron capture by siderophores is supported by the stimulation of fungal growth in the presence of coprogen B and dimerum acid [47]. In silico analysis ha revealed the presence of genes putatively involved in hydroxamate-type siderophore biosynthesis and transport [45]. Corroborating these data, it has been demonstrated by chrome azurol S assays that *Paracoccidioides* is a hydroxamate producer (Silva et al., personal communication).

In fungi, copper and iron homeostasis must be intrinsically linked since iron uptake requires ferroxidases, which are members of the multicopper oxidase family. Copper is first reduced by plasma membrane ferrireductases and then Cu^{1+} is internalized via a high-affinity permease [48]. In *C. albicans*, the ferrireductase c*fl1*/*fre1* is transcriptionally regulated in response to both iron and copper availability [49], indicating that this ferrireductase is also important in copper uptake. Furthermore, the mutant for the *ctr1* copper transporter displays deficient growth in medium low in copper and iron indicating that in *C. albicans* iron and copper homeostasis are linked [50].

As observed for iron, copper plays fundamental roles in several aspects of *C. neoformans* biology. For instance, the production of melanin pigment is dependent on a copper oxidase [51] and the copper-containing ferroxidases are necessary for iron uptake [44]. Copper is probably reduced in *C. neoformans* by the same enzymes that reduce iron at the cell surface [52]. Two copper transporters have been described in *C. neoformans*. The *ctr1* null mutant shows reduced growth in copper-depleted medium. Ctr4, by contrast, is not essential for cryptococcal development in low-copper medium. However, mutant cells lacking both *ctr1* and *ctr4* transporters display severe growth defects in copper-deprived environments [53•].

The *Paracoccidioides* genome contains genes that encode redundant ferrireductases [45] as cited above, suggesting that these enzymes could function as iron and copper reductases. Furthermore a high-affinity copper transporter, *ctr3*, is present at increased levels during copper shortage (Fig. 1b), reinforcing the view that Ctr3 could be involved in copper uptake in *Paracoccidioides* [45].

The zinc uptake system in most fungi comprises just high-affinity and low-affinity permeases belonging to the ZIP family [54, 55], since this metal does not need to be reduced before internalization. Eight genes encoding proteins of the ZIP family of zinc transporters have been described in *A. fumigatus* [56]. Expression of *zrfA*, *zrfB* and *zrfC* is regulated by both zinc and pH [56, 57]. ZrfA and ZrfB function under acidic, zinc-limiting conditions. It seems that ZrfB is a high-affinity zinc permease, since a *zrfB* transcript was downregulated under high zinc conditions [58]. ZrfC participates in zinc uptake in a neutral or alkaline, zinc-poor environment [56]. Aspf2 putatively contributes to zinc uptake as a zinc-binding protein localized in the fungal periplasm [56].

Paracoccidioides possesses two zinc permease homologues (*zrt1* and *zrt2*), indicating a specific zinc uptake system [45]. The transcriptional response of *zrt* homologues to zinc starvation has been demonstrated by quantitative RT-PCR (Fig. 1c). The *zrt2* transcript, but not *zrt1* transcript, is highly expressed at neutral to alkaline pH during zinc depletion (Fig. 1c), as observed to *A. fumigatus* ZrfC.

Host Iron Sources

A high proportion of circulating iron in humans exists as heme in hemoglobin and hemin, iron-containing porphyrins. *C. albicans* shows hemolytic activity, and membrane proteins capable of binding hemin/hemoglobin have been identified [1•]. *C. albicans* Rbt5, a glycosylphosphatidylinositol-anchored protein, is the major hemoglobin receptor [59]. *hmx1* encodes an intracellular heme oxygenase that breaks down iron-protoporphyrin IX to α-biliverdin and is required for heme-iron utilization [60]. In silico analysis has revealed that *Paracoccidioides* genome contains genes that encode *hmx1* and *rbt5* homologues, suggesting effective hemoglobin iron acquisition by this fungus [45].

Intracellular iron in humans is bound to ferritin. *C. albicans* hyphae are able to obtain iron from ferritin using Als3 protein as a receptor. Als3 is a multifunctional protein since it can also function as an adhesin and an invasin [1•]. Transferrin is a glycoprotein that transports iron in serum. *C. albicans* is able to take up iron from transferrin by the reductive pathway using the ferrous permease Ftr1 and ferrireductase Fre10 [61]. In *H. capsulatum*, ferrireductase activity is higher in the presence of hemin and transferrin, suggesting that this fungus uses the ferrireductases to obtain iron during infection [34]. *Paracoccidioides* is likely to be able to take up iron from transferrin since the fungus has five genes encoding ferrireductases in the genome [45].

Regulation of Iron, Copper and Zinc Homeostasis in Pathogenic Fungi

Fungi have evolved sophisticated control mechanisms for maintenance of optimal levels of iron, copper and zinc. These mechanisms include the regulation of genes involved in metal ion uptake, utilization and storage. In fungi, metal ion homeostasis is mainly achieved by transcriptional regulation of gene expression. A group of iron-responsive GATA-type transcription factors mediates repression of iron acquisition genes in response to iron

sufficiency [62]. These regulators have a cysteine-rich central domain located between two zinc fingers, which directly interact with iron [63].

A range of genes and regulators involved in the response of *C. albicans* to iron starvation have been described [64]. During iron sufficiency, the GATA-type regulator Sfu1 downregulates expression of *arn1* and *hap43* genes encoding a siderophore transporter and a transcription factor, respectively [64, 65]. Under iron-limited conditions, the Cap2 protein represses the expression of Sfu1, activating genes of iron uptake pathways [66•]. Sef1 and Rim101 were also identified as positive regulators of iron acquisition in *C. albicans* [67, 68].

A GATA-type factor, Sre1, has been described in *H. capsulatum*. Sre1 acts as a negative regulator of siderophore biosynthesis genes in response to iron excess [69]. Sre1 also regulates cellular processes other than iron acquisition, such as optimal filamentous growth [70]. The same occurs with the Sre1 homologue SreB in *Blastomyces dermatitidis*. SreB regulates siderophore biosynthesis and also governs phase transition and cell growth at 22 °C in *B. dermatitidis* [71].

During iron sufficiency, high-affinity iron uptake systems (reductive pathway and siderophore production) are repressed by SreA in *A. fumigatus* [72]. During iron starvation, the *A. fumigatus* bZIP-type regulator HapX represses iron-dependent pathways, such as respiration, TCA cycle and heme biosynthesis, to save iron, and activates iron uptake by siderophores [73•]. Thus the transcription factors SreA and HapX act in opposite ways within the cell depending on the environmental iron status. During iron excess, SreA is activated and represses HapX expression, while during iron paucity, HapX represses the expression of SreA. In *A. fumigatus* the transcription factor AcuM stimulates iron acquisition via HapX induction and SreA repression [74].

C. neoformans Cir1 possesses a cysteine-rich domain, but unlike other fungal GATA-type iron regulators, it has only a zinc finger motif [75]. Cir1 is a global transcription factor which senses iron levels and regulates positively and negatively the transcriptional response [75, 76]. The expression of *C. neoformans* virulence attributes, such as capsule formation, growth at host temperature and melanin production, are also controlled by Cir1 [75]. A post-translational mechanism for the control of the amount of Cir1 suggests that under conditions of iron starvation Cir1 protein levels decrease. In contrast, iron availability promotes Cir1 stabilization and consequent repression of iron acquisition genes [77]. The transcriptional response to iron in *C. neoformans* is also regulated by HapX. As well as Cir1, HapX has both a positive and negative influence in the regulation of gene expression. However, unlike Cir1, HapX plays a modest role during infection and probably is important during environmental iron acquisition [78].

Proteomic analysis has revealed that during iron starvation the metabolic status of the pathogenic fungus *Paracoccidioides* is altered. Glycolysis is upregulated while iron-consuming pathways, such as tricarboxylic and glyoxylate cycles, are repressed. It has been demonstrated that under iron-limited conditions the transcript level of the HapX increases [9]. However, the regulatory mechanisms that orchestrate the global changes in response to iron availability in this fungus have not been described and are the subject of current investigation.

Regulatory mechanisms that respond to copper availability among pathogenic fungi have been best studied in *C. neoformans*. The copper-dependent transcription factor, Cuf1, has a cysteine-rich sequence, which contains a putative copper binding motif [79]. Under conditions of copper limitation, Cuf1 induces the expression of the copper transporter encoding genes *ctr1* and *ctr4*. During copper excess, the metallothionein (copper binding and detoxifying protein) genes *cmt1* and *cmt2* are induced by Cuf1 [53•]. A copper-dependent transcriptional regulator, Mac1, found in *C. albicans*, is transcriptionally autoregulated and activates the expression of *ctr1* and *fre7* genes during copper paucity [80].

Although regulation of copper homeostasis has not yet been described in *Paracoccidioides*, studies have revealed that the high-affinity copper transporter, Ctr3, is upregulated under infection conditions [16, 81] and is also a potential adhesin [82]. Analysis of genes potentially involved in copper regulation has demonstrated the presence of a copper metalloregulatory transcription factor, Mac1, in *Paracoccidioides* [45], thus prompting further investigation.

As for copper, the regulation of zinc homeostasis in pathogenic fungi is poorly understood. A zinc-responsive transcription factor has been described in *C. albicans* [83]. The Zap1/Csr1 factor induces expression of the plasma membrane zinc transporters, Zrt1 and Zrt2, and is also involved in the control of efficient hyphae and biofilm matrix formation and production of quorum sensing molecules [83–86].

In *A. fumigatus* the expression of *zrfA* and *zrfB* is induced by the ZafA zinc-responsive transcriptional activator under zinc-limited conditions [87]. However, under neutral zinc-limited conditions, the expression of these transporters is repressed by the transcriptional regulator PacC [57]. Additionally, the expression of *zrfC* is upregulated by ZafA under zinc-limited conditions regardless of the environmental pH and downregulated by PacC under acidic growth conditions [56].

Although zinc metabolism regulation is not well understood in *Paracoccidioides* and *Cryptococcus* pathogens, a homologue of Zap1 zinc-regulated transcription factor has been found in their genomes [45]. Studies focusing on this potential transcriptional regulator are in progress.

Virulence

Despite the close correlation between metal availability and virulence, the fungal genes related to iron acquisition/homeostasis directly involved in host infection are poorly described [88, 89]. Table 1 lists metal acquisition/homeostasis genes and provides information on their role in virulence.

In *C. albicans,* mutants lacking the iron permease coding gene *ftr1* lose virulence [90]. The involvement of genes related to siderophore uptake in virulence was not observed [27, 28]. Moreover virulence attenuation was observed in *C. albicans* mutants lacking the iron-responsive transcriptional regulators *hap43*, *aft2*, *sef1* and *cap2* and the heme oxygenase coding gene *hmx1* [65, 66•, 91, 92, 93•], indicating that all these genes are important during *C. albicans* infection.

Table 1 Roles of genes involved in metal homeostasis and virulence of pathogenic fungi

Gene	Protein function	Role in virulence	Reference
Candida albicans			
ftr1	High-affinity iron permease	The *ftr1Δ* mutatation results in complete loss of the capacity to damage epithelial cells in vitro. Moreover mutants lacking *ftr1* are avirulent in mice infected with *C. albicans* during the early stationary phase	[90]
hap43	Transcriptional regulator	Deletion of *hap43* attenuates the virulence of *C. albicans* in a mouse model of disseminated infection	[65]
cap2	Transcriptional regulator	The *cap2Δ* mutant shows delayed virulence in a mouse model of *C. albicans* infection	[66•]
aft2	Transcriptional regulator	The *aft2Δ*/*aft2Δ* strain shows attenuated virulence in mice with disseminated infection	[91]
hmx1	Heme oxygenase	The homozygous mutant *hmx1Δ*/*hmx1Δ* shows reduced virulence in mice with disseminated infection	[93•]
sef1	Transcriptional regulator	The *sef1Δ* mutant shows significantly decreased virulence compared to wild-type strain in BALB/c mice with disseminated infection	[92]
Aspergillus			
sidA	Involved in siderophore biosynthesis	The *sidAΔ* mutant shows completely attenuated virulence in mice	[37, 94]
sidD	Involved in siderophore biosynthesis	The *sidDΔ* mutant shows severely attenuated virulence in neutropenic mice	[95]
sidF	Involved in siderophore biosynthesis	The *sidFΔ* mutant shows attenuated virulence in neutropenic mice infected intranasally	[95]
hapX	Transcriptional regulator	The *hapXΔ* mutant shows attenuated virulence in immunosuppressed mice	[73•]
acuM	Transcriptional regulator	The *acuMΔ* mutant shows attenuated virulence in neutropenic mice with disseminated infection and invasive pulmonary aspergillosis, resulting in significantly delayed mortality	[74]
zafA	Transcriptional regulator	The *zafAΔ* mutant shows reduced virulence in immunosuppressed mice infected intranasally	[87]
pacC	Transcriptional regulator	The *Aspergillus nidulans pacCΔ* mutant shows attenuated virulence in immunosuppressed mice	[99]
Histoplasma capsulatum			
sid1	Involved in siderophore biosynthesis	The *sid1Δ* strain shows a significant defect in pulmonary colonization compared to wild-type cells in mice infected intranasally	[31]
Cryptococcus neoformans			
cft1	High-affinity iron permease	The *cft1Δ* mutant shows attenuated virulence and reduced fungal burden	[43]
cfo1	Ferroxidase	The *cfo1Δ* mutant shows significantly attenuated virulence in mice	[44]
cir1	Transcriptional regulator	The *cir1Δ* mutant is avirulent in mice	[75]
ctr4	Copper transporter	The *ctr4Δ* null mutant shows reduced spread to tissues and is completely avirulent in infected mice	[79]
ctr1	Copper transporter	*ctr1Δ* mutant presented reduced melanization, reduced capsule and enhanced phagocytosis index	[97]
clc	Chloride channel	The *clc-A* mutant shows attenuated virulence in a mouse cryptococcosis model, since *clc-A* plays a role in capsule and laccase expression, important virulence factors	[98]
ccc2	Copper transporter	*ccc2* mutation results in absence of melanization, an important virulence factor	[18]
cuf1	Transcriptional regulator	The deletion of *cuf1* results in attenuated virulence in a mouse model of cryptococcosis	[79]

In *Aspergillus*, mutants lacking genes involved in siderophore biosynthesis (*sid*) have pointed to the relevance of this iron uptake pathway to virulence. *sidA* deletion in *A. fumigatus* abolishes siderophore biosynthesis and completely attenuates virulence [37, 94]. A similar effect was observed in mutants lacking the *sidA* homologue, *sid1*, in *H. capsulatum* [31]. Genes related to fusarinine C and triacetylfusarinine C production, *sidD* and *sidF,* significantly affect *A. fumigatus* virulence [95]. Furthermore, the deletion of the transcriptional regulators *hapX* and *acuM* causes significant attenuation of virulence in a murine model of infection [73•, 74].

The role of iron acquisition in *Cryptococcus* virulence has been extensively studied in recent years. Almost all *C. neoformans* genes involved in iron homeostasis that have been analyzed are related to cryptococcal virulence, as evaluated in murine models of cryptococcosis using null gene mutants [44, 75–78]. When considering the iron permeases Cft1 and Cft2, virulence attenuation and reduced fungal burden are observed in the *cft1* gene null mutant and in the *cft1*/*cft2* double mutant, but not in the *cft2* knockout strain [76]. The ferroxidase Cfo1 also plays a role in virulence, since null mutants are also attenuated in virulence [44]. In addition, *C. neoformans cir1* null mutants are completely avirulent in murine models of cryptococcosis, which is consistent with the hypocapsular phenotype and its reduced ability to proliferate at 37 °C [75].

Regarding copper, a pivotal biological role of this metal has been already described for *C. neoformans*, since two proteins involved in virulence, Cu/Zn-Sod1 and laccase, require copper as a cofactor for activity [51, 96]. The *ctr4* gene is expressed during infection and is directly associated with virulence, since null mutants show reduced spread to tissues and are completely avirulent in infected mice [79]. The *ctr1* gene also is associated with virulence, as mutants show reduced melanization, a reduced capsule, and an enhanced phagocytosis index [97]. Moreover, *C. neoformans* strains with mutation in genes encoding copper distribution transporters, such as the *clc* chloride channel and the *ccc2* secretory transporter, show reduced virulence or reduced expression of virulence factors [18, 98]. Furthermore, in a mouse model of cryptococcosis, transcriptional regulator *cuf1* null mutants display disruption of several virulence-linked characteristics, such as reduced laccase activity, severe growth defects in low-copper medium, and reduced virulence [79]. Cuf1 is required for infection of the brain but not of the lung in mouse models of cryptococcosis, suggesting that copper is limiting in neurological infections [79].

Studies investigating the role of zinc during pathogenesis are sparse. Investigations are restricted to the importance of zinc-responsive transcription factors during pathogenesis, such as *zaf*A. In *A. fumigatus,* ZafA regulates zinc homeostasis, and mutants lacking this gene show reduced virulence in mice [87]. A similar result was found for the pH-responsive transcriptional factor *pac*C, that plays an essential role in pulmonary infection by *A. fumigatus* [56, 99].

Conclusions

Iron, copper and zinc acquisition is a critical determinant in fungal pathogenesis. To circumvent metal sequestration by the host during infection, pathogenic fungi have evolved mechanisms of metal acquisition. Understanding of the roles of iron, copper and zinc in fungal pathogenicity has advanced in recent years. As discussed above, fungi demonstrate remarkable flexibility in gaining access to and utilizing the transition metals iron, copper and zinc. The sophisticated acquisition and regulation of homeostasis of these metals are surely an efficient weapon facilitating fungal survival within the human host, and represent an important component of virulence.

Acknowledgments Work at Universidade Federal de Goiás and Universidade Federal do Rio Grande do Sul was supported by grants from Financiadora de Estudos e Projetos (FINEP- 01.07.0552.00) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-558923/2009-7 and 478591/2010-1). E.F.L.C.B. and M.G.S.B. are supported by doctoral fellowships from Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). A.F.A.P. and L.K. are supported by postdoctoral fellowships from CAPES. We apologize to colleagues whose work we were not able to cite due to space limitations.

Disclosure E.F. Bailão: grants from Capes, CNPq and FINEP; A.F.A. Parente: grants from CNPq, CAPES and FINEP; J.A. Parente: none; M. Garcia Silva-Bailão: grant from CAPES; K. Castro: grants from FINEP and CNPq; L. Kmetzsch: grants from CAPES, CNPq and FINEP; C. Staats: grants from CNPq and FINEP; A. Schrank: grants from CNPq and FINEP; M. Vainstein: grants from CNPq and FINEP; C. Borges: grants from CNPq and FINEP; A. Bailão: grant from CNPq; C.M. Soares: grants from FINEP and CNPq.

References

Papers of particular interest, published recently, have been highlighted as:
- • Of importance

1. • Almeida RS, Wilson D, Hube B. Candida albicans iron acquisition within the host. FEMS Yeast Res. 2009;9:1000–12. *This article reviews the iron sources used by* C. albicans *during infection in the human host, that are xenosiderophores, hemoglobin, transferrin and ferritin*.
2. • Nairz M, Schroll A, Sonnweber T, Weiss G. The struggle for iron – a metal at the host-pathogen interface. Cell Microbiol. 2010;12:1691–702. *This article reviews the iron functions at the host–pathogen interface since mammalian and microbial cells have an essential demand for the metal. Microbial iron acquisition pathways are attractive targets for the development of new antimicrobial drugs*.

3. Theurl I, Fritsche G, Ludwiczek S, et al. The macrophage: a cellular factory at the interphase between iron and immunity for the control of infections. Biometals. 2005;18:359–67.
4. Ibrahim AS, Gebermariam T, Fu Y, et al. The iron chelator deferasirox protects mice from mucormycosis through iron starvation. J Clin Invest. 2007;117:2649–57.
5. Kaplan CD, Kaplan J. Iron acquisition and transcriptional regulation. Chem Rev. 2009;109:4536–52.
6. Kim BE, Nevitt T, Thiele DJ. Mechanisms for copper acquisition, distribution and regulation. Nat Chem Biol. 2008;4:176–85.
7. Lulloff SJ, Hahn BL, Sohnle PG. Fungal susceptibility to zinc deprivation. J Lab Clin Med. 2004;144:208–14.
8. Barluzzi R, Saleppico S, Nocentini A, et al. Iron overload exacerbates experimental meningoencephalitis by Cryptococcus neoformans. J Neuroimmunol. 2002;132:140–6.
9. Parente AF, Bailão AM, Borges CL, et al. Proteomic analysis reveals that iron availability alters the metabolic status of the pathogenic fungus Paracoccidioides brasiliensis. PLoS One. 2011;6:e22810.
10. Nevitt T. War-Fe-re: iron at the core of fungal virulence and host immunity. Biometals. 2011;24:547–58.
11. Al-Sheikh H. Effect of lactoferrin and iron on the growth of human pathogenic Candida species. Pak J Biol Sci. 2009;12:91–4.
12. Weinberg ED. Iron loading and disease surveillance. Emerg Infect Dis. 1999;5:346–52.
13. Weiss G. Modification of iron regulation by the inflammatory response. Best Pract Res Clin Haematol. 2005;18:183–201.
14. Winters MS, Chan Q, Caruso JA, Deepe Jr GS. Metallomic analysis of macrophages infected with Histoplasma capsulatum reveals a fundamental role for zinc in host defenses. J Infect Dis. 2010;202:1136–45.
15. Byrd TF, Horwitz MA. Regulation of transferrin receptor expression and ferritin content in human mononuclear phagocytes. Coordinate upregulation by iron transferrin and downregulation by interferon gamma. J Clin Invest. 1993;91:969–76.
16. Bailão AM, Schrank A, Borges CL, et al. Differential gene expression by Paracoccidioides brasiliensis in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. Microbes Infect. 2006;8:2686–97.
17. Samanovic MI, Ding C, Thiele DJ, Darwin KH. Copper in microbial pathogenesis: meddling with the metal. Cell Host Microbe. 2012;11:106–15.
18. Walton FJ, Idnurm A, Heitman J. Novel gene functions required for melanization of the human pathogen Cryptococcus neoformans. Mol Microbiol. 2005;57:1381–96.
19. Beisel WR. Herman Award Lecture, 1995: infection-induced malnutrition – from cholera to cytokines. Am J Clin Nutr. 1995;62:813–9.
20. Sohnle PG, Collins-Lech C, Wiessner JH. The zinc-reversible antimicrobial activity of neutrophil lysates and abscess fluid supernatants. J Infect Dis. 1991;164:137–42.
21. Haas H, Eisendle M, Turgeon BG. Siderophores in fungal physiology and virulence. Annu Rev Phytopathol. 2008;46:149–87.
22. Harrington JM, Crumbliss AL. The redox hypothesis in siderophore-mediated iron uptake. Biometals. 2009;22:679–89.
23. Renshaw JC, Robson GD, Trinci APJ, et al. Fungal siderophores: structures, functions and applications. Mycol Res. 2002;106:1123–42.
24. Jacobson ES, Petro MJ. Extracellular iron chelation in Cryptococcus neoformans. J Med Vet Mycol. 1987;25:415–8.
25. Jeeves RE, Mason RP, Woodacre A, Cashmore AM. Ferric reductase genes involved in high-affinity iron uptake are differentially regulated in yeast and hyphae of Candida albicans. Yeast. 2011;28:629–44.
26. • Ziegler L, Terzulli A, Gaur R, et al. Functional characterization of the ferroxidase, permease high-affinity iron transport complex from Candida albicans. Mol Microbiol. 2011;81:473–85. *This article demonstrates that Fe trafficking in* C. albicans *involves a complex Fet34-Ftr1 using* S. cerevisiae *as host for the functional expression of the* C. albicans *Fe-uptake proteins*.
27. Heymann P, Gerads M, Schaller M, et al. The siderophore iron transporter of Candida albicans (Sit1p/Arn1p) mediates uptake of ferrichrome-type siderophores and is required for epithelial invasion. Infect Immun. 2002;70:5246–55.
28. Hu CJ, Bai C, Zheng XD, et al. Characterization and functional analysis of the siderophore-iron transporter CaArn1p in Candida albicans. J Biol Chem. 2002;277:30598–605.
29. Zarnowski R, Cooper KG, Brunold LS, et al. Histoplasma capsulatum secreted gamma-glutamyltransferase reduces iron by generating an efficient ferric reductant. Mol Microbiol. 2008;70:352–68.
30. Timmerman MM, Woods JP. Ferric reduction is a potential iron acquisition mechanism for Histoplasma capsulatum. Infect Immun. 1999;67:6403–8.
31. Hwang LH, Mayfield JA, Rine J, Sil A. Histoplasma requires SID1, a member of an iron-regulated siderophore gene cluster, for host colonization. PLoS Pathog. 2008;4:e1000044.
32. Hilty J, George Smulian A, Newman SL. Histoplasma capsulatum utilizes siderophores for intracellular iron acquisition in macrophages. Med Mycol. 2011;49:633–42.
33. Howard DH, Rafie R, Tiwari A, Faull KF. Hydroxamate siderophores of Histoplasma capsulatum. Infect Immun. 2000;68:2338–43.
34. Timmerman MM, Woods JP. Potential role for extracellular glutathione-dependent ferric reductase in utilization of environmental and host ferric compounds by Histoplasma capsulatum. Infect Immun. 2001;69:7671–8.
35. Blatzer M, Binder U, Haas H. The metalloreductase FreB is involved in adaptation of Aspergillus fumigatus to iron starvation. Fungal Genet Biol. 2011;48:1027–33.
36. Charlang G, Ng B, Horowitz NH, Horowitz RM. Cellular and extracellular siderophores of Aspergillus nidulans and Penicillium chrysogenum. Mol Cell Biol. 1981;1:94–100.
37. Schrettl M, Bignell E, Kragl C, et al. Siderophore biosynthesis but not reductive iron assimilation is essential for Aspergillus fumigatus virulence. J Exp Med. 2004;200:1213–9.
38. Eisendle M, Schrettl M, Kragl C, et al. The intracellular siderophore ferricrocin is involved in iron storage, oxidative-stress resistance, germination, and sexual development in Aspergillus nidulans. Eukaryot Cell. 2006;5:1596–603.
39. Haas H. Molecular genetics of fungal siderophore biosynthesis and uptake: the role of siderophores in iron uptake and storage. Appl Microbiol Biotechnol. 2003;62:316–30.
40. Kragl C, Schrettl M, Abt B, et al. EstB-mediated hydrolysis of the siderophore triacetylfusarinine C optimizes iron uptake of Aspergillus fumigatus. Eukaryot Cell. 2007;6:1278–85.
41. • Haas H. Iron – a key nexus in the virulence of Aspergillus fumigatus. Front Microbiol. 2012;3:28. *This article reviews iron homeostasis and its participation in virulence in* Aspergillus *genus. The knowledge of the iron handling between host and fungus might improve therapy and diagnosis of fungal infections*.
42. Jacobson ES, Goodner AP, Nyhus KJ. Ferrous iron uptake in Cryptococcus neoformans. Infect Immun. 1998;66:4169–75.
43. Jung WH, Kronstad JW. Iron and fungal pathogenesis: a case study with Cryptococcus neoformans. Cell Microbiol. 2008;10:277–84.
44. Jung WH, Hu G, Kuo W, Kronstad JW. Role of ferroxidases in iron uptake and virulence of Cryptococcus neoformans. Eukaryot Cell. 2009;8:1511–20.
45. Silva MG, Schrank A, Bailão EF, et al. The homeostasis of iron, copper, and zinc in Paracoccidioides brasiliensis, Cryptococcus neoformans var. grubii, and Cryptococcus gattii: a comparative analysis. Front Microbiol. 2011;2:49.
46. Zarnowski R, Woods JP. Glutathione-dependent extracellular ferric reductase activities in dimorphic zoopathogenic fungi. Microbiology. 2005;151:2233–40.

47. Castaneda E, Brummer E, Perlman AM, et al. A culture medium for Paracoccidioides brasiliensis with high plating efficiency, and the effect of siderophores. J Med Vet Mycol. 1988;26:351–8.
48. Knight SA, Labbe S, Kwon LF, et al. A widespread transposable element masks expression of a yeast copper transport gene. Genes Dev. 1996;10:1917–29.
49. Hammacott JE, Williams PH, Cashmore AM. Candida albicans CFL1 encodes a functional ferric reductase activity that can rescue a Saccharomyces cerevisiae fre1 mutant. Microbiology. 2000;146 (Pt 4):869–76.
50. Marvin ME, Williams PH, Cashmore AM. The Candida albicans CTR1 gene encodes a functional copper transporter. Microbiology. 2003;149:1461–74.
51. Williamson PR. Biochemical and molecular characterization of the diphenol oxidase of Cryptococcus neoformans: identification as a laccase. J Bacteriol. 1994;176:656–64.
52. Nyhus KJ, Jacobson ES. Genetic and physiologic characterization of ferric/cupric reductase constitutive mutants of Cryptococcus neoformans. Infect Immun. 1999;67:2357–65.
53. • Ding C, Yin J, Tovar EM, et al. The copper regulon of the human fungal pathogen Cryptococcus neoformans H99. Mol Microbiol. 2011;81:1560–76. *This article describes a new* C. neoformans *Cu transporter, Ctr1, and some targets of the metalloregulatory transcription factor Cuf1*.
54. Zhao H, Eide D. The ZRT2 gene encodes the low affinity zinc transporter in Saccharomyces cerevisiae. J Biol Chem. 1996;271:23203–10.
55. Zhao H, Eide D. The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc Natl Acad Sci U S A. 1996;93:2454–8.
56. Amich J, Vicentefranqueira R, Leal F, Calera JA. Aspergillus fumigatus survival in alkaline and extreme zinc-limiting environments relies on the induction of a zinc homeostasis system encoded by the zrfC and aspf2 genes. Eukaryot Cell. 2009;9:424–37.
57. Amich J, Leal F, Calera JA. Repression of the acid ZrfA/ZrfB zinc-uptake system of Aspergillus fumigatus mediated by PacC under neutral, zinc-limiting conditions. Int Microbiol. 2009;12:39–47.
58. Vicentefranqueira R, Moreno MA, Leal F, Calera JA. The zrfA and zrfB genes of Aspergillus fumigatus encode the zinc transporter proteins of a zinc uptake system induced in an acid, zinc-depleted environment. Eukaryot Cell. 2005;4:837–48.
59. Weissman Z, Kornitzer D. A family of Candida cell surface haem-binding proteins involved in haemin and haemoglobin-iron utilization. Mol Microbiol. 2004;53:1209–20.
60. Santos R, Buisson N, Knight S, et al. Haemin uptake and use as an iron source by Candida albicans: role of CaHMX1-encoded haem oxygenase. Microbiology. 2003;149:579–88.
61. Knight SA, Vilaire G, Lesuisse E, Dancis A. Iron acquisition from transferrin by Candida albicans depends on the reductive pathway. Infect Immun. 2005;73:5482–92.
62. Rutherford JC, Bird AJ. Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. Eukaryot Cell. 2004;3:1–13.
63. Scazzocchio C. The fungal GATA factors. Curr Opin Microbiol. 2000;3:126–31.
64. Lan CY, Rodarte G, Murillo LA, et al. Regulatory networks affected by iron availability in Candida albicans. Mol Microbiol. 2004;53:1451–69.
65. Hsu PC, Yang CY, Lan CY. Candida albicans Hap43 is a repressor induced under low-iron conditions and is essential for iron-responsive transcriptional regulation and virulence. Eukaryot Cell. 2011;10:207–25.
66. • Singh RP, Prasad HK, Sinha I, et al. Cap2-HAP complex is a critical transcriptional regulator that has dual but contrasting roles in regulation of iron homeostasis in Candida albicans. J Biol Chem. 2011;286:25154–70. *This article describes the roles performed by Cap2 under iron limiting conditions: activation of genes in iron uptake pathways and repression of iron-utilizing and iron-storage genes*.
67. Homann OR, Dea J, Noble SM, Johnson AD. A phenotypic profile of the Candida albicans regulatory network. PLoS Genet. 2009;5: e1000783.
68. Baek YU, Li M, Davis DA. Candida albicans ferric reductases are differentially regulated in response to distinct forms of iron limitation by the Rim101 and CBF transcription factors. Eukaryot Cell. 2008;7:1168–79.
69. Chao LY, Marletta MA, Rine J. Sre1, an iron-modulated GATA DNA-binding protein of iron-uptake genes in the fungal pathogen Histoplasma capsulatum. Biochemistry. 2008;47:7274–83.
70. Hwang LH, Seth E, Gilmore SA, Sil A. SRE1 regulates iron-dependent and -independent pathways in the fungal pathogen Histoplasma capsulatum. Eukaryot Cell. 2012;11:16–25.
71. Gauthier GM, Sullivan TD, Gallardo SS, et al. SREB, a GATA transcription factor that directs disparate fates in Blastomyces dermatitidis including morphogenesis and siderophore biosynthesis. PLoS Pathog. 2010;6:e1000846.
72. Schrettl M, Kim HS, Eisendle M, et al. SreA-mediated iron regulation in Aspergillus fumigatus. Mol Microbiol. 2008;70:27–43.
73. • Schrettl M, Beckmann N, Varga J, et al. HapX-mediated adaption to iron starvation is crucial for virulence of Aspergillus fumigatus. PLoS Pathog. 2010;6:e1001124. *This article describes the functions of transcriptional regulator HapX of* A. fumigatus*, which is important to fungus adaptation in iron starvation conditions and is crucial for virulence in a murine model of infection*.
74. Liu H, Gravelat FN, Chiang LY, et al. Aspergillus fumigatus AcuM regulates both iron acquisition and gluconeogenesis. Mol Microbiol. 2010;78:1038–54.
75. Jung WH, Sham A, White R, Kronstad JW. Iron regulation of the major virulence factors in the AIDS-associated pathogen Cryptococcus neoformans. PLoS Biol. 2006;4:e410.
76. Jung WH, Sham A, Lian T, et al. Iron source preference and regulation of iron uptake in Cryptococcus neoformans. PLoS Pathog. 2008;4:e45.
77. Jung WH, Kronstad JW. Iron influences the abundance of the iron regulatory protein Cir1 in the fungal pathogen Cryptococcus neoformans. FEBS Lett. 2011;585:3342–7.
78. Jung WH, Saikia S, Hu G, et al. HapX positively and negatively regulates the transcriptional response to iron deprivation in Cryptococcus neoformans. PLoS Pathog. 2010;6:e1001209.
79. Waterman SR, Hacham M, Hu G, et al. Role of a CUF1/CTR4 copper regulatory axis in the virulence of Cryptococcus neoformans. J Clin Invest. 2007;117:794–802.
80. Woodacre A, Mason RP, Jeeves RE, Cashmore AM. Copper-dependent transcriptional regulation by Candida albicans Mac1p. Microbiology. 2008;154:1502–12.
81. Dantas SF, Vieira de Rezende TC, Bailão AM, et al. Identification and characterization of antigenic proteins potentially expressed during the infectious process of Paracoccidioides brasiliensis. Microbes Infect. 2009;11:895–903.
82. Bailão AM, Nogueira SV. Rondon Caixeta Bonfim SM, et al. Comparative transcriptome analysis of Paracoccidioides brasiliensis during in vitro adhesion to type I collagen and fibronectin: identification of potential adhesins. Res Microbiol. 2012;163:182–91.
83. Kim MJ, Kil M, Jung JH, Kim J. Roles of zinc-responsive transcription factor Csr1 in filamentous growth of the pathogenic yeast Candida albicans. J Microbiol Biotechnol. 2008;18:242–7.
84. Nobile CJ, Nett JE, Hernday AD, et al. Biofilm matrix regulation by Candida albicans Zap1. PLoS Biol. 2009;7:e1000133.
85. Finkel JS, Xu W, Huang D, et al. Portrait of Candida albicans adherence regulators. PLoS Pathog. 2012;8:e1002525.

86. Ganguly S, Bishop AC, Xu W, et al. Zap1 control of cell-cell signaling in Candida albicans biofilms. Eukaryot Cell. 2011;10:1448–54.
87. Moreno MA, Ibrahim-Granet O, Vicentefranqueira R, et al. The regulation of zinc homeostasis by the ZafA transcriptional activator is essential for Aspergillus fumigatus virulence. Mol Microbiol. 2007;64:1182–97.
88. Kehl-Fie TE, Skaar EP. Nutritional immunity beyond iron: a role for manganese and zinc. Curr Opin Chem Biol. 2010;14:218–24.
89. Weinberg ED. Iron availability and infection. Biochim Biophys Acta. 2009;1790:600–5.
90. Ramanan N, Wang Y. A high-affinity iron permease essential for Candida albicans virulence. Science. 2000;288:1062–4.
91. Liang Y, Wei D, Wang H, et al. Role of Candida albicans Aft2p transcription factor in ferric reductase activity, morphogenesis and virulence. Microbiology. 2010;156:2912–9.
92. Chen C, Pande K, French SD, et al. An iron homeostasis regulatory circuit with reciprocal roles in Candida albicans commensalism and pathogenesis. Cell Host Microbe. 2011;10:118–35.
93. • Navarathna DH, Roberts DD. Candida albicans heme oxygenase and its product CO contribute to pathogenesis of candidemia and alter systemic chemokine and cytokine expression. Free Radic Biol Med. 2010;49:1561–73. *This article describes the heme oxygenase Hmx1 as a virulence factor in* C. albicans*. The authors observed that mutants lacking hmx1 gene were not affected during initial kidney colonization, but Hmx1 absence clearly affects infection progression*.
94. Hissen AH, Wan AN, Warwas ML, et al. The Aspergillus fumigatus siderophore biosynthetic gene sidA, encoding L-ornithine N5-oxygenase, is required for virulence. Infect Immun. 2005;73:5493–503.
95. Schrettl M, Bignell E, Kragl C, et al. Distinct roles for intra- and extracellular siderophores during Aspergillus fumigatus infection. PLoS Pathog. 2007;3:1195–207.
96. Cox GM, Harrison TS, McDade HC, et al. Superoxide dismutase influences the virulence of Cryptococcus neoformans by affecting growth within macrophages. Infect Immun. 2003;71:173–80.
97. Chun CD, Madhani HD. Ctr2 links copper homeostasis to polysaccharide capsule formation and phagocytosis inhibition in the human fungal pathogen Cryptococcus neoformans. PloS One. 2010;5.
98. Zhu X, Williamson PR. A CLC-type chloride channel gene is required for laccase activity and virulence in Cryptococcus neoformans. Mol Microbiol. 2003;50:1271–81.
99. Bignell E, Negrete-Urtasun S, Calcagno AM, et al. The Aspergillus pH-responsive transcription factor PacC regulates virulence. Mol Microbiol. 2005;55:1072–84.

HEMOGLOBIN UPTAKE BY *Paracoccidioides* spp. IS RECEPTOR-MEDIATED

Elisa Flávia Luiz Cardoso Bailão[1,2,3], Juliana Alves Parente[1], Laurine Lacerda Pigosso[1], Kelly Pacheco de Castro[1], Fernanda Lopes Fonseca[4], Mirelle Garcia Silva-Bailão[1,3], Sônia Nair Báo[5], Alexandre Melo Bailão[1], Marcio L. Rodrigues[4,6], Orville Hernandez[7,8], Juan G. McEwen[7,9] and Célia Maria de Almeida Soares[1#].

[1]Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, Goiás, Brazil;
[2]Unidade Universitária de Iporá, Universidade Estadual de Goiás, Iporá, Goiás, Brazil;
[3]Programa de Pós Graduação em Patologia Molecular, Faculdade de Medicina, Universidade de Brasília, Brasília, Distrito Federal, Brazil;
[4]Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Brazil;
[5]Laboratório de Microscopia Eletrônica, Universidade de Brasília, Distrito Federal, Brazil;
[6]Fundação Oswaldo Cruz – Fiocruz, Centro de Desenvolvimento Tecnológico em Saúde (CDTS), Rio de Janeiro, Brazil.
[7]Unidad de Biología Celular y Molecular, Corporación para Investigaciones Biológicas (CIB), Medellín, Colombia.
[8]Facultad de Ciencias de la Salud, Institución Universitaria Colegio Mayor de Antioquia, Medellín, Colombia.
[9]Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia.

[#] Corresponding author:
Célia Maria de Almeida Soares
Laboratório de Biologia Molecular
Instituto de Ciências Biológicas II
Campus Samambaia
Universidade Federal de Goiás
74690-900
Goiânia, GO - Brazil
e-mail: cmasoares@gmail.com/ Tel./fax: +55 62 3521 1110.

Keywords: iron physiology, Rbt5, proteome, antisense RNA technology and *Agrobacterium tumefaciens*-mediated transformation.