UNIVERSIDADE FEDERAL DE GOIÁS

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA TROPICAL E SAÚDE PÚBLICA

GABRIEL BRUM TRISTÃO

Estudo da homeostase de cobre no fungo patogênico *Histoplasma capsulatum*

Goiânia

2018

sistema de bibliotecas ufg

PRPG
PRÓ-REITORIA DE PÓS-GRADUAÇÃO

UFG

TERMO DE CIÊNCIA E DE AUTORIZAÇÃO PARA DISPONIBILIZAR VERSÕES ELETRÔNICAS DE TESES E DISSERTAÇÕES NA BIBLIOTECA DIGITAL DA UFG

Na qualidade de titular dos direitos de autor, autorizo a Universidade Federal de Goiás (UFG) a disponibilizar, gratuitamente, por meio da Biblioteca Digital de Teses e Dissertações (BDTD/UFG), regulamentada pela Resolução CEPEC nº 832/2007, sem ressarcimento dos direitos autorais, de acordo com a Lei nº 9610/98, o documento conforme permissões assinaladas abaixo, para fins de leitura, impressão e/ou *download*, a título de divulgação da produção científica brasileira, a partir desta data.

1. Identificação do material bibliográfico: **[ ] Dissertação** **[x] Tese**

2. Identificação da Tese ou Dissertação:

Nome completo do autor: Gabriel Brum Tristão

Título do trabalho: Estudo da homeostase de cobre no fungo patogênico *Histoplasma capsulatum*

3. Informações de acesso ao documento:

Concorda com a liberação total do documento [X] SIM [ ] NÃO

Data: 16 de Outubro de 2018.

- Orientador(a) -

- Autor (a)-

Versão atualizada em setembro de 2017

GABRIEL BRUM TRISTÃO

Estudo da homeostase de cobre no fungo patogênico *Histoplasma capsulatum*

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Medicina Tropical e Saúde Pública da Universidade Federal de Goiás para obtenção do Título de Doutor em Medicina Tropical e Saúde Pública.

Orientador: Dr. Alexandre Melo Bailão

Goiânia

2018

Ficha de identificação da obra elaborada pelo autor, através do
Programa de Geração Automática do Sistema de Bibliotecas da UFG.

Brum Tristão, Gabriel
Estudo da homeostase de cobre no fungo patogênico Histoplasma capsulatum [manuscrito] / Gabriel Brum Tristão. - 2018.
100 f.

Orientador: Prof. Dr. Alexandre Melo Bailão .
Tese (Doutorado) - Universidade Federal de Goiás, Instituto de Ciências Biológicas (ICB), Programa de Pós-Graduação em Medicina Tropical e Saúde Pública, Goiânia, 2018.
Bibliografia. Anexos.
Inclui tabelas, lista de figuras.

1. Homeostase. 2. Cobre. 3. Metais. 4. Histoplasma. I. , Alexandre Melo Bailão, orient. II. Título.

CDU 579

UNIVERSIDADE FEDERAL DE GOIÁS
INSTITUTO DE PATOLOGIA TROPICAL E SAÚDE PÚBLICA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA TROPICAL E SAÚDE PÚBLICA
Rua 235, s/n – Setor Universitário - Goiânia/GO – CEP: 74.605-050
Fones: (62) 3209.6362 - 3209.6102 – Fax: (62) 3209.6363 - e-mail : ppgmtsp.ufg@gmail.com

ATA DA REUNIÃO DA BANCA EXAMINADORA DA DEFESA DE GABRIEL BRUM TRISTÃO - Aos dezoito dias do mês de julho do ano de 2018 (18/07/2018), às 13:00 horas, reuniram-se os componentes da Banca Examinadora: Profs. Drs. ALEXANDRE MELO BAILÃO, IRAN MALAVAZI, PATRÍCIA DE SOUSA LIMA, ELISA FLÁVIA LUIZ CARDOSO BAILÃO e JULIANO DOMIRACI PACCEZ, para, sob a presidência do primeiro, e em sessão pública realizada no INSTITUTO DE PATOLOGIA TROPICAL E SAÚDE PÚBLICA, procederem à avaliação da defesa de tese intitulada: **"ESTUDO DA HOMEOSTASE DE COBRE NO FUNGO PATOGÊNICO *Histoplasma capsulatum*"** em nível de **DOUTORADO**, área de concentração em **MICROBIOLOGIA**, de autoria de **GABRIEL BRUM TRISTÃO** discente do PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA TROPICAL E SAÚDE PÚBLICA, da Universidade Federal de Goiás. A sessão foi aberta pelo Orientador Prof. Dr. ALEXANDRE MELO BAILÃO, que fez a apresentação formal dos membros da Banca e orientou o Candidato sobre como utilizar o tempo durante a apresentação de seu trabalho. A palavra a seguir, foi concedida ao autor da tese que, em 30 minutos, procedeu à apresentação de seu trabalho. Terminada a apresentação, cada membro da Banca argüiu o Candidato, tendo-se adotado o sistema de diálogo seqüencial. Terminada a fase de argüição, procedeu-se à avaliação da defesa. Tendo-se em vista o que consta na Resolução nº. 1034/2014 do Conselho de Ensino, Pesquisa, Extensão e Cultura (CEPEC), que regulamenta o Programa de Pós-Graduação em Medicina Tropical e Saúde Pública a Banca, em sessão secreta, expressou seu Julgamento, considerando o candidato **Aprovado** ou **Reprovado**:

Banca Examinadora	**Aprovado / Reprovado**
Prof. Dr. Alexandre Melo Bailão	Aprovado
Prof. Dr. Iran Malavazi	Aprovado
Profa. Dra. Patrícia de Sousa Lima	Aprovado
Profa. Dra. Elisa Flávia Luiz Cardoso Bailão	Aprovado
Prof. Dr. Juliano Domiraci Paccez	Aprovado

Em face do resultado obtido, a Banca Examinadora considerou o candidato aprovado / habilitado (**Habilitado ou não Habilitado**), cumprindo todos os requisitos para fins de obtenção do título de **DOUTOR EM MEDICINA TROPICAL E SAÚDE PÚBLICA**, na área de concentração em **MICROBIOLOGIA**, pela Universidade Federal de Goiás. Cumpridas as formalidades de pauta, às 17 h 25 min, a presidência da mesa encerrou esta sessão de defesa de tese e para constar eu, KARINY VIEIRA SOARES E SILVA, secretário do Programa de Pós-Graduação em Medicina Tropical e Saúde Pública lavrei a presente Ata que depois de lida e aprovada, será assinada pelos membros da Banca Examinadora e por mim em duas vias de igual teor.

A Banca Examinadora aprovou a seguinte alteração no título da Tese:

Prof. Dr. Alexandre Melo Bailão - ICB/UFG

Prof. Dr. Iran Malavazi – UFSCAR/SP

Profa. Dra. Patrícia de Sousa Lima – UEG/GO

Profa. Dra. Elisa Flávia Luiz Cardoso Bailão – UEG/GO Bailão

Prof. Dr. Juliano Domiraci Paccez – ICB/UFG Juliano Domiraci Paccez

Secretário da Pós-Graduação: Karíny V Soares

SUMÁRIO

LISTA DE FIGURAS

RESUMO

Estudo da homeostase de cobre no fungo patogênico *Histoplasma capsulatum*.

Histoplasma capsulatum é um fungo patogênico termodimórfico causador da micose sistêmica conhecida como histoplasmose. Este fungo cresce como micélio a temperaturas próximas de 25 ºC e como levedura a 37 ºC. Durante o processo infeccioso, microrganismos patogênicos devem obter nutrientes do hospedeiro para sobreviver nos tecidos do mesmo. Dentre estes nutrientes, o cobre é um íon metálico essencial por participar de reações de oxidação/redução, no transporte de elétrons e é requerido para produção de energia e melanina. O cobre em excesso entretanto é tóxico, pois produz espécies reativas de oxigênio, desloca outros metais de metaloproteínas e causa danos ao DNA e devido a isso *H. capsulatum* deve manter a homeostase deste metal durante a infecção. Observamos aqui que *H. capsulatum*, através dos níveis transcricionais dos genes Ctr4, Mac1, que estão reprimidos, e Crp1 e Ace1 que estão induzidos durante o processo infeccioso em macrófagos, enfrenta um ambiente de excesso de cobre imposto pelas células hospedeiras via cobre ATPase ATP7a. Esse excesso de cobre realizado pelos macrófagos se mostrou INF-ɣ e tempo dependentes, pois quando macrófagos não são estimulados por INF-ɣ e em tempos maiores de infecção, estes passam a impor um ambiente restritivo de cobre durante a infecção. *H. capsulatum* usa a bomba de efluxo de cobre Crp1 para se defender desse ambiente tóxico de cobre, pois leveduras mutantes para Crp1 foram incapazes de crescer em níveis elevados do metal. Apesar da importância de Crp1 para o fungo neste contexto, parece que existem outras proteínas que também possam estar exercendo uma função igual a Crp1 em *H. capsulatum*. Fica claro aqui, mais uma vez, que na relação patógeno – hospedeiro o cobre tem um papel altamente dinâmico, complexo e dependente de certas variáveis biológicas.

Palavras chave: Cobre, Homeostase, Imunidade nutricional.

ABSTRACT

Copper homeostasis study in the pathogenic fungi *Histoplasma capsulatum*.

Histoplasma capsulatum is a thermodymorphic pathogenic fungus that causes systemic mycosis known as histoplasmosis. This fungus grows as mycelium at temperatures around 25°C and as yeast at 37°C. During the infectious process, pathogenic microorganisms must obtain nutrients from the host in order to survive in infected tissues. Among these nutrients, copper is an essential metal ion, because it participates in oxidation/reduction reactions, in energy production, in the transport of electrons, its cofactor of many enzymes and metalloproteins and is required for energy and melanin production. Copper excess however, it is toxic due to the fact that produces reactive oxygen species, dislocates other metals from metalloproteins, causes damage to lipids and DNA, so because of this *H. capsulatum* must maintain the homeostasis of this metal during infection. We observed here that *H. capsulatum*, through the transcriptional levels of Ctr4, Mac1, Crp1 and Ace1, during the infectious process in macrophages, faces an environment of copper overload imposed by the host cells via copper ATPase ATP7a. This copper excess shown to be INF-ɣ and time dependent, because when macrophages are not stimulated by INF-ɣ and in greater times of infection, they impose a restrictive copper environment instead, during the infection. *H. capsulatum* uses the Crp1 copper efflux pump in order to respond this toxic copper milieu, since mutant yeasts for Crp1 were unable to grow at high levels of the metal. Despite the importance of Crp1 for the fungus in this context, it appears that Crp1 is not strictly necessary for the total virulence of the fungus, leading us to infer that other proteins may also be exerting a Crp1-like function in *H. capsulatum*. It is clear once again that in the pathogen-host relationship copper plays a complex, highly dynamic and dependent on certain biological variables role.

Key words: Copper, Homeostasis, Nutritional Immunity.

CAPÍTULO 1: Revisão Bibliográfica

1.0 INTRODUÇÃO / REVISÃO DA LITERATURA

1.1 Infecções fúngicas e mecanismos de infecção

Uma pequena fração dentre as estimadas 5 milhões de espécies fúngicas conhecidas até o momento, é responsável por causar doenças graves que afetam a agricultura e a saúde humana. Devido a isso, doenças causadas por fungos vem sendo cada vez mais reconhecidas como ameaças pertinentes a saúde pública (BROWN et al., 2012), e sua incidência tem aumentado consideravelmente nas últimas décadas (RIVERA 2014).

Acredita-se que o aumento da incidência crescente das doenças fúngicas é devido muitas vezes a dispersão dessas doenças que eram anteriormente consideradas endemicamente fixas (BRASIER 2008). A dispersão destas micoses pode estar relacionada a atividades humanas em si, como por exemplo a modificação de ambientes naturais, a globalização e o aumento do fluxo de indivíduos entre países e regiões diferentes, e possivelmente pode estar correlacionada também com o impacto causado pela mudança climática do planeta (BRASIER 2008; RATINIEKS & CARRECK 2010).

Fungos ambientais podem entrar em hospedeiros humanos ocasionalmente ou acidentalmente tornando-se, assim, patogênicos (BRUNKE et al., 2016). Estes fungos, no ambiente, habitam micro nichos específicos semelhantes as condições encontradas nos tecidos humanos. Esta peculiaridade resulta em adaptações coevolutivas que auxiliam nos fatores de patogenicidade. De fato a maioria dos fungos patogênicos conhecidos tem origem ambiental, tais como fungos dos Gêneros *Cryptococcus*, *Histoplasma*, *Blastomyces*, *Aspergillus* dentre outros (BRUNKE et al., 2016).

Tais fungos tem como uma de suas características principais uma alta flexibilidade genética que facilita uma rápida coevolução e adaptação ao ambiente de seus hospedeiros (CALO et al., 2013). Essa característica, quando combinada com a seleção natural Darwiniana, propicia o surgimento de novas linhagens com virulência aumentada e com uma abrangência aumentada de espécies hospedeiras (CROLL & McDONALD, 2012). A alta adaptabilidade de sobrevivência em diversos ambientes é promovida pela imensa plasticidade do genoma destes fungos (CALO et al., 2013). Além disso estes também contam com a reprodução sexuada, com a presença de transposons, instabilidade

telomérica (STARNES et al., 2006), aneuploidia, transferência horizontal de genes, dentre outras estratégias para promover recombinações mitóticas, levando a esta alta adaptabilidade de nichos diferentes (SUDBERY et al., 2004).

Além das várias características proeminentes dos fungos patogênicos que os possibilitam causar doenças, alguns outros fatores também tem contribuído para o aumento destas doenças, principalmente aqueles que geram pacientes imunocomprometidos. Portanto, podem ser listados os fatores que geram grupos de risco frente as estas infecções: idade dos pacientes, doenças hematológicas malignas, cânceres de órgãos sólidos, síndrome da imunodeficiência adquirida, imunodeficiências primárias e secundárias; transplante de células hematopoiéticas e de órgãos sólidos, regimes de quimioterapia, administração de profilaxia antifúngica sistêmica, neutropenia, presença e inserção de cateteres, hemodiálise e cirurgias gastrointestinais (PFALLER & DIEKEMA 2007; SHOHAM & MARR 2012).

Durante o processo infeccioso, vários processos são necessários para a sobrevivência e replicação de fungos patogênicos e estes processos podem causar danos direta ou indiretamente ao hospedeiro sendo denominados de fatores de virulência (HUBE 2009). Os principais fatores de virulência que podem ser citados são a habilidade de crescer diretamente nos tecidos do hospedeiro, crescimento na temperatura do hospedeiro mamífero, estratégias de evasão do sistema imune, manipulação de microambientes no hospedeiro, fatores de adesão e evasão, mudanças de morfologia e dano direto ao hospedeiro (BRUNKE et al., 2016).

O crescimento durante a infecção caracteristicamente não é essencial durante a vida do patógeno. Fases transientes não replicativas porém podem ser vantajosas, como por exemplo, em biofilmes, cistos e estruturas granulosas que tendem a favorecer a persistência do patógeno no sitio infeccioso mesmo frente a antibióticos e antimicóticos (FANNING et al., 2012).

Por outro lado, a habilidade de crescer nos tecidos do hospedeiro, quando necessária, está diretamente correlacionada com a habilidade de manter uma homeostase metabólica adequada frente ao ataque do sistema imunitário (BRUNKE et al., 2016). Este processo se dá devido ao fato destes microrganismos crescerem nos tecidos de mamíferos com uma taxa metabólica ideal a 37 °C, e a habilidade de capturar e metabolizar nutrientes provenientes do hospedeiro (ENE et al., 2014). Neste contexto, alguns nutrientes devido a sua alta importância biológica, são ativamente extraídos das moléculas do hospedeiro,

tal como os metais de importância biológica e o nitrogênio extraído de proteínas circundantes por meio de proteases secretadas pelo patógeno (NAGLICK et al., 2003).

A estratégia de aquisição de micronutrientes tais como os íons metálicos, é de particular importância para fungos patogênicos, devido ao fato de que as células do hospedeiro desenvolveram mecanismos, ao longo da evolução, no intuito de negar o acesso do fungo a estes micronutrientes, como por exemplo o ferro ou o zinco, estratégia conhecida como imunidade nutricional (KEHL-FI et al., 2010). Em contrapartida fungos patogênicos desenvolveram também sofisticadas estratégias para ganhar acesso ao ferro, zinco e outros metais do hospedeiro (CRAWFORD & WILSON 2015). Além disso, para continuar sobrevivendo nos tecidos do hospedeiro, fungos patogênicos também devem apresentar capacidade de exibir uma robustez mecânica (conferida pela parede celular) e apresentar resistência ao estresse causado por espécies reativas de oxigênio (ROS) (JIMENEZ-LOPEZ & LORENZ 2013).

Estratégias de evasão, contra ataque e escape de respostas do sistema imune são muito utilizadas por fungos patogênicos (BRUNKE et al., 2016). Para evitar detecção, fungos se utilizam de mecanismos que consistem em esconder estruturas de superfícies imunogênicas da membrana ou parede celular (Pathogen Associated Molecular Pattern – PAMPs), que funcionam no intuito de modificar estas estruturas, cobrindo-as com cápsulas ou até mesmo com moléculas derivadas do próprio hospedeiro (CHAI et al., 2009).

Garfoot e colaboradores (2016) demostraram que o fungo patogênico *Histoplasma capsulatum* secreta uma enzima denominada Eng1 β-glicanase que acentua a virulência deste fungo por reduzir os níveis de β-glicana expostas na superfície celular do fungo, proporcionando com que as leveduras de *H. capsulatum* escapem da detecção de células do sistema imune, que possuem um receptor de reconhecimento para β-glicana chamado de Dectina 1. Como consequência, os níveis de fagocitose de leveduras deste fungo durante a infecção são reduzidos, levando a uma menor produção de citocinas pró-inflamatórias pelos fagócitos e um menor controle da infecção *in vivo* por parte do hospedeiro.

Substâncias antimicrobianas produzidas por células de defesa do hospedeiro tais como as espécies reativas de oxigênio, peptídeos antimicrobianos, fatores de complemento e anticorpos podem ser ativamente degradados por fungos patogênicos (STERKEL et al., 2016). Pigmentos variados, como por exemplo a melanina, são comuns

por proteger fungos contra muitos mecanismos de defesa do hospedeiro e até mesmo de antifúngicos (SCHARF et al., 2014).

A fagocitose durante o processo invasivo é a barreira primária que fungos patogênicos devem sobrepujar, e estes quando expostos aos fagócitos utilizam-se de estratégias sofisticadas de contra-ataque, tais como escape através da perfuração da membrana do fagócito, indução de lise celular e alteração do compartimento do fagolisossomo permitindo uma sobrevivência intracelular (SEIDER et al., 2010; GILBERT et al., 2015).

A adesão do patógeno aos tecidos hospedeiros é feita primariamente por proteínas ou outras moléculas denominadas de adesinas, que são praticamente encontradas em todos os microrganismos patogênicos (BRUNKE et al., 2016). O fato dos microrganismos patogênicos se aderirem diretamente às células hospedeiras, impede com que sejam retirados mecanicamente de superfícies mucosas, e também proporciona o contato íntimo necessário para manipular e invadir as células e tecidos hospedeiros (DALLE et al., 2010).

A invasão a células e tecidos hospedeiros é frequentemente descrito como uma atividade característica de microrganismos patogênicos, apesar de que alguns microrganismos simbiontes e comensais também são conhecidos por invadir células e causar infecções em momentos específicos (REINHARDT 2007). Enquanto que bactérias quase sempre invadem células hospedeiras induzindo a endocitose, fungos por sua vez fazem sua invasão através de atividades fisiológicas normais, que podem também ser exercidas quando o fungo está no meio ambiente, tais como crescimento de filamentos que podem causar a penetração de células e tecidos ou o aumento do turgor celular que também leva a uma penetração ativa (DALLE et al., 2010).

A translocação para tecidos mais profundos, entretanto, nem sempre requer uma invasão celular. Por exemplo, fungos patogênicos podem chegar a outros tecidos por conexões interepitelias rompidas ou por meio de necroses pré-estabelecidas (REINHARDT 2007). Além disso, fungos patogênicos tais como *Cryptococcus neoformans* podem utilizar as células hospedeiras, como os macrófagos por exemplo, como meio de transporte para sobrepujar as barreiras imunológicas naturais, sendo esta estratégia conhecida como “Cavalo de Tróia” (CASADEVALL 2010).

A manipulação do ambiente do hospedeiro por meio de proteínas efetoras também é uma estratégia comum entre microrganismos patogênicos. Estas proteínas especificamente interagem com moléculas hospedeiras no intuito de manipular a célula

hospedeira em favor do patógeno. Numa definição ampla, estas proteínas efetoras podem atravessar a membrana celular de células hospedeiras e manipular as atividades intracelulares. Esta estratégia é comum em bactérias e fungos patogênicos de plantas. Isso é possível devido a sistemas de secreção especializados para isso nas bactérias patogênicas, porém em fungos patogênicos de plantas o sistema ainda não é claro. Nenhuma proteína efetora foi ainda descrita em fungos patogênicos humanos (VANCE et al., 2009).

Alguns patógenos ambientais conseguem reconhecer condições típicas associadas ao hospedeiro e responder a elas, iniciando a expressão de fatores de virulência específicos. Por exemplo fungos dimórficos, tais como *H. capsulatum* e *Paracoccidioides brasiliensis*, mudam sua morfologia e todo o perfil de expressão gênica, em resposta a temperatura corporal de mamíferos (BOYCE et al., 2015). Outros sinais de contato com o hospedeiro, como o contato com células epiteliais por exemplo, podem também desencadear mudanças morfológicas e transcricionais em fungos patogênicos comensais (BRUNKE & HUBE 2014). Essas mudanças morfomoleculares servem para ajudar o patógeno a se preparar para possíveis situações hostis dentro do hospedeiro (BRUNKE et al., 2016).

Estes fatores de virulência podem diretamente causar danos ao hospedeiro durante a invasão celular, na permanência da infecção ou nos processos evasivos. Estes danos podem ser mediados por enzimas hidrolíticas (proteases ou lipases) ou toxinas na forma de peptídeos ou metabólitos (SCHARF et al., 2014). De fato, toxinas que causam patologias e doenças mesmo na ausência do microrganismo produtor vivo, são consideradas fatores de virulência clássicos especialmente em bactérias. Metabólitos secundários tóxicos são comumente encontrados em fungos filamentosos (VANCE et al., 2009).

Fungos com relevância clínica podem ser adquiridos por inalação de propágulos infectantes, tais como fragmentos de micélio e conídios, que resultam em infecções pulmonares e podem se disseminar para outros órgãos e sistemas (ROMANI 2011). Os gêneros *Aspergillus, Histoplasma* e *Paracoccidioides* exemplificam esse grupo de patógenos, de incidência elevada, que iniciam o processo infecioso pela inalação de propágulos (RESTREPO & TOBON 2005; RIVERA 2014). Outros possuem a forma infectante de levedura, como *Candida albicans*, por exemplo, a qual se destaca pela sua elevada incidência também em pacientes imunodeprimidos (PAGANO 2006). Além desses fungos, *C. neoformans*, *Trichosporium* spp., *Fusarium* spp., *Scedosporium* spp.,

Zygomycetes, e *Pneumocistis jirovecii* também têm sido descritos em pacientes com baixa imunidade (SOYSAL 2015).

Embora muitas espécies são inicialmente consideradas como patógenos oportunistas, os fungos podem desenvolver infecções em indivíduos imunocompetentes (PFALLER & DIEKEMA 2010). Casos fatais de paracoccidioidomicose, histoplasmose, meningite e mucormicose cutânea necrosante ilustram esta possibilidade (AGARWAL et al., 2015). Portanto, fungos dimórficos, os quais são classificados como patógenos primários, a saber, *Paracoccidioides* spp., *H. capsulatum, Coccidioides immitis*, *Blastomyces dermatitidis e Talaromyces marneffei,* infectam tanto indivíduos imunocomprometidos quanto imunocompetentes. Desta maneira o estudo dos aspectos que envolvem as bases da biologia, virulência e da interação fungo-hospedeiro tornam-se essenciais na pavimentação das vias para o desenvolvimento de novas terapias e diagnósticos para o controle das infecções fúngicas.

1.2 *Histoplasma capsulatum*

Histoplasma capsulatum é um fungo termodimórfico, que se apresenta na forma de levedura, quando cultivado a temperaturas de 35 a 37 °C ou quando presente como parasita em tecidos de mamíferos (ZANCOPÉ-OLIVEIRA et al., 2013). A forma de levedura do fungo é caracterizada por colônias de aspecto úmido, lisas e de coloração branco-amarelada. Microscopicamente mostra-se como pequenas leveduras, medindo de 2 a 4 µm de diâmetro, esféricas ou ovais, de paredes finas e unibrotantes (MUNIZ 2013; Figura 1).

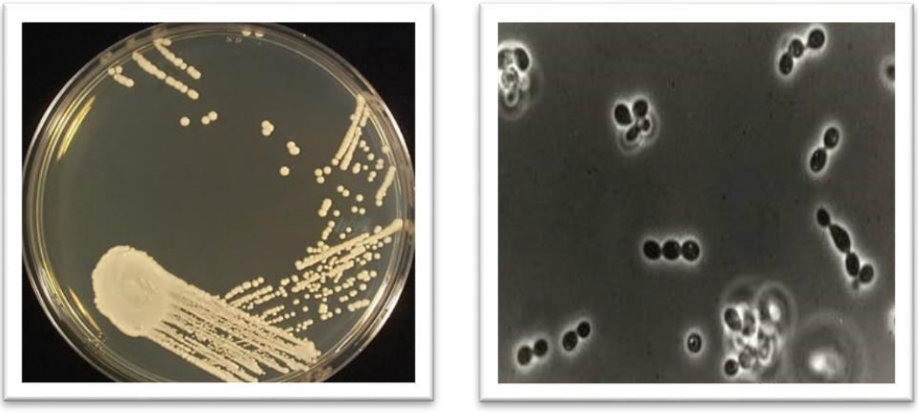


Figura 1. Levedura de *Histoplasma capsulatum*. Forma de levedura do fungo patogênico *H. capsulatum* crescido em meio BHI.

Quando este fungo é encontrado na natureza (saprofitismo), ou quando cultivado a temperaturas em torno de 22 °C, este se apresenta na forma de micélio, com morfologia filamentosa de colônias brancas, acastanhadas e algodonosas. Microscopicamente esta forma é caracterizada por hifas hialinas, septadas, ramificadas e de morfologia típica, contendo macroconídeos, medindo de 8 a 16 µm de diâmetro, geralmente esféricos e de parede celular lisa e microconídeos, menores em diâmetro, medindo de 2-5 a 16 µm, com paredes lisas ou irregulares (ZANCOPÉ-OLIVEIRA et al., 2013; Figura 2).

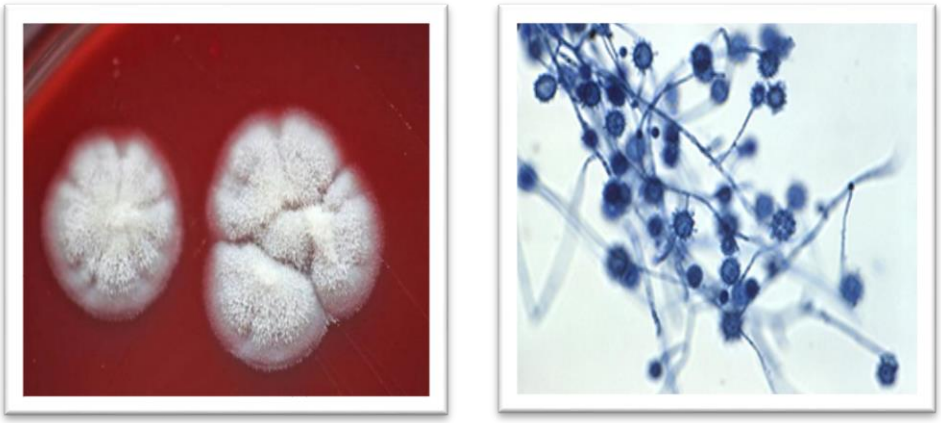


Figura 2. Micélio de *Histoplasma capsulatum*. Forma de micélio do fungo patogênico *Histoplasma capsulatum* em meio ágar sangue.

No ambiente a forma saprobiótica de *H. capsulatum* é encontrada em solos enriquecidos com matéria orgânica como fezes de galinhas, outras aves e morcegos. Acredita-se que esta relação é devida ao alto teor de ácido úrico encontrado nestes excrementos, e o fungo usa este componente como fonte de nitrogênio, importante metabolicamente para o seu crescimento e proliferação. Além disso tais micro nichos geralmente apresentam condições de temperatura, umidade e pH, ideais à sobrevivência deste microrganismo na natureza (CANO & HAJJEH 2001).

Locais fechados, com possíveis altas concentrações de excrementos e matéria orgânica, tais como cavernas, construções antigas ou abandonadas, forros, sótãos ou porões de casas, galinheiros, árvores ocas e campos cultiváveis são importantes fontes de infecção deste fungo. Além disso, o próprio contato ou movimentação direta do solo, proporciona a dispersão dos microconídeos pelo ar (WANKE 1985).

Muitos animais podem ser suscetíveis a infecção por este fungo, porém o morcego junto das aves, destacam-se por indiretamente exercerem o papel de disseminadores do

fungo na natureza. Devido ao intenso parasitismo do fungo nas células de sua mucosa intestinal, estes animais acabam naturalmente excretando conídeos do fungo em suas fezes, que podem ser respirados pelo hospedeiro, desempenhando um importante papel na manutenção do ciclo biológico de *H. capsulatum* na natureza (TAYLOR et al., 2000; DIAS et al., 2011; Figura 3). O fungo pode ser isolado, portanto, de quaisquer locais onde o solo possa estar enriquecido com excretas destes animais (FERREIRA 2009).

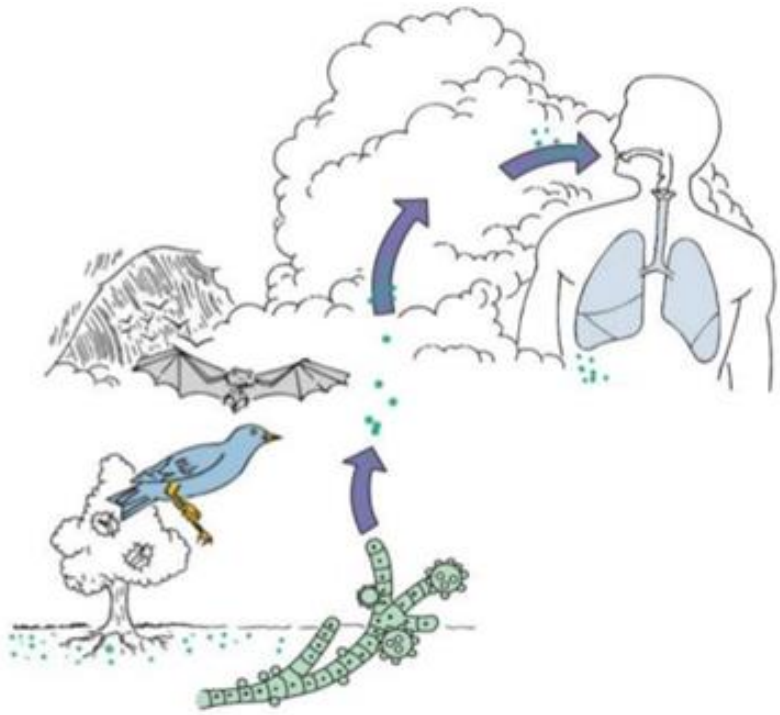


Figura 3. Ciclo biológico do fungo *Histoplasma capsulatum*. Solos ricos em excretas de aves e morcegos possuem alto teor de nitrogênio, que serve como fonte de nutriente para que o fungo *H. capsulatum* possa crescer na sua forma de micélio na natureza. Quando conídeos ou fragmentos destes micélios presentes no solo são inalados pelo hospedeiro, estes aderem ao epitélio pulmonar, transitando para a forma de levedura e assim dando início a infecção, acarretando posteriormente a doença conhecida por histoplasmose. Extraída e modificada de FERREIRA, 2009.

Atividades como paisagismo, limpeza de sótãos ou celeiros, demolição de prédios antigos e revolvimento de solos estão associadas a infecção por *H. capsulatum* e contribuem para a disseminação de suas partículas infectantes (KAUFFMAN 2009). Correntes de ar podem carrear os conídios por quilômetros de distância, expondo mesmo indivíduos que não estavam próximos e nem tiveram contato direto com áreas contaminadas (DEUS FILHO et al., 2009).

1.3 Taxonomia de *Histoplasma capsulatum*

Taxonomicamente, o fungo *H. capsulatum* é um eucarioto pertencente ao Reino Fungi, e encontra-se na divisão; Filo Ascomycota, Classe Eurotiomycetes, Ordem Onygenales, Família Ajellomycetaceae, Gênero *Histoplasma*, Espécie *capsulatum,* cuja forma teleomórfica denomina-se *Emmonsiella capsulata* (LACAZ et al., 2009; KASUGA et al., 2003). Mesmo tendo uma forma sexuada esta nunca foi observada na natureza e a magnitude da recombinação genética sexuada deste fungo ainda permanece não determinada (KWON-CHUNG 1972).

Baseada na morfologia e patogenicidade, historicamente a espécie *H. capsulatum* tem sido dividida em três variedades: *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii* e *H. capsulatum* var. *farciminosum*. A primeira variante, *H. capsulatum* var. *capsulatum* é considerada o agente etiológico da histoplasmose clássica, de distribuição universal. A segunda variedade *H. capsulatum* var. *duboisii*, causa a histoplasmose africana e é descrita por estar restrita a uma parte do continente africano. A terceira variante, *H. capsulatum* var. *farciminosum,* não foi encontrada parasitando o homem, tendo sido descrita apenas como patógeno de cavalos e mulas, causando linfagite epizoótica (GUEHO et al., 1997; LACAZ et al., 2009).

Estudos na área de taxonomia e genotipagem revelaram que estes três grupos clássicos são artificiais. Neste sentido, *H. capsulatum* parece ser composto por grupos genéticos distintos, que até o momento não tinham sido reconhecidos como novas espécies (KASUGA et al., 2003), mesmo diante do fato que um dos representantes deste grupo causa uma doença totalmente distinta dos outros, a linfagite epizoótica (GUGNANI & MUOTOE-OKAFOR 1997).

A identificação de espécies crípticas e a delimitação de uma espécie da outra em fungos patogênicos, já levou a descoberta de diferenças entre estratégias de virulência, susceptibilidade a drogas e diferenças nos sítios de infecção no hospedeiro que inicialmente não eram aparentes (SEPULVEDA et al., 2017). Com isso avanços na área da genotipagem molecular levaram a uma investigação mais criteriosa das relações filogenéticas de *H. capsulatum*. Técnicas como a de Restrição de Fragmentos Polimórficos (Restriction Fragment Length Polymorphisms - RFLP), hibridização do DNA, Amplificação Aleatória de DNAs Polimórficos (Random Amplified Polymorphic DNA – RAPD) e Sequenciamento ITS1/2, revelaram uma alta diversidade genética em *Histoplasma*, com indicações de associação geográfica (VITE-GARIN et al., 2014).

Duzentos e trinta e quatro isolados de *H. capsulatum* distribuídos entre 22 países foram analisados por Teixeira e colaboradores (2016) através de técnicas moleculares inseridas no método GCPSR (Genetic Concordance Phylogenetic Species Recognition) no intuito de se entender melhor a distrubuição filogenética deste fungo e sua distrubuição geográfica. Foi sugerido neste estudo, que *H. capsulatum* divide-se em pelo menos 8 clados com 7 espécies filogenéticas aparentemente distintas: América do Norte Clado 1 (NAm 1), América do Norte Clado 2 (NAm 2), América Latina Clado A (LAm A), América Latina Clado B (LAm B), Australia, Holanda e África (TEIXEIRA et al., 2016).

Sepulveda e colaboradores (2017) em um estudo abrangendo genética de populações e análises filogenéticas através das técnicas de PCA (Principal Allelic Frequencies), de RFPL, de análise do DNA mitocondrial (mtDNA), RAPD e da técnica de SNPs (Single Nucleotide Polymorphisms) demonstraram que *H. capsulatum* possui cinco linhagens geneticamente isoladas que podem ser consistentemente classificadas como novas espécies. Foi sugerido neste estudo que isolados do clado NAm1 passem a ser uma nova espécie, denominada *Histoplasma mississipiense*, do clado NAm2 a nova espécie *Histoplasma ohiense*, do clado LAm1 a nova espécie *Histoplasma suramericanum* e os representantes do clado LAm2 continuam a pertencer a espécie *Histoplasma capsulatum*.

Foi também proposto que os isolados dos clados Australia, Holanda e África formam um só clado, o clado África e que não foi possível ainda redefinir a taxonomia deste clado, devido a necessidade de um maior número de amostras a serem analisadas para essa afirmação (SEPULVEDA et al., 2017). No presente trabalho de tese de doutorado, foi estudada a espécie do clado LAm2 *Histoplasma capsulatum* G186A.

1.4 Histoplasmose

A histoplasmose é uma micose sistêmica cosmopolita, causada pelo fungo termodimórfico *H. capsulatum.* Esta micose conhecida também como doença de Darling ou doença das cavernas, foi descoberta no ano de 1906 por Samuel Taylor Darling quando examinava casos suspeitos de leishmaniose em tecidos de necropsia de homens que trabalhavam na construção do canal do Panamá, e haviam morrido com uma doença febril aguda, associada a anemia e hepatoesplenomegalia (HAGAN 2003). Darling encontrou no interior de macrófagos alveolares, numerosos corpos ovais e arredondados morfologicamente, semelhantes a protozoários do gênero *Leishmania*, de

aproximadamente 3 µm de diâmetro. Esse novo parasita encontrado então foi denominado de *Histoplasma capsulatum*. O nome foi baseado na característica do fungo de residir no interior de histiócitos e na presença aparente de uma cápsula circundante (KAUFFMAN 2007).

A histoplasmose é adquirida através da inalação de microconídios infectantes, presentes em solo contaminado com o fungo. Os conídios inalados chegam até os alvéolos pulmonares estimulando uma resposta inflamatória no hospedeiro, composta de células mononucleares em sua maioria macrófagos. Ocorre então a transição para a fase leveduriforme no parênquima pulmonar, onde uma série de mudanças genéticas, bioquímicas e físicas ocorrem no microrganismo, devido a mudança de temperatura ambiental para a do hospedeiro (DEEPE 1995). A levedura então é fagocitada pelos macrófagos alveolares residentes, e a consequência imediata da fagocitose das leveduras pelos macrófagos é a fusão dos lisossomos com o vacúolo fagocítico. Uma vez internalizadas, as leveduras sobrevivem e multiplicam-se dentro dos falolisossomos (NEWMAN 1999).

A maioria das infecções causadas por *H. capsulatum* são assintomáticas ou subagudas. Estes casos de histoplasmose subaguda resultam da infecção com pequeno inóculo observando-se quadro clínico semelhante a um estado gripal com tosse seca, febre e adinamia (ROSSINI 2006). Os casos sintomáticos manifestam-se comumente como infecções do trato respiratório (FERREIRA & BORGES 2009) e a gravidade e a evolução da doença são determinadas quase sempre pela quantidade de partículas inaladas, estado imunológico do hospedeiro e virulência da cepa infectante (AIDE 2009).

A histoplasmose sintomática apresenta três formas clínicas principais: Infecção pulmonar aguda, pulmonar crônica e infecção disseminada. A infecção pulmonar aguda consiste em tosse, dispneia, calafrios, e dor torácica com presença de infiltrados reticolonodulares observados por radiografia dos pulmões e é na maioria das vezes regressiva, sendo a regressão observada por volta de 85% dos pacientes (ROSSINI & GOULART 2006). A infecção pulmonar crônica ocorre geralmente em indivíduos tabagistas, por apresentarem maior disposição de lesões pulmonares que associadas a infecção do fungo, podem progredir lentamente para uma forma fibrocavitária crônica que acomete principalmente os lobos superiores dos pulmões, levando a perda de peso, sudorese noturna, dor torácica e tosse com expectoração hemóptica, quadro bastante similar ao da tuberculose pulmonar crônica (MUKHERJEE et al., 2010).

A partir do trato respiratório, a infecção pode ser disseminada pelas vias linfáticas e hematogênica. Essa infecção é caracterizada pela disseminação do fungo para todo o organismo, particularmente para órgãos ricos em macrófagos, tais como fígado, baço, linfonodos e medula óssea acarretando lesões aos órgãos acometidos (FERREIRA & BORGES 2009). A histoplasmose disseminada ocorre em indivíduos imunocomprometidos, especialmente em pacientes HIV positivos, sendo a forma clinica mais grave, em que ocorre intensa multiplicação dos fungos nos órgãos extrapulmonares, tendo uma taxa muito elevada de mortalidade (WHEAT et al., 2000).

A histoplasmose é indiscutivelmente a infecção fungica respiratória mais comum no mundo todo, sendo notificada todo ano somente nas Américas do Norte e Sul (HAGE et al., 2015). A frequência de ocorrência da histoplasmose pulmonar é a maior entre qualquer outra doença causada por fungos patogênicos (KAUFFMAN 2007; WHEAT et al., 2016). Essa doença é conhecida por apresentar distribuição mundial, não sendo mais considerada uma micose endêmica das regiões dos rios Mississipi e Ohio dos Estados Unidos (BAHR et al., 2015). Essa micose já foi descrita em vários países do globo, com maior prevalência em zonas tropicais e temperadas. (Figura 4).

No continente asiático, a China vem chamando a atenção pelo crescente número de casos da doença. De 1990 a 2011, 300 casos de histoplasmose foram reportados neste país, sendo que 257 casos eram da micose disseminada em pacientes HIV positivos (PAN et al., 2013). Na Tailândia, o grande número de indivíduos HIV positivos está sendo responsável por um surto de histoplasmose disseminada, sendo que mais de 1200 casos da doença foram reportados para o Ministério de Saúde Pública de 1984 a 2012 (NORKAEW et al., 2013).

Na Índia, 12,3% dos casos de micoses sistêmicas relatados em pacientes imunocompetentes, são de histoplasmose, e *H. capsulatum* já foi isolado diversas vezes com sucesso de solos próximos aos rios Calcutta e Delhi indicando que o fungo pode ser endêmico nessas regiões no país (GOPALAKRISHNAN et al., 2012). Na Malásia, *H. capsulatum* foi isolado de morcegos nativos da região de Kuala Lumpur, e testes de sensibilidade para histoplasmina possuem uma prevalência positiva de 10.5% na população desta região (JING et al., 1999). Casos de histoplasmose na Malásia vem sendo recentemente registrados em indivíduos HIV positivos e negativos e entre aqueles individuos que visitam o país temporariamente, porém ainda não se sabe o número exato de casos no país (OHNO et al., 2010).

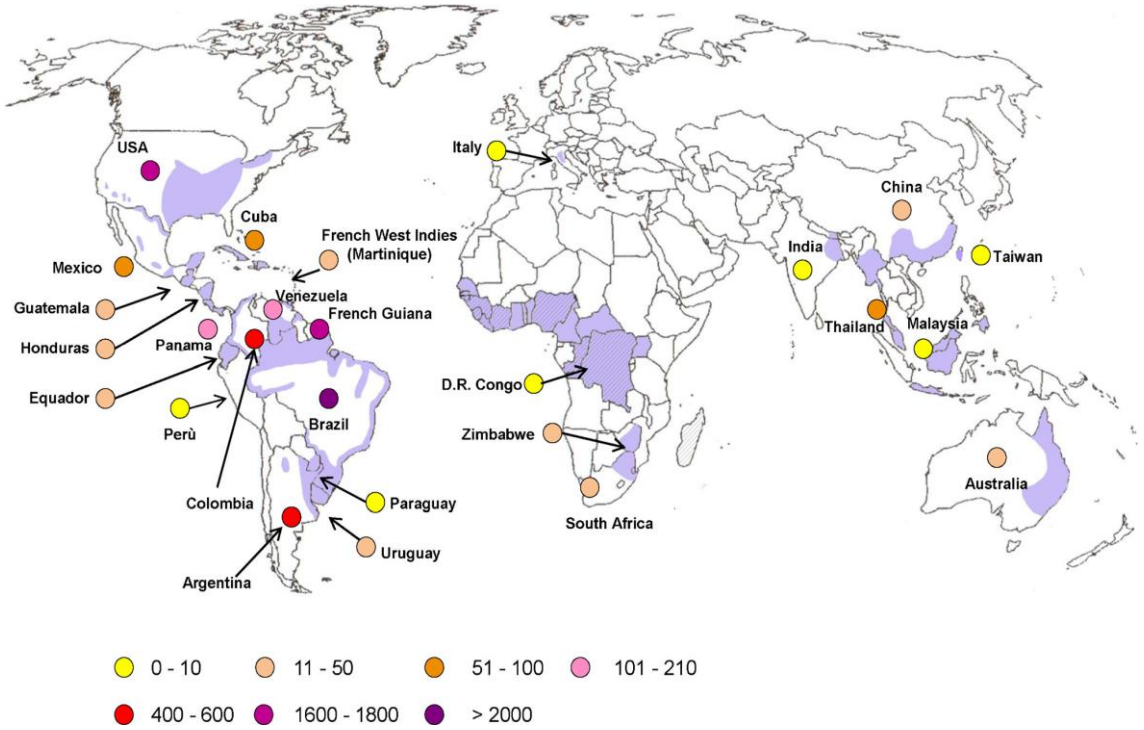


Figura 4. Distribuição geográfica de *Histoplasma capsulatum* var. *capsulatum* (cinza) e *Histoplasma capsulatum* var. *duboisii* (tracejado). Os círculos indicam o número de casos de histoplasmose associada ao HIV publicados via "Scopus query". Extraído de BAHR, et al., 2015.

Nas Américas do sul e central, um grande número de pacientes, a maioria HIV positivos, foram diagnosticados com hitoplasmose no Brasil, Guiana Francesa, Argentina, Colômbia, Venezuela e Panamá (BAHR et al., 2015). Na Colômbia, 70% dos pacientes com HIV adquiriram Histoplasmose, e a Argentina possui uma área endêmica da doença na região nordeste do país (ARANGO et al., 2011). Um estudo conduzido em Caracas na Venezuela, de 2000 a 2005 reportou que de todos os pacientes HIV postivos na região, 33,5% eram sintomáticos para histoplasmose.

No Brasil diversas microepidemias desta doença já foram relatadas desde 1958. Os principais fatores foram visitas a grutas com fezes de morcego, seguidas por visitas a minas abandonadas e contato com fezes de galinheiros (OLIVEIRA et al., 2006). O Brasil é responsável pelo maior número de casos de microepidemias relatadas na América Latina, sendo descritas mais de 18 microepidemias de histoplasmose na história do país (AIDE 2009). Prado e colaboradores (2009) demonstraram que entre os anos de 1996 a 2006 no Brasil foram descritos 3.583 casos de mortes causadas por micoses sistêmicas, onde 10,1% foram causadas pela histoplasmose (PRADO et al., 2009).

Guimarães e colaboradores (2016) observaram no estado do Rio de Janeiro que de todas as infecções invasivas causadas por fungos em pacientes recém submetidos a cirurgia de transplante de rins, 22,7% eram infecções causadas por *H. capsulatum*. Este número elevado está similar ao de regiões de países considerados altamente endêmicos para esta doença como, por exemplo, o estado do Mississippi nos Estados Unidos (GUIMARÃES et al., 2016).

Silva e colaboradores (2017) realizaram um levantamento dos casos relatados de histoplasmose em indivíduos HIV positivo em Goiânia, capital do estado de Goiás, dentre os anos de 2000 a 2012 e observaram que de 6330 pacientes com AIDS, 279 foram diagnosticados com histoplasmose, sendo que destes, 71.3% foram a óbito. Este foi o primeiro estudo epidemiológico da doença no estado. Os autores acreditam que a taxa de incidência pode ser muito maior se não fosse a grande negligencia de notificação da doença no estado (SILVA et al., 2017).

Devido ao número crescente de casos de histoplasmose sendo descritos no país, atualmente o Brasil é conhecido, entre os órgãos mundiais de saúde, como um país onde a histoplasmose é altamente endêmica, sendo que casos da doença já foram relatados em quase todos os seus estados. Nos municípios de maior risco nos estados do Amazonas, Roraima, Pará, Amapá, Ceará, Rio Grande do Norte, Bahia, Minas Gerais e São Paulo, a positividade do teste para histoplasmina chega a uma taxa assustadora de ~90% de positividade, frente aos indivíduos testados, demonstrando a importância da histoplasmose para a saúde pública do país brasileiro (BAHR et al., 2015).

1.5 Processo infeccioso e a homeostase de metais

Para uma colonização bem sucedida todo fungo patogênico deve inicialmente aderir a tecidos alvos do hospedeiro e ali obter nutrientes essenciais para seu desenvolvimento (WEINBERG 2009). Sabe-se que patógenos em geral tem pelo menos um desafio em comum: nutrição durante o processo infeccioso. Não apresentando habilidade para consumir os nutrientes disponíveis nos sítios do respectivo hospedeiro, um fungo não se estabelecerá como um patógeno (BROCK 2009).

A capacidade de captar nutrientes que os fungos patogênicos possuem é fundamental para seu crescimento, e a alteração na disponibilidade destes nutrientes (redução ou excesso), inclusive os íons metálicos, é um mecanismo de defesa do hospedeiro frente a microrganismos invasores (WINTERS et al., 2010). Elementos inorgânicos tal como os metais não podem ser criados nem destruídos, dessa maneira a sua homeostase dentro de sistemas biológicos deve ser estritamente regulada por ambos, patogêno e hospedeiro (FESTA & THIELE 2011). Desta maneira, a homeostase de metais de uma maneira geral, principalmente no processo infeccioso, se torna crucial pois todos os organismos se deparam com a necessidade de obtenção, bem como, com a de regulação das concentrações de metais em fluidos biológicos e compartimentos celulares, uma vez que a concentração destes micronutrientes em taxas elevadas na grande maioria das vezes é tóxico (VAN HO et al., 2002).

Os metais tem papéis importantes na função e integridade celular, tanto do patógeno quanto do hospedeiro (WINTERS et al., 2010; VIGNESH et al., 2013) sendo fundamentais para vários processos biológicos. O ferro (Fe), cobre (Cu), manganês (Mn) e o zinco (Zn) são micronutrientes ou metais biologicamente essenciais, e devido a isso células de quase todos os organismos desenvolveram mecanismos, como transportadores moleculares por exemplo, para adquirir estes metais do meio em que ocupam e assim disponibilizar os mesmos para sua utilização em processos metabólicos (VAN HO et al., 2002; MARINEZ-FINLEY et al., 2012).

Juntamente com o ferro, cobre e zinco, o sódio, potássio, magnésio, cálcio, manganês, cobalto, níquel, vanádio, molibdênio e tungstênio são também chamados de metais essenciais, devido a sua importância para os seres vivos (PERMYAKOV 2009). Os metais essenciais, frequentemente em pequenos níveis, tem ligação direta na sobrevivência dos microorganismos e sua permanencia nos nichos de infecção no hospedeiro. É estimado que por volta de 30% das proteínas conhecidas necessitam de um cofator metálico, denominando-se metaloproteínas, para exercer de forma adequada seus respectivos papéis catalíticos, regulatórios e estruturais (SUN et al., 2011). Os metais adquiridos pelos organismos na maioria das vezes tem como destino se associarem a essas proteínas (SEVCENCO et al., 2011).

Devido a sua importância biológica, a maioria dos patógenos desenvolveram mecanismos específicos para absorção de íons metálicos em seus hospedeiros, mesmo quando há baixas disponibilidades destes íons no tecido infectado. A quantidade de metais de importância biológica é essencial durante a evolução de uma doença infecciosa, o hospedeiro e o fungo irão gerar respostas diferentes, para poder controlar a homeostase desses metais (SILVA et al., 2011).

Diante disso fungos patogênicos desenvolveram estratégias sofisticadas para manter a homeostase destes metais e as células de defesa do hospedeiro também se utilizam de diferentes mecanismos para combater a infecção. Uma destas estratégias microbicidas dos hospedeiros mamíferos para evitar a infecção microbiana, é a já mencionada imunidade nutricional (APPELBERG 2006). O termo imunidade nutricional originalmente se referia a restrição da disponibilidade de ferro pelo hospedeiro, porém recentemente este termo passou a ser utilizado também para mecanismos de retenção de outros metais essenciais e também para mecanismos que utilizam o potencial tóxico do excesso destes metais contra microorganismos invasores (HOOD & SKAAR 2013).

Sendo assim para melhor entender o mecanismo da doença é preciso conhecer as vias metabólicas metal dependentes do patógeno, conhecendo assim suas necessidades nutricionais. Isso permitiria a ampliação do conhecimento dos mecanismos de virulência/sobrevivência de fungos patogênicos bem como a determinação de novos alvos de combate ao patógeno; análises estas que ajudariam no combate à infecções fúngicas. Avanços no conhecimento sobre imunidade nutricional e sobre a necessidade da homeostase de íons metálicos por patógenos já levaram a inúmeras aplicações clínicas e industriais (HOOD & SKAAR 2013).

Há uma clara necessidade de determinar a homeostase de metais em fungos patogênicos durante sua infecção, e durante suas fases de vida, pois estes fungos encontram diferentes ambientes com diferentes disponibilidades de micronutrientes essenciais. O fato de que a concentração disponível destes micronutrientes para os micro-organismos patogênicos é mantida fora da quantidade ideal para o desenvolvimento dos mesmos, através de mecanismos de defesa do hospedeiro, a habilidade em obter esses micronutrientes dessa maneira, contribui para a sua virulência.

1.6 Cobre (Cu) e sua homeostase

O cobre em sua forma elementar aparece naturalmente na crosta terrestre, no solo e em compostos minerais, sendo que pode transitar entre a forma reduzida Cu^{+} e a forma oxidada Cu^{2+} possuindo, desta maneira, um alto potencial redox. Este metal pode ser encontrado em todas as plantas, fungos e animais, exercendo a função de doador e receptor de elétrons em muitas proteínas associadas ao mesmo (MARTINEZ-FINLEY et al., 2012).

Este metal participa ativamente nas reações de óxido-redução constituindo enzimas responsáveis pelo transporte de elétrons, sendo que o processo cobre-dependente mais conservado em formas de vida aeróbicas é a redução do oxigênio pela citocromo *c* oxidase levando a produção de ATP, na mitocôndria (FESTA & THIELE 2012; VAN HO et al., 2002). O cobre participa ativamente também nos processos de produção de melanina, em oxidases, em superóxido dismutases que combatem o estresse oxidativo, em ferro oxidases que participam do transporte de ferro, no crescimento celular dentre outros (KORNITZER et al., 2009; FESTA & THIELE 2012).

Evidencias sugerem que o cobre livre no citoplasma é mantido em baixas concentrações, ressaltando a grande habilidade e necessidade das células e organismos de manterem os níveis deste metal estritamente regulados através de mecanismos homeostáticos (FESTA & THIELE 2012). Isso acontece devido ao fato de que o cobre livre em excesso pode causar dano celular por produzir espécies reativas de oxigênio (ROS) através de reações de Fenton e por deslocar competitivamente outros metais de metaloproteínas. O cobre livre também é capaz de destruir a estrutura de proteínas que contêm grupos ferro/enxofre comprometendo sua função além de causar danos diretos a lipídeos e ao DNA (BEAUDOIN & LABBE 2007).

De forma geral, os organismos procariotos não tem muita necessidade de cobre para os seus processos bioquímicos e fisiológicos. Quase todas as bactérias anaeróbicas são usuárias limitadas de cobre, talvez devido a disponibilidade limitada deste metal nessas condições. Porém enquanto esse metal não é muito utilizado por estes procariotos, em seus genomas existem muitos genes que codificam para exportadores e metalochaperonas de cobre, que protegem esses organismos contra o seu excesso e toxicidade. A homeostase

de cobre em bactérias então se resume a funções básicas, como o reconhecimento dos níveis de cobre por sensores específicos e a sua exportação para fora da célula (FESTA & THIELE 2011).

Dessa maneira, nos procariotos este processo é comumente codificado por um *operon* que opera em conjunto com um repressor transcricional (CueR ou CopY) que mantém o *operon* inativo na ausência de cobre. Na presença de cobre, proteínas do repressor se ligam a este metal causando assim a sua dissociação de sítios promotores específicos do DNA, e levando a expressão de genes que codificam para chaperonas e exportadores de cobre (P-type ATPases) que proporcionam a proteção contra o excesso tóxico de cobre através de seu sequestro e transporte para fora da célula (FESTA & THIELE 2011; FESTA & THIELE 2012).

Com a evolução de eucariotos unicelulares, surgiu um novo desafio no sentido da captação de cobre e a sua relocação para metaloproteínas localizadas em várias organelas, tais como as mitocôndrias, cloroplastos, complexo de Golgi e compartimentos secretórios, aumentando assim a complexidade da homeostase deste metal. Além disso, com o subsequente aparecimento de organismos multicelulares veio a necessidade de regular o cobre em locais e tecidos específicos, de acordo com necessidades metabólicas específicas (BOAL & ROSENZWEIG 2009). Eucariotos portanto, tem uma relação com o cobre mais complexa do que a existente em bactérias, pois estes devem adquirir o cobre do meio externo, internaliza-lo, distribuí-lo para enzimas e proteínas no citosol, mitocondria e compartimentos secretórios, além de manter seus níveis dentro de limites não tóxicos (GARCIA-SANTAMARINA et al., 2018).

Em fungos, a primeira descrição da homeostase de cobre foi realizada em *Saccharomyces cerevisiae*. Nesta levedura o cobre é adquirido por dois transportadores de cobre de alta afinidade, Ctr1 e Ctr3. As proteínas da família Ctr apresentam três domínios transmembrânicos ricos em metioninas e são encontradas na membrana da célula regulando o transporte de cobre do meio extracelular para o intracelular (BEAUDOIN & LABBE 2007). Este transporte se dá depois que este metal é reduzido da forma Cu^{2+} para a forma Cu^{+} por uma metaloredutase, Fre1, localizada também na membrana celular (FESTA & THIELE 2011; Figura 5).

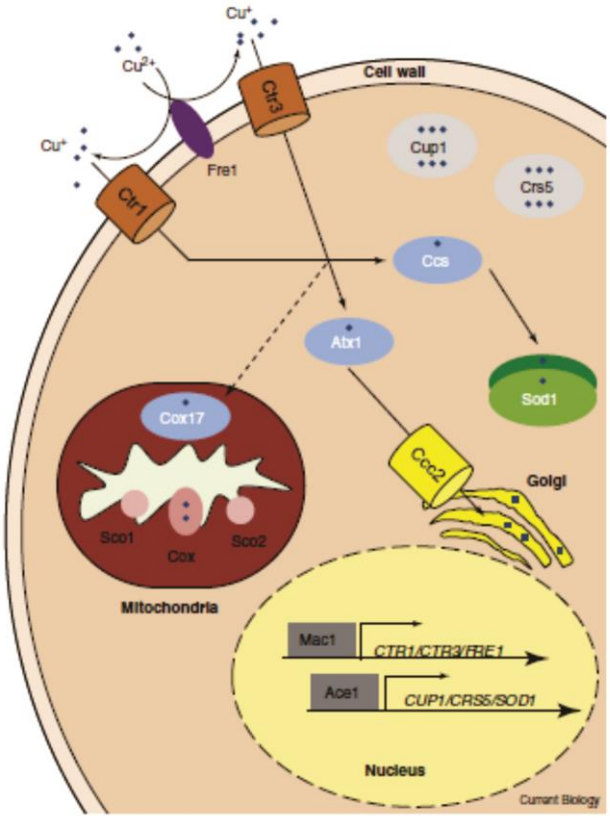


Figura 5. Homeostase de cobre descrita em *Saccharomyces cerevisiae*. Organismos tal como as leveduras tem uma grande complexidade de distribuição de cobre entre proteínas e compartimentos intracelulares. Notoriamente chaperonas são utilizadas para proteger a célula do cobre livre e para entregar o cobre para meloproteínas que necessitam deste metal. Ccs são cobrechaperonas que entregam o cobre depois de ser importado pelos importadores Ctr1 e Ctr3 para superóxido disumutases Sod1 para proteção contra o estress oxidativo. Atx1 é uma chaperona que entrega cobre para o transportador Ccc2 que o importa para o complexo de golgi para ser utilizado em cuproenzimas. Cup1 e Crs5 são metalotioeninas que armazenam cobre. Mac1 e Ace1 são fatores de transcrição que respondem para a ausência e excesso de cobre respectivamente. Fonte: FIESTA e THIELE 2011.

O cobre, uma vez dentro da célula, se liga a proteínas de transporte e metalochaperonas, Ccs e Atx1, que auxiliam na regulação do cobre livre, impedindo com que este metal atinja níveis muito altos no citosol. Estas moléculas também realizam a entrega do cobre para metaloproteínas que necessitam desse metal como as superóxido dismutases e laccases, ou podem transferir este metal para a mitocôndria ou para o complexo de Golgi para armazenamento (HODGKINSON & PETRIS 2012).

Existem também proteínas chamadas de metalotioeninas, Cup1 e Crs5, que se ligam ao cobre livre, diminuindo sua disponibilidade e evitando que o metal se ligue com outras proteínas não específicas, além de permitir sua estocagem (HODGKINSON & PETRIS 2012). Apesar de estocarem cobre, as metalotioeninas no entanto são incapazes de transferir o cobre para metaloproteínas que necessitam de cobre. Esse papel, é exercido por metalochaperonas como já descrito (MARTINEZ-FINLEY et al., 2012).

Em eucariotos, o importe e exporte de cobre intracelular é regulado por fatores de transcrição que respondem aos níveis de cobre, e ativam genes que atuam na homeostase deste metal. Quando células de *S. cerevisiae* enfrentam ambientes com baixa concentração do metal, o fator de transcrição sensível a cobre Mac1 ativa a transcrição de genes que codificam para os sistemas de aquisição de cobre, como por exemplo os importadores de cobre Ctr1 e Ctr3 (SERPE et al., 1999). A regulação transcricional cobre específica mediada por Mac1, é realizada através da sua ligação em regiões específicas no promotor dos genes sob seu controle, regiões estas denominadas de "CuREs" (Cu Response Elements) que possuem a sequência específica 5'-TTTGC(T/G)C(A/G)-3' (BEAUDOIN & LABBE 2001). Em condições de elevado nível de cobre, íons deste metal se ligam diretamente no fator de transcrição Mac1, que se desliga das regiões CuREs (LABBE et al., 1997) e sofre uma mudança conformacional, tornando-se inativa (BEAUDOIN & LABBE 2001).

Por outro lado, quando *S. cerevisiae* enfrenta um ambiente com alta concentração de cobre, o fator de transcrição Ace1 por sua vez ativa a transcrição de genes que codificam para a detoxificação de cobre, como os que codificam para a expressão da metalotioenina Cup1, metalochaperona Crs5 e superóxido dismutases cobre dependentes (KELLER et al., 2005). Quando íons de cobre se ligam a Ace1, este sofre uma mudança conformacional se tornando ativo, e assim se ligando por sua vez, a regiões CuREs na região promotora de genes que estão sobre seu controle transcricional (SAYERS et al., 1999). A sequência CuREs reconhecida por Ace1 é 5'-(A/C/T)T(A/C/T)NNGCTG(A/G/T)- 3', onde N = qualquer nucleotídeo (BEAUDOIN & LABBE 2001).

Recentemente foi demonstrado por Kusuya e colaboradores (2017) que o fungo patogênico *A. fumigatus* possui um fator de transcrição homólogo a Mac1 de *S.*

cerevisiae, que controla a importação de cobre em condições de privação do metal, chamado de Afmac1. O fator de transcrição Afmac1 ativa a transcrição dos importadores de cobre CtrA1, CtrA2 e CtrC neste fungo (KASUYA et al., 2017). Wiemann e colaboradores (2017) demonstraram que *A. fumigatus* também possui o fator de transcrição AfAce1 homólogo a Ace1 de *S. cerevisiae*. Foi demonstrado neste estudo que AfAce1 em *A. fumigatus* ativa a transcrição de um exportador de cobre de membrana CrpA, e que este fator de transcrição é importante para este fungo durante o processo infeccioso em macrófagos murinos, pois parece que neste contexto este fungo enfrenta um ambiente de excesso de cobre (WIEMANN et al., 2017).

Apesar de *S. cerevisiae,* como o modelo mais bem estudado, apresentar a existência de dois fatores de transcrição pra cobre, Mac1 e o Ace1, essa não é uma característica comum dentre a maioria dos fungos. *Schizosaccharomyces pombe e C. neoformans,* por exemplo, possuem um único fator de transcrição (Cuf1) que regula a homeostase de cobre nestes fungos (NEVITT et al., 2012; GARCIA-SANTAMARINA et al., 2018), ativando genes que atuam para o reestabelecimento da homeostase de cobre tanto no excesso quanto em níveis baixos do metal. Cuf1 em *S. pombe* ativa a expressão de genes para a importação de cobre (Ctr4 e Ctr5) em condições de baixa disponibilidade do metal, porém quando esta levedura está em condições de excesso de cobre, Cuf1 ativa a expressão de genes de detoxificação (Cup1) ativando com isso genes relacionados a homeostase de cobre em ambas as situações (BEAUDOIN et al., 2013). Cuf1 neste fungo apresenta alta homologia tanto para Mac1 quanto para Ace1 de *S. cerevisiae,* sendo considerado um fator de transcrição de dupla função (BEAUDOIN et al., 2013).

Em *C. neoformans*, Cuf1 age de maneira similar ao do fungo *S. pombe,* estando ativo tanto na ausência quanto na presença de cobre. Quando *C. neoformans* está em condições de ausência do metal, Cuf1 ativa a expressão de importadores de membrana (Ctr1 e Ctr4) e quando está em condições de excesso do metal Cuf1 ativa genes que expressam para metalotioeninas cobre específicas Cmt1 e Cmt2 promovendo assim a detoxificação do excesso do metal (DING et al., 2013). Experimentalmente já foi comprovado que mutantes para Cuf1 em *C. neoformans* não conseguem crescer tanto em condições de privação quanto em condições de excesso do metal, mostrando o duplo papel deste fator de transcrição (GARCIA-SANTAMARINA et al., 2018). Mutantes para Cuf1 são avirulentos em modelos murinos de infecção (WATERMAN et al., 2012; SUN et al., 2014), demonstrando a importância deste fator de transcrição para *C. neoformans* durante o estabelecimento da infecção.

Sabe-se há muito tempo que a deficiência de cobre em mamíferos compromete a imunidade celular e sensibiliza o hospedeiro diante de infecções causadas por micro-organismos. Ainda não se sabe bem o motivo dessa dependência de cobre nas respostas imunes de hospedeiros mamíferos, porém alguns estudos foram recentemente feitos, visando a importância de cobre na resposta de defesa de macrófagos, pois estas células são frequentemente a primeira linha de células de combate a patógenos (FESTA & THIELE 2012).

Em contraste com a limitação imposta por macrófagos a microrganismos patogênicos, durante o processo infeccioso, de metais essenciais tais como o ferro, zinco e manganês, o cobre durante a infecção é acumulado no fagolisossomo (WHITE et al., 2009). Esse acúmulo é coordenado pelo aumento da expressão do importador de cobre de membrana de macrófagos Ctr1 e do importador de cobre para o fagolisossomo ATP7a (WHITE et al., 2009).

Estudos *in vitro* demonstraram que o cobre realmente se relaciona com a atividade antimicrobiana de macrófagos. Uma das primeiras evidências neste sentido surgiram através da análise elementar de fagossomos de macrófagos; as citocinas interferon-ɣ (INTɣ) e fator de necrose tumoral (TNFα) promoveram o acúmulo de cobre nos fagossomos de macrófagos infectados com a bactéria *Mycobacterium avium*, reforçando a hipótese que o cobre tem um papel importante durante a infecção (STAFFORD et al., 2013). Estudos anteriores também já demonstraram em laboratório, que macrófagos tanto de linhagem eternizada como de linhagem primária, têm seus níveis de expressão para o importador de cobre Ctr1 aumentados quando tratados com INT- ɣ e Lipopolissacarídeos (LPS) (FESTA & THIELE 2012).

Neste mesmo sentido, foi observado que a infecção de macrófagos primários oriundos de camundongos com *Salmonella typhimurium* durante um período de 14 horas, promoveu o acúmulo de cobre em vesículas intracelulares nestes macrófagos. Quando estas células foram tratadas durante a infecção com um quelante de cobre, o ácido batocuprosulfônico (BCS), estas foram incapazes de eliminar de forma ideal a bactéria patogênica (STAFFORD et al., 2013).

Mackie e colaboradores (2016) demonstraram que tecidos do hospedeiro tendem a remanejar a concentração de cobre contra patógenos invasores. Isso foi observado por análises do perfil de expressão gênica de *C. albicans* durante infecções disseminadas no fígado e rins de camundongos. Durante a infecção por 24 horas nos rins, este fungo sofre um excesso de cobre imposto por células hospedeiras, pois os níveis de expressão da bomba de efluxo de cobre Crp1, neste momento estão elevados. Porém na infecção a longo prazo, 48 e 96 horas, os níveis de expressão de Crp1 caem, e em contrapartida os níveis do importador de cobre Ctr1 aumentam, dando a entender que neste dado tempo, o fungo enfrenta um ambiente de escassez do metal (MACKIE et al., 2016).

Durante a fagocitose de um microorganismo patogênico, o fagolisossomo do macrófago transita para um ambiente com alta capacidade microbicida, onde a presença de cobre auxilia na composição deste ambiente, acidificando o pH, gerando um maior número de espécies reativas de oxigênio e nitrogênio devido a sua capacidade de óxido/redução e otimizando a função de proteases presentes no lúmen (HODGKINSON & PETRI 2012; FESTA & THIELE, 2012; FESTA & THIELE 2011). Além disso, já foi descrito que durante a fagocitose na presença da citocina INT-ɣ há uma grande liberação de cobre livre no fagolisossomo, na tentativa de matar o patógeno por intoxicação diante dos grandes níveis deste metal (RAJA et al., 2013; STAFFORD et al., 2013). Os potenciais mecanismos utilizados por macrófagos dos quais o cobre pode contribuir no combate a patógenos são a limitação ou o aumento dos níveis deste íon dentro do fagolisossomo. Estes mecanismos estão resumidamente demonstrados e descritos na figura 6.

Diante disso, alguns fungos desenvolveram estratégias de contra-ataque como *C. neoformans* que consegue armazenar grandes quantidades de cobre, até níveis 5 vezes acima de suas necessidades normais, para evitar a intoxicação (RAJA et al., 2013). Outra estratégia utilizada por fungos de uma forma geral, como resposta das células aos níveis elevados de cobre é reprogramar a transcrição de genes que são importantes para a captura de cobre e sua exportação para fora da célula, para manter as concentrações do mesmo dentro dos limites homeostáticos, permitindo que a quantidade deste metal seja suficiente para servir como cofator e prevenindo sua citotoxicidade (BEAUDOIN et. al., 2011; BEAUDOIN & LABBE 2007).

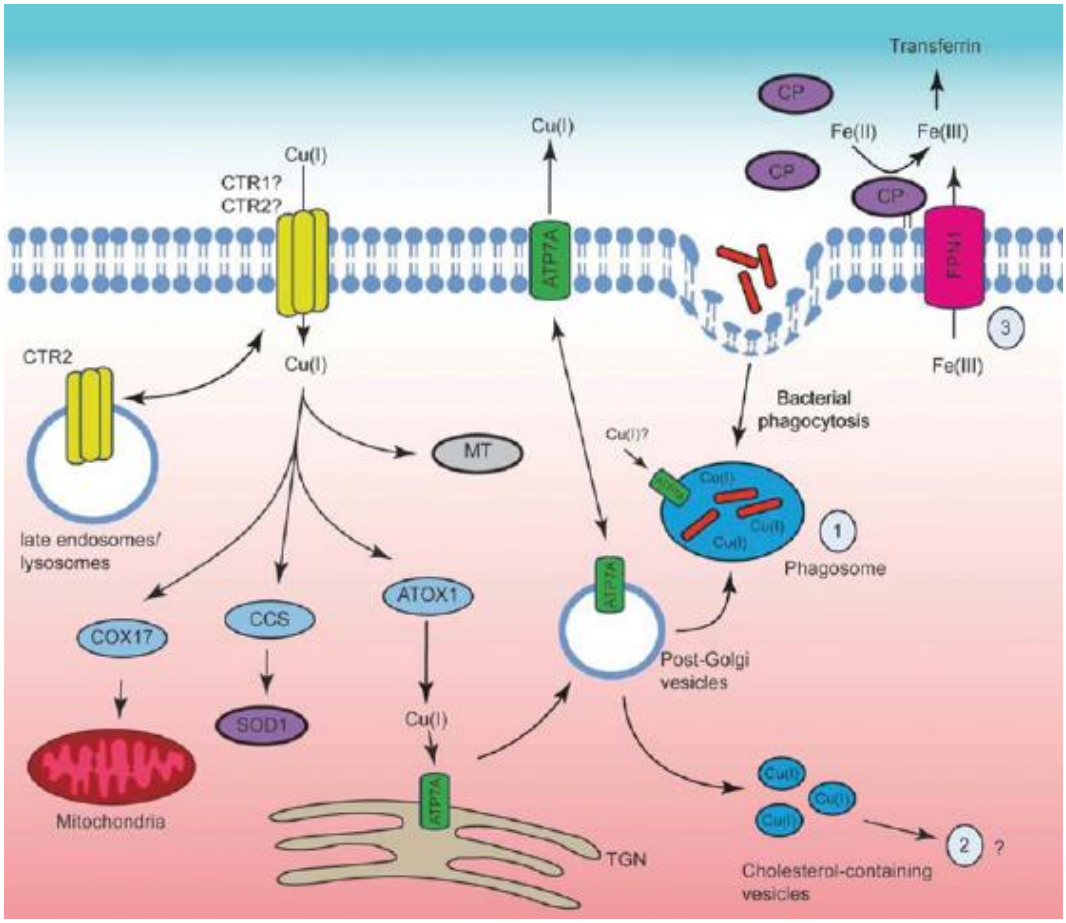


Figura 6. Possíveis mecanismos dos quais o cobre pode contribuir em respostas antimicrobianas de macrófagos. Cobre é importado para o macrófago através dos transportadores CTR1 e/ou CTR2. Dentro da célula ele se liga as chaperonas (COX17, CCS e ATOX1) ou a proteínas de ligação a metais como as metalotioeninas (MT). O cobre pode ser importado para mitocondria para produção de energia, para superóxido dismutases (SOD) ou para o transportador vesicular ATP7A. INTγ e ou LPS up-regulam a expressão de vários genes de transporte de cobre em macrófagos de camundongo e promovem a importação deste metal nestas células. Possíveis mecanismos com efeitos antimicrobianos que utilizam cobre: (1) toxicidade direta do acumulo de cobre no fagolisossomo; (2) acumulo vesicular para geração de espécies reativas de oxigênio e (3) uso do cobre nas ferro-oxidades para o transporte de ferro para fora do fagossomo, indisponibilisando o ferro para o patógeno intracelularmente. Extraído de STAFFORD et al., 2013.

Futuros estudos que investiguem o papel do cobre durante a infecção de fungos patogênicos são requeridos, para uma maior compreensão do papel que o cobre desempenha durante a infecção (SAMANOVIC et al., 2012). Pouco se sabe entretanto, sobre a homeostase de cobre em *H. capsulatum*, que mecanismos o fungo se utiliza para manter esta homeostase, e qual o papel dos genes diretamente ligados a este processo.

Devido a isso, este trabalho teve o intuito de caracterizar a homeostase de cobre em *H. capsulatum*, explorando quais genes estão envolvidos na aquisição e detoxificação de cobre neste fungo, *in vitro* e durante o processo infeccioso.

2.0 JUSTIFICATIVA

A histoplasmose nos dias de hoje é considerada uma doença de incidência global, diferentemente do que se achava no passado e mesmo assim o conhecimento sobre sua distribuição no globo ainda é incompleta. Pandemias causadas pelo vírus HIV tornam a histoplasmose um risco mundial, fazendo surgir casos da doença mesmo em regiões que não eram endêmicas. Adicionalmente, o aumento de viagens internacionais e o aumento do uso de medicamentos imunossupressores aumentam o risco de aquisição da doença. Áreas de grande risco existem e o Brasil é considerado um dos países de maior incidência da doença dentre as américas, fazendo com que a histoplasmose seja uma importante doença para a saúde pública no país.

Fungos patogênicos tais como o *H. capsulatum*, durante a infecção, utilizam diversos mecanismos para manutenção da homeostase de nutrientes, dentre eles os diversos metais responsáveis pelo funcionamento adequado dos sistemas biológicos. Estes mecanismos são importantes para fungos patogênicos, pois ao utiliza-los estes fungos conseguem infectar, crescer, se proliferar em tecidos hospedeiros, se defender de células de defesa hospedeiras e causar enfermidades.

Dentre os íons metálicos importantes para os metabolismos biológicos, o cobre se destaca pela sua imensa importância tanto para o patógeno, quanto para uma resposta imune adequada, visto que hospedeiros com deficiência em cobre não conseguem combater infecções causadas por microrganismos invasores, e patógenos que não conseguem controlar os níveis, tanto na limitação quanto no excesso deste metal, não conseguem se estabelecer nos sítios da infecção. Apesar da importância do cobre, os mecanismos moleculares de sua homeostase durante a interação patógeno/hospedeiro ainda permanece pouco estudada, e em fungos dimórficos, grupo do qual *H. capsulatum* faz parte, quase que desconhecida.

É fato que *H. capsulatum* codifica maquinarias homeostáticas de regulagem e de captação de cobre durante o processo infeccioso, pois este fungo consegue se estabelecer como patógeno, porém este mecanismo ainda é desconhecido. A homeostase de cobre necessária para o estabelecimento deste patógeno durante o processo infeccioso é controlada diretamente por genes que respondem tanto a limitação quanto para o excesso

deste íon. Futuros estudos que investiguem o papel do cobre durante a infecção de fungos patogênicos são requeridos, para uma maior compreensão do papel que o cobre desempenha durante a infecção, principalmente em patógenos fúngicos (SAMANOVIC, et al., 2012). Elucidando-se este processo, os mecanismos que o fungo utiliza-se para manter sua homeostase e causar a doença se tornam mais claros, e com isso, consequentemente, surgem novos alvos para o desenvolvimento de futuros fármacos, procedimentos clínicos e tratamentos.

Além disso, os dados disponíveis sobre o papel do cobre durante a infecção, na literatura, são dúbios, mostrando que as estratégias de defesa das células hospedeiras se utilizam tanto da toxicidade causada pelo excesso do metal quanto da limitação do mesmo para o patógeno. Está claro então a necessidade de se desvendar o real papel que o cobre tem em *H. capsulatum* e quais mecanismos transcricionais este fungo se utiliza, para manter a homeostase deste metal e dessa maneira se estabelecer como um patógeno. Existe com isso a necessidade de elucidar a homeostase de cobre em *H. capsulatum*, quais as ferramentas genéticas que este fungo se utiliza tanto *in vitro*, quanto durante o processo infeccioso. Estes estudos virão a esclarecer a interação patógeno/hospedeiro de *H. capsulatum* tendo o cobre como alvo, e também virão a trazer maior esclarecimento sobre os papéis deste metal para a biologia e a doença causada por este fungo.

3.0 OBJETIVOS

3.1 Objetivo Geral

- Caracterizar a homeostase de cobre no fungo patogênico humano *H. capsulatum*,

Objetivos específicos

- Identificar genes relacionados a homeostase de cobre em *H. capsulatum;*
- Analisar a expressão de genes relacionados a homeostase de cobre em *H. capsulatum*, em condições *in vitro* e *ex vivo*;
- Analisar a expressão de genes relacionados a homeostase de cobre em macrófagos durante a infecção com *H. capsulatum;*
- Caracterizar a dinâmica do cobre durante a infecção;
- Identificar genes relacionados com a homeostase de cobre de *H. capsulatum* durante a infecção;
- Obter e caracterizar cepas mutantes de *H. capsulatum* para genes que tem papel importante na homeostase de cobre neste fungo.

CAPÍTULO 2: Manuscrito

4.0 RESULTADOS (Manuscrito a ser submetido)

The human pathogen *Histoplasma capsulatum* face a high copper environment during macrophage infection.

Gabriel Brum Tristão[1], Leandro do Prado Assunção[1], Juliano Domiraci Paccez[1], Rosely Maria Zancopé-Oliveira[2], Célia Maria de Almeida Soares[1], Chad Reppleye[3] and Alexandre Melo Bailão[1*].

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

[2] Laboratório de Micologia, Instituto Nacional de Infectologia Evandro Chagas, Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil.

[3] Department of Microbiology, Department of Microbial Infection and Immunity, Ohio State University, Columbus, Ohio, United States.

*alexandre.bailao@gmail.com

Abstract

Histoplasma capsulatum is a thermodymorphic pathogenic fungus that causes systemic mycosis known as histoplasmosis. This fungus grows as mycelium at temperatures around 25°C and as yeast at 37°C. During the infectious process, pathogenic microorganisms must obtain nutrients from the host in order to survive in infected tissues. Among these nutrients, copper is an essential metal ion, because it participates in oxidation/reduction reactions, in energy production, in the transport of electrons, its cofactor of many enzymes and metalloproteins and is required for the production of melanin. Copper excess however, it is toxic due to the fact that produces reactive oxygen species, dislocates other metals from metalloproteins, causes damage to lipids and DNA, so because of this *H. capsulatum* must maintain the homeostasis of this metal during infection. We observed here that *H. capsulatum*, through the transcriptional levels of Ctr4, Mac1, Crp1 and Ace1, during the infectious process in macrophages, faces an environment of copper overload imposed by the host cells via copper ATPase ATP7a. This copper excess shown to be INF-ɣ and time dependent, because when macrophages are not stimulated by INF-ɣ and in greater times of infection, they impose a restrictive copper environment instead, during the infection. *H. capsulatum* uses the Crp1 copper efflux pump in order to respond this toxic copper milieu, since mutant yeasts for Crp1 were unable to grow at high levels of the metal. Despite the importance of Crp1 for the fungus in this context, it appears that Crp1 is not strictly necessary for the total virulence of the fungus, leading us to infer that other proteins may also be exerting a Crp1-like function in *H. capsulatum*. It is clear once again that in the pathogen-host relationship copper plays a complex, highly dynamic and dependent on certain biological variables role.

INTRODUCTION

Histoplasma capsulatum is a thermodimorphic human pathogenic fungus, the etiological agent of the systemic mycosis histoplasmosis. *H. capsulatum* can be found as mycelium in nature, at temperatures of 18 to 28 °C in nitrogen/phosphate rich soils generally associated with birds and bats guano (Ajello et al., 1964; Teixeira et al., 2016). In this form, the fungus presents a filamentous morphology of white and brownish, cottony colonies. Microscopically is characterized by septate hyaline hyphae, with the presence of macroconidia, measuring from 8 to 16 μm in diameter, generally spherical and usually of smooth cell wall and microconidia, that are smooth or rough-walled and smaller in diameter (2-5 to 16 μm; Zancopé-Oliveira et al., 2013). The macroconidia and microconidia that together or with mycelium fragments, may be inhaled by several vertebrates, including humans, that when in pulmonary alveoli differentiate into yeast initiating the infectious process (Maresca & Kobayashi 1989; Teixeira et al., 2016). The yeast form of the fungus, on the other hand, is characterized by humid, smooth-looking, yellowish-white colonies in solid media cultures. Microscopically it is shown as small spherical or oval yeasts with a thin-wall, measuring from 2 to 4 μm in diameter (Goodwin et al., 1981). From the respiratory tract, the infection can disseminate through both lymphatic and hematogenous pathways. The fungus may spread to whole body, particularly to macrophages rich organs such as liver, spleen, lymph nodes and bone marrow causing lesions in the infected sites (Ferreira & Borges 2009).

Individuals with acquired immunodeficiency syndrome (AIDS) are the group which histoplasmosis has the highest morbidity and mortality rates (Adenis et al., 2014). Also, the recent increase in immunosuppressive therapies due to organ transplantation, autoimmune diseases, chronic inflammatory disorders and the smoking habit, which leads to a greater disposition of pulmonary lesions that can facilitate the fungus infection, histoplasmosis is becoming more frequent around the globe (Mukherjee et al., 2010; Nacher et al., 2013; Pulot et al., 2015). Currently, this disease has a worldwide distribution and has been described in more than 50 countries, with a higher prevalence in tropical and temperate zones. This ringworm is widely distributed in the American continent, occurring in countries such as the United States, Mexico, Honduras, Guatemala, Panama, Venezuela, Colombia, Peru, Argentina, Uruguay and Brazil, being an important disease for public health in these countries (Bahr et al. 2015).

During the infection, several processes are necessary for the survival and replication of pathogenic fungi and, such processes, may cause directly or indirectly damages to the host, being denominated virulence factors (Hube 2009). The main virulence factors that can be mentioned, are the ability to grow directly in the host tissues, growth in the mammalian host temperature, immune system evasion strategies, adhesion factors, morphology changes and nutrient acquisition, such as metal ions (Brunke et al., 2016). The strategy of acquiring metallic micronutrients is of particular importance for pathogenic fungi, because the host cells have developed mechanisms, along the evolution, in order to restrict or overload the fungus access to these metals, a phenomenon known as nutritional immunity (Kehl-Fi et al., 2010).

The concept of nutritional immunity has been described for decades, as the nutritional limitation imposed by host cells to a given pathogen, when observing the host-mediated iron limitation during infectious process (Ballou & Wilson 2016). However new findings have been changing this concept, pointing out that there is not only a limitation, but also an excess imposed by the host as form of defense, because the excess of metallic ions is toxic (Sun et al., 2014; Mackie et al., 2016; Wiemann et al., 2017). Additionally the concept of nutritional immunity, covering both excess and nutritional limitation, is not only limited to iron, covering other metallic ions such as zinc, manganese and copper for example (Hood et al., 2012).

H. capsulatum has the specific ability to survive and replicate inside the hostile environment of macrophage phagosomes. For that the fungus inhibit phagosomal acidification, counteract reactive oxygen species by producing and secreting antioxidant enzymes CatB and CatP (Smith & May 2013). Intraphagosomal pathogenic microorganisms undergo iron deficiency as a result of the host microbicidal mechanism that keep iron away from invaders (Holbrook et al.; 2013). In order to circumvent this limitation, *H. capsulatum* developed mechanisms for iron acquisition from host Fe-containing proteins such as transferrin, ferritin; via hydroxamates siderophore production or by secretion of ferric reductases (Newman & Smulian 2013).

In addition to iron, it has also been demonstrated that *H. capsulatum* also suffers a zinc limitation within the phagolysosome. Vignesh et al. (2013) demonstrated that macrophages stimulated with granulocyte macrophage colony stimulating factor (GM-CSF) sequestrates zinc from phagolysosome via Slc30a4 and Slc30a7 exporters, limiting the availability of this metal inside phagosomes of infected macrophages (Vignesh et al.,

2013). Dade et al. (2016) also demonstrated that *H. capsulatum* has the Zrt2 gene that codes for the zinc importer Zip2. Yeast cells silenced for Zrt2 gene presents a decrease in the intracellular zinc accumulation, growth deficiency in zinc-free medium and also impaired virulence (Dade et al., 2016).

Copper, together with iron and zinc, is also biologically essential, because it plays cofactor functions in several enzymes such as cytochrome c oxidase, superoxide dismutases, laccases, ferric reductases; thus acting in metabolic processes such as energy production, iron acquisition, melanin production and antioxidant defense (Tristão et al., 2015). On the other hand, the free copper in cytoplasm is toxic at high levels, due to its ability to generate reactive oxygen species by Fenton Reactions (Achard et al., 2012; Ding et al., 2013; Wiemann et al., 2017). Copper-toxicity is also based in the competitive displacement of native metals from non-copper metalloproteins, as well as in the destruction of the structure of proteins containing iron/sulfur groups (Beaudoin & Labbe 2007). Based on that, host uses the copper poisoning as a weapon against invaders microorganisms (White et al., 2009; Sun et al., 2014; Mackie et al., 2016; Wiemann et al., 2017).

A prompt response to the dynamism of copper homeostasis requires a coordinated and accurate transcriptional response (Ballou & Wilson 2016). In this way fungi use various classes of proteins to regulate copper homeostasis, such as copper-binding transcription factors, Cu importers and exporters, copper-binding metallothioenins, and copper-specific metallochaperones (Jungman et al., 1993; Graden and Winge 1997; Cyert & Philpott 2013). In *Saccharomyces cerevisiae* copper deficiency is sensed by the Mac1 copper-binding transcription factor, which activates the expression of the copper importers Ctr1 and Ctr3 (Cyert & Philpott 2013). The specific copper transcriptional regulation mediated by Mac1 is carried out by binding to specific regions in the promoter of the genes under its control, regions known as Cu Response Elements (CuREs) having the specific sequence 5’-TTTGC(T/G)C(A/G)-3’ (Beaudoin & Labbe 2001). The copper excess, on the other hand, in *S. cerevisiae* is sensed by the transcription factor Ace1 that activates expression of the metallothioenines Cup and Crs5 (Culotta et al., 1994; Thiele 1988). The CuRE sequence recognized by Ace1 is 5'-(A/C/T)T(A/C/T)NNGCTG(A/G/T) -3 ', where N = any nucleotide (Beaudoin & Labbe 2001).

Candida albicans responds to low copper levels via CaMac1 transcription factor in a similar way to that observed in *S. cerevisiae* (Ballou & Wilson 2016). CaCup2 (*S.*

cerevisiae Ace1 orthologous) responds to high copper levels, and induces expression of the metallothioneins Cup1 and Crd2 (Homann et al., 2009), as well as a copper efflux pump Crp that plays a critical role in Cu-detoxification (Douglas et al., 2011; Mackie et al., 2016). It has been demonstrated that *Aspergillus fumigatus* also has two main regulators of copper homeostasis AfMac1 (Kusuya et al., 2017) and AfAce1 which coordinates fungal adaptation to Cu-limitation and copper excess, respectively. The transcription factor AfAce1 plays a critical role in fungal pathogenesis, since it induces CrpA production during murine infection avoiding fungal killing by Cu-poisoning (Wiemann et al., 2017).

In *Cryptococcus neoformans*, there is only one copper-responsive transcription factor, Cuf1, which is required for growth on both low and high copper availability conditions (Ballou and Wilson 2016). Cuf1 regulates the expression of copper importer Ctr4 as well as the two metallothioenins Cmt1 and Cmt2 involved in copper detoxification (Ding et al., 2011; Waterman et al., 2007). While Ctr1 is constitutively expressed in this fungus, low copper specifically induces Ctr4 in a Cuf1 dependent manner, and during growth on high copper, Cuf1 mediates Cmt1 and Cmt2 expression (Ding et al., 2013).

In contrast to the nutritional immunity observed in iron and zinc, which mainly consists of the sequestration of these micronutrients by the host, the nutritional immunity of copper is a highly dynamic system (Potrykus et al., 2014; Hood et al., 2012). Two studies conducted with *Salmonella typhimurium* and three species of *Mycobacterium* demonstrated that macrophages stimulated with interferon ɣ during infection with these bacteria, increases the expression of the copper transporter Ctr1 and relocate the golgi coplex ATP7a copper pump to the phagosome membrane. Then ATP7a transporter pumps copper into the phagossome, raising the levels of this metal in this compartment and killing the pathogen by Cu-mediated poisoning (White et al., 2009; Achard et al., 2012).

Mackie et al. (2016) through gene expression profiling from infected mouse tissues, showed readjustments in hepatic, splenic and renal copper homeostasis during disseminated *C. albicans* infections. During systemic infection in mouse kidneys, this fungus faces a rich copper environment at the early infection (24 hours). At this time point, *C. albicans* induces a copper efflux pump expression and decreases copper importer expression. In the long-term infection (48 and 96 hours), the copper efflux pump is down-regulated and the copper importer is up-regulated, suggesting that at this time

point *C. albicans* faces a scarce Cu environment (Mackie et al., 2016). Sun et al. (2014) showed that *C. neoformans* has high transcriptional levels of the metallothioneins genes Cmt1 and Cmt2, during mouse lung infection, indicating that in this tissue the fungus suffers copper overload. However, when the fungus migrates do the brain, Cmt1 and Cmt2 are down-regulated while the Ctr4 is up-regulated, suggesting *C. neoformans* faces a low Cu environment (Sun et al., 2014).

Little is known about copper homeostasis in *H. capsulatum*, mainly during the infectious process, what mechanisms the fungus uses to optimize its homeostasis in this process and what are the cooper-mediated strategies that are used by host cells to fight this pathogen. Because of this we explored which genes are involved in the acquisition and detoxification of copper in *H. capsulatum*. As the role of copper in the host - pathogen interaction is highly dynamic and complex we also explored the dynamics of this metal during macrophage cell line infection.

RESULTS

Copper homeostasis genes encoded by *H. capsulatum* genome

Although mechanisms of iron and zinc homeostasis in *H. capsulatum* have been initially studied (Chao et al., 2008, Hilty et al., 2008, Newman & Smulian 2013), the knowledge related to copper is still absent. On this way, genes potentially related to copper homeostasis were sought at *H. capsulatum* G186A database using as templates sequences of copper homeostasis related genes from *S. cerevisiae*, *C. neoformans* and *A. fumigatus* (Table S1). Data mining approach and orthology comparisons identified 8 putative copper homeostasis genes in the fungus: 2 high affinity copper membrane transporters Ctr1 (EEH10721.1) and Ctr4 (EEH05121.1), a copper efflux membrane pump Crp1 (EEH08652.1), the regulator for copper excess adaptation Ace1 (EEH09540.1), the regulator for low copper adaptation Mac1 (EEH06208.1), a copper transporter localized at Golgi membrane Ccc2 (EEH07254.1), the metallochaperone Atx1 (EEH03921.1) and the copper binding melallothioneine Crs5 (EEH09689.1; Table 1; Supp. Table 1). Overall identity values obtained by BLAST comparison ranged from 60% to 86%. The *C. neoformans* gene Ctr4 presented the highest identity while the Crs5 gene of *S. cerevisiae* was the less conserved one. Also using the OrthoVenn tool, it was observed that all of the genes of *H. capsulatum* found here so far, group with their

respective orthologues in other fungi (Table S2; *Blastomyces dermatitidis, C. neoformans, A. fumigatus, Paracoccidioides brasiliensis* and *S. cerevisiae*).

Table 1. Copper responsive genes in *H. capsulatum* resulting from data mining from *A. fumigatus, C. neoformans* and *S. cerevisiae* genomes.

Accession Number	Gene	Function
EEH10721.1	Ctr1	Copper membrane transporter
EEH05121.1	Ctr4	Copper membrane transporter
EEH08652.1	Crp1	Copper membrane efflux pump
EEH09540.1	Ace1	Copper responsive transcription factor
EEH06208.1	Mac1	Copper responsive transcription factor
EEH07254.1	Ccc2	Golgi complex copper transporter
EEH03921.1	Atx1	Copper metallochaperone
EEH09689.1	Crs5	Copper metallothionein

The copper sensing transcription factors Ace1 and Mac1 promote their regulatory role by binding to promoter elements, (A/C/T)T(A/C/T)NNGCTG(A/G/T) where N = any nucleotide for Ace1 and TTTGC(T/G)C(A/G) for Mac1, in the target genes (Beaudoin and Labbe 2001). Those copper responsive elements were searched in the promoter regions of Cu-related *H. capsulatum* genes (Fig. 1). It was observed that all genes potentially related to copper homeostasis found here have Ace1 and/or Mac1 binding motifs in their promoter regions, suggesting that these genes are regulated by these transcription factors. Structurally, fungal copper responsive transcription factors maintain a copper responsive motif (R/K)GRP and a conserved N-terminus Copper-fist DNA-binding motif (Balou & Wilson 2016), which were found in the two transcription factors identified in this study. In addition, *S. cerevisiae* Ace1 encode CXC and CX_2C (tetra-copper) motifs, and *S. cerevisiae* Mac1 is characterized by dual cysteine rich C-terminal motifs, REP-I ($CXCX_4CXCX_2CX_2H$) and REP-II ($CXCX_4CXCX_2CX_2H$; Beaudoin et al., 2001; Keller et al., 2000). The Ace1 of *H. capsulatum* presented the characteristic motifs CXC and CX_2C such as *S. cerevisiae* Ace1, but interesting *H. capsulatum* Mac1, together with *B. dermatitidis, P. brasiliensis, Coccidioides immitis, A. fumigatus, Talaromyces marneffei* and *Sporothrix schenkii* has the REPI and REPII structure similar to the single REP found in *Schizosaccharomyces pombe* Cuf1 ($CXCX_3CXCX_2CX_2H$), which governs adaptation to both copper excess and deprivation (Beaudoin et al., 2013;

Fig. 2a). This peculiarity at the Mac1 REPI and REPII motifs is shared among dimorphic fungi (Fig. 2a and b).

Growth and viability analysis of *H. capsulatum*

The effect of copper availability on *H. capsulatum* growth and viability was evaluated (Fig. 3). For that the fungus was grown in copper limiting condition (with 30μM of copper chelator BCS), medium copper level (30μM $CuSO_4$) and in high copper availability (250 μM and 500 μM $CuSO_4$). It was observed that the copper deprivation did not affect fungal growth and survival. On the other hand, Cu at 250 μM slightly affects fungal grown and 500 μM completely impaired *Histoplasma* growth. Those results suggest the fungus keeps efficient mechanisms to support growth at low and high metal availability. It is, also, shown that Cu levels above 250 μM are beyond *Histoplasma* capacity to fight copper-poisoning.

Transcriptional profile of copper related genes in *in vitro* conditions

Mammalian host defense cells use antimicrobial strategies that focus directly on trying to interrupt the metal homeostasis from invasive pathogens, a strategy referred to as nutritional immunity. In terms of copper, fungal pathogens may suffer either copper limitation or excess depending on tissue or time of infection (Sun et al., 2014, Mackie et al., 2016). In order to evaluate the copper homeostasis of *H. capsulatum* the expression profiles of copper-related genes were evaluated during *in vitro* conditions (Fig. 4a). Transcripts of *Histoplasma* genes encoding to high affinity copper transporters Ctr1 and Ctr4 were differentially induced in copper deprived cells. Ctr4 presented a stronger induction in both 3 h and 24 h when compared with Ctr1. At the later time point Ctr4 was induced more than 30 times when compared to cells grown in 30 μM Cu. On the other hand, only at 24 hours Ctr1 was induced 2.5 times. Thus, the expression data suggest Ctr4 plays a more effective role in Cu uptake in copper limiting scenario. In a complementary way, the expression of the copper efflux pump Crp1 was repressed in low Cu availability. The transcripts encoding to proteins related to intracellular metal storage and distribution Ccc2, Crs5 and Atx1 accumulated in yeast cells treated with copper chelator BCS. Regarding the putative regulators of copper homeostasis, Ace1 expression was repressed in Cu deprivation while Mac1 was induced. These results indicate that Ace1 coordinates fungal adaptation to high Cu availability conditions while Mac1 orchestrates fungal

survival in metal limiting environments, corroborating with the sequence characterization data (Fig. 2).

H. capsulatum is exposed to high copper levels in macrophage phagosomes

Since *Histoplasma* is an intraphagosomal organism, the expression profiles of Cu-related genes were accessed during fungal growth in macrophage phagosomal environment (Fig. 4b). Yeast cells recovered from macrophages decreased transcript levels of both Ctr1 and Ctr4 in comparison to control cells. However, the Crp1 expression was up-regulated in host milieu. Transcripts encoding the regulator Ace1 accumulated during macrophage infection while Mac1 encoding transcripts decreased. The Ccc2 and Crs5 expressions were down regulated, but metallochaperone Atx1 expression did not change in phagocytized cells. The down-regulation of genes related to adaptation to copper limiting conditions (Ctr1, Ctr4 and Mac1) and the induction of that ones related to survival in high metal availability suggest *H. capsulatum* experience a high copper content in phagosome environment. Thus, likely copper poisoning composes the microbicidal macrophage arsenal against *H. capsulatum.* To test the hypothesis that macrophage imposes copper toxic levels to *Histoplasma*, the influence of copper availability in fungal survival in macrophages was analyzed by CFU assays. Macrophages pre-treated with 30 μM $CuSO_4$ were more efficient to kill fungal cells then untreated phagocytes (Fig. 5). Complimentary, copper chelation hampered macrophages fungicidal activity against *Histoplasma*. The decrease in fungal burden in macrophage exposed to 30 μM Cu reinforces the hypothesis that the fungus is exposed to high copper levels in phagosomes.

ATP7a is responsible for *H. capsulatum* copper poisoning in phagosomes

The joint action of ATP7a copper ATPase and importer Ctr1 is implicated in host-mediated copper accumulation in phagosome during bacterial infections (Wagner et al., 2005, White et al., 2009). Also, the accumulation of ATP7a in phagolysosomes is linked to an increase in copper concentration in *A. fumigatus* spores recovered from macrophages (Wiemann et al., 2017). Thus, we reasoned that this conserved host defense strategy my also be used against *H. capsulatum*. On this way, ATP7a silenced J774A macrophage cell line was obtained. The transfection of host cells with siRNAs against ATP7a transcripts promoted a 15 times reduction in copper ATPase mRNA levels (Fig. 6a) and such treatment did not affect macrophage viability (Supp. Fig. 1). Next, infection

assays were performed in silenced and non-silenced phagocytes. ATP7a silencing caused an increase in fungal burden in macrophages (Fig. 6b). This observation may be due to the role of ATP7a in mediating fungal Cu-poisoning. Thus, Crp1 expression levels in yeasts during silenced ATP7a macrophage infection were assessed. The mRNAs levels encoding to Crp1 decreased to control cell levels (yeast grown in medium only) in ATP7a silenced cells, showing that the fungus is not exposed to a high Cu environment in ATP7a silenced macrophages (Fig. 6c).

It is already known that IFN-ɣ stimulated macrophages increase the uptake and cellular internalization of copper via Ctr1 transporter and redirect this copper to the phagolysosomes via ATP7a, thus creating an environment that is toxic to pathogens (White et al., 2009). As it has been observed so far, *H. capsulatum* may be suffering an copper excess during the macrophage infectious process, and we tested to see if this Cu excess is mediated by the cytokine IFN-ɣ. In this way Crp1 mRNA levels were assessed during infection in IFNɣ-treated and non-treated macrophages (Fig 7). As expected, Crp1 transcriptional levels were more expressed as compared to Ctr4 levels during intraphagosomal growth in INFɣ activated macrophages. On the other hand, in non-treated macrophages, yeast cells increase Ctr4 transcripts at 24, 48 and 96h post infection while decreased Crp1 mRNAs at 48 and 96h. Intringuily, Crp1 transcripts also increased at 24h post infection in untreated macrophages. Altogether, these data strongly suggest that *H. capsulatum* experience a copper poisoning milieu in macrophages treated with INFɣ and that ATP7a is responsible for Cu accumulation in phagosomes. On the other hand, macrophages not treated with INF-ɣ appear to be limiting copper during infection, because in this scenario the transcriptional levels of Ctr4 are significantly higher than those of Crp1. Quantitative PCR results have shown that macrophage ATP7a transcripts did not change upon *H. capsulatum* infection or interferon gamma treatment (Supp. Fig. 2), implying that Cu ATPase function, in this case, may be related to its migration from Golgi to phagolysosomes (White et al., 2009; Rupp et al., 2017).

H. capsulatum Crp1 function analysis

The data collected so far indicate *Histoplasma* is challenge with cu-poisoning levels in phagolysosomes of INFɣ-treated macrophages. In this scene the pathogen must have mechanisms to avoid Cu toxicity and survive in phagosomes. As Crp1 is a copper transporter that pumps out the metal in toxic conditions, we further characterized this gene. Crp1 transcript measurements shown an increasing in mRNAs coupled to the

increased in copper availability (Fig. 8a). Thus, Crp1 likely is responsible for copper efflux in fungal cells exposed to Cu-toxic environments. In order to expand the knowledge about this gene in pathobiology of *H. capsulatum*, Crp1 knockdown strains were generated. Silenced fungal cells are more sensitive to Cu levels then wild type cells by about 4 fold (Fig. 8b). We also, evaluated the impact of CRP1-silencing in the virulence of the fungus. Crp1-knockdown yeasts present a slightly lower virulence in macrophage model of infection (Fig. 8c).

Because the mutant strain for Crp1 did not have its virulence highly altered in comparison to the wild strain, fungus genome was sought for additional putative copper efflux genes that could be playing a Crp1-like function. Blast tool-based analysis identified two genes with similarity to Crp1, a hypothetical protein (EEH07254.1) and a cation transport ATPase (EEH06861.1) referred herein as Crp2 and Crp3, respectively. RT-PCR data showed that both genes are more expressed in copper containing environment than in copper deprivation condition (Fig. 9), thus we hypothesized that these genes may play role in fungal copper detoxification during macrophage infection.

DISCUSSION

Pathogenic fungi possess genetic machinery for regulation and uptake of metal ions, maintaining their homeostasis during the infectious process, as they can establish themselves, grow and proliferate in host tissues. We have seen here that for *H. capsulatum* this is not different, that in relation to copper, this fungus has genes responsible for capturing this ion under limitation conditions (Ctr1 and Ctr4), genes responsible for avoiding the toxicity of this metal when in excess (Crp1 and Crs5), and genes that maintains the flow of copper intracellularly (Atx1 and Ccc2). Differently from *S. pombe* and *C. neoformans* that possess only one transcription factor, Cuf1, *H. capsulatum*, similarly to *S. cerevisiae*, *C. albicans* and *A. fumigatus* has two copper sensitive transcription factors, Ace1 that regulates genes that have a role in copper excess and Mac1 that regulates those that act in this metal limitation. When analyzing the copper binding motifs of these transcription factors, we find here that the REPI and REPII motifs of *H. capsulatum* Mac1 and all the dimorphic fungi also analyzed here, interestingly are the same as the REP Cuf1 motif of *S. pombe* and not the REPI and REPII Mac1 motifs of *S. cerevisiae*. As these REPI and REPII motifs similar to REP of *S. pombe* appear in all dimorphic fungi and are well preserved among them, it can be inferred that this

conservation maybe occurred at some point during the evolutionary process of these fungi (Fig 2b).

During the yeast growth under copper presence and absence conditions, the genes analyzed so far had the expected expression profile according to their functions (Fig 4a). The copper importers Ctr1 and Ctr4 were more expressed in the absence of copper and the Crp1 pump more expressed in the presence, confirming their roles of copper uptake and detoxification respectively (Ding et al., 2013; Mackie et al., 2016; Wiemann et al., 2017). Ccc2, Atx1 and Crs5 were more expressed in the absence of copper, probably indicating an attempt to harvest intracellular copper at a time of lack (Coyle et al., 2002). Regarding the Mac1 and Ace1 transcription factors, the expression profile it was not different from the roles previously described in other fungi (Ballou & Wilson 2016). *H. capsulatum* Mac1 was more expressed in the absence of the metal, probably regulating genes that have roles in this condition, and Ace1 was more expressed in Cu presence, also regulating genes that have roles in this specific condition. The transcriptional behavior observed here strongly indicates that these copper related *H. capsualtum* genes are being regulated by their respective transcription factors, corroborating with the characterization data of Mac1 and Ace1 (Fig 2a).

When we set out to analyze the transcriptional profile of these genes during fungal infection in macrophages (Fig. 4b), strong evidence emerges that *H.* capsulatum may be facing an environment of copper excess imposed by the host cells. This is observed by the expression levels of Ace1 and Crp1 that are up regulated during infection while Mac1, Ctr1 and Ctr4 are down regulated. A transcriptional profile similar to this has already been observed in other fungi that have been proven to undergo, at some point, an excess of copper during the infectious process such as *C. neoformans*, *C. albicans* and *A. fumigatus* (Sun et al., 2014; Mackie et al., 2016; Wiemann et al., 2017; Garcia-Santamarina et al., 2018). When we observed that macrophages pretreated with copper had a greater fungicidal activity in *H. capsulatum* yeasts than the untreated macrophages (Fig. 5), it was clear that copper is used by these host cells to defend against this fungus during the infectious process.

Findings in the literature demonstrate that macrophages stimulated by INF-ɣ tend to increase copper levels within the phagolysosome. This process occurs by an increase of Ctr1 expression and an expression increase and/or a reallocation of the copper importer ATP7a from the golgi complex to the phagolysosomes, making ATP7a a key role of

copper overload macrophage strategy (Wagner et al., 2005, White et al., 2009, Stafford et al., 2013; Wiemann et al., 2017). In order to investigate the hypothesis of copper overload observed so far, we constructed silenced ATP7a macrophage cells (Fig. 6a). When evaluating the infection in these macrophages, we observed that the levels of fungi Crp1 were down regulated in comparison with infection in non-silenced macrophages, plus the fungal burden of silenced macrophages was higher than the non-silenced ones (Fig 6). These findings once again led us to confirm the hypothesis that the macrophages during the infection with *H. capsulatum* uses the toxicity caused by the excess of copper to their favor, via ATP7a.

Nutritional immunity is currently described as a mechanism by which host cells manipulate the concentration of micronutrients during an infection caused by microorganisms (Mackie et al., 2016). It is already well documented that regarding iron and zinc, the main strategy used by host cells is the limitation of these ions on infected sites (Hood & Skaar 2012; Potrykus et al., 2014). In *H. capsulatum* inclusive, it has already been described that macrophages when stimulated by GMCSF, tend to limit the concentrations of zinc within the phagolysosome, when infected by this fungus (Vignesh et al., 2013). The available literature data, regarding the copper role during infection however, shows that for this metal, the strategies used by the host are much more dynamic. Host cells can limit or increase the Cu levels at the sites of infection, depending on the tissue where the pathogen is infecting and also on the time elapsed from the infectious process (Sun et al., 2014; Mackie et al., 2016).

Because it is already described that INF-ɣ stimulated macrophages tend to create an environment of copper excess, we hypothesized that the copper excess faced by *H. capsulatum* during macrophage infection maybe be INF-ɣ dependent. As we were already observing so far, yeast cells when infecting INF-ɣ stimulated macrophages, even in greater times than 24 hours (48 and 96 hours) continued to face an Cu overload environment. During infection in INF-ɣ non-treated macrophages however, we observed that the fungus might be suffering a copper limitation in 48 and 96 hours of infection, because in this point the expression levels of Ctr4 are increased whereas those of Crp1 are decreased. This findings demonstrates that in the same way observed in *C. neoformans* and *C. albicans*, the copper nutritional immunity imposed by the host cells to *H. capsulatum* shows a highly dynamic process, having situations where copper is limited and others where their levels are increased. Additionally within 24 hours of infection,

even in untreated macrophages, there is evidence that the fungus undergoes a copper excess condition demonstrating that perhaps the copper excess caused by the macrophages besides being INF-ɣ dependent, although it may be time dependent as well. This may be in some way related to the innate immunity of the host, being a primary response of macrophages, but this statement still depends on future studies and confirmations.

In the same way as occurs in *C. albicans* and *A. fumigatus* (Mackie et al., 2016; Wiemann et al., 2017*)*, the copper export pump Crp in *H. capsulatum* appears to be the main response tool to face copper excess. In order to confirm this we constructed *H. capsulatum* Crp1 silenced strains and we observed, as expected, that the Crp1 mutant is more sensitive than the wild type when grown under high copper conditions, however its virulence is subtly attenuated comparing to the wild-type strain. Due to the small difference in the virulence found in the Crp1 mutants and the need for the fungus to detoxify the copper excess, since this seems to be the main strategy used by the macrophages cells during the infection, we look for other proteins that may be excercising a Crp1-like function. In doing so, we found two proteins that we herein called Crp2 and Crp3, that may be performing a Crp1-like function, relieving the Cu high-level condition, influencing the lack of virulence of Crp1 mutants and helping the *H. capsulatum* survival during the infection. Crp2 and Crp3 are more expressed in the copper presence condition, and according to OrthoVenn analysis (Supp. Table S3) this two proteins have orthologs in other fungi. This proteins will be targets for future studies and investigations.

In conclusion, in this work we clearly demonstrate that copper plays a key role in the pathogen-host relationship of *H. capsulatum*. Macrophages use the copper overload to kill the fungus cells during infection via ATP7a, and this strategy appears to be INF-ɣ and time dependent. In addition, we observed that Crp1 is important for the survival of the fungus in high concentrations of copper and we can say that to a lesser extent for its virulence. It seems however, that Crp1 is not the only protein that can be exercising this role in *H. capsulatum*. It is clear once again with this work, that nutritional immunity does not only apply to the limitation of a certain micronutrient, and when copper is the main target, this process is complex, highly dynamic and dependent on certain biological variables.

METHODS

In silico search of copper homeostasis related genes

Amino acid sequences of proteins related to copper homeostasis (copper uptake, distribution, storage, detoxification and the copper sensitive transcription factors) were used in the search of orthologues in *H. capsulatum* G186A genome available at NCBI data bank (https://www.ncbi.nlm.nih.gov). The organisms *A. fumigatus* (http://www.aspergillusgenome.org), *C. neoformans* (http://fungidb.org) and *S. cerevisiae* (http://www.yeastgenome.org) were used as models, and their copper homeostasis related proteins sequences were obtained at the respective databases. The homologous sequences were obtained by the BlastP tool. The orthologs shown in Table S2 were identified using the web plataform Ortho Venn (Wang et al., 2015) and the phylogenetic tree was constructed using the IQ-TREE web service (http://iqtree.cibiv.univie.ac.at). The DNA Pattern Find tool from Sequence Manipulation Suite (http://www.bioinformatics.org) was used in the search for consensus sequences at promoters of each selected genes.

Strain and growth conditions

H. capsulatum strain G186A (ATCC 26029) yeast cells were maintained in Histoplasma Macrophage Medium (HMM) agar plates at 37 °C in 5% CO^2 atmosphere. To exponential growth the cells were transferred to HMM broth at 37 °C, in 5% CO_2 atmosphere for 72 hours under rotation. After this, except for expression analysis under infection conditions, all the experiments were performed with strains cultivated in chemically defined medium MVM supplemented with 0,2 and 2 µM of $CuSO_4$ (for basal copper condition), 30 µM of $CuSO_4$ (for copper medium condition), 250 µM and 500 µM of $CuSO_4$ (for copper excess condition) and with 30 µM of BCS (Bathocuproinedisulfonic acid, Sigma-Aldrich; for copper limitation condition).

RNA isolation and expression analysis by qRT-PCR

After *H. capsulatum* cells growth in MVM medium in the presence and limitation copper conditions, in the times of 3 and 24 hours, the cells were harvested by centrifugation, washed twice with phosphate saline buffer (PBS) and total RNA was isolated using Trizol (Sigma-Aldrich, St. Louis, MO) and mechanical cell rupture (Beadbeater – Biospec Products Inc., Bartlesville, OK). The quantitative qRT-PCR was

performed as previously described (Silva-Bailão et al., 2014). The sequences of forward and reverse primers used are listed in Table S3. At least one primer of each pair spanned an intron, preventing amplification of genomic DNA. The transcript of actin (EEH03071.1) were used for normalization of transcript amplification.

Macrophage infection experiments

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

For gene expression infection analysis, *H. capsulatum* yeasts was grown in HMM broth for 72 h, then washed three times with PBS1X. The number of viable yeasts cells was adjusted for 5 x 10^6 and then incubated with $1x10^6$ of previously activated macrophage cells, being the infection proportion 5 yeast cells for each macrophage. Cells were co-cultivated for 3 and 24 hours at 37 °C in 5% CO_2 in 24 well tissue plate. Each well was washed twice with 1 ml of 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 *H. capsulatum* yeast cells cultured in RPMI 1640 medium, were obtained as control. After reverse transcription, cDNAs were submitted to qRT-PCR.

For fungal burden analysis, $1x10^6$ macrophage cells already treated with INF-ɣ were incubated with RPMI medium containing 30 µM of $CuSO_4$ and 30 µM of BCS for 1 hour in 24 well tissue plate, washed extensively in PBS 1x, refilled with RPMI medium and then co-cultivated with $5x10^6$ yeast cells of *H. capsulatum*. The cells were co-cultivated for 24 hours at 37 °C in 5% CO_2 to allow fungal internalization. Each well was washed twice with 1 ml of PBS 1x in order to get rid of non-internalized yeasts. Infected macrophages were lysed with ice-cold ultrapure sterile water, and dilutions of the lysates containing the phagocytized yeasts were plated in HMM agar and incubated at 37 °C in 5% CO_2 atmosphere. After 7 days of incubation the number of CFU was determined.

Generation of J774 macrophages ATP7a-silenced cells

ATP7a silenced macrophage cells were generated, in 24 well tissue plates, by stable transfection of a double-stranded siRNA against ATP7a gene (Silencer siRNA mouse ATP7a Cat. No. AM16708, ThermoFisher Scientific, Waltham, MA), using Lipofectamine 2000 as transfection reagent, following the Lipofectamine 2000 protocol (Invitrogen, Cat. No. 11668-027), in RMPI 1640 medium without any supplementation. A scrumble siRNA was used as transfection negative control (Silencer Negative Control Cat. No. AM4611, ThermoFisher Scientific, Waltham, MA). After 24, 48 and 96 hours the macrophages viability was confirmed by microscopy through Trypan blue dye, Trizol was added in each well and total RNA was isolated. RNAs from non-silenced macrophages were obtained as control. After reverse transcription, the ATP7a silencing quality was evaluated by qRT-PCR, through TaqMan gene expression assay (ThermoFisher Scientific, Waltham, MA; ATP7a TaqMan Cat. No. 437663). The transcript of α-tubulin (TaqMan Cat. No. 492936) were used for normalization of transcript amplification. The best processing time for subsequent transfections was set to 48 hours, after the quality analysis of 24, 48 and 96 hours of ATP7a silencing transcriptional profiles.

For *H. capsulatum* Crp1 gene expression in ATP7a silenced macrophages, *H. capsulatum* yeasts was grown in HMM broth for 72 h, then washed three times with PBS1X. The number of viable yeasts cells was adjusted for 5 x 10^6 and then incubated with 1x10^6 of previously activated ATP7a silenced macrophage cells, being the infection proportion 5 yeast cells for each macrophage. Cells were co-cultivated for 3 and 24 hours at 37 °C in 5% CO_2 in 24 well tissue plate. Each well was washed twice with 1 ml of 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 non-silenced macrophages were obtained as control. After reverse transcription, cDNAs were submitted to qRT-PCR.

For fungal burden analysis in ATP7a silenced macrophages, 1x10^6 silenced and non-silenced macrophage cells were co-cultivated with 5x10^6 yeast cells of *H. capsulatum* in RMPI 1640 medium with no supplementation. The cells were co-cultivated for 24 hours at 37 °C in 5% CO_2 to allow fungal internalization. Each well was washed twice with 1 ml of PBS 1x in order to get rid of non-internalized yeasts. Infected macrophages were lysed with ice-cold ultrapure sterile water, and dilutions of the lysates containing

the phagocytized yeasts were plated in HMM agar and incubated at 37 °C in 5% CO_2 atmosphere. After 7 days of incubation the number of CFU was determined.

Depletion of Crp1 gene function by RNAi

Oligonucleotides specific for the coding regions of the Crp1 gene were designed to produce fragments of approximately 300 pair bases. Specific restriction sites were added to the oligonucleotides for cloning of inverted fragments in the vector Histoplasma URA5-based RNAi vector pCR473 with the aim of favoring the formation of double stranded RNAs. This vector contains inverted sequences complementary to GFP (Green Flourescent Protein) flanking the region in which the specific RNAi gene sequences will be introduced and also contains an auxotrophic selection mark URA5. The vector with the silencing sequence was used to transform the bacterium *Agrobacterium tumefaciens*. Cells receiving the recombinant vector were selected by kanamycin resistance and used for yeast transformation of *H. capsulatum.*

Transformed *A. tumefaciens* cells were inoculated in liquid LC medium containing kanamycin for growth for 2-3 days at room temperature. When the culture obtained an optical density of 0.6-0.7 the cells was co-cultivated with $1x10^8$ cel/ml yeasts of *H. capsulatum* fungi expressing GFP, previously constructed called *Histoplasma* GFP-fluorescent sentinel strain OSU22 (Youseff & Reppleye 2012), previously grown in liquid HMM medium for 48 hours. A volume of 1: 1 (400 ul of bacterial culture with 400 ul of *Histoplasma* culture) were mixed in fresh HMM medium, centrifuged at 500 g, the pellet resuspended in fresh HMM medium and plated on solid IM medium containing the respective inducer. The plates were incubated for up to 10 days at a temperature of 37 °C to 5% CO_2. Control cells were obtained by transformation with *A. tumefaciens* carrying the vector pCR473 containing only silencing sequences for GFP and not the target gene. The transformed *H. capsulatum* yeasts were selected for their ability to grow in the absence of uracil. Subsequently, the mutants were screened for fluorescence emitted by GFP through ImageJ software coupled to a transluminator. The transformants that showed the greatest decrease in the fluorescence emitted by GFP were selected. Mutants that presented a silence score of 70% or more were used for the phenotypic assays.

Phenotypic analysis

The Crp1 mutants selected for having minimal silencing rate underwent phenotypic analysis. These were grown in chemically defined MVM liquid medium under

conditions of copper excess (25 µM $CuSO_4$) at median concentrations (30 µM $CuSO_4$) at basal concentrations (0.2 and 2 µM $CuSO_4$) and in copper deprivation (30 µM of BCS chelator) for growth analysis. Fungal growth was measured by optical density.

Virulence assays

C57BL/6 male mice with 6 to 8 weeks were co-infected intranasally with 5 x 10^6 cells of the mutant strains expressing GFP and with 5x10^6 cells of a wild type strain expressing a Red Fluorescent Protein. The lungs and liver were removed after 12 days of infection, homogenized, plated in solid HMM medium and incubated at 37 °C in 5% CO_2 atmosphere. After 9 days of incubation the number of CFU of wild types cells (red) and the Crp1 mutant cells (green) was determined.

REFERENCES

ACHARD, M. E.; STAFFORD, S. L.; BOKIL, N. J.; CHARTRES, J.; BERNHARDT, P. V.; SCHEMBRI, M. A.; SWEET, M. J.; McEWAN, A. G. Copper redistribution in murine macrophages in response to *Salmonella* infection. **Biochem.** 2012.

ADENIS, A.; NACHER, M.; HANF, M.; VANTILCKE, V.; BOUKHARI, R.; BLACHET, D.; DEMAR, M.; AZNAR, C.; CARME, B.; COUPPIE, P. HIV-associated histoplasmosis early mortality and incidence trends: from neglect to priority. **PLoS Negl Trop Dis.** 2014.

BAHR, N. C.; ANTINORI, S.; WHEAT, L. J.; SAROSI, G. A. Histoplasmosis infections worldwide: thinking outside of the Ohio River Valley. **Curr Trop Med Rep.** 2015.

BALLOU, E. R.; WILSON, D. The roles of zinc and copper sensing in fungal pathogenesis. **Curr Opin Microb.** 2016.

BEAUDOIN, J.; LABBE, S. The fission yeast copper sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1 like recognition sequence. **Journal of Biological Chemistry.** 2001.

BEAUDOIN, J.; LABBE, S. Crm1-mediated nuclear export to the *Schizosaccharomycies pombe* transcription factor Cuf1 during a shift from low to high copper concentrations. **Eukaryotic Cell.** 2007.

BRUNKE, S.; MOGAVERO, S.; KASPER, L.; HUBE, B. Virulence factor in fungal pathogens of man. **Current Opinion in Microbiology.** 2016.

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.** 2008.

CULLOTA, V. C., HOWARD, W. R.; LIU, X. F. CRS5 encodes a metallothionein-like protein in *Saccharomyces cerevisiae*. **J Biol Chem.** 1994.

CYERT, M. S.; PHILPOTT, C. C. Regulation of cation balance in *Saccharomyces cerevisiae*. **Genetics.** 2013.

DADE, J.; DUBOIS, J. C.; PASULA, R.; DONNELL, A. M.; CARUSO, J. A.; SMULIAN, A. G.; DEEPE Jr, G. S. HcZrt2, a zinc responsive gene, is indispensable for the survival of *Histoplasma capsulatum in vivo.* **Medical Mycology.** 2016.

DING, C.; YIN, J.; TOVAR, E. M.; FITZPATRICK, D. A.; HIGGINGS, D. G.; THIELE, D. J. The copper regulon of the human fungal pathogen *Cryptococcus neoformans* H99. **Mol Microbiol.** 2013.

DOUGLAS, L. M.; WANG, H. X.; KEPPLER-ROSS, S.; DEAN, N.; KONOPKA, J. B. Sur7 promotes plasma membrane organization and is needed for resistance to stressful conditions and to the invasive growth and virulence of *Candida albicans*. **MBio.** 2011.

FERREIRA, M. S.; BORGES, A. S. Histoplasmosis. **Rev Soc Bras Med Trop.** 2009.

GOODWIN, R. A.; LOYD, J. E.; DES PREZ, R. M. Histoplasmosis in normal hosts. **Medicine.** 1981.

GRADEN, J. A. and WINGE, D. R. Copper mediated repression of the activation domain in the yeast Mac1 transcription factor. **Proc. Natl. Acad. Sci.** 1997.

HILTY, J.; SMULIAN, A. G.; NEWMAN, S. L. The *Histoplasma capsulatum* vacuolar ATPase is required for iron homeostasis, intracellular replication in macrophages and virulence in a murine model of histoplasmosis. **Mol. Microbiol.** 2008.

HOLBROOK, E.; SMOLNYCKI, K.; YOUSEFF, B.; RAPPLEYE, C. Redundant catalases detoxify phagocyte reactive oxygen and facilitate *Histoplasma capsulatum* pathogenesis. **Infect Immun.** 2013.

HOOD, M. I.; SKAAR, E. P. Nutritional immunity: transition metals at the pathogen-host interface. **Nat Rev Microbiol.** 2012.

HOMANN, O. R.; DEA, J.; NOBLE, S. M.; JOHNSON, A. D. A phenotypic profile of the *Candida albicans* regulatory network. **PLoS Genetic.** 2009.

HUBE, B. Fungal adaptation to the host environment. **Curr Opin Microbiol.** 2009.

INGLIS, D. O.; VOORHIES, M.; HOCKING, D. R.; SIL, A. Comparative transcriptomics of infectious spores from the fungal pathogen *Histoplasma capsulatum* reveals a core set of transcripts that specify infectious and pathogenic states. **Eukaryot Cell.** 2013.

JUNGMANN, J., REINS, H. A.; LEE, J.; ROMEO, A.; HASSET, R.; KOSMAN, D.; JENTSCH, S. Mac1, a nuclear regulatory protein related to Cu-dependent-transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. **EMBO.** 1993.

KEHL-FIE, T. E.; SKAAR, E. P. Nutritional Immunity beyond iron: a role for manganese and zinc. **Curr. Opin. Chem. Biol.** 2010.

KUSUYA, Y.; HAGIWARA, D.; SAKAI, K.; YAGUSHI, T.; GONOI, T.; TAKAHASHI, H. Transcription factor AfMac1 controls copper import machinery in *Aspergillus fumigatus*. **Curr. Genet.** 2017.

MACKIE, J.; SZABO, E. K.; URGAST, D. S.; BALLOU, E. R.; CHILDERS, S.; BROWN, A. J. P. Host imposed copper poisoning impacts fungal micronutrient acquisition during systemic *Candida albicans* infections. **PloS One.** 2016.

MARESCA, B. and KOBAYASHI, G. S. Dimorphism in *Histoplasma capsulatum*: a model for the study of cell differentiation in pathogenic fungi. **Microbiological reviews.** 1989.

MUKHERJEE, A.; TANGRI, R.; VERMA, N.; GAUTAM, D. Chronic disseminated Histoplasmosis boné marrow involvement in an immunocompetent patient. **Indian J Hematol Blood Transfus.** 2010.

MUNIZ, M. M. LOTT, T. J.; MAYER, L. W. Molecular cloning, characterization and expression of the M antigen of *Histoplasma capsulatum*. **Infect Immun.** 2013.

NACHER, M.; ADENIS, A.; McDONALD, S.; GOMES, M.; SINGH, S.; LOPES LIMA, I. Disseminated histoplasmosis in HIV-infected patients in South America: a neglected killer continues on its rampage. **PLoS Negl Trop Dis.** 2013.

NEWMAN, S. L. and SMULIAN, A. G. Iron uptake and virulence in *Histoplasma capsulatum*. **Curr. Op. Microbiology.** 2013.

POTRYKUS, J.; BALLOU, E. R.; CHILDERS, D. S.; BROWN, A. J. Conflicting interests in the pathogen-host tug of war: fungal micronutrient scavenging versus mammalian nutritional immunity. **PLoS Pathog.** 2014.

PULOT, A.; PERRIN, S.; JOLIVET, A.; VANTICKE, V. HIV-associated histoplasmosis in western French Guiana, 2002-2012. **Mycoses.** 2015.

RUPP, J. C.; LOCATELLI, M.; GRIESER, A.; RAMOS, A.; CAMPBELL, P. J.; YI, H.; STEEL, J.; BURKHEAD, J. L.; BORTZ, E. Host cell copper transporters Ctr1 and ATP7a are important for influenza A virus replication. **Virol Journal.** 2017.

SUN, T.; JU, X.; GAO, H.; WANG, T.; THIELE, D. J.; LI, Y.; WANG, Z.; DING, C. Reciprocal functions of *Cryptococcus neoformans* copper homeostasis machinery during pulmonary infection and meningoencephalitis. **Nature Communications.** 2014.

SMITH, L. M. and MAY, R. C. Mechanisms of microbial escape from phagocyte killing. **Biochem Soc Trans.** 2013.

TEIXEIRA, M. M.; PATANÉ, J. S. L.; TAYLOR, M. L.; GOMEZ, B. L.; THEODORO, R. C.; HOOG, S.; ENGELTHALER, D. M.; ZANCOPÉ-OLIVEIRA, R. M. Z.; FELIPE, M. S. S.; BARKER, B. M. Worldwide phylogenetic distribution and population dynamics of the genus *Histoplasma*. **PLoS One.** 2016.

THIELE, D. J. ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. **Mol Cell Biol.** 1988.

TRISTÃO, G. B.; ASSUNÇÃO, L. P.; SANTOS, L. P.; BORGES, C. L.; SILVA-BAILÃO, M. G.; SOARES, C. M.; CAVALARO, G.; BAILÃO, A. M. Predicting copper, iron and zinc binding proteins in pathogenic species of the *Paracoccidioides* genus. **Frontiers in Microbiology.** 2015.

VIGNESH, K. S.; LANDERO-FIGUEROA, J. A.; PAROLLO, A.; CARUSO, J. A.; DEEPE, G. S. Granulocyte Macrophage-Colony Stimulating Factor induced zinc

sequestration enhances macrophage superoxide and limits intracellular pathogen survival. **Immunity.** 2013.

WAGNER, D.; MASER, J.; LAI, B.; CAI, Z.; BARRY, C. E.; HONER, K.; RUSSEL, D. G.; BERMUDEZ, L. E. Elemental analysis of *Mycobacterium avium, Mycobacterium tuberculosis,* and *Mycobacterium smegmatis* containing phagosomes indicates pathogen induced microenvironments within the host cells´s endosomal system. **J Immunol.** 2005.

WANG, Y.; COLEMAN-DERR, D.; CHEN, G.; GU, Y. Q. OrthoVenn: a web server for genome wide comparison and annotation of orthologous clusters across multiple species. **Nucleic Acids Res.** 2015.

WATERMAN, S. R.; HACHAM, M.; HU, G.; ZHU, X.; PARK, Y. D.; SHIN, S.; PANEPINTO, J.; VALYL-NAGY, T.; BEAM, C.; HUSAIN, S. Role of a CUF1/CTR4 copper regulatory axis in the virulence of *Cryptococcus neoformans*. **J Clin Invest.** 2007.

WHEAT, J.; SASORI, G.; MCKINSEY, D.; HAMILL, R.; BRADSHER, R.; JOHNSON, P.; LOYD, J. Practice guidelines for the management of patients with histoplasmosis. **Clin Infect Dis.** 2000.

WHITE, C.; LEE. J.; KAMBE, T.; FRITSCHE, L.; PETRIS, M. J. A role for the ATP7a copper-transporting ATPase in macrophage bactericidal activity. **J Biol Chem.** 2009.

WIEMANN, P.; PEREVITSKY, A.; LIM, F. Y.; HUTTENLOCHER, A.; OSHEROV, N.; KELLER, N. P. *Aspergillus fumigatus* copper export machinery and reactive oxygen intermediate defense counter host copper-mediated oxidative antimicrobial offense. **Cell Reports.** 2017.

YOUSEFF, B. H.; RAPPLEYE, C. A. RNAi based gene silencing using a GFP sentinel system in *Histoplasma capsulatum*. **Host-fungus interactions: Methods and protocols.** 2012.

ZANCOPÉ-OLIVEIRA, R. M.; MUNIZ, M. M.; WANKE, B. Genetic diversity of *Histoplasma capsulatum* strains in Brazil. **FEMS Imunnol Med Microbiol.** 2013.

FIGURES

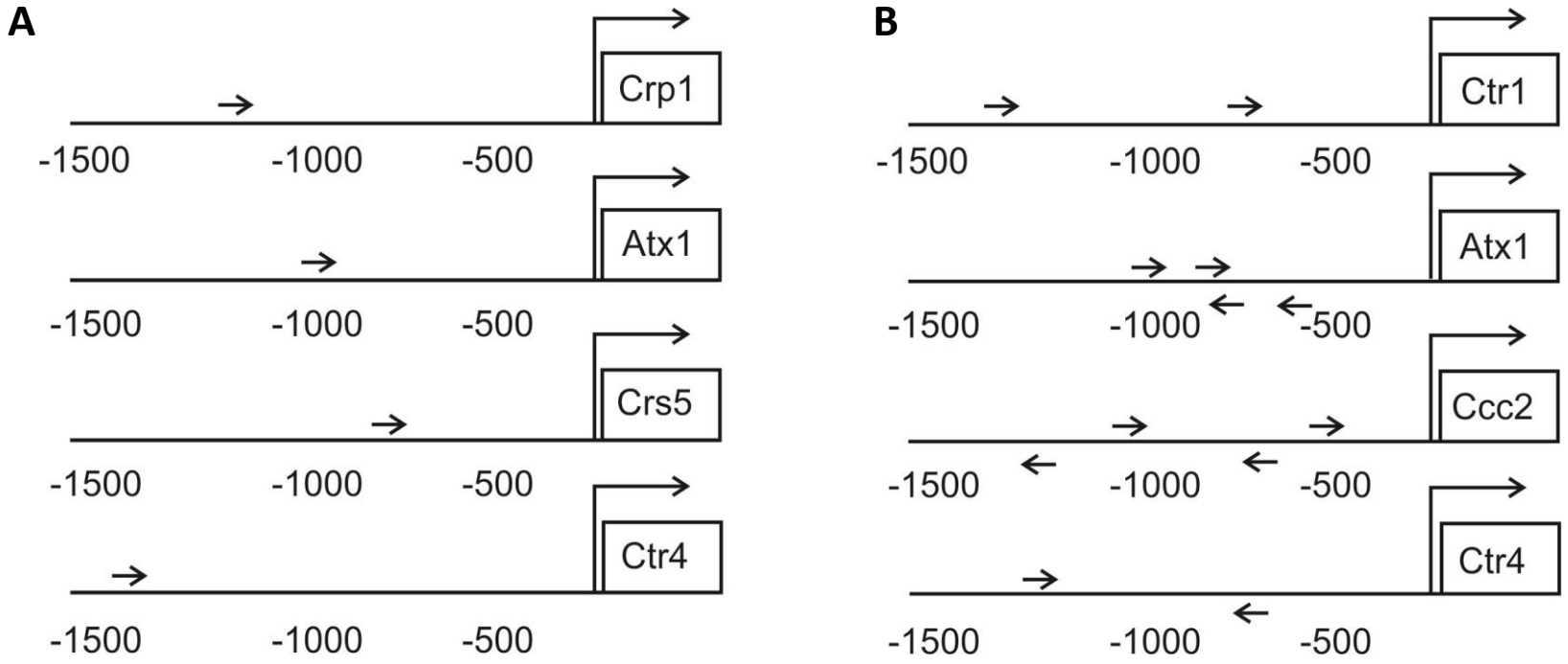
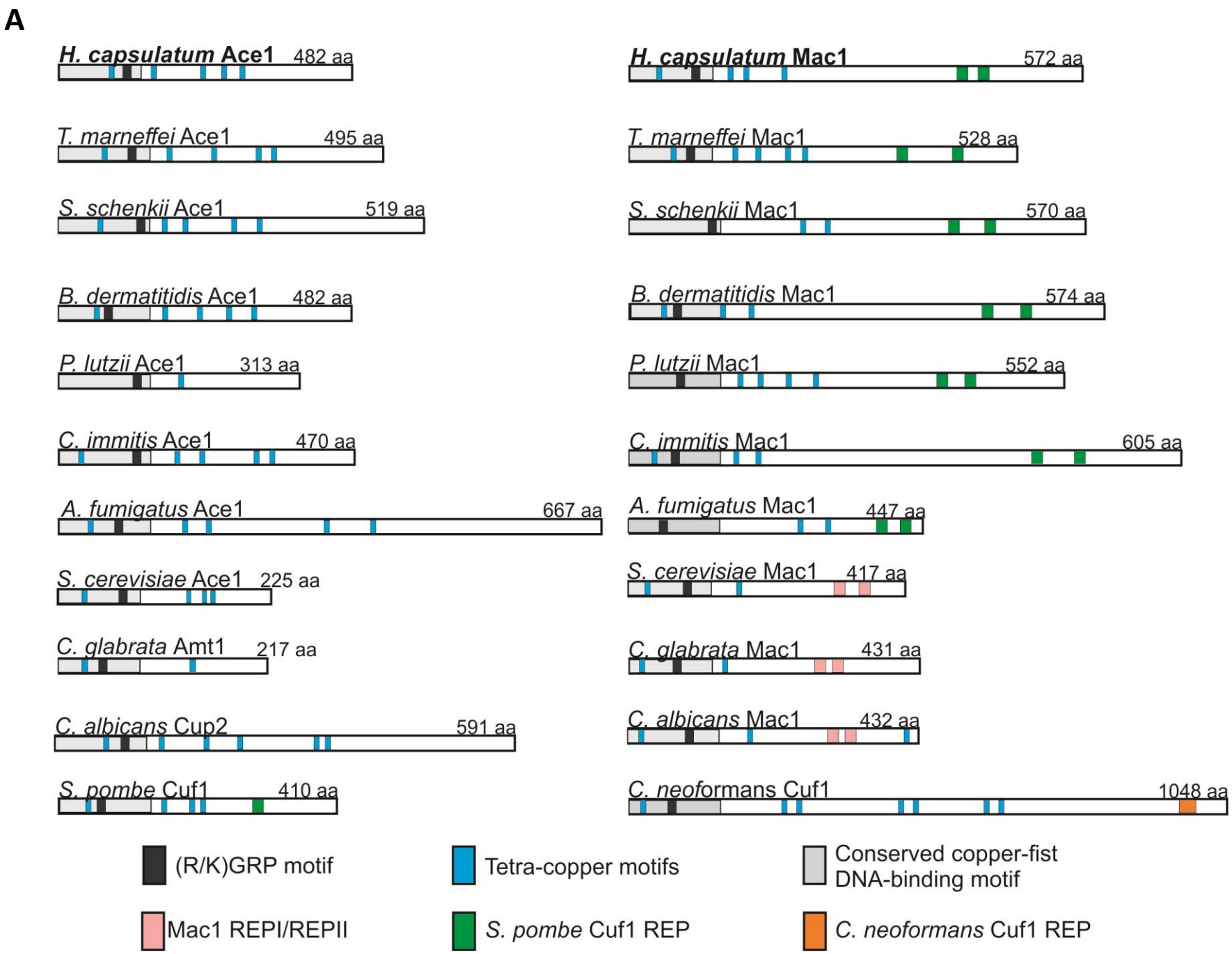


Figure 1. Mapped Copper Responsive Elements (CuREs) in the promoter regions of *H. capsulatum* Ctr1, Ctr4, Crp1, Ace1, Mac1, Atx1, Ccc2 and Crs5 genes. The Ace1 responsive motif (A/C/T)T(A/C/T)NNGCTG(A/G/T) which N = any residue are shown in **A** and the Mac1 motif (TTTGC(T/G)C(A/G) are shown in **B**. -500, -1000 and -1500 represents the base pairs upstream the start codon of each gene. Bended arrows in the figure represent the transcription start sites. The straight arrows represent the corresponding CuREs sequence in the promoter region of the gene.


A
H. capsulatum Ace1 482 aa
T. marneffei Ace1 495 aa
S. schenkii Ace1 519 aa
B. dermatitidis Ace1 482 aa
P. lutzii Ace1 313 aa
C. immitis Ace1 470 aa
A. fumigatus Ace1 667 aa
S. cerevisiae Ace1 225 aa
C. glabrata Amt1 217 aa
C. albicans Cup2 591 aa
S. pombe Cuf1 410 aa
H. capsulatum Mac1 572 aa
T. marneffei Mac1 528 aa
S. schenkii Mac1 570 aa
B. dermatitidis Mac1 574 aa
P. lutzii Mac1 552 aa
C. immitis Mac1 605 aa
A. fumigatus Mac1 447 aa
S. cerevisiae Mac1 417 aa
C. glabrata Mac1 431 aa
C. albicans Mac1 432 aa
C. neoformans Cuf1 1048 aa
(R/K)GRP motif
Tetra-copper motifs
Conserved copper-fist DNA-binding motif
Mac1 REPI/REPII
S. pombe Cuf1 REP
C. neoformans Cuf1 REP

B

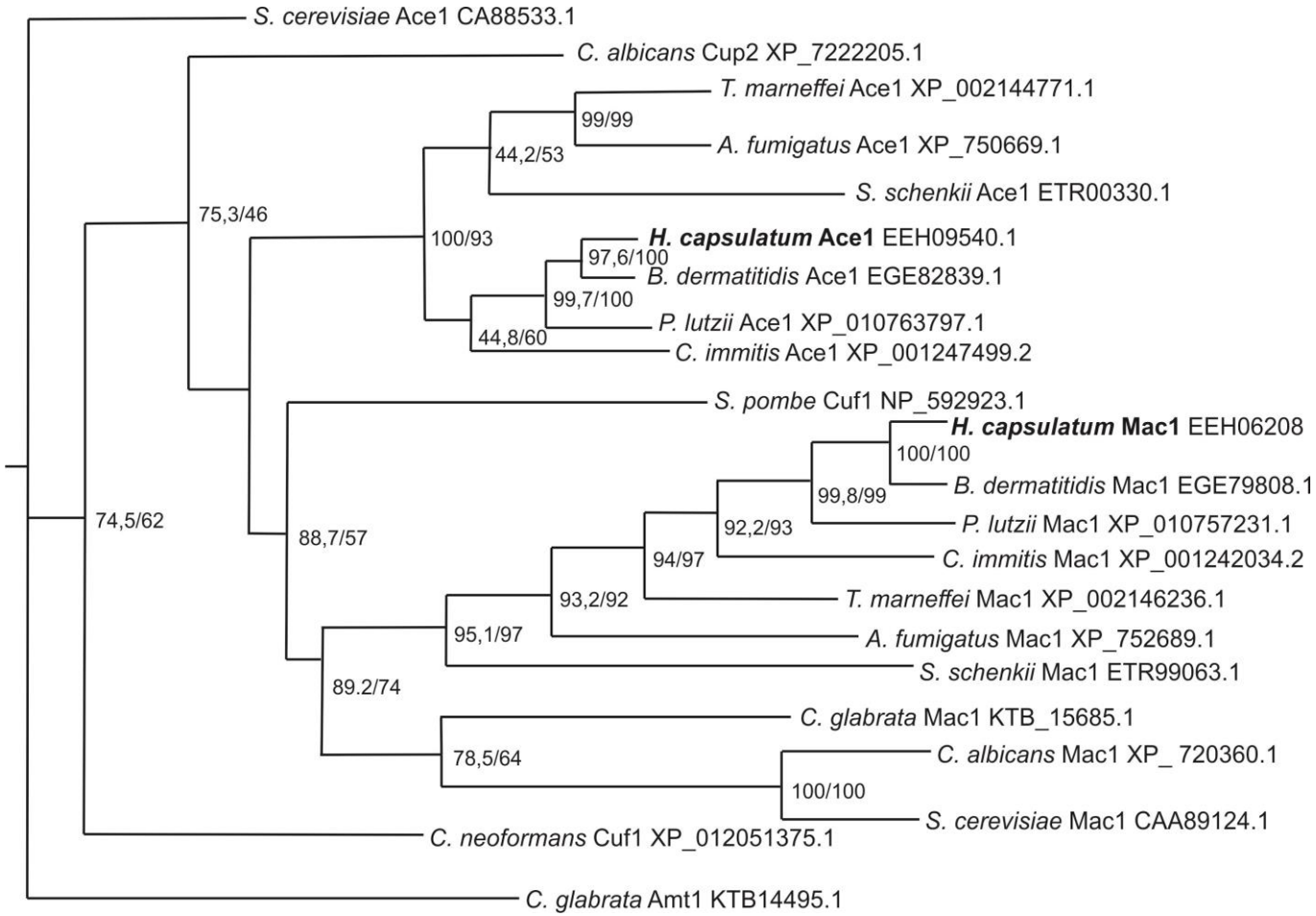


Figure 2. ***H. capsulatum*** **Ace1 and Mac1 have characteristic classical motifs of copper responsive transcription factors**. **A)** Structural features of Ace1 and Mac1 copper binding motifs. **B**) Phylogenetic relationships of Ace1 and Mac1 genes in fungi. Mac1 REPI/REPII (CXCX4CXCX2CX2H), *S. pombe* Cuf1 REP (CXCX3CXCX2CX2H), *C. neoformans* Cuf1 REP (CCX3CX4CXCX3CCXCCXC).

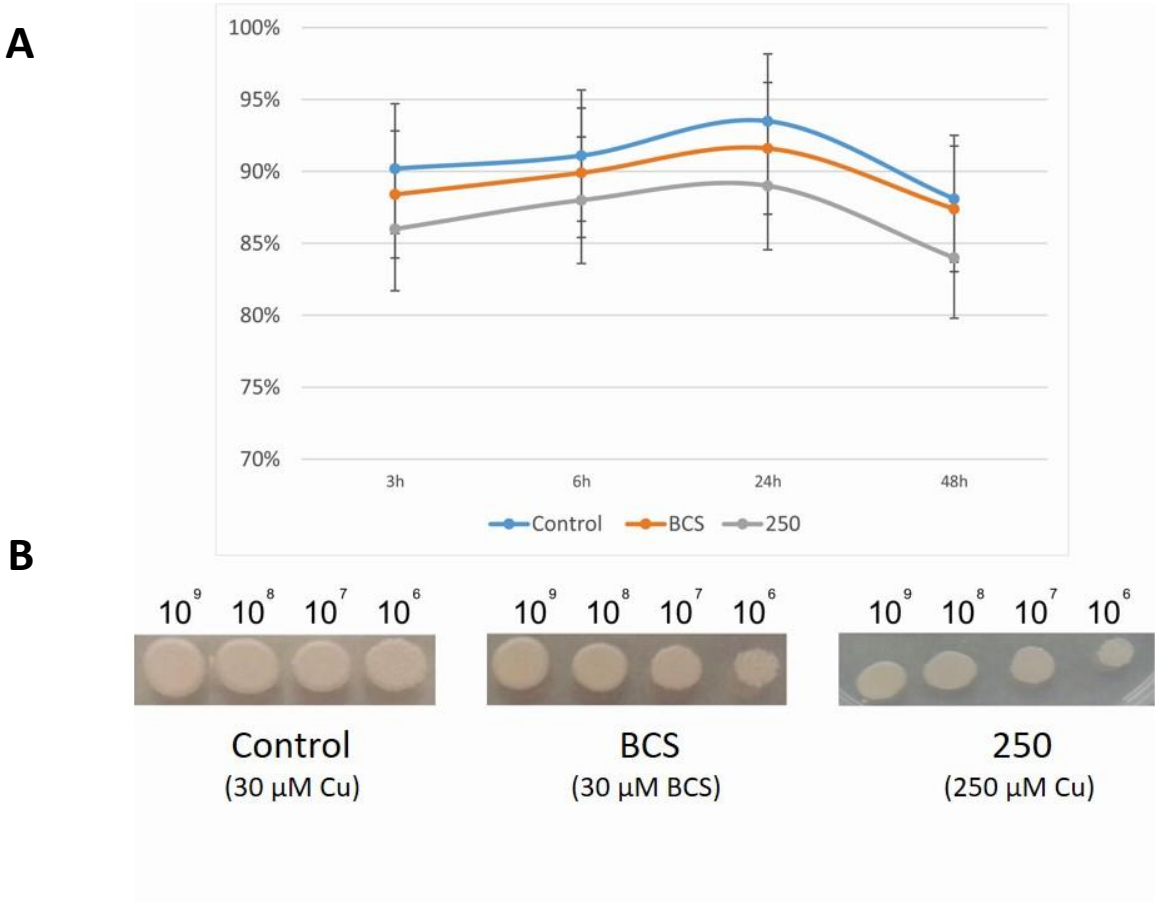
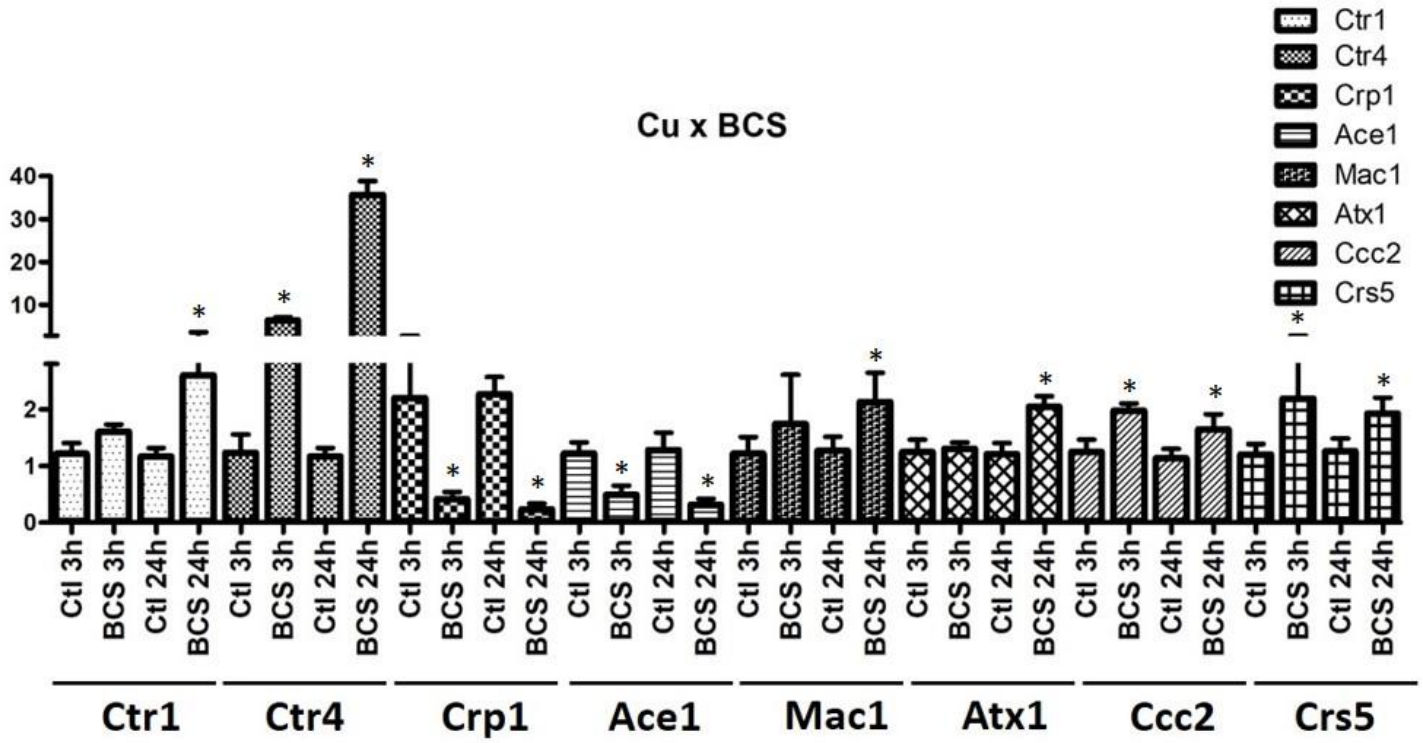


Figure 3. ***H. capsulatum*** **has the hability to grow under conditions of limitation and copper excess.** Viability and growth curves of *H.capsulatum* yeasts (**A**) in $CuSO_4$ 30 µM (Control condition), BCS 30 µM (Bathocuprosulphonic acid; copper limitation condition) and $CuSO_4$ 250 µM (copper excess condition). (**B**) Plate growth of *H. capsulatum* yeasts in $CuSO_4$ 30 µM , BCS 30 µM and $CuSO_4$ 250 µM conditions.

A


Cu x BCS
Ctr1
Ctr4
Crp1
Ace1
Mac1
Atx1
Ccc2
Crs5
40
30
20
10
2
1
0
Ctl 3h
BCS 3h
Ctl 24h
BCS 24h
Ctr1
Ctr4
Crp1
Ace1
Mac1
Atx1
Ccc2
Crs5

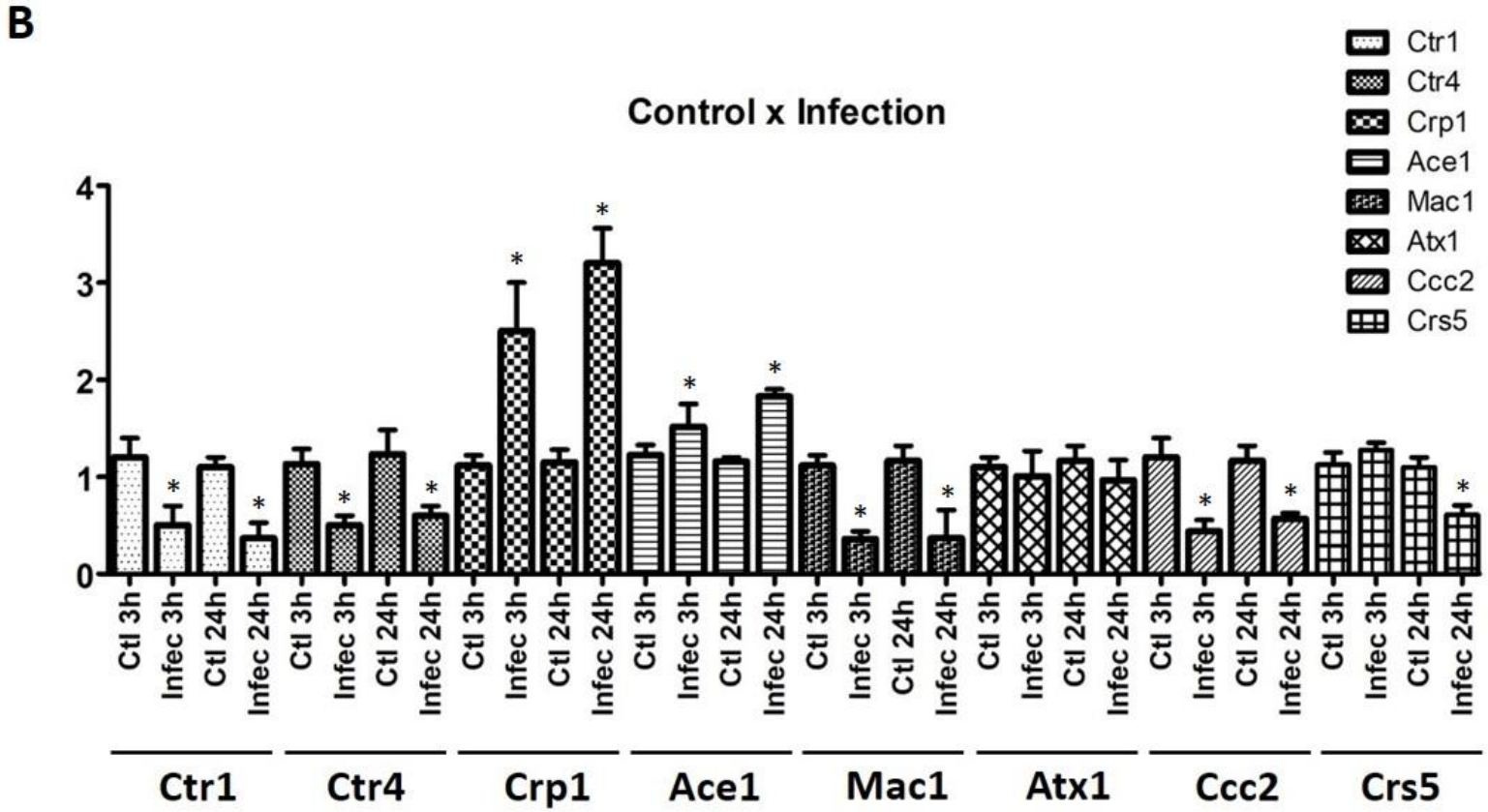


Figure 4. Macrophage infection induces the copper excess response genes Crp1 and Ace1 in *H.* capsulatum. Quantitative RT-PCR was performed with transcripts of *H. capsulatum* yeasts and the transcription levels of Ctr1, Ctr4, Crp1, Ace1, Mac1, Atx1, Ccc2 and Crs5 genes were evaluated during: (**A)** 3 and 24 hours in vitro growth at copper control concentration of $CuSO_4$ 30 µM (Ctl) and poor copper levels 30 µM of BCS (BCS). (**B**) 3 and 24 hours of macrophage infection. Infec - yeast transcripts during infection. Ctl - yeast transcripts of yeast growth in macrophage medium only. * - Significant difference $p < 0,05$.

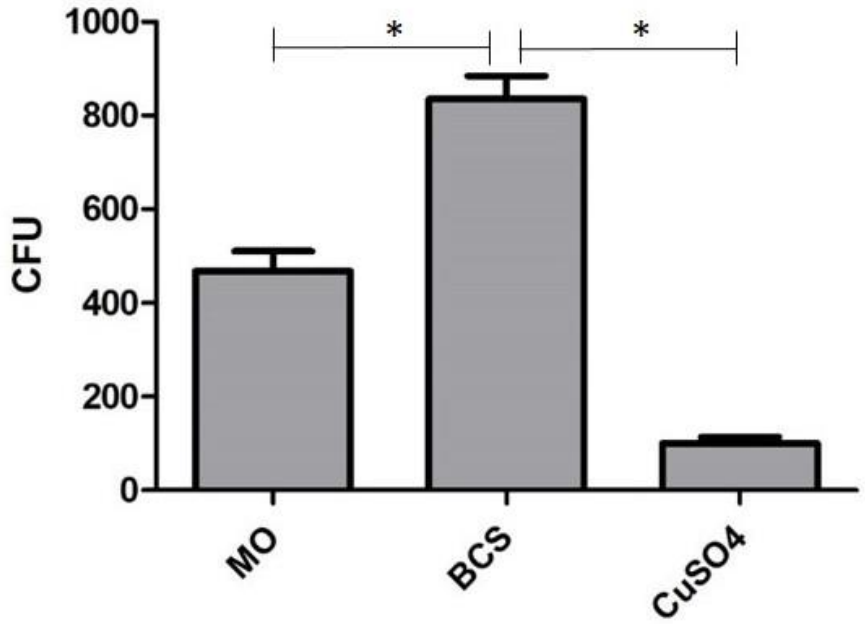


Figure 5. Copper enhances fungal killing by macrophage cells. Colony forming units of *H. capsulatum* cells after 24 hours of infection in J774 macrophages pre-treated with copper and BCS. MO – Macrophages without supplementation, BCS – Macrophages treated with 30 µM of BCS and $CuSO_4$ – Macrophages treated with 30 µM of copper sulphate. * - Significant difference $p < 0,05$.

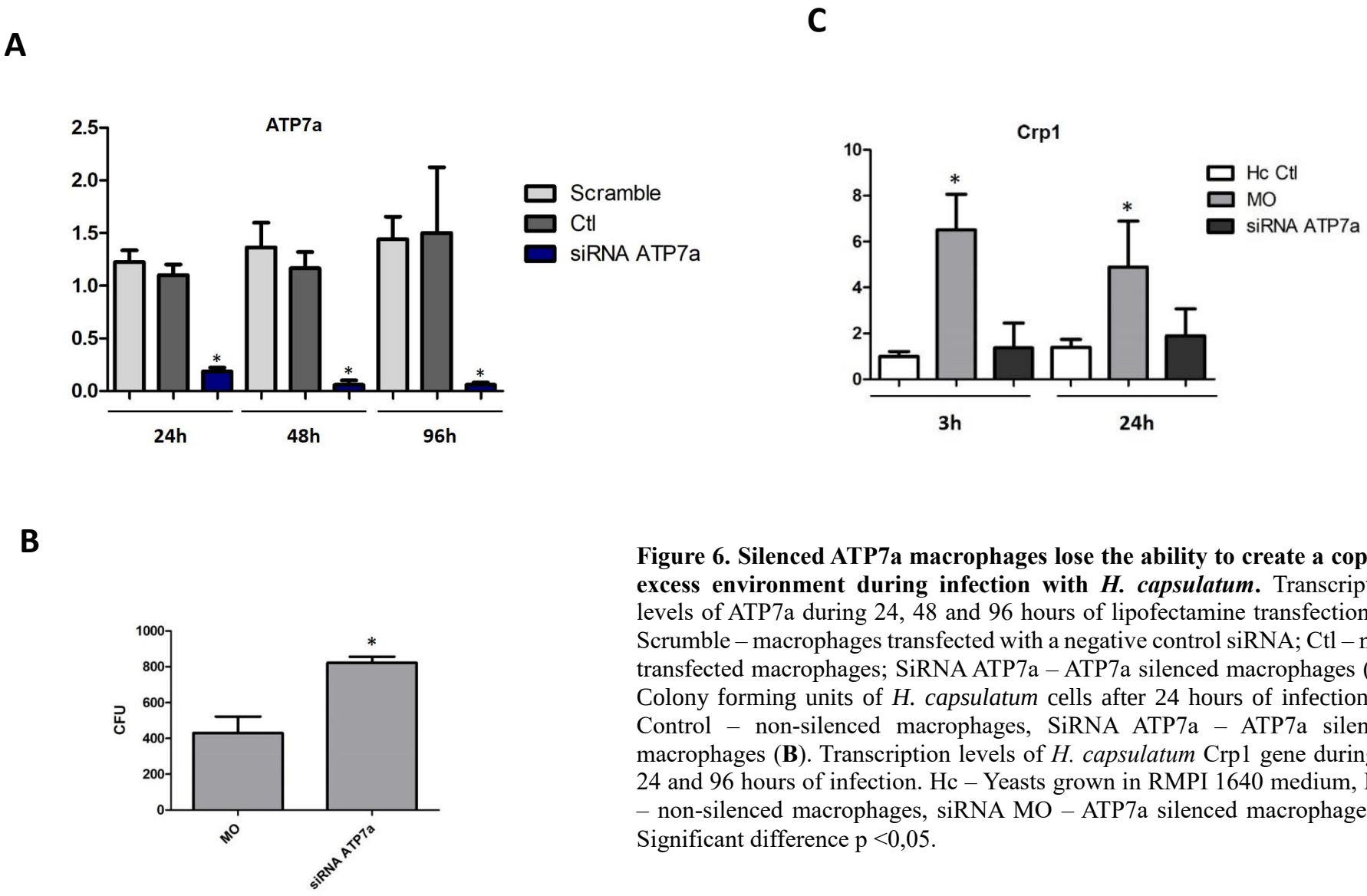
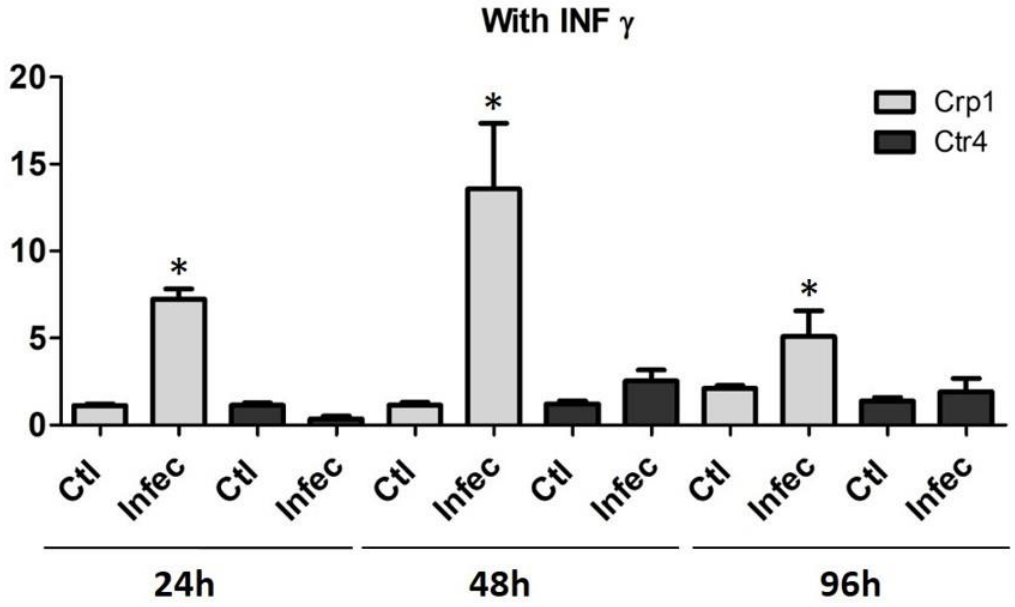


Figure 6. Silenced ATP7a macrophages lose the ability to create a copper excess environment during infection with *H. capsulatum*. Transcription levels of ATP7a during 24, 48 and 96 hours of lipofectamine transfection in: Scrumble – macrophages transfected with a negative control siRNA; Ctl – non-transfected macrophages; SiRNA ATP7a – ATP7a silenced macrophages (**A**). Colony forming units of *H. capsulatum* cells after 24 hours of infection in: Control – non-silenced macrophages, SiRNA ATP7a – ATP7a silenced macrophages (**B**). Transcription levels of *H. capsulatum* Crp1 gene during 3, 24 and 96 hours of infection. Hc – Yeasts grown in RMPI 1640 medium, MO – non-silenced macrophages, siRNA MO – ATP7a silenced macrophages. * Significant difference p <0,05.

A


With INF γ
Crp1
Ctr4
20
15
10
5
0
*
*
*
Ctl
Infec
Ctl
Infec
Ctl
Infec
Ctl
Infec
Ctl
Infec
Ctl
Infec
24h
48h
96h

B

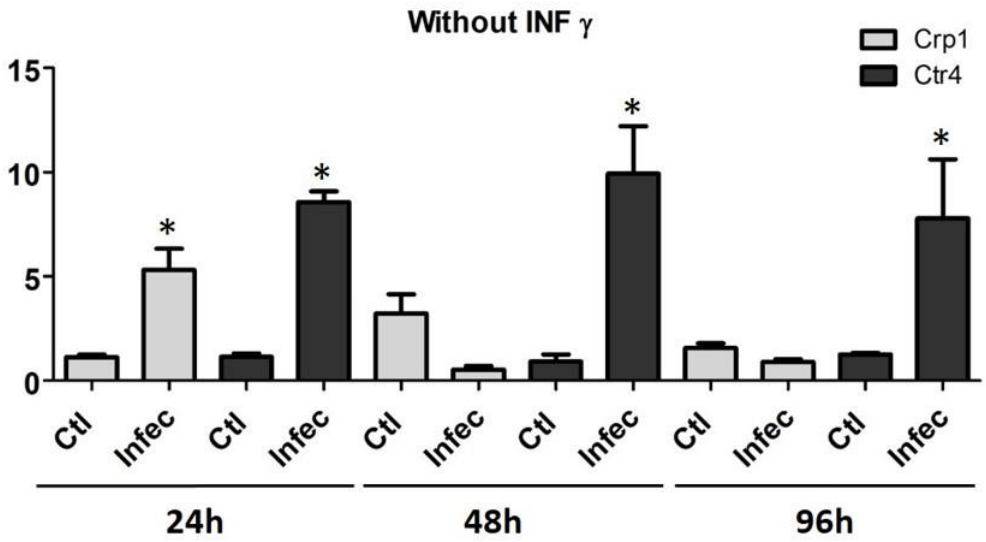
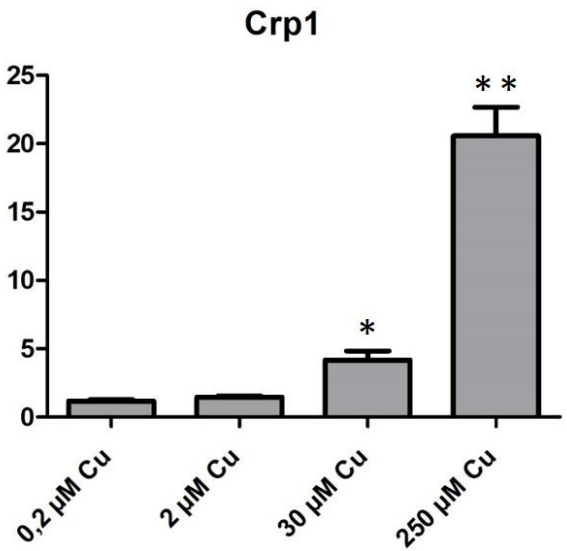
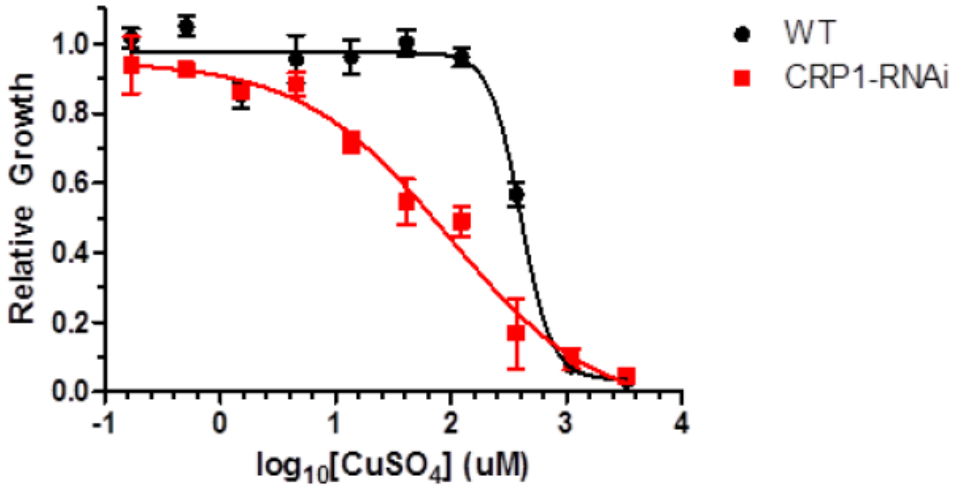


Figure 7. The copper excess environment created by macrophages during infection appears to be time and INF-ɣ dependent. Transcription levels of *H. capsulatum* Crp1 and Ctr4 genes during 24, 48 and 96 hours of infection in INF-ɣ treated macrophages (**A**) and untreated macrophages (**B**). * Significant difference p <0,05.

A


Crp1
25
20
15
10
5
0
* *
*
0,2 µM Cu
2 µM Cu
30 µM Cu
250 µM Cu

B


Relative Growth
1.0
0.8
0.6
0.4
0.2
0.0
-1
0
1
2
3
4
log10[CuSO4] (uM)
WT
CRP1-RNAi

C

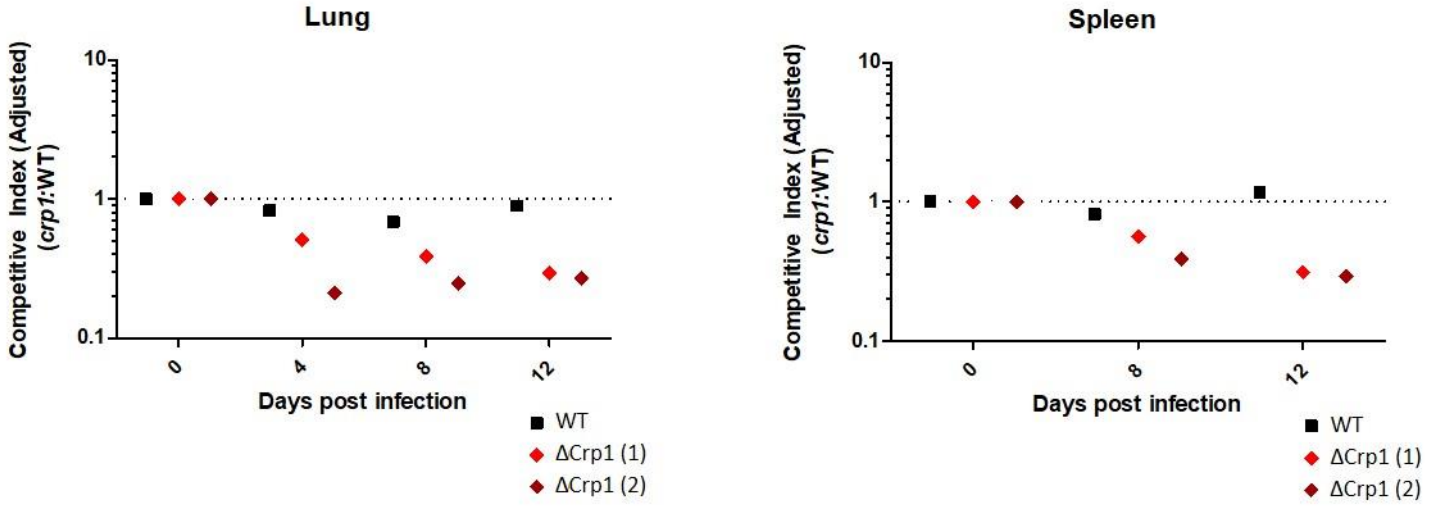


Figure 8. *H. capsulatum* Crp1 mutants lose their ability to withstand high copper concentrations and also has its virulence affected. *H. capsulatum* Crp1 transcription levels in a concentration gradient of 0.2, 2, 30 and 250 µM of copper sulphate, during its growth in chemically defined MvM broth (**A**). Crp1 silenced strains growth in different copper concentrations (0.2, 2, 30, 250 and 500 µM of copper sulphate) (**B**). Fungal burden of Crp1 silenced strains, from lung and mouse spleen, during 12 days of murine infection (**C**). * $p < 0,05$.

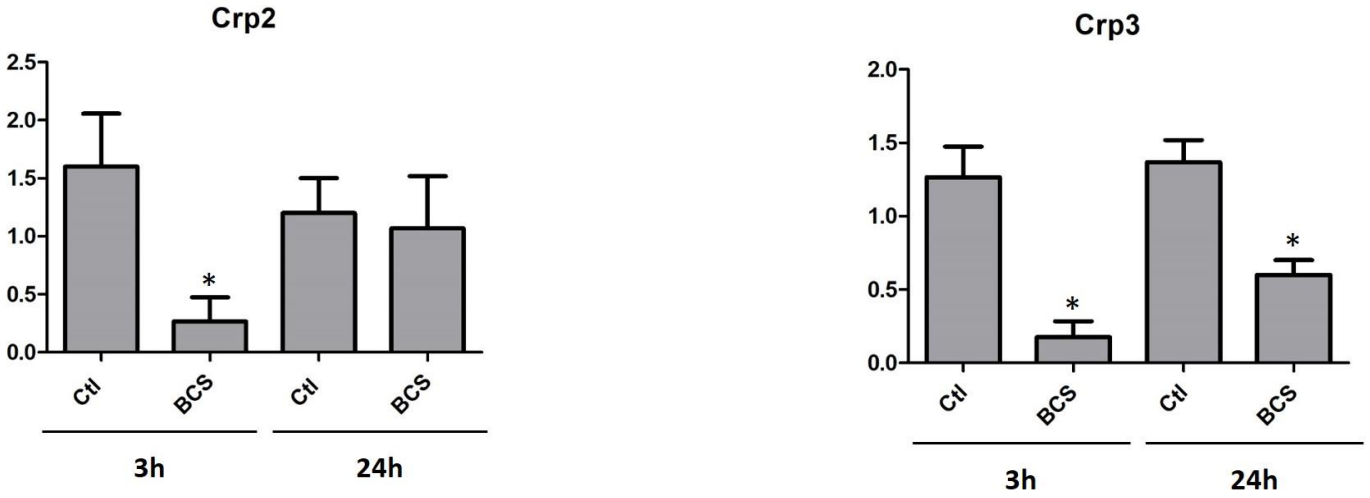


Figure 9. Possible proteins with Crp1 function have a Crp1-like transcriptional profile in the presence and absence of copper. Transcription levels of Crp2 and Crp3 genes of *H. capsulatum* in vitro growth at homeostatic (Ctl) and poor copper (BCS) levels. *$p < 0,05$.

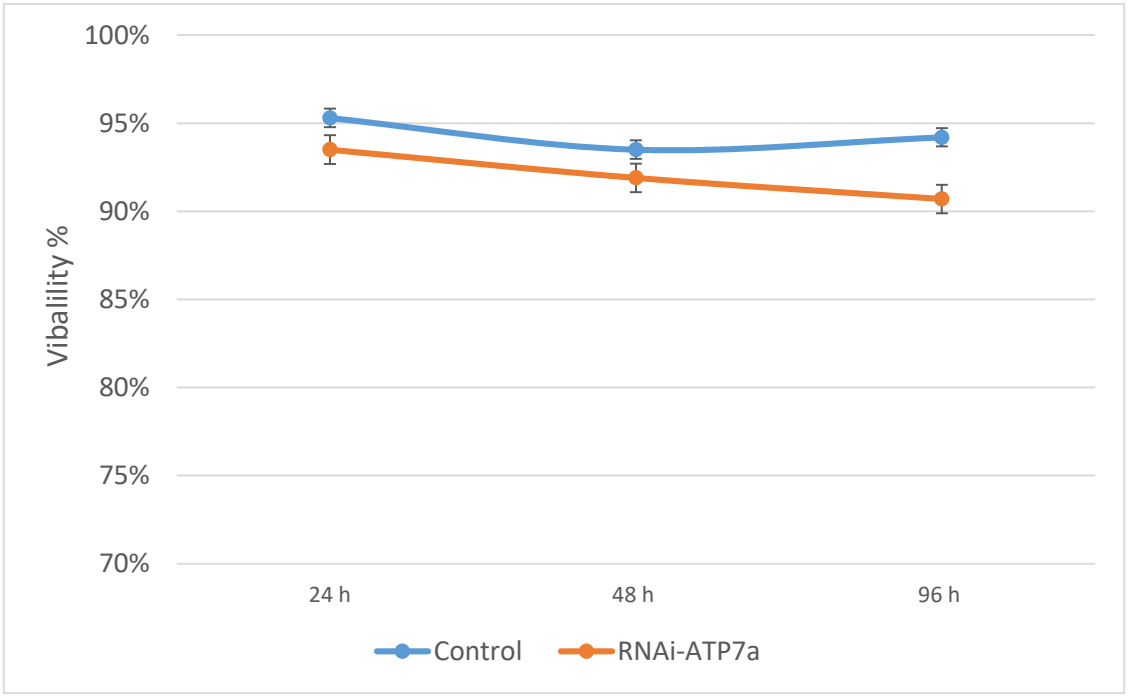
SUPPLEMENTARY FIGURES

Supplementary table 1. Genes of *S. cerevisiae*, *C. neoformans* and *A. fumigatus* used in blast search.

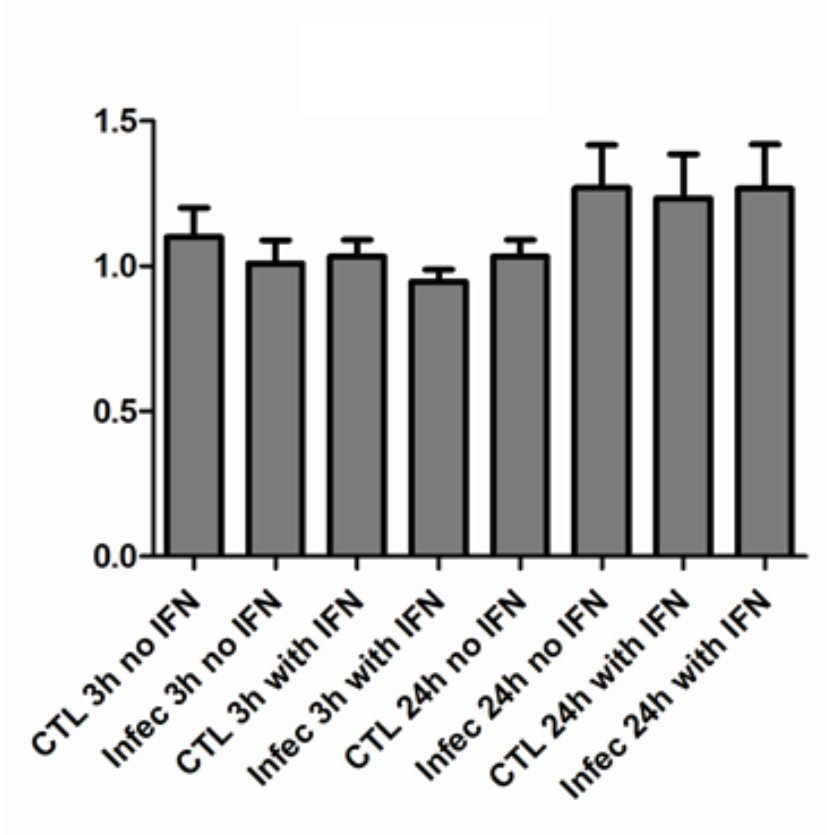
Genes	***H. capsulatum***	*S. cerevisiae*	*C. neoformans*	*A. fumigatus*
Ctr1	Ctr1 (EEH10721.1)	Ctr1 (NP_012045.3)	-	Ctr (XP_754796.1)
Ctr4	Ctr4 (EEH05121.1)	Ctr3 (NP_013515.3)	Ctr4 (XP_012049224.1)	Ctr (XP_749505.1)
Crp1	Crp1 (EEH08652.1)	-	-	CrpA (XP_754347.1)
Ace1	Ace1 (EEH09540.1)	Cup2 (NP_011349.3)	Cuf1 (XP_012051375.1)	Ace1 (XP_750669.1)
Mac1	Mac1 (EEH06208.1)	Mac 1 (NP_013734.1)	-	Mac1 (XP_752689.1)
Atx1	Atx1 (EEH03921.1)	Ccs1 (NP_013752.1)	-	Atx1 (XP_755303.2)
Ccc2	Ccc2 (EEH07254.1)	-	-	Ccc2 (XP_751569.1)
Crs5	Crs5 (EEH09689.1)	Crs5 (NP_116709.3)	Crs (XP_012049528.1)	Crs (XP_752952.1)

Supplementary table 2. Oligonucleotides used for qRT-PCR expression analysis of copper homeostasis related genes in *H. capsulatum*.

Oligonucleotides	Sequence	Access Number
Actin – s	GTCCTCGCCATCATGGTATTA	EEH03071.1
Actin - as	CTCAGGAGCGACACGGAGT	
Ctr1 – s	GGTCAATGTAGCATGAATATGC	EEH10721.1
Ctr1 - as	TCAACGCCAATGCATGCCGT	
Crp1 – s	CCCTTGAAACGGACGGTTGA	EEH08652.1
Crp1 - as	TTGGCAGATTCGATAACAGCAA	
Ctr4 – s	GGACTGTATTGAATGCCTGTTT	EEH05121.1
Ctr4 – as	ATCGTCCGGTATAGAAGTTGC	
Ace1 – s	GGCAAGCGATAATCAAGTTGG	EEH09540.1
Ace1 - as	TTAGTGTGGCGGGTCGGGA	
Ccc2 – s	ATCGAGCTAAGGGCCAAACAT	EEH07254.1
Ccc2 - as	GGAGGCAGACGATATCACCA	
Atx1 – s	AGCGGGTCCTCAAACAATGC	EEH03921.1
Atx1 - as	ATCTCCCATATCACGTACTGTA	
Mac1 – s	TCAGAATTCCGAAGGGGTTCA	EEH06208.1
Mac1 - as	GGCTGCTCTGGCGTCGTG	
Crs5 – s	AGCCACTAAGCATATATGACCT	EEH09689.1
Crs5 - as	GCCCTCAGGAGAACGTCGA	



Supplementary figure 1. Viability curve of siRNA-ATP7a macrophages compared to non-transfected ones, at time of 24, 48 e 96 hours.



Supplementary figure 2. Macrophage ATP7a transcription levels in INF-ɣ treated and non-treated J774 macrophages.

5.0 CONCLUSÃO

Os dados obtidos neste estudo, permitem as seguintes conclusões:

- *H. capsulatum* possui em seu genoma a maquinaria gênica necessária para regular sua homeostase de cobre, possuindo dois importadores de cobre, Ctr1 e Ctr4, uma bomba de exportação de cobre Crp1, fatores de transcrição sensíveis a cobre, Mac1 e Ace1, uma metalochaperona, uma metalotioenia e um transportador de cobre intracelular, Atx1, Crs5 e Ccc2 respectivamente;
- Os genes relacionados a homeostase de cobre no fungo, tiveram o comportamento transcricional esperado nas análises *in vitro*, podendo-se sugerir que estes genes estão sob a regulação dos fatores de transcrição Ace1 e Mac1;
- O perfil transcricional dos genes Ace1, Crp1, Ctr4 e Mac1 durante a infecção de *H. capsulatum* em macrófagos, leva a concluir que durante o processo infeccioso o fungo sofre um excesso de cobre como forma de defesa do hospedeiro;
- Os dados obtidos do silenciamento de ATP7a em macrófagos juntamente com os dados da expressão de Crp1 do fungo, durante a infecção em macrófagos silenciados, reforça a afirmação de que durante a infecção o fungo enfrenta um ambiente com altos níveis de cobre;
- Os macrófagos criam o ambiente com elevado nível de cobre durante a infecção via ATP7a, e este processo parece ser Interferon-ɣ (INT-ɣ) e tempo dependente, pois os dados obtidos da expressão de Crp1 e Ctr4, na infecção com macrófagos não tratados com INT-ɣ junto com a infecção em tempos mais elevados, levam a crer que o fungo passa a enfrentar uma condição de limitação de cobre durante a infecção nestas condições;
- Cepas mutantes de *H. capsulatum* silenciadas para Crp1 são incapazes de crescer em altas concentrações de cobre e tem a sua virulência levemente atenuada em modelo de infecção em camundongos;
- Outras proteínas parecem estar exercendo a função de bomba de efluxo de cobre, tal como Crp1, em *H. capsulatum*, ajudando o fungo a combater o excesso de cobre imposto pelas células hospedeiras durante a infecção.

6.0 PERSPECTIVAS

- As possíveis novas Crp´s encontradas neste estudo, aqui chamadas de Crp2 e Crp3, passarão por análises de caracterização para confirmar sua função durante o processo infeccioso de *H. capsualtum;*
- Será realizado a construção de cepas mutantes de *H. capsulatum* para os fatores de transcrição Mac1 e Ace1;
- Será realizada a caracterização fenotípica das cepas mutantes construídas;
- No intuito de caracterizar os processos celulares regulados por estes fatores de transcrição, será realizado o sequenciamento de RNA dos genes regulados por Mac1 e Ace1, durante o crescimento do fungo em condições de limitação, na presença e no excesso de cobre;
- A caracterização do regulon de cobre no fungo *H. capsulatum* será realizado com os dados obtidos do RNA-seq;
- Por fim, através de ferramentas bioinformáticas, será identificado sequencias reconhecidas por Mac1 e Ace1 nos promotores dos genes alvo destes fatores de transcrição.

5. REFERÊNCIAS BIBLIOGRÁFICAS

AGARWAL, P.; AJELLO, L.; POLONELLI, L. (2015). An unusual presentation of disseminated histoplasmosis: case report and review of pediatric immunocompetent patients from India. *Mycopathologia.* doi: 10.1007/s11046-015-9917-y.

AIDE, M. A. (2009). Histoplasmosis. *Jornal Brasileiro de Pneumologia.* doi: 10.1590/S1806-37132009001100013.

APPELBERG, R. (2006). Macrophage nutriptive antimicrobial mechanisms. *Journal of Leukocyte Biology.*

ARANGO, M.; CASTANEDA, E.; AGUDELO, C. I. et al. (2011). Histoplasmosis: results of the Colombian national survey. *Biomedica.* doi: 10.1590/S0120-41572011000300007.

BAHR, N. C.; ANTINORI, S.; WHEAT, L. J.; SAROSI, G. A. (2015). Histoplasmosis infections worldwide: Thinking outside of the Ohio River valley. *Tropical Mycosis.* doi: 10.1007/s40475-015-0044-0.

BEAUDOIN, J.; EKICI, S.; DALDAL, F. et al. (2013). Copper transport and regulation in *Schizosaccharomyces pombe*. *Biochem Soc Trans.* doi: 10.1042/BST2013089.

BEAUDOIN, J. & LABBE, S. (2007). Crm1-mediated nuclear export to the *Schizosaccharomycies pombe* transcription factor Cuf1 during a shift from low to high copper concentrations. *Eukaryotic Cell.* doi: 10.1128/EC.00002-07.

BEAUDOIN, J.; LABBE, S. (2011). The fission yeast copper sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1 like recognition site. *J Bio Chem.* doi: 10.1074/jbc.M011256200.

BOAL, A. K.; ROSENZWEIG, A. C. (2009). Structural biology of copper trafficking. *Chemical Reviews.* doi: 10.1021/cr900104z.

BOYCE, K. J.; ANDRINOPOULOUS, A. (2015). Fungal dimorphis: the switch from hyphae to yeast is a specialized morphogenetic adaptation allowing colonization of a host. *FEMS Microbiol Rev.* doi: 10.1093/femsre/fuv035.

BRASIER, C. M. (2008). The biosecurity threat to the UK and global environment from international trade in plants. *Plant. Pathol.* https://doi.org/10.1111/j.1365-3059.2008.01886.x.

BROCK, M. (2009). Fungal metabolism in host niches. *Curr. Opin. Microbiol*. doi: 10.1016/j.mib.2009.05.004.

BROWN, G. D.; DENNING, D. W.; GOW, N. A.; LEVITZ, S. M.; NETEA, M. G.; WHITE, T. C. (2012). Hidden killers: human fungal infections. *Sci Trans/Med.* doi: 10.1126/scitranslmed.3004404.

BRUNKE, S.; HUBE, B. (2014). Adaptive prediction as a strategy in microbial infections. *PLoS Pathog.* doi: 10.1371/journal.ppat.1004356.

BRUNKE, S.; MOGAVERO, S.; KASPER, L.; HUBE, B. (2016). Virulence factor in fungal pathogens of man. *Current Opinion in Microbiology*. doi: 10.1016/j.mib.2016.05.010.

CALO, S.; BILLMYRE, R. B.; HEITMAN, J. (2013). Generators of phenotypic diversity in the evolution of pathogenic microrganisms. *PLoS Pathog.* doi: 10.1371/journal.ppat.1003181.

CANO, M. V.; HAJJEH, R. A. (2001). The epidemiology of histoplasmosis: a review. *Semin Respir Infec.* PMID: 11521243.

CASADEVALL, A. (2010). Cryptococci at the brain gate: break and enter or use a Trojan horse? *J Clin Invest.* doi: 10.1172/JCI42949.

CHAI, L. Y.; NETEA, M. G.; VONK, A. G.; KULLBERG, B. J. (2009). Fungal strategies for overcoming host innate immune response. *Med. Mycol.* doi: 10.1080/13693780802209082.

CRAWFORD, A.; WILSON, D. (2015). Essential metals at the host-pathogen interface: nutritional immunity and micronutrient assimilation by human fungal pathogens. *FEMS Yeast.* doi: 10.1093/femsyr/fov071.

CROLL, D.; McDONALD, B. A. (2012). The accessory genome as a cradle for adaptive evolution in pathogens. *PLoS Pathog.* doi: 10.1371/journal.ppat.1002608.

DALLE, F.; WATCHTLER, B.; HOLLAND, G.; BONNIN, A. (2010). Cellular interactions of *Candida albicans* with human oral ephitelial cells. *Cell Microbiol.* doi: 10.1111/j.1462-5822.2009.01394.x.

DEEPE, G. S.; DUROSE, G. G. (1995). Immunological activity of recombinant H antigen from *Histoplasma capsulatum*. *Infect Immun.* PMID: 7622243.

DEUS FILHO, A.; WANKE, B.; SALMITO CAVALCANTI, A.; SOARES MARTINS, L. M.; CASTELO, A. (2009). Histoplasmosis in the northeast of Brazil. Report of three cases. *Rev Port Pneumol.* PMID: 19145393.

DIAS, M. A.; OLIVEIRA, R. M.; GIUDICE, M. C.; NETTO, H. M.; JORDAO, L. R.; GRIGORIO, I. M. (2011). Isolation of *Histoplasma capsulatum* from bats in the urban area of São Paulo state, Brazil. *Epidemiol Infect.* doi: 10.1017/S095026881000289X.

DING, C.; YIN, J.; TOVAR, E. M.; FITZPATRICK, D. A.; HIGGINGS, D. G.; THIELE, D. J. (2013). The copper regulon of the human fungal pathogen *Cryptococcus neoformans* H99. *Mol Microbiol.* doi: 10.1111/j.1365-2958.2011.07794.x.

ENE, V.; BRUNKE, S.; BROWN, A. J.; HUBE, B. (2014). Metabolism in fungal pathogenesis. *Perspect. Med.* doi: 10.1101/cshperspect.a019695.

FANNING, S.; MITCHEL, A. P. (2012). Fungal biofilms. *PLoS Pathog.* doi: 10.1371/journal.ppat.1002585.

FERREIRA, M. S. (2009). Histoplasmosis. *Rev Soc Bras Med Trop.* PMID: 19448941.

FERREIRA, M. S.; BORGES, A. S. (2009). Histoplasmosis. *Rev Soc Bras Med Trop.* PMID: 19448941.

FESTA, R. A. & THIELE, D. J. (2012). Copper at the front line of the host-pathogen battle. *Plos Pathogens.* doi: 10.1371/journal.ppat.1002887.

FESTA, R. A.; THIELE, D. J. (2011). Copper: An essential metal in biology. *Current Biology.* doi: 10.1016/j.cub.2011.09.040.

FISHER, M. C.; KOENIG, G. L.; WHITE, T. J.; TAYLOR, J. W. (2002). Molecular and phenotypic description of *Coccidioides posadaii* previously recognized as the non-California population of *Coccidioides immitis*. *Mycologia.* PMID: 21156479.

GARCIA-SANTAMARINA, S.; FESTA, R. A.; SMITH, A. D.; YU, C.; PROBST, C.; DING, C.; HOMER, C. M.; YIN, J.; NOONAN, J. P.; MADHANI, H.; PERFECT, J. R.; THIELE, D. J. (2018). Genome-Wide analysis of the regulation of Cu metabolism in *Cryptococcus neoformans*. *Molecular Microbiology.* doi: 10.1111/mmi.13960.

GARFOOT, A. L.; QIAN, S.; WUTHRICH, M.; KLEIN, B. S.; RAPPLEY, B. A. (2016). The Eng1 B-Glucanase enhances *Histoplasma* virulence by reducing B-Glucan exposure. *Mbio.* doi: 10.1128/mBio.01388-15.

GILBERT, A. S.; WHEELER, R. T.; MAY, R. C. (2015). Fungal pathogens: survival and replication within macrophages. *Cold Spring Harb Perspect Med.* doi: 10.1101/cshperspect.a019661.

GOPALAKRISHNAN, R.; NAMBI, P. S.; RAMASUBRAMANIAN, V.; ABDUL-GAFHUR K.; PARAMESWARAN, A. (2012). Histoplasmosis in India: truly uncommon or uncommonly recognized? *J Assoc Physicians India.* PMID: 23777021.

GUEHO, E.; LECLERC, M. C.; HOOG, G. S.; DUPONT, B. (1997). Molecular taxonomy and epidemiology of *Blastomyces* and *Histoplasma* species. *Mycoses.* PMID: 9375491.

GUGNANI, H. C.; MUOTOE-OKAFOR, F. (1997). African histoplasmosis: a review. *Rev. Iberoam Micol.* PMID: 15538817.

GUIMARÃES, L. F.; HAELPERN, M.; LEMOS, A. S.; GOUVEA, R. T. et al. (2016). Invasive fungal disease in renal transplant recipients at a brazilian center: local epidemiology matters. *Transplant Proc.* doi: 10.1016/j.transproceed.2016.06.019.

HAGAN, T. (2003). The discovery and naming of histoplasmosis: Samuel Taylor Darling. *Antimicrobe.*

HAGE, C. A.; AZAR, M. M.; BAHR, N.; LOYD, J.; WHEAT, L. J. (2015). Histoplasmosis: Up-to-Date evidence based approach to diagnosis and management. *Crit Care Med.* doi: 10.1055/s-0035-1562899.

HODGKINSON, V. & PETRI, M. J. (2012). Copper homeostasis at the host-pathogen interface. *Journal of Biological Chemistry.* doi: 10.1074/jbc.R111.316406.

HOOD, M. I. & SKAAR, E. P. (2013). Nutritional immunity: Transition metals at the pathogen-host interface. *Nat. Rev. Microbiol.* doi: 10.1038/nrmicro2836.

HUBE, B. Fungal adaptation to the host environment. (2009). *Current Opinion in Microbiology.* doi: 10.1016/j.mib.2009.06.009.

JIMENEZ-LOPEZ, C.; LORENZ, M. C. (2013). Fungal immune evasion in a model host-pathogen interaction: *Candida albicans* versus macrophages. *PLoS Pathog.* doi: 10.1371/journal.ppat.1003741.

JING, W.; ISMAIL, R. (1999). Mucocutaneous manifestations of HIV infection: a retrospective analysis of 145 cases in a Chinese population in Malaysia. *Int J Dermatol.* advanced HIV infection. PMID: 10397587.

KASUGA, T.; WHITE, T. J.; KOENIG, G.; McEWEN, J.; RESTREPO, A. (2003). Phylogeography of the fungal pathogen *Histoplasma capsulatum*. *Mol Ecol.* PMID: 14629354.

KASUYA, Y.; DAISUKE, H.; SAKAI, K.; YAGUSHI, T. et al. (2017). Transcription factor *Afmac1* controls copper import machinery in *Aspergillus fumigatus*. *Current Genetics.* doi: 10.1007/s00294-017-0681-z.

KAUFFMAN, C. A. (2009). Histoplasmosis. *Clin Chest Med.* doi: 10.1016/j.ccm.2009.02.002.

KAUFFMAN, C. A. (2007). Histoplasmosis: a clinical and laboratory update. *Clinical microbiology reviews.* doi: 10.1128/CMR.00027-06.

KEHL-FIE, T. E.; SKAAR, E. P. (2010). Nutritional Immunity beyond iron: a role for manganese and zinc. *Curr. Opin. Chem. Biol.* doi: 10.1016/j.cbpa.2009.11.008.

KELLER, G.; BIRD, A.; WINGE, D. R. (2005). Independent metalloregulation of Ace1 and Mac1 in *Saccharomyces cerevisiae*. *Eukaryot Cell.* doi: 10.1128/EC.4.11.1863-1871.2005.

KIM, M. S.; KIM, S. Y.; YOON, J. K.; LEE, Y. W.; BAHN, Y. S. (2009). An gene-disruption method in *Cryptococcus neoformans* by double joint PCR with NAT-split markers. *Biochem Biophys Res Commun.* doi: 10.1016/j.bbrc.2009.10.089.

KORNITZER, D. (2009). Fungal mechanisms of host iron acquisition. *Curr. Opin. Microbiol.* doi: 10.1016/j.mib.2009.05.005.

KWON-CHUNG, K. J. (1972). *Emmonsiella capsulate*: perfect state of *Histoplasma capsulatum*. *Science.* PMID: 5035491.

LABBE, S.; ZHU, Z.; THIELE, D. J. (1997). Copper specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. *J Bio Chem.* PMID: 9188496.

LACAZ, C. S.; PORTO, E.; MARTINS, J. E. C.; HEINS-VACCARI, E. M.; MELO, N. T. (2009). Tratado de micologia médica. *Ver Soc Med Trop.*

LONDERO, A. T.; WANKE, B. (1998). Histoplasmose capsulata. *J Bras Med.*

MACKIE, J.; SZABO, E. K.; URGAST, D. S.; BALLOU, E. R.; CHILDERS, S.; BROWN, A. J. P. (2016). Host imposed copper poisoning impacts fungal micronutrient acquisition during systemic *Candida albicans* infections. *PloS One.* doi: 10.1371/journal.pone.0158683.

MARTINEZ-FINLEY, E. J.; CHAKRABORTY, S.; FRETHAM, S. J. B.; ASCHNER, M. (2012). Cellular transport and homeostasis of essential and non essential metals. *Metallomics.* doi: 10.1042/BJ20091909.

MUKHERJEE, A.; TANGRI, R.; VERMA, N.; GAUTAM, D. (2010). Chronic disseminated Histoplasmosis boné marrow involvement in an immunocompetent patient. *Indian J Hematol Blood Transfus.* doi: 10.1007/s12288-010-0022-6.

MUNIZ, M. M. LOTT, T. J.; MAYER, L. W. (2013). Molecular cloning, characterization and expression of the M antigen of *Histoplasma capsulatum*. *Infect Immun.* PMID: 10085041.

NAGLICK, J. R.; CHALLACOMBE, S. J.; HUBE, B. (2003). *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol. Mol. Biol. Rev.* PMID: 12966142.

NEVITT, T. (2012). War-Fe-re: Iron at the core of fungal virulence and hos immunity. *Biometals.* doi: 10.1007/s10534-011-9431-8.

NEWMAN, S. L. (1999). Macrophages in host defense against *Histoplasma capsulatum*. *Trends Microbiol.* PMID: 10081083.

NORKAEW, T.; OHNO, H.; SRIBUREE, P, et al. (2013). Detection of environmental sources of *Histoplasma capsulatum* in Chiang Mai, Thailand by nested PCR. *Mycopathologia.* doi: 10.1007/s11046-013-9701-9.

OHNO, H.; OGATA, Y.; SUGURO, H, et al. (2010). An outbreak of histoplasmosis among healthy young Japanese women after traveling to Southeast Asia. *Intern Med.* PMID: 20190491.

OLIVEIRA, F. M.; UNIS, G.; SEVERO, L. C. (2006). An outbreak of histoplasmosis in the city of Blumenau, Santa Catarina. *J Bras Pneumol.* PMID: 17268739.

PAGANO, L. (2006). The epidemiology of fungal infections in patients with hematologic malignancies. *Haematologica.* PMID: 16885047.

PAN, B.; CHEN, M.; PAN, W.; LIAO, W. (2013). Histoplasmosis: a new endemic fungal infection in China? Review and analysis of cases. *Mycoses.* doi: 10.1111/myc.12029.

PERMYAKOV, E. (2009). Metalloproteomics. *Jonh Wiley & Sons, Inc. Ed. 1. Pg: 105-122.*

PFALLER, M. A.; DIEKEMA, D. J. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. *Clinical Microbiology Reviews.* doi: 10.1128/CMR.00029-06.

PRADO, M.; SILVA, MB.; LAURENTI, R.; TRAVASSOS, LR.; TABORDA, CP. (2009). Mortality due to systemic mycosis as a primary cause of death or in association with AIDS in Brazil: A review from 1996 to 2006. *Mem. Inst. Oswaldo Cruz.* PMID: 19547881.

RAJA, M. R.; WATERMAN, S. R.; Q. I. U, J.; BLEHER, R.; WILLIAMSON, P. R.; HALLORAN, V. O. (2013). A copper hyperaccumulation phenotype correlates with pathogenesis in *Cryptococcus neoformans*. *Metallomics.* doi: 10.1039/c3mt20220h.

RATINIEKS, F. L.; CARRECK, N. L. (2010). Ecology and the hooney bee collapse? *Science.* doi: 10.1126/science.1185563.

REINHARDT, D. (2007). Programing good relations – development of the arbuscular mycorrhizal symbiosis. *Curr Opin Plant Biol.* doi: 10.1016/j.pbi.2006.11.001.

RESTREPO, A.; TOBON, A. (2005). Principles and practices of infectious diseases. *Medical Mycology.*

RIVERA, A. (2014). Protective immune responses to fungal infections. *Parasite imunnology.* doi: 10.1111/pim.12098.

ROMANI, L. (2011). Immunity to fungal infections. *Nature Reviews.* doi: 10.1038/nri2939.

ROSSINI, F. T.; GOULART, L. S. (2006). Classic histoplasmosis. *RBAC.*

SAMANOVIC, M. I.; DING, C.; THIELE, D. J.; DARWIN, H. K. (2012). Copper in microbial pathogenesis: Meddling with the metal. *Cell Host & Microbe.* doi: 10.1016/j.chom.2012.01.009.

SAYERS, Z.; BROUILLON, P.; SVERGUN, D. I.; ZIELENKIEWICZ, P.; KOCH, M. H. (1999). Biochemical and structural characterization of recombinant copper metallothioenin from *Saccharomyces cerevisiae*. *Eur J Biochem.* PMID: 10411649.

SCHARF, D. H.; HEINEKAMP, T.; BRAKHAGE, A. A. (2014). Human and plant fungal pathogenesis: the role of secondary metabolites. *PLoS Pathog.* doi: 10.1371/journal.ppat.1003859.

SEIDER, K.; HEYKEN, A.; LUTTICH, A.; MIRAMON, P.; HUBE, B. (2010). Interaction of pathogenic yeast with phagocytes: survival persistence and escape. *Curr. Opin. Microbiol.* doi: 10.1016/j.mib.2010.05.001.

SEPULVEDA, V. E.; MARQUEZ, R.; TURISSINI, D. A.; GOLDMAN, W. E.; MATUTE, D. R. (2017). Genome sequences reveal cryptic speciation in the human pathogen *Histoplasma capsulatum*. *M Bio.* doi: 10.1128/mBio.01339-17.

SERPE, M.; JOSHI, A.; KOSMAN, D. J. (1999). Structure function analysis of the protein binding domains of Mac1, a copper dependent transcriptional activator of copper uptake in *Saccharomyces cerevisiae*. *J Biol Chem.* PMID: 10506178.

SEVCENCO, A. M.; PINKSE, M. W. H.; WOLTERBEEK.; H. T. H.; VERHAERT, PDEM.; HAGEN, W. R.; HAGEDORN, P. L. (2011). Exploring the microbial metalloproteome using MIRAGE. *The Royal Society of Chemistry*. doi: 10.1039/c1mt00154j.

SHOHAM, S.; MARR, K. A. (2012). Invasive fungal infections in solid organ transplant recipients. *Future Microbiology.* doi: 10.2217/fmb.12.28.

SILVA, M. G.; SCHRANK, A.; BAILÃO, E. F. L. C.; BAILÃO, A. M.; BORGES, C. L.; STAATS, C. C.; PARENTE, J. A.; PEREIRA, M.; SALEM-IZAAC, S. M.; MENDES-GIANINNI, M. J. S.; OLIVEIRA, R. M. Z.; SILVA, L. K. R.; NOSANCHUCK, J. D.; VAINSTEIN, M. H.; SOARES, C. M. A. (2011). The homeostasis of iron, copper and zinc in *Paracoccidioides brasiliensis, Cryptococcus*

neoformans var. *grubii*, and *Cryptococcus gattii*: a comparative analysis. *Frontiers in Microbiology.* doi: 10.3389/fmicb.2011.00049.

SILVA, T. C.; TREMEA, C. M.; ZARA, A. L.; MENDONÇA, A. F. et al. (2017). Prevalence and lethality among patients with histoplasmosis and AIDS in the Midwest region of Brazil. *Mycoses.* doi: 10.1111/myc.12551.

SOYSAL, A. Prevention of invasive fungal infections in immunocompromised patients: the role of delayed release posaconazole. **Infect Drug Resist.** 2015.

STAFFORD, L. S.; BOKIL, N. J.; ACHARD, M. E. S.; KAPETANOVIC, R.; SCHEMBRI, N. A.; McEWIN, A. G.; SWEET, M. J. (2013). Metal ions in macrophage antimicrobial pathways: emerging roles for zinc and copper. *Bioscience Reports.* doi: 10.1042/BSR20130014.

STARNES, G. L.; JEWETT, T. J.; CARRUTHERS, V. B.; SIBLEY, L. D. Two separate, conserved acidic amino acid domains within the *Toxoplasma gondii* MIC2 cytoplasmic tail are required for parasite survival. **J Biol Chem.** 2006.

STERKEL, A. K.; LORENZINI, J. L.; FITES, J. S.; VIGNESH, K.; KLEIN, B. S. Fungal mimicry of a mammalian aminopeptidase disables innate immunity and promotes pathogenicity. **Cell Host Microbe.** 2016.

SUDBERY, P.; GOW, N.; BERMAN, J. The distinct morphogenic states of *Candida albicans*. **Trends Microbiol.** 2004.

SUN, T.; JU, X.; GAO, H.; WANG, T.; THIELE, D. J.; LI, Y.; WANG, Z.; DING, C. Reciprocal functions of *Cryptococcus neoformans* copper homeostasis machinery during pulmonary infection and meningoencephalitis. **Nature Communications.** 2014.

SUN, X.; XIAO, C.; GE, R.; YIN, X.; LI, H.; LI, N.; YANG, X.; ZHU, H.; HE, X.; HE, KY. Putative copper – and zinc-binding motifs in *Streptococcus pneumoniae* indentified by immobilized metal affinity chromatography and mass spectrometry. **Proteomics.** 2011.

TAYLOR, M. L.; CHAVEZ-TAPIA, C. B.; REYES-MONTES, M. R. (2000). Molecular typing of *Histoplasma capsulatum* isolated from infected bats, captured in Mexico. *Fungal Genet Biol.* doi: 10.1006/fgbi.2000.1219.

TEIXEIRA, M. M.; PATANÉ, J. S. L.; TAYLOR, M. L.; GOMEZ, B. L.; THEODORO, R. C.; HOOG, S.; ENGELTHALER, D. M.; ZANCOPÉ-OLIVEIRA, R. M. Z.; FELIPE, M. S. S.; BARKER, B. M. Worldwide phylogenetic distribution and population dynamics of the genus *Histoplasma*. **PLoS One.** 2016.

TURISSINI, D. A.; GOMEZ, O. M.; TEIXEIRA, M. M.; MCWEN, J. G.; MATUTE, D. R. Species boundaries in the human pathogen *Paracoccidioides.* **Fungal Genet Biol.** 2017.

VAN HO, A.; WARD, D. M.; KAPLAN, J. Transition metal transport in yeast. **Anu. Rev. Microbiol.** 2002.

VANCE, R. E.; ISBERG, R. R.; PORTNOY, D. A. Patterns of pathogenesis: discrimination of pathogenic and non-pathogenic microbes by the innate immune system. **Cell Host Microbe.** 2009.

VIGNESH, K. S.; LANDERO-FIGUEROA, J. A.; PAROLLO, A.; CARUSO, J. A.; DEEPE, G. S. Granulocyte Macrophage-Colony Stimulating Factor induced zinc sequestration enhances macrophage superoxide and limits intracellular pathogen survival. **Immunity.** 2013.

VITE-GARIN, T.; BARCENAS, D. A.; CIFUENTES, J.; TAYLOR, M. L. The importance of molecular analyses for understanding the genetic diversity of *Histoplasma capsulatum*. **Revista Iberoamericana de Micologia.** 2014.

WANKE, B. (1985). Histoplasmose, estudo epidemiológico, clínico e ambiental. *Rev UFRJ.* doi: 10.1590/S0037-86822009000200020.

WATERMAN, S. R.; PARK, Y.; RAJA, M.; QIU, J.; HAMMOUND, T.; WILLIANSOM, R. (2012). Role of CTR4 in the virulence of *Cryptococcus neoformans. Mbio.* doi: 10.1128/mBio.00285-12.

WEINBERG, E. D. (2009). The role of iron in protozoan and fungal infectious diseases. *Eukaryot Microbiol.* PMID: 10377984WHEAT, J.; SASORI, G.; MCKINSEY, D.; HAMILL, R.; BRADSHER, R.; JOHNSON, P.; LOYD, J. Practice guidelines for the management of patients with histoplasmosis. **Clin Infect Dis.** 2000.

WHEAT, J.; SASORI, G.; MCKINSEY, D.; HAMILL, R.; BRADSHER, R.; JOHNSON, P.; LOYD, J. (2000). Clinical practice guidelines for the management of patients with histoplasmosis. *Clin Infect Dis.* 10.1086/521259.

WHITE, C.; LEE, J.; KAMBE, T. et al. (2009). A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. *J Bio Chem.* doi: 10.1074/jbc.M109.070201.

WIEMANN, P.; PEREVITSKY, A.; LIM, F. Y.; HUTTENLOCHER, A.; OSHEROV, N.; KELLER, N. P. *Aspergillus fumigatus* copper export machinery and reactive oxygen intermediate defense counter host copper-mediated oxidative antimicrobial offense. **Cell Reports.** 2017.

WINTERS, M. S.; CHAN, Q.; CARUSO, J. A.; DEEP, G. S. Jr. Metallomic analysis of macrophages infected with *Histoplasma capsulatum* reveals a fundamental role for zinc in host defences. **The Journal of Infectious Diseases.** 2010.

ZANCOPÉ-OLIVEIRA, R. M.; MUNIZ, M. M.; WANKE, B. (2013). Genetic diversity of *Histoplasma capsulatum* strains in Brazil. *FEMS Imunnol Med Microbiol.* doi: 10.1016/j.femsim.2005.05.01.

CAPÍTULO 3: Anexos

Artigo publicado durante o período do Doutorado

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 09 January 2015
doi: 10.3389/fmicb.2014.00761

Predicting copper-, iron-, and zinc-binding proteins in pathogenic species of the *Paracoccidioides* genus

Gabriel B. Tristão[1], Leandro do Prado Assunção[1], Luiz Paulo A. dos Santos[1], Clayton L. Borges[1], Mirelle Garcia Silva-Bailão[1], Célia M. de Almeida Soares[1], Gabriele Cavallaro[2] and Alexandre M. Bailão[1]*

[1] *Biochemistry and Molecular Biology, Laboratório de Biologia Molecular, Universidade Federal de Goiás, Goiânia, Brazil*
[2] *Magnetic Resonance Center, University of Florence, Sesto Fiorentino, Italy*

Edited by:
Joshua D. Nosanchuk, Albert Einstein College of Medicine of Yeshiva University, USA

Reviewed by:
Marcio Rodrigues, Oswaldo Cruz Foundation, Brazil
Charley Christian Staats, Universidade Federal do Rio Grande do Sul, Brazil

****Correspondence:***
Alexandre M. Bailão, Laboratório de Biologia Molecular, Departamento de Bioquímica, Instituto de Ciências Biológicas II, Universidade Federal de Goiás, Goiânia-GO, Estrada do campus, Campus Samambaia, ICB2, room 206, Goiania, Brazil
e-mail: alexandre.bailao@gmail.com

Approximately one-third of all proteins have been estimated to contain at least one metal cofactor, and these proteins are referred to as metalloproteins. These represent one of the most diverse classes of proteins, containing metal ions that bind to specific sites to perform catalytic, regulatory and structural functions. Bioinformatic tools have been developed to predict metalloproteins encoded by an organism based only on its genome sequence. Its function and the type of metal binder can also be predicted via a bioinformatics approach. *Paracoccidioides* complex includes termodimorphic pathogenic fungi that are found as saprobic mycelia in the environment and as yeast, the parasitic form, in host tissues. They are the etiologic agents of Paracoccidioidomycosis, a prevalent systemic mycosis in Latin America. Many metalloproteins are important for the virulence of several pathogenic microorganisms. Accordingly, the present work aimed to predict the copper, iron and zinc proteins encoded by the genomes of three phylogenetic species of *Paracoccidioides* (*Pb*01, *Pb*03, and *Pb*18). The metalloproteins were identified using bioinformatics approaches based on structure, annotation and domains. Cu-, Fe-, and Zn-binding proteins represent 7% of the total proteins encoded by *Paracoccidioides* spp. genomes. Zinc proteins were the most abundant metalloproteins, representing 5.7% of the fungus proteome, whereas copper and iron proteins represent 0.3 and 1.2%, respectively. Functional classification revealed that metalloproteins are related to many cellular processes. Furthermore, it was observed that many of these metalloproteins serve as virulence factors in the biology of the fungus. Thus, it is concluded that the Cu, Fe, and Zn metalloproteomes of the *Paracoccidioides* spp. are of the utmost importance for the biology and virulence of these particular human pathogens.

Keywords: metalloproteome, bioinformatics, Paracoccidioidomycosis, metal homeostasis, virulence

INTRODUCTION

Metal ions such as copper, iron, and zinc and others play an essential role in living organisms primarily by virtue of their association with proteins, which are referred to as metalloproteins (Frausto Da Silva and Williams, 1991; Bertini et al., 2007; Festa and Thiele, 2011). Approximately one-third of all proteins studied are associated with a metal ion (Shi and Chance, 2008) whose presence is most commonly needed for the catalytic mechanism of enzymes and/or the stabilization of the tertiary or quaternary structure of proteins (Andreini et al., 2006a).

Many metals play important roles in organisms as a free ion or coupled to proteins. However, the function of Cu, Fe, and Zn is primarily related to metalloprotein dependence on those elements. Copper essentiality retains both its activity in structural stabilization and its redox ability, which is used by metalloenzymes that catalyze electron transfer reactions (Festa and Thiele, 2012). In this respect, this metal functions in a broad range of metabolic activities including, for example, energy production, iron acquisition, melanin production and antioxidant defense (Kim et al., 2008). Iron is also a redox-active element and is essential as a cofactor in the form of heme and iron-sulfur clusters in a variety of cellular processes such as respiration, amino acid metabolism, biosynthesis of sterols and DNA, peroxide detoxification, and DNA replication (Nevitt, 2011; Schrettl and Haas, 2011; Netz et al., 2012). Zinc constitutes the catalytic and/or structural core of many proteins involved, among other functions, in transcriptional control, reactive oxygen species (ROS) detoxification, carbohydrate oxidation and alcoholic fermentation (Murakami and Hirano, 2008; Wilson et al., 2012).

Although metals are fundamental for the correct functioning of cells, their excess is toxic. Thus, metal availability is tightly controlled (Valko et al., 2005; Bleackley and Macgillivray, 2011). During infection there is a battle for micronutrients where a host can either decrease metal availability to the invader or increase the metal concentration to toxic levels, and pathogens must keep metal homeostasis in host tissues to promote a successful infection (Ammendola et al., 2007; Samanovic et al., 2012; Wilson et al., 2012; Cassat and Skaar, 2013). A prerequisite to

understanding the homeostatic mechanisms that maintain constant levels of the essential metals and removing the unwanted metals in an organism is the knowledge, as complete as possible, of the metalloproteins encoded by that organism. With the advent of genome sequencing, the entire proteome of several species has become available. For the majority of these data, however, there is no functional information available, as a thorough functional characterization of whole proteomes is not yet routinely possible. Thus, a systematic approach to search metalloproteins in protein sequence databases has been developed (Andreini et al., 2004) and used in a systematic description of copper, iron and zinc proteins through the three domains of life (Andreini et al., 2006b, 2007, 2008).

Paracoccidioidomycosis is a systemic mycosis restricted to Latin American countries. This disease is caused by the fungi of the species complex *Paracoccidioides* spp. The complex is composed of two species: *Paracoccidioides lutzii* and *Paracoccidioides brasiliensis*; the latter has four phylogenetic species, S1, PS2, PS3, and PS4, with a different geographic distribution (Matute et al., 2006a,b; Carrero et al., 2008; Teixeira et al., 2009, 2013; Bocca et al., 2013). *Paracoccidioides* spp. are thermodimorphic fungi that present as mycelium in the environment at temperatures of 18–25°C and as yeast in mammalian hosts at 36°C. Metal homeostasis has been well described as a determinant factor in fungal pathogenesis (Bailao et al., 2006; Schrettl and Haas, 2011; Festa and Thiele, 2012; Schneider Rde et al., 2012; Wilson et al., 2012), and *Paracoccidioides* spp. presents several metal homeostasis genes that encode for molecules that have been described as important virulence factors in fungi (Silva et al., 2011). In the present work, we used the abovementioned bioinformatics approach to predict the Cu-, Fe-, and Zn-binding proteins encoded by the genomes of the genus *Paracoccidioides*, with the aim of advancing our comprehension of the fungal metal homeostasis and virulence.

MATERIALS AND METHODS

IDENTIFICATION OF Cu-, Fe-, AND Zn-BINDING PROTEINS IN THE *PARACOCCIDIOIDES* SPP. GENOME

Metalloproteins were identified by using the RDGB tool (Andreini et al., 2011) with default options. In the RDGB strategy, the protein domains defined in the Pfam library are used to identify putative homologs in any desired genome or list of genomes. Copper-, iron-, and zinc-binding Pfam domains were initially identified in the sequence of copper-, iron-, and zinc-binding proteins of known 3D structures, which are available from the Protein Data Bank (PDB). When a particular metal is present within the 3D structure of the protein, this information can be readily extracted from the PDB database along with the pattern of amino acids that are involved in the interaction of the protein with the metal.

The latter is referred to as the ligand binding pattern (LBP) and is defined by the identity and spacing of the amino acids, e.g., CX(4)CX(2)H, where X is any amino acid. This pattern is usefully applied as a filter to reduce the number of false positives (i.e., of the proteins predicted to bind the metal, which in reality are unable to bind it) by rejecting the proteins that lack the LBP. The LBP filter is applied by imposing that the predicted protein contains all of the ligands of the LBP with a spacing in sequence that it is maintained within ±20% (or ±1 amino acid for short spacing). The lists of copper-, iron-, and zinc-binding Pfam domains were manually refined before being used in the RDGB protocol by (I) removing the domains that did not bind the metal(s) physiologically, and (II) adding domains that were known to bind the metal(s) physiologically, although no 3D structure was available. The latter refinement is based on the annotation present in the Pfam database, which is sufficiently detailed to allow users to evaluate the actual relevance of a domain to the biochemistry under investigation (in this case, the metal-binding properties). In further detail, for each domain in the list, the annotation is examined to confirm that the domain physiologically binds that metal. If this is not the case (e.g., due to adventitious binding during crystallization or purification procedures), then the domain is rejected. When the Pfam annotation alone is not sufficient to establish whether a metal is physiologically bound, the relevant literature is analyzed. Additionally, the Pfam is queried for those domains whose annotation contains the name and/or the symbol of the metal. This typically provides additional domains that lack structural characterization but have been identified as binding a specific metal. Again, the relevant literature is also investigated.

As reported in the original RDGB paper (Andreini et al., 2011), the RDGB performance parameters are evaluated as follows: sensitivity [TP/(TP + FN)] = 97.7%; specificity [TN/(TN + FP)] = 78.8%; precision [TP/(TP + FP)] = 85.9%; and accuracy [(TP + TN)/(TP + TN + FP + FN)] = 89.6%.

FUNCTIONAL CLASSIFICATION, LOCALIZATION AND COMPARISONS OF Cu-, Fe-, AND Zn-BINDING PROTEINS FROM *PARACOCCIDIOIDES* SPP.

The predicted metalloproteins of *P. lutzii Pb*01 and *P. brasiliensis Pb*18 and *Pb*03 were functionally classified with the FunCat2 scheme accessed on the Pedant database (http://pedant.gsf.de/) (Walter et al., 2009). The WolfPsort system was used to predict the putative cell localization of the proteins. The homology comparison among the metalloproteomes of the three species was performed using the BLAST tool. The metalloproteins of one phylogenetic species were compared to the corresponding metalloproteome of the two other species to find the orthologs. The structural analysis was performed to ascertain that the bound metal was completed with the I-Tasser algorithm (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). BLAST and I-Tasser were used in the analysis of the species-exclusive metalloproteins. When the BLAST comparison of the metalloprotein databank results indicated a specific protein, the sequence was compared against the whole proteome and the structure of the protein was predicted. To study the potential functional associations among the copper proteins having representatives in all *Paracoccidioides* spp., a COG identification number was associated with these proteins. The list of COG identification numbers was then used to query the STRING database (Von Mering et al., 2003).

CULTURE CONDITIONS AND EXPRESSION ANALYSIS BY qRT-PCR

Yeast cells of *P. lutzii* were maintained in a solid Fava Neto's medium at 37°C. The yeasts were grown in a liquid medium

for 72 h at 37°C. The cells were harvested by centrifugation and washed twice with a phosphate saline buffer and then transferred to the chemically defined medium MMcM (Restrepo and Jimenez, 1980). The iron limiting condition was generated without the addition of iron sources and the supplementation of the iron chelator BPS (bathophenanthroline-disulfonic acid) at 50 μM. The iron excess condition was induced by adding ammonium ferrous sulfate at 50 and 100 μM. Control cells were incubated in a MMcM medium with 3.5 μM of iron. The RNA extraction and quantitative RT-PCR was performed as previously described (Silva-Bailao et al., 2014). The oligonucleotides used in the PCR amplification were: Ca-transporter FW 5′- GTCGAACGAGGCAATGAGAGAG-3′; Ca-transporter RV 5′- TTGTAAAATCTTGCGCTTCGGG-3′; α-tubulin FW 5′-ACAGTGCTTGGGAACTATACC-3′; and α-tubulin RV 5′-GGGACATATTTGCCACTGCC-3′.

RESULTS

IDENTIFICATION OF COPPER-, IRON-, AND ZINC-BINDING PROTEINS IN *PARACOCCIDIOIDES* SPP. GENOMES

The genomes of three phylogenetic species of the *Paracoccidioides* spp. complex were analyzed for Cu, Fe, and Zn proteins using two complementary approaches: all of the metal binding patterns (MBP) were retrieved from the PDB and used along with sequence analysis to scan the proteins; additionally, a library of metal-binding protein domains based on multiple sequence alignments of known metalloproteins was used (taken from Pfam) to browse the predicted proteome (Andreini et al., 2006a). In total, 25,753 proteins from the three completely predicted proteomes were subjected to analysis and 1952 metalloproteins were detected. The results also show that those proteins represent, on average, 7.6% of the predicted proteome of these fungi (**Table 1**).

The most abundant metalloproteins were zinc-binding followed by iron-binding and copper-binding (Supplementary Tables 1–3). A higher frequency of Zn-binding proteins was expected because zinc is one of the most abundant metal ions in living organisms, playing two major roles: catalytic and structural. Some of the identified metalloproteins presented ambiguity between their annotation and the identified metal domain (e.g., proteins PAAG_06410, PADG_01717, PAAG_03944, and PAAG_02157). These molecules were subjected to structural analysis and 70% were found to have the same metal (Supplementary Table 4), whereas 30% presented other putative metals, such as Mn and Ca. Some proteins presented two possible ligand metals. The most frequent combinations were Fe/Zn followed by Cu/Zn and Cu/Fe (**Table 2**). To investigate the results of the metalloproteins with two bound metals, the protein structure was predicted using the I-Tasser software. This approach revealed that 11.9% (Pb01), 17.5% (Pb03) and 13.5% (Pb18) of the proteins present motifs for two metals and most of them corroborate with the MBP-based prediction. The remaining metalloproteins presented only one ligand metal, and 62.1 to 81.8% of the structures were in consonance with the results based on the MBP and Pfam domains (Supplementary Table 4). The list of the metalloproteins identified in this work is available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

COPPER PROTEINS

The bioinformatics analysis showed that 26 Cu-binding proteins were found in the *P. lutzii* and Pb03 genomes, whereas 25 were found in the Pb18 genome. Functional classification of the Cu proteins revealed that ion transport and melanin synthesis are the most enriched functional categories because 6 copper transporters and 3 laccases were found (**Figure 1**). Not surprisingly, the high-affinity copper transporter whose expression is induced in infection-like conditions (Bailao et al., 2006; Costa et al., 2007) belongs to the predicted copper proteome. Although only two proteins related to stress response were found, it is important to highlight that the two superoxide dismutases are copper dependent. Additionally, three amino oxidases contain a Cu domain, suggesting the relevance of this metal for their catalysis. *In silico* analysis also showed that 5 hypothetical proteins (Supplementary Table 1) present a Cu-binding domain. The copper proteins present in the three PS were analyzed in search of potential functional associations. To accomplish this, a COG identification number was associated with each ortholog group. According to the STRING database, 11 of 19 copper proteins are interconnected by high functional associations (score = 0.99) as shown in **Figure 2**. The interaction network represents a combination of different pieces of evidence that there are relationships of a functional nature among the proteins. In fact, the linkages between the pairs reveals that Cu-chaperone and Cu-transporting ATPase are core proteins that are functionally essential to metal homeostasis because the former binds to and distributes copper in the cell and the latter transports and keeps copper level in the Golgi apparatus for copper proteins such as laccases. Additionally, cu-superoxide dismutase and laccase interact with many cu-binding molecules, suggesting their participation in Cu homeostasis (**Figure 2**).

The cell localization prediction analysis corroborates the functional analysis and annotation of Cu-proteins (Supplementary Tables 1–3). The results indicate a high frequency of such proteins at the membranes as a result of the high numbers of copper transporters. The number of secreted proteins was also high. Among the molecules predicted to reach the extracellular environment are laccases, SOD, amine oxidase, polyphenoloxidase, and

Table 1 | Number of metalloproteins identified in the *Paracoccidioides* spp. genome.

Paracoccidioides sp.	Predicted proteome	Cu proteins (%)	Fe proteins (%)	Zn proteins (%)
*P. lutzii (Pb*01)	9136	26 (0.28)	115 (1.25)	522 (5.71)
*Pb*18	8741	25 (0.28)	115 (1.31)	511 (5.84)
*Pb*03	7876	26 (0.33)	111 (1.40)	501 (6.38)

Table 2 | Number of proteins with two metal-binding domains.

Paracoccidioides spp.	Cu × Fe	Cu × Zn	Fe × Zn
P. lutzii	2	6	34
Pb18	2	5	30
Pb03	2	7	31

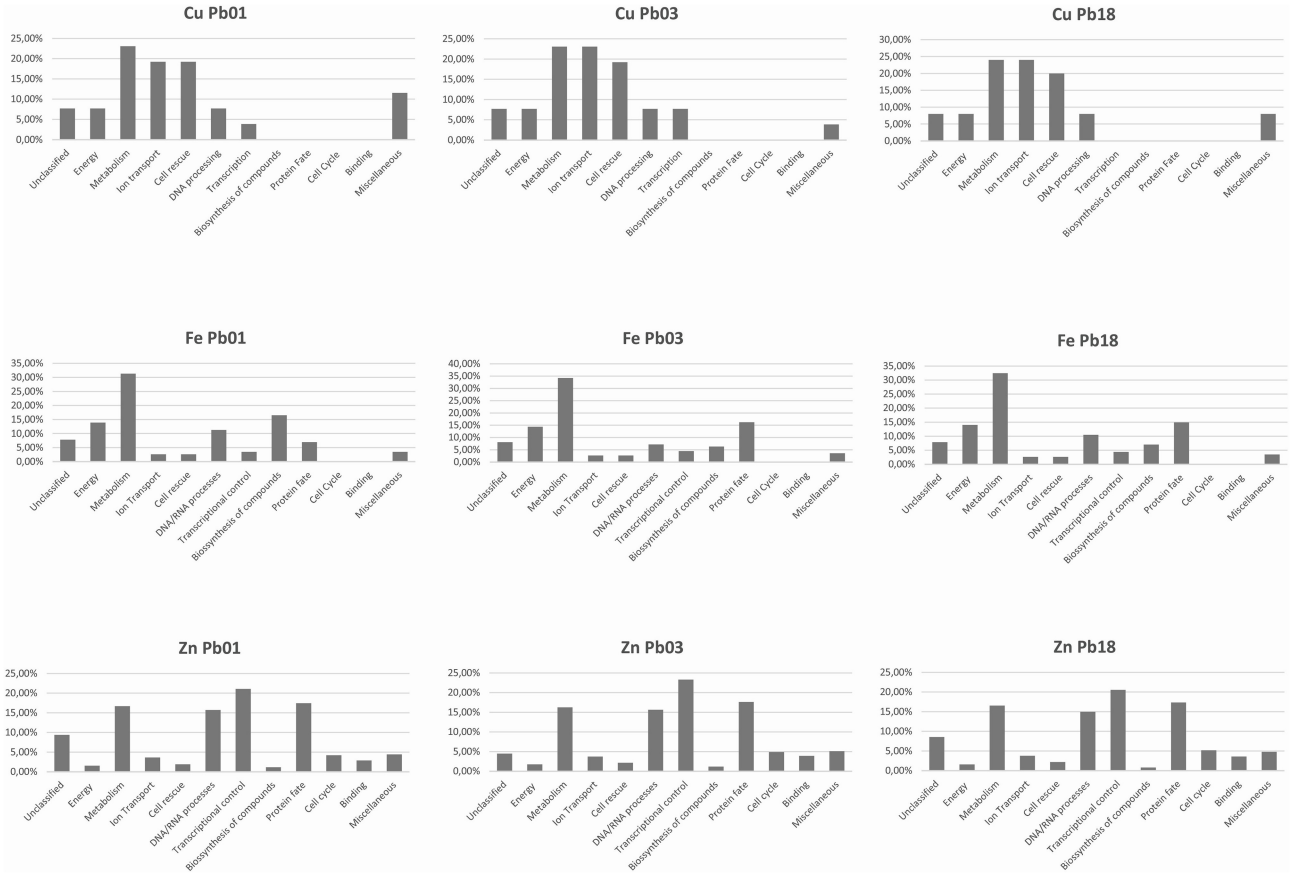


FIGURE 1 | Functional classification of Cu-, Fe-, and Zn- proteins encoded by *Paracoccidioides* spp. genomes. The metalloproteins were classified following the FunCat2 scheme available at the Pedant database.

tyrosinase. As expected, the majority of the Cu-containing proteins were localized in cytoplasm for general metabolism-related enzymes. Because of its function in the electron-transfer chain, some copper proteins were localized at the mitochondrion.

IRON PROTEINS

The three genomes encode for 115, 111, and 115 proteins in Pb01, Pb03, and P18, respectively. The general cell metabolism functions were the most enriched category; the primary reason is explained by the presence of many dehydrogenases that catalyze oxide-reduction reactions that use iron to promote electron-transfer processes. Of special note, the metabolism of amino acids and vitamins were highly represented inside this class. Additionally, many protein phosphatases were associated to iron, as expected. Iron proteins related to TCA and electron transfer chains that use iron-sulfur clusters that are essential in aerobic energy production were also found (**Figure 1**). Accordingly, iron-sulfur cluster assembling proteins compose the iron-proteome of the genus *Paracoccidioides*. Intriguingly, no iron transporter was found using *in silico* analysis. Actually, a calcium transporter (PAAG_07762; PABG_00362; PADG_02775) was found and, consequently, the 3D modeling was built to solve this ambiguity. The analysis showed that this transporter presents iron as a ligand (**Figure 3**) suggesting that this transporter could act in iron homeostasis in this fungus because no specific iron transporters were found in the genomes available (Silva et al., 2011). To confirm that, expression levels of this transporter were analyzed in conditions with low iron availability and with iron excess. The transcript levels encoding the transporter were decreased in iron deprivation and increased in iron excess suggesting this protein is probably related to iron detoxification more than iron uptake (**Figure 4**). A urease containing iron as a ligand was also found. However, most of the urease structures available in the PDB are Ni-dependent proteins and only two ureases from bacteria are iron dependent (Carter et al., 2011). *Paracoccidioides* spp. ureases present a higher identity with iron-binding enzymes (57%; PDB ID 3QGA) than with Ni-dependent enzymes (41%; PDB ID 1E9Y; data not shown). Among the iron-binding proteins, three superoxide dismutases were detected.

Most of the iron proteins were predicted to localize in the mitochondrion and cytoplasm. This observation corroborates with the cell processes performed by such molecules. The energy production and electron transfer reactions classically occur in the mitochondrion of eukaryotes. Additionally, proteins related to general metabolic events (amino acid metabolism, carbohydrate metabolism) are predicted to localize in cytosol. Moreover, the

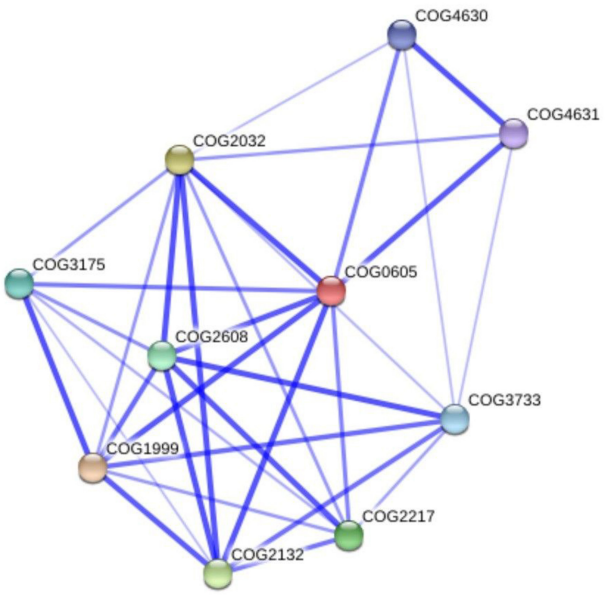


FIGURE 2 | Functional interaction graph of copper proteins from *P. lutzii* predicted proteome. The graph was constructed in the STRING tools using standard parameters. Cu-proteins found in the present study: COG3733 copper amine oxidase; COG2608 copper-transporting P-type ATPase; COG2217 copper-transporting ATPase; COG1999 mitochondrial metallochaperone Sco1; COG2132 laccase IV; COG2032 superoxide dismutase; COG0605 cytosolic Cu/Zn superoxide dismutase; COG4631 xanthine dehydrogenase; COG4630 xanthine dehydrogenase; COG3175 cytochrome c oxidase assembly protein ctaG.

plasma membrane and nucleus are cell organelles that contain iron proteins.

ZINC PROTEINS

Prediction of the *Paracoccidioides* spp. Zn proteomes revealed that zinc proteins are the most abundant metalloproteins encoded by their genome in comparison to Fe and Cu proteins. More than 500 zinc-binding proteins were found to be encoded for each genome (Supplementary Table 1). The most enriched functional categories in the Zn proteome were those related to transcription, cell cycle, and DNA processing, as well as protein fate and modification. Many zinc finger domain containing transcription factors were identified, which contributed to the functional enrichment. Regarding protein fate and modification, many peptidases and proteases were found to be zinc dependent. Additionally, proteins related to phosphorylation and ubiquitination were abundant in this category (**Figure 1**). Unlike the Cu- and Fe-proteomic data, several metal transporters compose the predicted zinc proteome of the complex *Paracoccidioides* spp. Accordingly, zinc-, metal specific- (other than zinc), heavy metal-, and cation-transporters were found to be zinc binding molecules. A considerable portion of the Zn proteome did not present describe function, reinforcing the fact that the role of metals in several cellular events is beyond the current knowledge. Many Zn proteins are related to nucleic acid related processes and metabolism. Consequently, the nucleus localization is the most frequent protein target in the zinc proteome. Additionally, cytoplasm and mitochondrion localization

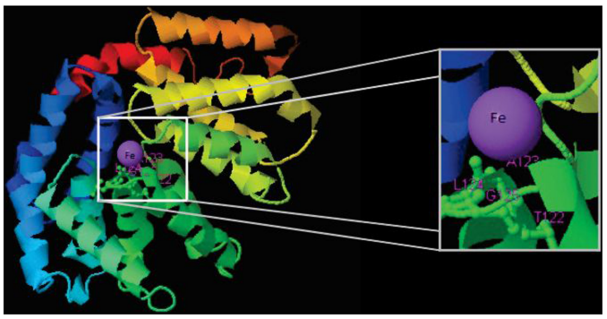


FIGURE 3 | Structural model of a calcium transporter showing that Fe is the putative ligand. Structural model was obtained by using the I-Tasser software.

was also frequent. Corroborating with the high number of metal-transporters, the predicted localization of proteins at the plasma membrane is expressive.

COMPARISON OF PREDICTED METALLOPROTEOMES OF THREE ISOLATES FROM THE *PARACOCCIDIOIDES* COMPLEX

The metalloprotein datasets were compared among the three phylogenetic species (PS) by using the BLAST tool. As expected, the comparison showed that most of the proteins are common among the species. Additionally, the specific proteins of each species were identified as well as the specific proteins for two species (**Figure 5**). *Paracoccidioides lutzii* presented the highest number of specific metalloproteins, which corroborates with the fact that it belongs to a new species in the genus and it has the greater genome. The metal-binding molecules specific to one or two PSs may be a result of different situations: the encoding gene is specific or the encoding gene is not specific but the metal-binding motif is. In the latter group orthologs with no metal-binding domain or orthologs that bind to a different metal were identified, as revealed by the structural prediction.

A comparison of the copper-proteins revealed that each isolate has one exclusive protein and the other five molecules are specific for two isolates according to the BLAST analysis. The exclusive proteins are not encoded by exclusive genes: the protein from Pb01 is not exclusive (orthologs with a Cu-binding domain were found) and the proteins from Pb03 and Pb18 presented orthologs with no metal binding domain (Supplementary Table 5). Regarding the iron proteome, the results revealed that most of the proteins (102 orthologs) are present in the three phylogenetic species. The Pb18 and Pb01 presented six iron-binding molecules that were not found in Pb03. However, Pb03 and Pb18 genomes encode five specific orthologs, whereas Pb01 and Pb03 have 3 iron proteins not found in Pb18. Although the Pb18 iron proteome has no specific protein, Pb01 and P03 present three specific proteins and one specific protein, respectively. None of these proteins are encoded by specific genes. The BLAST search coupled to structural prediction analysis revealed three different situations: a specific iron protein (there are orthologs in other phylogenetic species but a metal-binding protein was not found in one or two species); the iron proteins are not specific (a structural I-Tasser analysis developed iron-dependent models in other

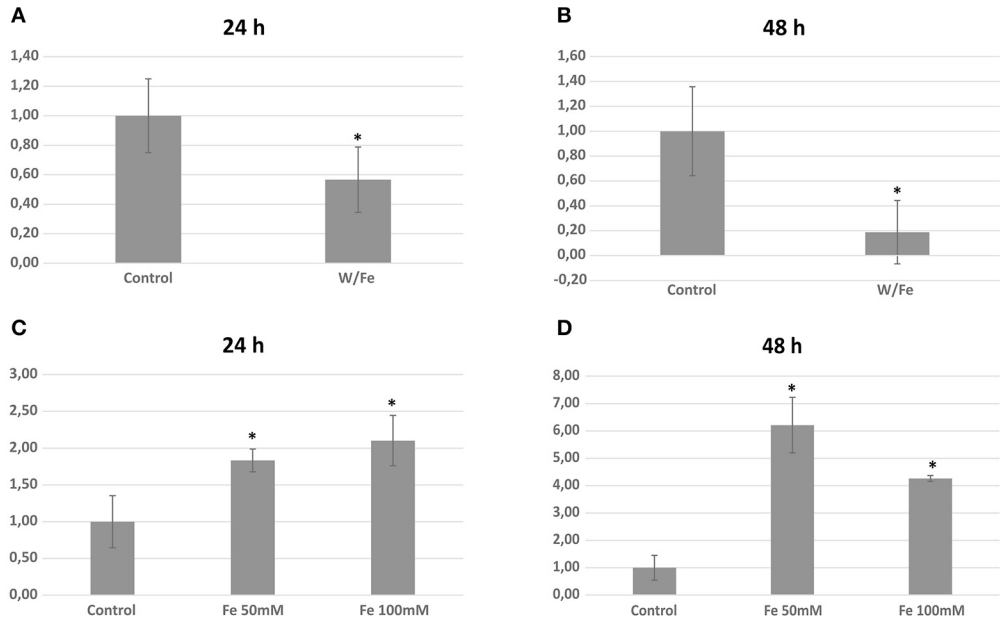


FIGURE 4 | Expression analysis of the calcium (iron) transporter (PAAG_07762) during iron limitation and iron excess. Relative transcript level of the transporter in iron limiting condition during 24 h **(A)** and 48 h **(B)**. Relative transcript level of the transporter in iron excess during 24 h **(C)** and 48 h **(D)** *statistically significant differences with $p < 0.05$.

species); or the protein presents many orthologs in the other species that an arbitrary selection could not drive a reliable conclusion. The comparison of the zinc-binding proteomes of three genomes shows that a high portion is conserved among them (Supplementary Table 5). Regarding Pb01 and Pb03, most of the specific Zn proteins are not encoded by specific genes and, therefore, there are peptides with Zn-binding domains specific to a single phylogenetic species. Proteins encoded by species-specific genes were also found, as well as, proteins that are not specific.

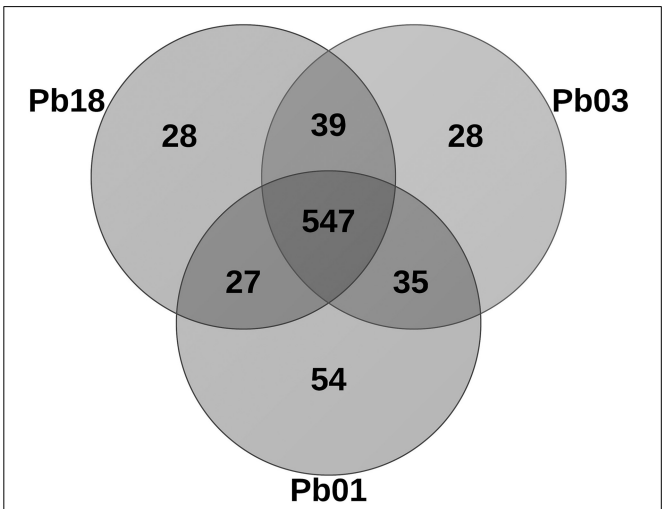


FIGURE 5 | Venn diagram showing the homology comparisons of metalloproteins found in the three species of *Paracoccidioides* spp. The numbers represent the how many proteins are specific or common among the species. The comparison was performed using the Blast tool.

METAL DEPENDENT VIRULENCE FACTORS

Metal essentiality and toxicity is used by a host to defend against invaders, thus metal uptake and homeostasis by pathogens in mammal host tissues are essential for virulence (Hood and Skaar, 2012). Some metalloproteins are essential for the intracellular regulation of metals, as well as for the control of their import and export (Sun et al., 2011). Pathogens must acquire and control metal utilization during infection to survive in their hosts (Silva et al., 2011). Several metalloproteins identified in this work play roles in fungal pathogenesis (**Table 3**). Classical virulence factors compose the predicted metalloproteomes in *Paracoccidioides* spp. For example, five superoxide dismutases were found: two are Cu and Zn-dependent and three are Fe-dependent. Thus, these metals become essential elements to an effective response against ROS production by the host. Laccase enzymes are related to melanin production and iron uptake, which contributes to the virulence of pathogenic fungi present in copper-binding domains. Urease is another enzyme that contributes primarily to fungal pathogenesis and was identified as an iron protein. This protein promotes changes in pH and toxic effects to increase the fungal pathogenicity (Mirbod-Donovan et al., 2006). Alcohol dehydrogenases (ADH) are related to the *Paracoccidioides* spp. response to host mimicking conditions. Five ADH genes were found to be zinc-binding molecules and one as iron-binding. Additionally, other Zn-binding enzymes related to central carbon metabolism such as fructose 1,6-biphosphate aldolase, triose phosphate isomerase and enolase have moonlight functions related to the adhesion to host cells and tissue dissemination. Protease production

Table 3 | Metalloproteins described as virulence factors in fungi.

Pb01 access number	Pb03 access number	Pb18 access number	Protein description	Virulence factor's reference	Metal bound
PAAG_00610	PABG_05322	PADG_06931	GATA transcription factor	Hwang et al., 2012	Zn
PAAG_02358	PABG_04857	PADG_05497	GATA factor SREP	Hwang et al., 2012	Zn
PAAG_04164	PABG_03954	PADG_07418	superoxide dismutase	Cox et al., 2003	Zn
PAAG_02971	PABG_00431	PADG_02842	cytosolic Cu/Zn superoxide dismutase	Cox et al., 2003	Cu/Zn
PAAG_06363	PABG_02770	PADG_01263	superoxide dismutase	Cox et al., 2003	Fe
PAAG_02725	PABG_03204	PADG_01755	superoxide dismutase	Cox et al., 2003	Fe
PAAG_02926	PABG_03387	PADG_01954	superoxide dismutase	Cox et al., 2003	Fe
PAAG_03681	PABG_00738	PADG_03184	laccase-1	Zhu et al., 2001	Cu
PAAG_06004	PABG_05667	PADG_05994	laccase-IV	Zhu et al., 2001	Cu
PAAG_00163	PABG_05183	PADG_07092	laccase-3	Zhu et al., 2001	Cu
PAAG_00954	PABG_01291	PADG_03871	urease	Mirbod-Donovan et al., 2006	Fe
PAAG_00243	None	None	alcohol dehydrogenase IV	Pancholi and Chhatwal, 2003	Fe
PAAG_08903	PABG_05423	PADG_05734	alcohol dehydrogenase	Pancholi and Chhatwal, 2003	Zn
PAAG_06916	PABG_02939	PADG_01454	alcohol dehydrogenase	Pancholi and Chhatwal, 2003	Zn
PAAG_06596	PABG_02619	None	alcohol dehydrogenase	Pancholi and Chhatwal, 2003	Zn
PAAG_06715	PABG_02727	PADG_01174	alcohol dehydrogenase	Pancholi and Chhatwal, 2003	Zn
PAAG_05227	PABG_07631	PADG_05031	alcohol dehydrogenase	Pancholi and Chhatwal, 2003	Zn
PAAG_04541	PABG_04316	PADG_04701	alcohol dehydrogenase GroES domain-containing protein	Pancholi and Chhatwal, 2003	Zn
PAAG_06104	PABG_06552	PADG_08012	fructose-biphosphate aldolase	Pancholi and Chhatwal, 2003	Zn
PAAG_01995	PABG_02260	PADG_00668	fructose-biphosphate aldolase	Pancholi and Chhatwal, 2003	Zn
PAAG_02152	PABG_02388	PADG_00743	Class II aldolase family protein	Pancholi and Chhatwal, 2003	Zn
PAAG_00557	PABG_03558	PADG_02132	mannose-6-phosphate isomerase	Pancholi and Chhatwal, 2003	Zn
PAAG_00771	PABG_01457	PADG_04059	enolase	Pancholi and Chhatwal, 2003	Zn
PAAG_07076	PABG_03073	PADG_01601	M6 family metalloprotease	Puccia et al., 1999	Zn
PAAG_05251	None	None	High affinity copper transporter	Bailao et al., 2006; Waterman et al., 2012	Cu
PAAG_07053	PABG_03057	PADG_01582	Copper-transporting ATPase	Walton et al., 2005	Cu
PAAG_07154	PABG_02495	PADG_00917	Copper-transporting ATPase	Walton et al., 2005	Cu
PAAG_07762	PABG_00362	PADG_02775	Calcium transporter (iron transporter)	Ramanan and Wang, 2000; Jung et al., 2008; Silva et al., 2011	Fe

is a strategy used by several pathogens in host colonization and many proteases are metal-dependent proteins as found in our analysis. Also we found that many peptidases and proteases were related to protein processing rather than virulence. As metals are essential to the molecules of live organisms, the components that control their uptake and utilization have been described as factors that contribute to virulence. Accordingly, several cation/metal transporters were identified in the zinc metalloproteome of the genus *Paracoccidioides*. Among several zinc-dependent transcription factors the GATA-type Cir1/SREA (PAAG_00610, PABG_05322, PADG_06931/PAAG_02358, and PABG_04857) related to iron homeostasis and virulence in fungi was found. Previous analysis suggested that *Paracoccidioides* spp. are devoid of a classical high affinity iron transport Ftr (Silva et al., 2011). In the present work, a putatively iron-transporter that contributes to the virulence of *C. albicans* and *C. neoformans* was found (Ramanan and Wang, 2000; Jung et al., 2008). Additionally, copper homeostasis plays an essential role in fungal pathogenesis. High affinity copper transporters (CTR) are key elements in copper uptake in Cu-limiting conditions (Waterman et al., 2007, 2012). The CTR3 of *Paracoccidioides* spp. was identified in our analysis and its transcripts are induced during infectious mimicking conditions (Bailao et al., 2006; Costa et al., 2007). Other copper transporters (copper-transporting ATPases) are localized at the Golgi complex and a copper-chaperone participates in melanin synthesis in *C. neoformans*, suggesting their role in virulence (Walton et al., 2005).

DISCUSSION

The essentiality of metals in biology, such as Cu, Fe, and Zn, is most related to metal-dependent proteins that play fundamental roles in cells. However, the intracellular levels of these elements have to be tightly controlled because their excess is toxic (Yannone et al., 2012). Regarding pathogenic microorganisms, the uptake, storage, use and distribution of metals are key factors for virulence. To establish infection, fungal pathogens must acquire and use metals in host tissues to cope with the metal scarcity induced by the immune system (Vignesh et al., 2013a,b).

In this perspective the pathogen also has to adapt to a low level metal environment based on its metalloproteome, changing its metabolism, as much as possible, toward metal independent pathways (Parente et al., 2011).

Not unexpectedly, copper protein functions are most related to ion transport, virulence and general metabolism. The latter include metabolic oxidases such as amine oxidases, glyoxal oxidase, laccases and other oxidases that use the copper redox ability in oxidoreduction/electron-transfer reactions. The dependence of copper by amine oxidases and nitrite reductases suggests that nitrogen metabolism of these fungi is supported by Cu (Laliberte and Labbe, 2006). The *Paracoccidioides* spp. Cu proteomes present the primary molecular components of copper uptake and distribution. Among those found, the copper-transporting ATPases are important in metal detoxification and trafficking, as well as, in delivering metal to copper proteins. The laccases are ferroxidases, essential in the reductive iron uptake system and in melanin synthesis, were also found. Other factors important in copper homeostasis are the high affinity Cu-transporting and copper chaperones (Festa and Thiele, 2011, 2012). Additionally, the functional interaction network for Cu-protein suggests a clear linkage among those that orchestrate several Cu-dependent cellular mechanisms, including activities that sustain pathogenesis. It is important to highlight the strong functional association among copper-chaperones, copper-transporting ATPases, and laccases that shows the central roles of those proteins in cellular copper equilibrium. The functional interactions among these proteins are highly confident, suggesting that Cu acquisition and distribution in the fungal cells are tightly controlled processes preventing increases in free-copper levels.

The analysis of the functional repertoire performed by the putative Fe proteins revealed that the primary roles played by the Fe proteins are the catalysis of redox and electron transfer reactions. At a relatively general level, metabolism is the most representative functional category; it is composed of proteins that belong to amino acid metabolism, biosynthesis of vitamins, nucleotide metabolism, carbohydrate metabolism and others. The amplitude of these metabolic processes is evidence as to how important iron is for basal fungal metabolism. Corroborating that importance, a proteomic analysis in iron starvation condition suggests *Paracoccidioides* spp. decrease iron dependent enzymes and consequently increase proteins of iron independent pathways (Parente et al., 2011).

Previous work, based on genome-mining, shows that *Paracoccidioides* spp. genomes encode proteins related to the reductive iron uptake system (Silva et al., 2011). However, no iron transporter was found in this study, and it was assumed that the iron uptake would be performed by a non-specific metal transporter. In the present work, among the iron proteins is a predicted iron-transporter formerly annotated as a calcium transporter. Structural modeling of this transporter suggested that iron is the metal ligand. The quantitative PCR results show that the expression of this transporter is regulated by iron and strongly suggests that it plays a role in metal detoxification rather than in uptake. Moreover, this ambiguity is common in metalloproteome studies because protein metal affinity is a multifactorial event and wrong annotations or structures are frequently detected (Maret, 2010). Future functional studies should be conducted to better elucidate the specific role of this molecule in iron homeostasis. It is well established that defects in iron-sulfur cluster biogenesis or transport induce the expression of iron uptake genes (Chen et al., 2004) and that Fe-S containing proteins participate in sensing iron availability (Muhlenhoff et al., 2010). The presence of many iron-sulfur cluster assembly proteins in the predicted iron proteome described here provides new molecules that may perform roles in Fe homeostasis.

The Zn-proteome of *Paracoccidioides* spp. represents the majority of the metalloproteins described in the present work. 511 proteins were identified with at least one metal-binding motif, on average. The number of putative zinc proteins found in each species directly correlates with their predicted proteome sizes. As expected, proteins related to gene expression were the most abundant (20% on average) because transcription factors use zinc for structural reasons to bind to DNA. Thus, this enrichment is responsible for the high frequency of Zn proteins found in eukaryotes (Andreini et al., 2006b). Among them is the GATA-type transcription factor SREP (accession numbers PAAG_02358, PABG_04857, and PADG_05497), which inhibits the expression of iron-uptake related genes in iron-sufficient conditions, and also regulates some iron-independent genes. Additionally, SREP mutants presented abrogated virulence in pathogenic fungi (Gauthier et al., 2010; Hwang et al., 2012). The most populated functional categories include protein folding, processing and degradation. Additionally, some protein importers, kinases/phosphatases and many proteases and peptidases are zinc dependent. Regarding pathogens, the protease activity is intimately connected with virulence (Parente et al., 2005, 2010; Tacco et al., 2009). Thus, zinc homeostasis during infection supports protease based virulence, as well as protein transport and processing in the genus *Paracoccidioides.*

The Zn proteome of *Paracoccidioides* spp. presents at least 17 transporters related to Zn translocation. The *Paracoccidioides* spp. Zinc proteome contains many metal transporters including Zrt1 and Zrt2, which are localized at the plasma membrane and are related to metal uptake during Zn-limiting and Zn-replete conditions, respectively. The expression of both genes is induced at the fungal cells (Parente et al., 2013). Vacuolar transporters that regulate the cytoplasmic levels of the metal were also identified (Amich et al., 2010). A metallochaperone previously described as a copper-binding protein was identified in this study as a Zn-binding molecule. This is not surprising as the same has been observed in bacterial chaperones (Dainty et al., 2010). Considering *Paracoccidioides* spp. have no predicted metallothionein (Silva et al., 2011), this molecule could somehow play a role in metal level maintenance.

Recently, a metallomics-based study unveiled that the host immune response against a fungal pathogen promotes zinc limitation in fungal-infected cells. GM-CSF treated macrophages promote zinc uptake to generate ROS as a fungicidal mechanism, and at the same time decrease Zn availability by metallothionein production and zinc Golgi sequestration (Vignesh et al., 2013a). Thus, metalloproteins that allow *Paracoccidioides* spp. to uptake and distribute zinc during infection is an essential event for survival in host niches. *Paracoccidioides* spp. induce

the production of SODs during oxidative stress, as demonstrated by the proteomic approach (De Arruda Grossklaus et al., 2013). These enzymes are metal dependent and are classified in accordance to their metal as Cu/ZnSOD, MnSOD, FeSOD, and NiSOD (Broering et al., 2013). *Paracoccidioides* spp. encode for five different SODs: two are copper- and zinc-binding proteins and the other three are Fe-SODs. As these metalloenzymes are important components in the arsenal of fungal virulence factors, an efficient combat against host microbicide ROS rely on a virtuous copper, iron and zinc uptake and homeostasis during infection. Melanization is a virulence factor of many pathogenic fungi (Nosanchuk and Casadevall, 2006; Taborda et al., 2008). *Paracoccidioides* spp present one tyrosinase and three putative laccases that are key copper-oxidase enzymes in melanin synthesis. Furthermore, metallochaperones and copper transporters play a critical role on melanin production as both proteins participate in Cu-loading in laccases (Festa and Thiele, 2012). The urease production contributes to the virulence of microorganisms (Rutherford, 2014). Although nickel is the most common metal found in urease structures, an iron-binding urease was found in the present analysis. Moreover, as no fungal urease structure is available, these data can be used in future structural studies. Additionally, the enolase, a Zn-binding protein of these pathogens, interacts with host plasminogen favoring the invasion and dissemination steps during host tissue infection (Nogueira et al., 2010).

CONCLUSION

A previous study described the association of zinc metabolism and fungal virulence based on the identification of zinc-binding proteins and a literature review. The authors used an annotation based approach only, which is not a first line technique for identifying metalloproteins in databases (Andreini et al., 2006b). The present work was used a systematic bioinformatic approach based on domains and conserved structures available in the PDB to predict the Cu, Fe, and Zn proteomes of a pathogenic fungus. The results show that these metals are cofactors of enzymes related to central metabolism and thus are essential for the biology of *Paracoccidioides* spp. It was also noted that many metalloproteins have no characterized function, indicating that the roles of many metals in biological systems are still unknown. Additionally, some metalloproteins belong to the arsenal of virulence factors taken by *Paracoccidioides* spp. to be able to infect hosts. Although bioinformatics based studies are important to identify metalloproteins, experimental techniques will be used to characterize the metalloproteins of these pathogenic fungi.

AUTHOR CONTRIBUTIONS

Gabriel B. Tristão, Mirelle Garcia Silva-Bailão, Clayton Luiz Borges, Gabriele Cavallaro and Leandro do Prado Assunção performed the analysis, data analysis and writing. The following contributed to financial support: Alexandre M. Bailão, Gabriele Cavallaro, Célia M. de Almeida Soares and Clayton Luiz Borges. Leandro do Prado Assunção and Luiz Paulo Araújo dos Santos analyzed the metalloproteome data. Alexandre M. Bailão and Gabriele Cavallaro conceived the ideas, performed the experimental design and wrote the paper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00761/abstract

REFERENCES

Amich, J., Vicentefranqueira, R., Leal, F., and Calera, J. A. (2010). *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* 9, 424–437. doi: 10.1128/EC.00348-09

Ammendola, S., Pasquali, P., Pistoia, C., Petrucci, P., Petrarca, P., Rotilio, G., et al. (2007). High-affinity Zn2+ uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of *Salmonella enterica*. *Infect. Immun.* 75, 5867–5876. doi: 10.1128/IAI.00559-07

Andreini, C., Banci, L., Bertini, I., Elmi, S., and Rosato, A. (2007). Non-heme iron through the three domains of life. *Proteins* 67, 317–324. doi: 10.1002/prot.21324

Andreini, C., Banci, L., Bertini, I., and Rosato, A. (2006a). Counting the zinc-proteins encoded in the human genome. *J. Proteome Res.* 5, 196–201. doi: 10.1021/pr050361j

Andreini, C., Banci, L., Bertini, I., and Rosato, A. (2006b). Zinc through the three domains of life. *J. Proteome Res.* 5, 3173–3178. doi: 10.1021/pr0603699

Andreini, C., Banci, L., Bertini, I., and Rosato, A. (2008). Occurrence of copper proteins through the three domains of life: a bioinformatic approach. *J. Proteome Res.* 7, 209–216. doi: 10.1021/pr070480u

Andreini, C., Bertini, I., Cavallaro, G., Decaria, L., and Rosato, A. (2011). A simple protocol for the comparative analysis of the structure and occurrence of biochemical pathways across superkingdoms. *J. Chem. Inf. Model.* 51, 730–738. doi: 10.1021/ci100392q

Andreini, C., Bertini, I., and Rosato, A. (2004). A hint to search for metalloproteins in gene banks. *Bioinformatics* 20, 1373–1380. doi: 10.1093/bioinformatics/bth095

Bailao, A. M., Schrank, A., Borges, C. L., Dutra, V., Molinari-Madlum, E. E. W. I., Felipe, M. S. S., et al. (2006). Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: representational difference analysis identifies candidate genes associated with fungal pathogenesis. *Microbes Infect.* 8, 2686–2697. doi: 10.1016/j.micinf.2006.07.019

Bertini, I., Gray, R. B., Stiefel, E. I., and Valentini, J. S. (2007). *Biological Inorganic Chemistry: Structure and Reactivity.* Sausalito, CA: University Science Book.

Bleackley, M. R., and Macgillivray, R. T. (2011). Transition metal homeostasis: from yeast to human disease. *Biometals* 24, 785–809. doi: 10.1007/s10534-011-9451-4

Bocca, A. L., Amaral, A. C., Teixeira, M. M., Sato, P. K., Shikanai-Yasuda, M. A., and Soares Felipe, M. S. (2013). Paracoccidioidomycosis: eco-epidemiology, taxonomy and clinical and therapeutic issues. *Fut. Microbiol.* 8, 1177–1191. doi: 10.2217/fmb.13.68

Broering, E. P., Truong, P. T., Gale, E. M., and Harrop, T. C. (2013). Synthetic analogues of nickel superoxide dismutase: a new role for nickel in biology. *Biochemistry* 52, 4–18. doi: 10.1021/bi3014533

Carrero, L. L., Nino-Vega, G., Teixeira, M. M., Carvalho, M. J., Soares, C. M., Pereira, M., et al. (2008). New *Paracoccidioides brasiliensis* isolate reveals unexpected genomic variability in this human pathogen. *Fungal Genet. Biol.* 45, 605–612. doi: 10.1016/j.fgb.2008.02.002

Carter, E. L., Tronrud, D. E., Taber, S. R., Karplus, P. A., and Hausinger, R. P. (2011). Iron-containing urease in a pathogenic bacterium. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13095–13099. doi: 10.1073/pnas.1106915108

Cassat, J. E., and Skaar, E. P. (2013). Iron in infection and immunity. *Cell Host Microbe* 13, 509–519. doi: 10.1016/j.chom.2013.04.010

Chen, O. S., Crisp, R. J., Valachovic, M., Bard, M., Winge, D. R., and Kaplan, J. (2004). Transcription of the yeast iron regulon does not respond directly to iron but rather to iron-sulfur cluster biosynthesis. *J. Biol. Chem.* 279, 29513–29518. doi: 10.1074/jbc.M403209200

Costa, M., Borges, C. L., Bailao, A. M., Meirelles, G. V., Mendonca, Y. A., Dantas, S. F., et al. (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. doi: 10.1099/mic.0.2007/009332-0

Cox, G. M., Harrison, T. S., McDade, H. C., Taborda, C. P., Heinrich, G., Casadevall, A., et al. (2003). Superoxide dismutase influences the virulence of *Cryptococcus neoformans* by affecting growth within macrophages. *Infect. Immun.* 71, 173–180. doi: 10.1128/IAI.71.1.173-180.2003

Dainty, S. J., Patterson, C. J., Waldron, K. J., and Robinson, N. J. (2010). Interaction between cyanobacterial copper chaperone Atx1 and zinc homeostasis. *J. Biol. Inorg. Chem.* 15, 77–85. doi: 10.1007/s00775-009-0555-z

De Arruda Grossklaus, D., Bailao, A. M., Vieira Rezende, T. C., Borges, C. L., De Oliveira, M. A., Parente, J. A., et al. (2013). Response to oxidative stress in *Paracoccidioides* yeast cells as determined by proteomic analysis. *Microbes Infect.* 15, 347–364. doi: 10.1016/j.micinf.2012.12.002

Festa, R. A., and Thiele, D. J. (2011). Copper: an essential metal in biology. *Curr. Biol.* 21, R877–R883. doi: 10.1016/j.cub.2011.09.040

Festa, R. A., and Thiele, D. J. (2012). Copper at the front line of the host-pathogen battle. *PLoS Pathog.* 8:e1002887. doi: 10.1371/journal.ppat.1002887

Frausto Da Silva, J. J. R., and Williams, R. J. P. (1991). *The Biological Chemistry of the Elements.* Oxford: Clarendon Press.

Gauthier, G. M., Sullivan, T. D., Gallardo, S. S., Brandhorst, T. T., Vanden Wymelenberg, A. J., Cuomo, C. A., et al. (2010). SREB, a GATA transcription factor that directs disparate fates in *Blastomyces dermatitidis* including morphogenesis and siderophore biosynthesis. *PLoS Pathog.* 6:e1000846. doi: 10.1371/journal.ppat.1000846

Hood, M. I., and Skaar, E. P. (2012). Nutritional immunity: transition metals at the pathogen-host interface. *Nat. Rev. Microbiol.* 10, 525–537. doi: 10.1038/nrmicro2836

Hwang, L. H., Seth, E., Gilmore, S. A., and Sil, A. (2012). SRE1 regulates iron-dependent and -independent pathways in the fungal pathogen *Histoplasma capsulatum*. *Eukaryot. Cell* 11, 16–25. doi: 10.1128/EC.05274-11

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

Kim, B. E., Nevitt, T., and Thiele, D. J. (2008). Mechanisms for copper acquisition, distribution and regulation. *Nat. Chem. Biol.* 4, 176–185. doi: 10.1038/nchembio.72

Laliberte, J., and Labbe, S. (2006). Mechanisms of copper loading on the *Schizosaccharomyces pombe* copper amine oxidase 1 expressed in *Saccharomyces cerevisiae*. *Microbiology* 152, 2819–2830. doi: 10.1099/mic.0.28998-0

Maret, W. (2010). Metalloproteomics, metalloproteomes, and the annotation of metalloproteins. *Metallomics* 2, 117–125. doi: 10.1039/b915804a

Matute, D. R., McEwen, J. G., Puccia, R., Montes, B. A., San-Blas, G., Bagagli, E., et al. (2006a). Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by gene genealogies. *Mol. Biol. Evol.* 23, 65–73. doi: 10.1093/molbev/msj008

Matute, D. R., Sepulveda, V. E., Quesada, L. M., Goldman, G. H., Taylor, J. W., Restrepo, A., et al. (2006b). Microsatellite analysis of three phylogenetic species of *Paracoccidioides brasiliensis*. *J. Clin. Microbiol.* 44, 2153–2157. doi: 10.1128/JCM.02540-05

Mirbod-Donovan, F., Schaller, R., Hung, C. Y., Xue, J., Reichard, U., and Cole, G. T. (2006). Urease produced by *Coccidioides posadasii* contributes to the virulence of this respiratory pathogen. *Infect. Immun.* 74, 504–515. doi: 10.1128/IAI.74.1.504-515.2006

Muhlenhoff, U., Molik, S., Godoy, J. R., Uzarska, M. A., Richter, N., Seubert, A., et al. (2010). Cytosolic monothiol glutaredoxins function in intracellular iron sensing and trafficking via their bound iron-sulfur cluster. *Cell Metab.* 12, 373–385. doi: 10.1016/j.cmet.2010.08.001

Murakami, M., and Hirano, T. (2008). Intracellular zinc homeostasis and zinc signaling. *Cancer Sci.* 99, 1515–1522. doi: 10.1111/j.1349-7006.2008.00854.x

Netz, D. J., Stith, C. M., Stumpfig, M., Kopf, G., Vogel, D., Genau, H. M., et al. (2012). Eukaryotic DNA polymerases require an iron-sulfur cluster for the formation of active complexes. *Nat. Chem. Biol.* 8, 125–132. doi: 10.1038/nchembio.721

Nevitt, T. (2011). War-Fe-re: iron at the core of fungal virulence and host immunity. *Biometals* 24, 547–558. doi: 10.1007/s10534-011-9431-8

Nogueira, S. V., Fonseca, F. L., Rodrigues, M. L., Mundodi, V., Abi-Chacra, E. A., Winters, M. S., et al. (2010). *Paracoccidioides brasiliensis* enolase is a surface protein that binds plasminogen and mediates interaction of yeast forms with host cells. *Infect. Immun.* 78, 4040–4050. doi: 10.1128/IAI.00221-10

Nosanchuk, J. D., and Casadevall, A. (2006). Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. *Antimicrob. Agents Chemother.* 50, 3519–3528. doi: 10.1128/AAC.00545-06

Pancholi, V., and Chhatwal, G. S. (2003). Housekeeping enzymes as virulence factors for pathogens. *Int. J. Med. Microbiol.* 293, 391–401. doi: 10.1078/1438-4221-00283

Parente, A. F., Bailao, A. M., Borges, C. L., Parente, J. A., Magalhaes, A. D., Ricart, C. A., et al. (2011). Proteomic analysis reveals that iron availability alters the metabolic status of the pathogenic fungus *Paracoccidioides brasiliensis*. *PLoS ONE* 6:e22810. doi: 10.1371/journal.pone.0022810

Parente, A. F., De Rezende, T. C., De Castro, K. P., Bailao, A. M., Parente, J. A., Borges, C. L., et al. (2013). A proteomic view of the response of *Paracoccidioides* yeast cells to zinc deprivation. *Fungal Biol.* 117, 399–410. doi: 10.1016/j.funbio.2013.04.004

Parente, J. A., Costa, M., Pereira, M., and Soares, C. M. (2005). Transcriptome overview of *Paracoccidioides brasiliensis* proteases. *Genet. Mol. Res.* 4, 358–371. Available online at: http://www.funpecrp.com.br/gmr/year2005/vol2-4/Pb10_abstract.htm

Parente, J. A., Salem-Izacc, S. M., Santana, J. M., Pereira, M., Borges, C. L., Bailao, A. M., et al. (2010). A secreted serine protease of *Paracoccidioides brasiliensis* and its interactions with fungal proteins. *BMC Microbiol.* 10:292. doi: 10.1186/1471-2180-10-292

Puccia, R., Juliano, M. A., Juliano, L., Travassos, L. R., and Carmona, A. K. (1999). Detection of the basement membrane-degrading proteolytic activity of *Paracoccidioides brasiliensis* after SDS-PAGE using agarose overlays containing Abz-MKALTLQ-EDDnp. *Braz. J. Med. Biol. Res.* 32, 645–649. doi: 10.1590/S0100-879X1999000500019

Ramanan, N., and Wang, Y. (2000). A high-affinity iron permease essential for *Candida albicans* virulence. *Science* 288, 1062–1064. doi: 10.1126/science.288.5468.1062

Restrepo, A., and Jimenez, B. E. (1980). Growth of *Paracoccidioides brasiliensis* yeast phase in a chemically defined culture medium. *J. Clin. Microbiol.* 12, 279–281.

Rutherford, J. C. (2014). The emerging role of urease as a general microbial virulence factor. *PLoS Pathog.* 10:e1004062. doi: 10.1371/journal.ppat.1004062

Samanovic, M. I., Ding, C., Thiele, D. J., and Darwin, K. H. (2012). Copper in microbial pathogenesis: meddling with the metal. *Cell Host Microbe* 11, 106–115. doi: 10.1016/j.chom.2012.01.009

Schneider Rde, O., Fogaca Nde, S., Kmetzsch, L., Schrank, A., Vainstein, M. H., and Staats, C. C. (2012). Zap1 regulates zinc homeostasis and modulates virulence in *Cryptococcus gattii*. *PLoS ONE* 7:e43773. doi: 10.1371/journal.pone.0043773

Schrettl, M., and Haas, H. (2011). Iron homeostasis–Achilles' heel of *Aspergillus fumigatus*? *Curr. Opin. Microbiol.* 14, 400–405. doi: 10.1016/j.mib.2011.06.002

Shi, W., and Chance, M. R. (2008). Metallomics and metalloproteomics. *Cell. Mol. Life Sci.* 65, 3040–3048. doi: 10.1007/s00018-008-8189-9

Silva, M. G., Schrank, A., Bailao, E. F. L. C., Bailao, A. M., Borges, C. L., Staats, C. C., et al. (2011). The homeostasis of iron, copper and zinc in *Paracoccidioides brasiliensis*, *Cryptococcus neoformans* var. *grubi,* and *Cryptococcus gatii*: a comparative analysis. *Front. Microbiol.* 2:49. doi: 10.3389/fmicb.2011.00049

Silva-Bailao, M. G., Bailao, E. F., Lechner, B. E., Gauthier, G. M., Lindner, H., Bailao, A. M., et al. (2014). Hydroxamate production as a high affinity iron acquisition mechanism in *Paracoccidioides* spp. *PLoS ONE* 9:e105805. doi: 10.1371/journal.pone.0105805

Sun, X., Xiao, C. L., Ge, R., Yin, X., Li, H., Li, N., et al. (2011). Putative copper- and zinc-binding motifs in *Streptococcus pneumoniae* identified by immobilized metal affinity chromatography and mass spectrometry. *Proteomics* 11, 3288–3298. doi: 10.1002/pmic.201000396

Taborda, C. P., Da Silva, M. B., Nosanchuk, J. D., and Travassos, L. R. (2008). Melanin as a virulence factor of *Paracoccidioides brasiliensis* and other dimorphic pathogenic fungi: a minireview. *Mycopathologia* 165, 331–339. doi: 10.1007/s11046-007-9061-4

Tacco, B. A., Parente, J. A., Barbosa, M. S., Báo, S. N., Góes, T. D., Pereira, M., et al. (2009). Characterization of a secreted aspartyl protease of the fungal pathogen *Paracoccidioides brasiliensis*. *Med. Mycol.* 47, 845–854. doi: 10.3109/13693780802695512

Teixeira, M. D., Theodoro, R. C., Oliveira, F. F., Machado, G. C., Hahn, R. C., Bagagli, E., et al. (2013). *Paracoccidioides lutzii* sp. nov.: biological and clinical implications. *Med. Mycol.* 52, 19–28. doi: 10.3109/13693786.2013.794311

Teixeira, M. M., Theodoro, R. C., De Carvalho, M. J., Fernandes, L., Paes, H. C., Hahn, R. C., et al. (2009). Phylogenetic analysis reveals a high level of

speciation in the *Paracoccidioides* genus. *Mol. Phylogenet. Evol.* 52, 273–283. doi: 10.1016/j.ympev.2009.04.005

Valko, M., Morris, H., and Cronin, M. T. (2005). Metals, toxicity and oxidative stress. *Curr. Med. Chem.* 12, 1161–1208. doi: 10.2174/09298670537 64635

Vignesh, K. S., Landero Figueroa, J. A., Porollo, A., Caruso, J. A., and Deepe, G. S. Jr. (2013a). Granulocyte macrophage-colony stimulating factor induced Zn sequestration enhances macrophage superoxide and limits intracellular pathogen survival. *Immunity* 39, 697–710. doi: 10.1016/j.immuni.2013. 09.006

Vignesh, K. S., Landero Figueroa, J. A., Porollo, A., Caruso, J. A., and Deepe, G. S. Jr. (2013b). Zinc Sequestration: arming phagocyte defense against fungal attack. *PLoS Pathog.* 9:e1003815. doi: 10.1371/journal.ppat.1003815

Von Mering, C., Huynen, M., Jaeggi, D., Schmidt, S., Bork, P., and Snel, B. (2003). STRING: a database of predicted functional associations between proteins. *Nucleic Acids Res.* 31, 258–261. doi: 10.1093/nar/gkg034

Walter, M. C., Rattei, T., Arnold, R., Guldener, U., Munsterkotter, M., Nenova, K., et al. (2009). PEDANT covers all complete RefSeq genomes. *Nucleic Acids Res.* 37, D408–D411. doi: 10.1093/nar/gkn749

Walton, F. J., Idnurm, A., and Heitman, J. (2005). Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans*. *Mol. Microbiol.* 57, 1381–1396. doi: 10.1111/j.1365-2958.2005.04779.x

Waterman, S. R., Hacham, M., Hu, G., Zhu, X., Park, Y. D., Shin, S., et al. (2007). Role of a CUF1/CTR4 copper regulatory axis in the virulence of *Cryptococcus neoformans*. *J. Clin. Invest.* 117, 794–802. doi: 10.1172/JCI30006

Waterman, S. R., Park, Y. D., Raja, M., Qiu, J., Hammoud, D. A., O'halloran, T. V., et al. (2012). Role of CTR4 in the Virulence of *Cryptococcus neoformans*. *MBio* 3:e00285-12. doi: 10.1128/mBio.00285-12

Wilson, D., Citiulo, F., and Hube, B. (2012). Zinc exploitation by pathogenic fungi. *PLoS Pathog.* 8:e1003034. doi: 10.1371/journal.ppat.1003034

Yannone, S. M., Hartung, S., Menon, A. L., Adams, M. W., and Tainer, J. A. (2012). Metals in biology: defining metalloproteomes. *Curr. Opin. Biotechnol.* 23, 89–95. doi: 10.1016/j.copbio.2011.11.005

Zhu, X., Gibbons, J., Garcia-Rivera, J., Casadevall, A., and Williamson, P. R. (2001). Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. *Infect. Immun.* 69, 5589–5596. doi: 10.1128/IAI.69.9.5589-5596.2001

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: 06 October 2014; accepted: 13 December 2014; published online: 09 January 2015.

Citation: Tristão GB, Assunção LP, dos Santos LPA, Borges CL, Silva-Bailão MG, Soares CMA, Cavallaro G and Bailão AM (2015) Predicting copper-, iron-, and zinc-binding proteins in pathogenic species of the Paracoccidioides genus. Front. Microbiol. ***5****:761. doi: 10.3389/fmicb.2014.00761*

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