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

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Iron, copper, and zinc are essential for all living organisms. Moreover, the homeostasis of these metals is vital to microorganisms during pathogenic interactions with a host. Most pathogens have developed specific mechanisms for the uptake of micronutrients from their hosts in order to counteract the low availability of essential ions in infected tissues. We report here an analysis of genes potentially involved in iron, copper, and zinc uptake and homeostasis in the fungal pathogens Paracoccidioides brasiiliensis, Cryptococcus neoformans var. grubii, and Cryptococcus gattii. Although prior studies have identified certain aspects of metal regulation in Cryptococcus species, little is known regarding the regulation of these elements in P. brasiiliensis. We also present amino acid sequences analyses of deduced proteins in order to examine possible conserved domains. The genomic data reveals, for the first time, genes associated to iron, copper, and zinc assimilation and homeostasis in P. brasiiliensis. Furthermore, analyses of the three fungal species identified homologs to genes associated with high-affinity uptake systems, vacuolar and mitochondrial iron storage, copper uptake and reduction, and zinc assimilation. However, homologs to genes involved in siderophore production were only found in P. brasiiliensis. Interestingly, in silico analysis of the genomes of P. brasiiliensis Pb01, Pb03, and Pb18 revealed significant differences in the presence and/or number of genes involved in metal homeostasis, such as in genes related to iron reduction and oxidation. The broad analyses of the genomes of P. brasiiliensis, C. neoformans var. grubii, and C. gattii for genes involved in metal homeostasis provide important groundwork for numerous interesting future areas of investigation that are required in order to validate and explore the function of the identified genes and gene pathways.

**INTRODUCTION**

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

Ferric iron is generally insoluble at physiological pH in the presence of oxygen. Thus, the common mechanisms of iron-assimilation include the reduction of ferric (Fe³⁺) to ferrous (Fe²⁺),...
and solubilization of Fe$^{3+}$ by binding siderophores (Kornitzer, 2009). The reductive system in fungi is regulated by three different mechanisms. First, a low-affinity iron reductase that functions in iron-rich environments generates Fe$^{2+}$, which is transported into the cell by a non-specific low-affinity iron permease. Second, a regulated high-affinity ferric reductase operates in low iron conditions, such as those present in a mammalian host. The produced Fe$^{2+}$ is further oxidized to Fe$^{3+}$ by a membrane multi-copper-oxidase before being transported across the cell membrane by a high-affinity iron permease. The third mechanism is a non-enzymatic reduction, such as that promoted by 3-hydroxyanthranilic acid (3HAA), which is known to maintain a reduced environment to facilitate the release and sustain the presence of Fe$^{2+}$ at the fungal membrane until transport occurs (Howard, 1999).

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

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

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

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

**MATERIALS AND METHODS**

**Sequences of genes related to iron, copper and zinc uptake, as well as to siderophore biosynthesis and uptake were used in the search for orthologs of P. brasilensis and Cryptococcus species genomes. The P. brasilensis database** includes the genomes of three isolates (Pb01, Pb03, and Pb18) and the cryptococcal database includes genomes of C. neoformans var. grubii and C. gattii. The sequences used in

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1. http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html
2. http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html
3. http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans_b/MultiHome.html
the in silico analysis were obtained from the NCBI databank, and they are primarily from \textit{S. cerevisiae}, but also include genes from other fungi, such as \textit{Aspergillus fumigatus, Aspergillus nidulans, C. albicans} and \textit{H. capsulatum}. The search by orthologs was based on sequence similarity by using the BLAST tool. The expectation value adopted in the databases search was $E$-value $\leq 10^{-5}$.

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

**RESULTS AND DISCUSSION**

**IRON**

**Uptake of iron at the cell surface by the reductive system**

To better understand how \textit{P. brasiliensis} could acquire iron by the reductive system, in silico analyses were performed utilizing \textit{S. cerevisiae} and \textit{C. albicans} sequences. The data showed that \textit{Pb}01 contains four metalloreductase (Frep) homologs, \textit{Pb}03 five homologs, and \textit{Pb}18 three homologs (Table 1). The genes encoding metalloreductases were \textit{fre}1, \textit{fre}3, \textit{fre}5, \textit{fre}7 and \textit{fre}p1. Also, \textit{Pb}01 and \textit{Pb}03 have two homologs each of the ferroxidase \textit{Fetp} and \textit{Fetb} has one. The reductive uptake system was first described in \textit{S. cerevisiae} (Lesuisse et al., 1987). The enzymatic reduction step in \textit{S. cerevisiae} is catalyzed by members of the FRE family of metalloreductases. The products of the \textit{frem} genes are not specific for iron reduction, since they can also promote copper reduction. \textit{S. cerevisiae} \textit{Frem}p and \textit{Frem}p2 are required for growth on media with low concentrations of ferric iron salts. \textit{Frem}p and \textit{Frem}p catalyze uptake of iron from siderophores and \textit{Frem}p7 is under the control of the copper-dependent transcription factor \textit{Mac}1p (Philpott and Protchenko, 2008). The expression of \textit{C. albicans} ferric reductase \textit{Frp}lp1 is upregulated by alkaline pH and iron-limited conditions (Li et al., 2009). Future studies are required to dissect the roles of the different \textit{P. brasiliensis} reductases, especially in in vivo conditions.

Homologs for iron permeases (\textit{Ftp}p and \textit{Fth}p) were not found in \textit{P. brasiliensis} genomes, corroborating the hypothesis that iron is transported by the zinc permeases, as previously suggested by transcriptional analyses (Balbão et al., 2006, 2007, Costa et al., 2007). However, in the present in silico analysis, we identified five zinc transporters (Table 1). These permeases could be coupled with one or more of the ferroxidases homologs (\textit{Fet}tp5, \textit{Fet}tp3p and \textit{Fet}tp3p) identified in the \textit{P. brasiliensis} genome database. In \textit{S. cerevisiae}, reduced iron is taken up through a high-affinity transport complex that consists of \textit{Fet}p3, a multi-copper ferroxidase, and \textit{Ft}tp1p, a permease. Independent studies have demonstrated that \textit{Fet}p3 produced by \textit{S. cerevisiae} \Delta\textit{ftt}1 mutant cells is retained in a cytoplasmic compartment in a copper-free, inactive form. Correspondingly, \textit{Ft}tp1p produced by \textit{S. cerevisiae} \Delta\textit{ftt}3 mutant cells fails to reach the plasma membrane (Stearman et al., 1996). These observations are in agreement with a model in which the two proteins form a heterodimer or higher order structure for correct maturation and trafficking to the plasma membrane (Kosman, 2003).

The \textit{P. brasiliensis} genomes analysis revealed the presence of a \textit{ggt}1 homolog. This gene is presumably responsible for the glutathione (GSHI)-dependent iron reduction activity previously identified in functional studies (Zarnowski and Woods, 2005). The proposed mechanism comprises secretion of a glutathione-dependent ferric reductase (GSH–FeR), named \textit{Ggt}1p, that purportedly utilizes siderophores and Fe$^{2+}$-binding proteins as substrates, enhancing the enzymatic activity under iron-limiting conditions, which is consistent with the function of a high-affinity uptake system, as described in \textit{H. capsulatum} (Timmerman and Woods, 2001).

Homologs of permease genes involved in low-affinity iron reductive systems, such as \textit{snf}, were not detected in our analysis. Hence, the low-affinity permease utilized by \textit{P. brasiliensis} to acquire iron could be one of the zinc permeases, as suggested (Table 1). Despite the absence of iron permease \textit{fth}1 gene homologs, \textit{P. brasiliensis} has one \textit{ccc}1 gene homolog that could drive iron vacuolar transport. \textit{P. brasiliensis} also has homologs of the mitochondrial iron transporters genes \textit{mrs}3 and \textit{mrs}4 and the mitochondrial iron chaperone \textit{Yfh}1p, suggesting mitochondrial iron homeostasis in this pathogen (Table 1). Since mitochondria are major users of iron, it follows that they should contain machinery required for its transport. \textit{Mrs}3p and \textit{Mrs}4p are homologous and functionally redundant proteins found in the inner mitochondrial membrane of \textit{S. cerevisiae}, which are involved in transport under iron-limiting conditions (Fouhy and Roganti, 2002). \textit{Yfh}1p, a homolog of human frataxin, is also involved in mitochondrial iron homeostasis (Babcock et al., 1997). While \textit{Mrs}3p and \textit{Mrs}4p mediate iron delivery from the outside to the inside of mitochondria, the frataxin homolog facilitates the use of iron within this organelle, functioning as a mitochondrial matrix iron chaperone (Zhang et al., 2006; Froschauer et al., 2009).

Cryptococcal genomic databases analysis revealed both \textit{S. cerevisiae} and \textit{C. albicans} homologs for proteins related to iron metabolism (Table 1). Remarkably, the \textit{C. neoformans} var. \textit{grubii} database contains four metalloreductase homologs, while the \textit{C. gattii} genome has three similar homologs. The reason for the multiplicity of metalloreductases isoenzymes is not clear, although it is speculated that some sets of genes are expressed under specific conditions for iron acquisition (Kornitzer, 2009). Concerning the ferroxidases, \textit{C. neoformans} var. \textit{grubii} has three homologs and \textit{C. gattii} contains one. Both genomes possess two iron permeases homologs, whose presence is supported by prior functional analyses (Jung et al., 2008). Two iron permeases, gene orthologs of \textit{S. cerevisiae} \textit{ftr}1, have been identified in \textit{C. neoformans}, namely \textit{Cft}1p and \textit{Cft}2p (Jung et al., 2008). The expression of the \textit{cfl}1 gene is down-regulated at high iron concentrations, suggesting that its product functions as a high-affinity iron permease. The role of \textit{cfl}2 is still unclear, although it supposedly encodes a low-affinity iron permease or a vacuolar permease that could transport stored iron to the cytoplasm, similar to what occurs in \textit{S. cerevisiae} with the iron permease \textit{Fth}1p. One of the iron permeases here identified is probably a \textit{Fth}1p homolog, which

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\(^{4}\)http://www.ncbi.nlm.nih.gov/guide/
\(^{5}\)http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
\(^{6}\)http://smart.embl-heidelberg.de/
\(^{7}\)http://mobile.pasteur.fr/cgi-bin/portal.py?form=toppred
\(^{8}\)http://www.yeastgenome.org/
\(^{9}\)http://www.candidageneome.org/
Table 1 | Orthologs to genes related to iron, copper and zinc uptake by reductive systems in *P. brasiliensis* and *Cryptococcus* species.

| Gene | Organism/accession number | Predicted function | Orthologs in *Pb* 01, 03 and 18 (accession numbers)$^1$ | E-value$^*$ | Orthologs in *Cryptococcus* species (accession numbers)$^1$ | E-value$^*$ |
|------|----------------------------|--------------------|-------------------------------------------------------------|------------|----------------------------------------------------------|------------|
| fre1 | *S. cerevisiae* NP_013315 | Metalloreductase    | PAAG_05370.1                                              | e-22       | Not identified                                           | –          |
| fre3 | *S. cerevisiae* NP_015026 | Metalloreductase    | PAAG_02079.1                                              | e-35       | Not identified                                           | –          |
| fre5 | *S. cerevisiae* NP_015029 | Metalloreductase    | PABG_06003.1                                              | e-19       | Not identified                                           | –          |
| fre7 | *S. cerevisiae* NP_014489 | Metalloreductase    | PABG_02329.1                                              | e-35       | Not identified                                           | –          |
| fre8 | *S. cerevisiae* NP_013148 | Metalloreductase    | Not identified                                            | –          | CNAG_00876.2                                            | e-37       |
| fre9 | *C. albicans* XP_711543  | Metalloreductase    | Not identified                                            | –          | CNBG_6082.2                                             | e-37       |
| fre10| *C. albicans* XP_711263  | Metalloreductase    | PAAG_05667.1                                              | e-40       | CNAG_07334.2                                            | e-10       |
| cfl4 | *C. albicans* XP_711263  | Metalloreductase    | Not identified                                            | –          | CNAG_06821.2                                            | e-34       |
| frp1 | *C. albicans* XP_713315  | Metalloreductase    | PAAG_04493.1                                              | e-26       | Not identified                                           | –          |
| fet3 | *S. cerevisiae* NP_013774 | Ferroxidase         | Not identified                                            | –          | CNAG_06241.2                                            | 0.0        |
| fet5 | *S. cerevisiae* NP_116612 | Ferroxidase         | PABG_05994.1                                              | e-37       | CNBG_4942.2                                             | 0.0        |
| fet31| *C. albicans* XP_711263  | Ferroxidase         | PAAG_06004.1                                              | e-39       | CNAG_02958.2                                            | 0.0        |
| fet33| *C. albicans* XP_711265  | Ferroxidase         | PAAG_00163.1                                              | e-33       | Not identified                                           | –          |
| fr1/fr2| *C. albicans* XP_715020/ XP_715031 | Iron permease | Not identified                                            | –          | CNAG_06242.2                                            | 0.0        |
| smf1 | *S. cerevisiae* NP_014519 | Low-affinity permease | Not identified                                            | –          | CNAG_06242.2                                            | 0.0        |
| fth1 | *C. albicans* XP_723298  | Vacuolar transporter | Not identified                                            | –          | CNBG_4943.2                                             | 0.0        |
| ccc1 | *S. cerevisiae* NP_013321 | Vacuolar transporter | PAAG_07762.1                                              | e-31       | CNAG_05154.2                                            | e-23       |
| mrs3/ mrs4 | *S. cerevisiae* NP_012402/ NP_012976 | Mitochondrial iron transporter | PAAG_05053.1                                              | 0.0        | CNAG_05011.2                                            | e-18       |
| yfh1 | *S. cerevisiae* NP_010163 | Mitochondrial matrix chaperone | PAAG_02608.1                                              | e-15       | CNBG_4670.2                                             | e-18       |

(Continued)
| Gene | Organism/accession number | Predicted function | Orthologs in Pb 01, 03 and 18 (accession numbers)† | E-value* | Orthologs in Cryptococcus species (accession numbers)† | E-value* |
|------|--------------------------|--------------------|-----------------------------------------------|---------|-------------------------------------------------|---------|
| ggt1 | H. capsulatum EGC49121 | Secreted glutathione-dependent ferric reductase | PAAG_06130.1, PABG_06527.1, PADG_07986.1 | 0.0 0.0 0.0 | CNAG_02888.2, CNBG_35372.2 | 0.0 0.0 |
| mac1 | S. cerevisiae NP_013734 | Copper metalloregulatory transcription factor | PAAG_08210.1, PABG_07429.1 | e-5 e-5 | CNAG_07724.2, CNBG_2252.2 | e-7 e-7 |
| ctr3 | S. cerevisiae NP_013515 | High-affinity copper transporter of the plasma membrane | PAAG_05251.1, PABG_07607.1, PADG_05084.1 | e-22 e-21 e-21 | CNAG_00979.2, CNBG_0560.2 | e-14 e-14 |
| ctr1 | S. cerevisiae NP_015449 | High-affinity copper transporter of the plasma membrane | Not identified | | Not identified | |
| ctr2 | S. cerevisiae NP_012045 | Putative low-affinity copper transporter of the vacuolar membrane | PABG_01536.1, PADG_04146.1 | e-14 e-14 | CNAG_01872.2 | e-13 |
| atx1 | S. cerevisiae NP_14140 | Cytosolic copper metallochaperone | PAAG_00326.1, PABG_06615.1, PADG_02352.1 | e-12 e-12 e-12 | CNAG_02434.2, CNBG_4136.2 | e-10 e-11 |
| ccc2 | S. cerevisiae NP_010556 | Cu²⁺ transporting P-type ATPase | PAAG_07053.1, PABG_03057.1, PADG_01582.1 | 0.0 0.0 0.0 | CNAG_06415.2, CNBG_5045.2 | 0.0 0.0 |
| cup1 | S. cerevisiae NP_011920 | Metallothionein | Not identified | | Not identified | |
| cup2 | S. cerevisiae NP_011922 | Metallothionein | Not identified | | Not identified | |
| sod1 | S. cerevisiae NP_012638 | Cytosolic superoxide dismutase | PAAG_04164.1, PABG_03954.1, PADG_07418.1 | 0.0 0.0 0.0 | CNAG_01019.2, CNBG_0899.2 | 0.0 0.0 |
| sod2 | S. cerevisiae NP_011872 | Mitochondrial superoxide dismutase | PAAG_02725.1, PABG_03204.1, PADG_01756.1 | 0.0 0.0 0.0 | CNAG_04388.2, CNBG_2661.2 | 0.0 0.0 |
| zrt1 | S. cerevisiae NP_011259 | High-affinity zinc transporter of the plasma membrane | PAAG_08727.1, PABG_07725.1, PADG_08567.1 | 0.0 0.0 0.0 | CNAG_03398.2, CNBG_2209.2 | e-40 e-41 |
| zrt2 | S. cerevisiae NP_013231 | Low-affinity zinc transporter of the plasma membrane | PAAG_03419.1, PABG_05498.1, PADG_06417.1 | e-27 e-26 e-28 | CNAG_00895.2 | 0.0 |
Table 1 | Continued

| Gene | Organism/accession number | Predicted function | Orthologs in Pb 01, 03 and 18 (accession numbers) | E-value* | Orthologs in Cryptococcus species (accession numbers) | E-value* |
|------|---------------------------|--------------------|-----------------------------------------------|----------|------------------------------------------------|----------|
| zrc1 | S. cerevisiae NP_013970   | Vacuolar membrane zinc transporter | PAAG_00702.1 | e-41 | Not identified | – |
| cot1 | S. cerevisiae NP_014961   | Vacuolar membrane zinc transporter | PAAG_07885.1 | e-44 | CNAG_02806.2 | e-40 |
| zrt3 | S. cerevisiae NP_012746   | Vacuolar membrane zinc transporter | PAAG_08074.1 | e-23 | Not identified | – |
| msc2 | S. cerevisiae NP_010491   | Cation diffusion facilitator protein of the endoplasmic reticulum and nucleus | PABG_07115.1 | e-40 | CNAG_05394.2 | e-23 |
| zap1 | S. cerevisiae NP_012479   | Zinc-regulated transcription factor | PAAG_03645.1 | e-20 | CNAG_05392.2 | e-40 |
|      |                           |                    | PABG_03305.1 | e-18 | CNBG_4460.2 | e-28 |
|      |                           |                    | PADG_01870.1 | e-24 | CNBG_4458.2 | e-24 |

*Similarities with E-values <10^-4 were considered significant.

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

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

**Conserved domains in proteins related to the reductive iron metabolism**

Amino acid sequence analyses of orthologs proteins found in the P. brasiliensis isolates and Cryptococcus species may support the assumption of conserved functions. Searching for conserved domains in all the analyzed sequences (Table A1 in Appendix) revealed that most of the P. brasiliensis and Cryptococcus deduced proteins codified by the genes related to reductive iron metabolism contain conserved domains related to specific functions. Regarding to metalloreductases, the presence of a ferric reductase domain and a FAD- and/or a NAD-binding domain can be essential for functional enzymatic activity, since they are responsible for electron donation, as described in other organisms (De Luca and Wood, 2000). A schematic diagram presenting the cited motifs in a metalloreductase Frep is shown in Figure 2. An HPFTXXS motif is believed to be a site for FAD-binding and a glycine-rich motif and a cysteine–glycine couple are thought to be involved in NADPH binding (Shatwell et al., 1996). As well, copper-oxidase domains are required for ferroxidase activity. S. cerevisiae Fet3p is a multi-copper-oxidase and, like other copper proteins, possesses three distinct types of Cu²⁺-binding sites. Oxidation of Fe²⁺ occurs at the type 1 copper site followed by the reduction of molecular oxygen to 2H₂O at the other two copper sites (Hassett et al., 1998; Kosman, 2003). The ferroxidases in the P. brasiliensis isolates and Cryptococcus species present such domain, suggesting they are functional proteins.

**Siderophore production**

Culture supernatants of P. brasiliensis grown in media with low iron concentrations display higher iron binding capacity when compared with culture supernatants from iron-rich media (Arago and Restrepo, 1988), which has suggested that siderophores are involved in iron acquisition in this fungus. Furthermore, in silico analysis of P. brasiliensis structural genomes indicates that this fungus can potentially produce siderophores. The three sequenced P. brasiliensis genomes show sequences that potentially encode all the necessary enzymes for siderophore synthesis: sidA, sidF, sidC and sidD (A. fumigatus orthologs), as shown in Table 2 and Figure 1. This biosynthetic pathway may lead to the production of hydroxamate-type siderophores. The first committed step in siderophore biosynthesis is the N³-hydroxylation of ornithine catalyzed by ornithine-N³-oxygenase. The sid1 gene of Ustilago maydis, the etiologic agent of corn smut, was the first characterized fungal ornithine-N³-oxygenase-encoding gene (Mei et al., 1993).
Orthologs of *sid1* have been identified in *A. fumigatus* (*sidA*) and *H. capsulatum* (*sid1*). In the latter, disruption of *sid1* causes poor growth under low iron conditions and loss of siderophore production, suggesting an important role of siderophore production in iron-limiting conditions (Schrettl et al., 2004; Hwang et al., 2008). The formation of the hydroxamate group consists of the transfer of an acyl group from acyl-coenzyme A to *N*<sub>5</sub>-hydroxyornithine. Different acyl group usage results in the production of distinct siderophores.

**FIGURE 1** | Schematic comparison of iron metabolism in *P. brasiliensis* isolates and *Cryptococcus* species. Sit1p, MirAp, MirBp and MirCp are membrane transporters that traffic siderophores bound to ferric iron into the intracellular environment. SidAp, SidFp, SidCp and SidDp are enzymes from the biosynthetic pathway of hydroxamate-type siderophores. Ccc1p is a vacuolar membrane iron transporter. Mrs3/4p are iron transporters found in the inner mitochondrial membrane and Yfh1p is a mitochondrial matrix iron chaperone.
siderophores. Acetyl is used for rhodotorulic acid and ferrichrome synthesis, while anhydromevalonyl is utilized in the fusarines and coprogens pathway (Haas et al., 2008). A. fumigatus sidF encodes an N²-hydroxyornithine: cis anhydromevalonyl coenzyme A-N²-transacylase involved in the synthesis of fusarines and triacetylfusarine (Schrettl et al., 2007). The sidF ortholog of H. capsulatum, sid3 gene, is transcriptionally induced under iron restricted conditions (Hwang et al., 2008). Hydroxamates are covalently linked via peptide (rhodotorulic acid, ferrichromes, coprogens) or ester bonds (fusarines, coprogens) carried out by non-ribosomal peptide synthetases (NRPSs; Finking and Marahiel, 2004). In A. fumigatus, sidC and sidD encodes two NRPSs involved in ferricrocin (intracellular siderophore) and triacetylfusarine C (TAFc) biosynthesis, respectively. Some siderophores additionally require acetylation at the N²-amino group, such as coprogen and TAFc. For example, sidG deletion in A. fumigatus results in the abrogation of the TAFc siderophore production (Schrettl et al., 2007). Given that our in silico analysis of P. brasiliensis identified sequences capable of coding for SidAp, SidFp, SidCp and SidDp, it is reasonable to hypothesize that P. brasiliensis may be able to synthesize both intracellular and extracellular siderophores.

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

**Conserved domains in proteins related to siderophore biosynthesis**

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

**Siderophore uptake**

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

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

**Analysis of transmembrane domains in siderophore–iron transporters**

Amino acid sequences of siderophore transporter orthologs found in P. brasiliensis isolates and Cryptococcus species were analyzed in the TopPred server to predict their transmembrane domain topologies. Figure 3B presents the transmembrane segments of Sit1p in S. cerevisiae, P. brasiliensis isolates, C. neoformans var. grubii and C. gattii.
hemoglobin-binding protein (Weissman et al., 2008). Although there is no experimental evidence regarding the utilization of iron from the heme group by *P. brasiliensis*, there are genes that show similarity with Hmx-1p (Pendrak et al., 2004), and exhibit a heme oxygenase domain (PAAG_06626.1 in Pb01; PABG_02644.1 in Pb03; PADG_01082.1 in Pb18) in each of the *P. brasiliensis* isolates. These genes are annotated as conserved hypothetical or as predicted proteins.

*C. neoformans* var. *grubii* is also able to utilize heme and hemoglobin as iron sources, but the mechanism(s) of heme utilization by this fungus are still unclear (Jung et al., 2008).

Transferrin has also been shown to be an iron source for both *C. albicans* and *C. neoformans* var. *grubii*. These fungi employ high-affinity permeases to acquire iron from transferrin in mammalian serum. The number of segments varies between 12 and 15. Identical topology was found in Sit1p from *P. brasiliensis* isolates Pb03 and Pb18, whereas Pb01 has a different topology. Transmembrane domains were also identified in all the other siderophore transporters, as shown in Table A2 in Appendix. These transporters also contain a MFS1 domain, which indicates that they belong to the MFS of transporters.

### Iron source preferences

Several fungal pathogens utilize heme or hemoglobin as sources of iron (Foster, 2002; Jung et al., 2008). *C. albicans* expresses surface receptors for hemoglobin and hemolytic factors (Manns et al., 1994). Interestingly, heme–iron utilization in *C. albicans* is facilitated by Rbt5p, an extracellular glycosylphophatidylinositol (GPI)-anchored hemoglobin-binding protein (Weissman et al., 2008). Although there is no experimental evidence regarding the utilization of iron from the heme group by *P. brasiliensis*, there are genes that show similarity with Hmx-1p (Pendrak et al., 2004), and exhibit a heme oxygenase domain (PAAG_06626.1 in Pb01; PABG_02644.1 in Pb03; PADG_01082.1 in Pb18) in each of the *P. brasiliensis* isolates. These genes are annotated as conserved hypothetical or as predicted proteins. *C. neoformans* var. *grubii* is also able to utilize heme and hemoglobin as iron sources, but the mechanism(s) of heme utilization by this fungus are still unclear (Jung et al., 2008).

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

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

| Gene | Organism/accession number | Predicted function | Orthologs in Pb01, 03 and 18 (accession numbers)* | E-value* | Orthologs in *Cryptococcus* species (accession numbers)† | E-value* |
|------|--------------------------|-------------------|-----------------------------------------------|--------|-----------------------------------------------|--------|
| *sidA* | *A. fumigatus*/XP_755103 | Ornithine-N⁵- monooxygenase | PAAG_01682.1 | 0.0 | Not identified | – |
| *sidF* | *A. fumigatus*/XM_743567 | N⁵-transacylases | PAAG_01680.1 | 0.0 | Not identified | – |
| *sidC* | *A. fumigatus*/XP_753088 | Non-ribosomal peptide synthetase | PAAG_08527.1 | 0.0 | Not identified | – |
| *sidD* | *A. fumigatus*/XP_748662 | Non-ribosomal peptide synthetase | PAAG_01679.1 | 0.0 | CNAG_03588.2 | e-40 |
| *sidG* | *A. fumigatus*/XP_748685 | N⁵-transacetylase | Not identified | – | CNAG_04355.2 | 2e-5 |
| *sit1/arn3* | *S. cerevisiae*/NP_010849 | Siderophore transporter | PAAG_06516.1 | 0.0 | CNAG_00815.2 | 0.0 |
| *mirA* | *A. nidulans*/AY027565 | Siderophore transporter | PAAG_01685.1 | 0.0 | CNAG_07751.2 | 0.0 |
| *mirB* | *A. nidulans*/XP_681809 | Siderophore transporter | PAAG_01685.1 | 0.0 | CNAG_07751.2 | 0.0 |
| *mirC* | *A. nidulans*/AY135152 | Siderophore transporter | PAAG_02233.1 | 0.0 | CNAG_07519.2 | 0.0 |

| Gene | Organism/accession number | Predicted function | Orthologs in Pb01, 03 and 18 (accession numbers)* | E-value* | Orthologs in *Cryptococcus* species (accession numbers)† | E-value* |
|------|--------------------------|-------------------|-----------------------------------------------|--------|-----------------------------------------------|--------|
| *sidA* | *A. fumigatus*/XP_755103 | Ornithine-N⁵- monooxygenase | PAAG_01682.1 | 0.0 | Not identified | – |
| *sidF* | *A. fumigatus*/XM_743567 | N⁵-transacylases | PAAG_01680.1 | 0.0 | Not identified | – |
| *sidC* | *A. fumigatus*/XP_753088 | Non-ribosomal peptide synthetase | PAAG_08527.1 | 0.0 | Not identified | – |
| *sidD* | *A. fumigatus*/XP_748662 | Non-ribosomal peptide synthetase | PAAG_01679.1 | 0.0 | CNAG_03588.2 | e-40 |
| *sidG* | *A. fumigatus*/XP_748685 | N⁵-transacetylase | Not identified | – | CNAG_04355.2 | 2e-5 |
| *sit1/arn3* | *S. cerevisiae*/NP_010849 | Siderophore transporter | PAAG_06516.1 | 0.0 | CNAG_00815.2 | 0.0 |
| *mirA* | *A. nidulans*/AY027565 | Siderophore transporter | PAAG_01685.1 | 0.0 | CNAG_07751.2 | 0.0 |
| *mirB* | *A. nidulans*/XP_681809 | Siderophore transporter | PAAG_01685.1 | 0.0 | CNAG_07751.2 | 0.0 |
| *mirC* | *A. nidulans*/AY135152 | Siderophore transporter | PAAG_02233.1 | 0.0 | CNAG_07519.2 | 0.0 |

*Similarities with E-values < 10⁻⁵ were considered significant.

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

See Table A2 in Appendix for full details.
hosts through the reductive system (Knight et al., 2005; Jung et al., 2008). In the P. brasiliensis genome databases, genes were found (PAAG_04670.1; PABG_00038.1; PADG02428.1, respectively for isolates P. brasiliensis isolates and Cryptococcus species. White boxes represent putative segments, according to cutoff parameters (cut-off for certain transmembrane segments 1.00; cut-off for putative transmembrane segments 0.60). E: extracellular environment; C: cytosol. The topology prediction was performed using the TopPred server. Accession numbers in A: AtXp (NP_010849), Pb01 (PAAG_08527.1), Pb03 (PAAG_04670.1), Pb18 (PADG_05295.1). Accession numbers in B: Sc (XP_753088), Pb01 (PAAG_08527.1), Pb03 (PAAG_04670.1), Pb18 (PADG_05295.1), Cn (CNAG_00819.2), and Cg (CNBG_1123.2).

**COPPER**

**Copper uptake by the reductive system**

Little is known about copper metabolism in P. brasiliensis. However, our in silico analyses of the S. cerevisiae copper metabolism–related genes in comparison to P. brasiliensis genomic databases revealed genes related to the copper reduction metalloreguctase, frc. Copper transport is well described in S. cerevisiae where it is reduced from Cu (II) to Cu (I) by several cell surface metalloductases encoded by several frc genes. These metalloductases are regulated by iron and copper availability, mediated by the transcriptional factor Mac1p (Jungmann et al., 1993). Homologs of the copper metalloregulatory transcription factor gene (mac1) are present in both Pb01 and Pb03 genomes, but not in Pb18. Additionally, the high-affinity copper transporter (Ctr3p) was found in all three genome isolates. In S. cerevisiae, after reduction, copper is transported by the high-affinity copper transporter comprised by Ctrlp and Ctr1p, which are functionally redundant, although they have distinct amino acid sequences. Ctrl3p is an integral membrane protein that assembles as a trimer to form a competent copper uptake permease at the plasma membrane. S. cerevisiae Ctrl1p is localized at the plasma membrane and exists as an oligomer in vivo. These two high-affinity copper transport proteins are induced by copper deprivation and repressed by copper excess (Dancis et al., 1994a; Pena et al., 2000). In our in silico analyses, genes for the high-affinity copper transporter of the plasma membrane (ctr1) were not found, suggesting that high-affinity copper transport is performed only by the Ctrl3p protein.

Genes related to metallochaperone (atxl), Cu{sup +} transporting P-type ATPase (ccc2) and superoxide dismutases (sod1 and sod2; Table 1) were also found in P. brasiliensis genomes. In the cell, copper is transported by Atx1p, a cytosolic copper metallochaperone protein, that transports Cu (I) to Ccc2p, a transporting P-type ATPase containing a cytoplasmic region containing two distinct soluble metal-binding domains that interact with Atx1p (Banci et al., 2007). Ccc2p mediates the export of copper from the cytosol and distributes it to cupric proteins (Yuan et al., 1997). S. cerevisiae also has a detoxification pathway formed by Cup1p and Cup2p, metallothioneins (Table 1), that protect against copper poisoning (Hamer et al., 1985). An alternative copper transport system is mediated by Ctr2p, a vacular membrane protein of S. cerevisiae, that mobilizes vacuolar copper stores to cytosolic copper chaperones (Rees et al., 2004). Homologs of the low-affinity copper transporter of the vacular membrane (Ctr2p) are in Pb03 and Pb18, but not in Pb01. Additionally, the metallothioneins (encoded by cup1 and cup2 genes) were not identified in P. brasiliensis isolates Pb01, Pb03 and Pb18.

In silico analysis (Table 1) revealed that Cryptococcus species have orthologs encoding ferric/cupric reductases, suggesting that the copper reduction process is similar to that described for S. cerevisiae. Homologs of the high-affinity copper transporter ctr3 gene and copper metalloregulatory transcription factor gene (mac1) have previously been identified (Waterman et al., 2007). Also, proteins with similarity to the cytosolic copper metallochaperone (atxl gene), the Cu{sup +} transporting P-type ATPase (ccc2 gene) and the cytosolic and mitochondrial superoxide dismutases (sod1 and sod2 genes) have also identified, suggesting that copper distribution in Cryptococcus species occurs as described in S. cerevisiae. A homolog of the ctr2 gene was identified only in C. neoformans var. grubii. Recently it was demonstrated that Ctr2p links copper homeostasis to polysaccharide capsule production in C. neoformans. The lack of this protein resulted in increased phagocytosis by murine macro-
phage, sensitivity to copper starvation and defects in polysaccharide capsule formation and melanization (Chun and Madhani, 2010).

The gene *ctr1* for the high-affinity copper transporter of the plasma membrane and the genes *cup1* and *cup2* for metallothioneins were not found in *Cryptococcus* species. These analyses suggest that the high-affinity copper transport in cryptococcal cells is primarily performed by the protein encoded by *ctr3*.

**Analysis of conserved motifs present in copper transporters**

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

**ZINC**

**Zinc uptake**

Comparisons to the *S. cerevisiae* genes related to zinc metabolism performed in *P. brasiliensis* genomes are presented in Table 1. Analyses demonstrate that *P. brasiliensis* has homologs to zinc transporters described in *S. cerevisiae* that are localized in the plasmatic, vacular and endoplasmic reticulum membranes. Importantly, five genes encoding to transporters of the ZIP family, with homology to *S. cerevisiae* Zrt1p or Zrt2p, are in the *P. brasiliensis* genomic database. In *S. cerevisiae*, zinc is transported by proteins belonging to the ZIP family, which is composed by a zinc high-affinity transporter protein encoded by the *zrt1* gene and a low-affinity transporter encoded by the *zrt2* gene (Gaither and Eide, 2001). We have previously identified homologs of zinc transporters by transcriptional analysis of *P. brasiliensis* yeast cells after incubation in human blood and plasma (Balão et al., 2006, 2007). Interestingly, *P. brasiliensis* isolate Pb01 has two vacuolar membrane zinc transporters, encoded by the *zrc1* and *cot1* genes, whereas isolates Pb03 and Pb18 contain only the *cot1* homolog. Intracellularly, zinc is in vacuoles in association with the vacuolar membrane proteins Zrc1p and Cot1p, members of the cation diffusion facilitator (CDF) family (MacDiarmid et al., 2002). A homolog of the transcription factor Zap1p is also present in the three *P. brasiliensis* isolates. The expression of the genes associated with zinc homeostasis is positively regulated in *S. cerevisiae* by the transcription factor Zap1p, which regulates the expression of *zrt1*, *zrt2*, *zrt3*, *fet4*, and *zcr1* under zinc limiting conditions (Wu et al., 2008). Therefore, zinc assimilation in *P. brasiliensis* may be similar to that of *S. cerevisiae*.

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

![FIGURE 4](image_url) **Conserved features found in the primary structure of Ctr3p of *P. brasiliensis* isolates and *Cryptococcus* species.** Ctr3p from *P. brasiliensis* isolates contains three putative transmembrane domains (TMD1-3, shown in black) while Ctr3p from *Cryptococcus* species presents only two TMDs. All species contain putative copper binding motifs (Mets motifs) arranged as MXXX and/or MXM. MXXXM motif in TMD3 in *P. brasiliensis* isolates and TMD2 in *Cryptococcus* species are represented in white characters. The length of each protein, in amino acids, is shown on the right. Accession numbers: Pb01 (PAAG_05251.1), Pb03 (PAAG_05084.1), Pb18 (PADG_05084.1), Cn (CNAG_00979.2) and Cg (CNBG_0560.2).
FIGURE 5 | Alignment of amino acid sequences of Zrt1p from *S. cerevisiae*, *P. brasiliensis* isolates and *Cryptococcus* species. The predicted transmembrane domains are shown in gray boxes. The black boxes inside the transmembrane segment contain conserved histidine-serine and glycine residues. The histidines found in the amino-terminal region of Zrt1p from *Cryptococcus* species and in the loop between transmembrane domains III and IV in *P. brasiliensis* and *S. cerevisiae* are boxed. Asterisks indicate amino acid identity and dots represent conserved substitutions. Accession numbers: Pb03 (PABG_07725.1), Pb18 (PADG_08567.1), Pb01 (PAAG_08727.1), Sc (NP_011259), Cn (CNAG_03398.2) and Cg (CNBG_2209.2).
could be responsible for the zinc transport to this organelle. This analysis suggests that *C. neoformans* var. *grubii* and *C. gattii* could obtain zinc via routes similar to that described for *S. cerevisiae*.

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

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

**CONCLUSION**

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

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

| Gene product | Predicted function | Organism/accession number | Conserved domains* | Transmembrane domains* | Signal peptide* |
|--------------|--------------------|---------------------------|-------------------|-----------------------|----------------|
| Fre1 Metalloreductase | *P. brasiliensis* 01/PAAG_05370.1 | Ferric reductase domain | 7 | Yes |
| Fre1 Metalloreductase | *P. brasiliensis* 03/PABG_06003.1 | FAD-binding domain | 6 | No |
| Fre2 Metalloreductase | *P. brasiliensis* 01/PAAG_02079.1 | Ferric reductase domain | 6 | Yes |
| Fre2 Metalloreductase | *P. brasiliensis* 03/PABG_02329.1 | FAD-binding domain | 6 | Yes |
| Fre2 Metalloreductase | *P. brasiliensis* 18/PADG_00813.1 | NAD-binding domain | 6 | Yes |
| Fre3 Metalloreductase | *P. brasiliensis* 01/PAAG_02079.1 | Ferric reductase domain | 6 | Yes |
| Fre3 Metalloreductase | *P. brasiliensis* 03/PABG_02329.1 | FAD-binding domain | 6 | Yes |
| Fre3 Metalloreductase | *P. brasiliensis* 18/PADG_00813.1 | NAD-binding domain | 6 | Yes |
| Fre5 Metalloreductase | *P. brasiliensis* 03/PABG_07812.1 | Ferric reductase domain | 6 | No |
| Fre5 Metalloreductase | *P. brasiliensis* 03/PABG_07812.1 | FAD-binding domain | 6 | No |
| Fre5 Metalloreductase | *P. brasiliensis* 18/PADG_00813.1 | NAD-binding domain | 6 | Yes |
| Fre6 Metalloreductase | *P. brasiliensis* 01/PAAG_06164.1 | Ferric reductase domain | 8 | No |
| Fre6 Metalloreductase | *P. brasiliensis* 03/PABG_06497.1 | FAD-binding domain | 8 | No |
| Fre6 Metalloreductase | *P. brasiliensis* 18/PADG_07967.1 | NAD-binding domain | 8 | No |
| Fre8 Metalloreductase | *C. neoformans* CNAG_00876.2 | NAD-binding domain | 7 | No |
| Fre8 Metalloreductase | *C. neoformans* CNAG_07334.2 | Ferric reductase domain | 6 | No |
| Fre8 Metalloreductase | *C. gattii* CNBG_6082.2 | Ferric reductase domain | 8 | No |
| Fre10 Metalloreductase | *C. neoformans* CNAG_06821.2 | Ferric reductase domain | 4 | No |
| Fre10 Metalloreductase | *C. gattii* CNBG_8882.2 | Ferric reductase domain | 4 | No |
| Cfl4 Metalloreductase | *C. neoformans* CNAG_06524.2 | Ferric reductase domain | 5 | No |
| Cfl4 Metalloreductase | *C. gattii* CNBG_2116.2 | Ferric reductase domain | 5 | No |
| Frp1 Metalloreductase | *P. brasiliensis* 01/PAAG_04493.1 | Ferric reductase domain | 5 | No |
| Frp1 Metalloreductase | *P. brasiliensis* 03/PABG_04278.1 | FAD-binding domain | 6 | No |
| Frp1 Metalloreductase | *P. brasiliensis* 18/PADG_04652.1 | NAD-binding domain | 5 | No |
| Fet3 Ferroxidase | *C. neoformans* CNAG_06241.2 | Copper-oxidase domain | 1 | Yes |
| Fet5 Ferroxidase | *P. brasiliensis* 03/PABG_06667.1 | Copper-oxidase domain | – | No |
| Fet31 Ferroxidase | *P. brasiliensis* 01/PAAG_06004.1 | Copper-oxidase domain | 1 | Yes |
| Fet33 Ferroxidase | *P. brasiliensis* 01/PAAG_00163.1 | Copper-oxidase domain | – | No |
| Fet33 Ferroxidase | *P. brasiliensis* 03/PABG_05183.1 | Copper-oxidase domain | – | Yes |
| Ftr1/Ftr2 Iron permease | *C. neoformans* CNAG_06242.2 | FTR1 domain | 7 | Yes |
| Fth1 Iron permease | *C. neoformans* CNAG_02959.2 | FTR1 domain | 7 | Yes |
| Fth1 Iron permease | *C. gattii* CNBG_4943.2 | FTR1 domain | 7 | Yes |

(Continued)
| Gene product | Predicted function | Organism/accession number† | Conserved domains* | Transmembrane domains* | Signal peptide* |
|--------------|-------------------|---------------------------|-------------------|-----------------------|----------------|
| Smf1         | Low-affinity Permease | C. neoformans/CNAG_05640.2 | Nramp domain | 11 | No |
|              |                   | C. gattii/CNBG_6162.2 |                   |                       |                |
| Ccc1         | Vacuolar transporter | P. brasiliensis 01/PAAG_07762.1 | DUF125 domain | 4 | No |
|              |                   | P. brasiliensis 03/PABG_00362.1 |                   |                       |                |
|              |                   | P. brasiliensis 18/PADG_02775.1 |                   |                       |                |
|              |                   | C. neoformans/CNAG_05154.2 |                   |                       |                |
|              |                   | C. gattii/CNBG_4540.2 |                   |                       |                |
| Mrs3/Mrs4    | Mitochondrial iron transporter | P. brasiliensis 01/PAAG_05053.1 | Mitochondrial carrier | – | No |
|              |                   | P. brasiliensis 03/PABG_04509.1 | domain | – | No |
|              |                   | P. brasiliensis 18/PADG_04903.1 |                   |                       |                |
|              |                   | C. neoformans/CNAG_02522.2 |                   |                       |                |
|              |                   | C. gattii/CNBG_4218.2 |                   |                       |                |
| Yfh1         | Mitochondrial matrix iron chaperone | P. brasiliensis 01/PAAG_02608.1 | Frataxin domain | – | No |
|              |                   | P. brasiliensis 03/PABG_03095.1 |                   |                       |                |
|              |                   | P. brasiliensis 18/PADG_01626.1 |                   |                       |                |
|              |                   | C. neoformans/CNAG_05011.2 |                   |                       |                |
|              |                   | C. gattii/CNBG_4670.2 |                   |                       |                |
| Ggt1         | Secreted glutathione-dependent ferric reductase | P. brasiliensis 01/PAAG_06130.1 | Gamma- | 1 | Yes |
|              |                   | P. brasiliensis 03/PABG_06527.1 | glutamyltranspeptidase | 1 | Yes |
|              |                   | P. brasiliensis 18/PADG_07986.1 | domain | 1 | Yes |
|              |                   | C. neoformans/CNAG_05011.2 |                   |                       |                |
|              |                   | C. gattii/CNBG_35372 |                   |                       |                |
| Mac1         | Copper metalloregulatory transcription factor | P. brasiliensis 01/PAAG_08210.1 | Copper fist domain | – | No |
|              |                   | P. brasiliensis 03/PABG_07429.1 |                   |                       |                |
|              |                   | P. brasiliensis 18/PADG_01626.1 |                   |                       |                |
|              |                   | C. neoformans/CNAG_07724.2 |                   |                       |                |
|              |                   | C. gattii/CNBG_2252.2 |                   |                       |                |
| Ctr3         | High-affinity copper transporter of the plasma membrane | P. brasiliensis 01/PAAG_05251.1 | Ctr domain | 3 | No |
|              |                   | P. brasiliensis 03/PABG_07607.1 |                   |                       |                |
|              |                   | P. brasiliensis 18/PADG_05084.1 |                   |                       |                |
|              |                   | C. neoformans/CNAG_0979.2 |                   |                       |                |
|              |                   | C. gattii/CNBG_0560.2 |                   |                       |                |
| Ctr2         | Putative low-affinity copper transporter of the vacuolar membrane | P. brasiliensis 03/PABG_01536.1 | Ctr domain | 3 | No |
|              |                   | P. brasiliensis 18/PADG_04146.1 |                   |                       |                |
|              |                   | C. neoformans/CNAG_01872.2 |                   |                       |                |
| Atx1         | Cytosolic copper metallochaperone | P. brasiliensis 01/PAAG_00326.1 | HMA domain | – | No |
|              |                   | P. brasiliensis 03/PABG_06615.1 |                   |                       |                |
|              |                   | P. brasiliensis 18/PADG_02352.1 |                   |                       |                |
|              |                   | C. neoformans/CNAG_02434.2 |                   |                       |                |
|              |                   | C. gattii/CNBG_4136.2 |                   |                       |                |
| Ccc2         | Cu²⁺ transporting P-type ATPase | P. brasiliensis 01/PAAG_07053.1 | HMA domain | 7 | No |
|              |                   | P. brasiliensis 03/PABG_03057.1 |                   |                       |                |

(Continued)
| Gene product | Predicted function | Organism/accession number† | Conserved domains* | Transmembrane domains* | Signal peptide* |
|--------------|-------------------|---------------------------|-------------------|------------------------|----------------|
| Sod1         | Cytosolic superoxide dismutase | *P. brasiliensis* 01/PAAG_04164.1 | Hydrolase domain 8 | No | - |
|              |                   | *C. neoformans* CNAG_06415.2 | E1-E2 ATPase domain 8 | No | - |
|              |                   | *C. gattii* CNBG_0599.2 | - | No | - |
| Sod2         | Mitochondrial superoxide dismutase | *P. brasiliensis* 01/PAAG_02725.1 | SOD domain – | No | - |
|              |                   | *P. brasiliensis* 03/PABG_03204.1 | SOD N-terminal domain – | No | - |
|              |                   | *C. neoformans* CNAG_03398.2 | 9 | Yes | - |
|              |                   | *C. gattii* CNBG_2209.2 | 9 | Yes | - |
| Zrt1         | High-affinity zinc transporter of the plasma membrane | *P. brasiliensis* 01/PAAG_08727.1 | Zip domain 8 | No | - |
|              |                   | *P. brasiliensis* 03/PABG_07725.1 | 8 | No | - |
|              |                   | *P. brasiliensis* 18/PADG_08567.1 | 8 | No | - |
|              |                   | *C. neoformans* CNAG_00895.2 | 6 | Yes | - |
|              |                   | *C. gattii* CNBG_2209.2 | 9 | Yes | - |
| Zrt2         | Low-affinity zinc transporter of the plasma membrane | *P. brasiliensis* 01/PAAG_03419.1 | Zip domain 8 | Yes | - |
|              |                   | *P. brasiliensis* 03/PABG_05498.1 | 8 | Yes | - |
|              |                   | *P. brasiliensis* 18/PADG_06417.1 | 8 | Yes | - |
|              |                   | *C. neoformans* CNAG_00895.2 | 8 | Yes | - |
| Zrc1         | Vacular membrane zinc transporter | *P. brasiliensis* 01/PAAG_00702.1 | Cation efflux domain 6 | Yes | - |
| Cot1         | Vacular membrane zinc transporter | *P. brasiliensis* 01/PAAG_07885.1 | Cation efflux domain 5 | Yes | - |
|              |                   | *P. brasiliensis* 03/PABG_07467.1 | 4 | No | - |
|              |                   | *P. brasiliensis* 18/PADG_08196.1 | 5 | Yes | - |
|              |                   | *C. neoformans* CNAG_02806.2 | 6 | Yes | - |
|              |                   | *C. gattii* CNBG_3460.2 | 4 | Yes | - |
| Zrt3         | Vacular membrane zinc transporter | *P. brasiliensis* 01/PAAG_09074.1 | Zip domain 6 | No | - |
|              |                   | *P. brasiliensis* 03/PABG_04697.1 | 6 | No | - |
|              |                   | *P. brasiliensis* 18/PADG_05322.1 | 6 | No | - |
| Msc2         | Cation diffusion facilitator protein of the endoplasmic reticulum and nucleus | *P. brasiliensis* 03/PABG_07115.1 | Cation efflux domain 10 | No | - |
|              |                   | *P. brasiliensis* 18/PADG_06381.1 | 10 | No | - |
|              |                   | *C. neoformans* CNAG_05394.2 | 11 | No | - |
|              |                   | *C. gattii* CNBG_4458.2 | 10 | No | - |
| Zap1         | Zinc-regulated transcription factor | *P. brasiliensis* 01/PAAG_03645.1 | Zinc finger C2H2 domain – | No | - |
|              |                   | *P. brasiliensis* 03/PABG_03305.1 | – | No | - |
|              |                   | *P. brasiliensis* 18/PADG_01870.1 | – | No | - |
|              |                   | *C. neoformans* CNAG_05392.2 | – | No | - |
|              |                   | *C. gattii* CNBG_4460.2 | – | No | - |

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

*Amino acid sequence analysis was performed using the online software SMART.*
Table A2 | Conserved domains in proteins related to siderophore biosynthesis and to iron uptake by the non-reductive siderophore transport system in *P. brasiliensis* isolates and *Cryptococcus* species.

| Gene product | Predicted function | Organism/accession number† | Conserved domains* | Transmembrane domains* | Signal peptide* |
|--------------|-------------------|----------------------------|-------------------|------------------------|----------------|
| SidA         | Ornithine-N^5-     | *P. brasiliensis* 01/PAAG_01682.1 | Pyr_redox_2 domain | –                      | No             |
|             | monoxygenase       | *P. brasiliensis* 03/PABG_03730.1 | –                 | –                      | No             |
|             |                   | *P. brasiliensis* 18/PADG_00097.1 | –                 | –                      | No             |
| SidF         | N^5-transacylases  | *P. brasiliensis* 01/PAAG_01680.1 | AlcB domain       | –                      | No             |
|             |                   | *P. brasiliensis* 03/PABG_03728.1 | –                 | –                      | No             |
|             |                   | *P. brasiliensis* 18/PADG_00100.1 | –                 | –                      | No             |
| SidC         | Non-ribosomal      | *P. brasiliensis* 01/PAAG_08527.1 | Adenylation domain | –                      | No             |
|             | peptide synthetase | *P. brasiliensis* 03/PABG_04670.1 | Peptidyl carrier domain | –                      | No             |
|             |                   | *P. brasiliensis* 18/PADG_05295.1 | Condensation domain | –                      | No             |
| SidD         | Non-ribosomal      | *P. brasiliensis* 01/PAAG_01679.1 | Adenylation domain | –                      | Yes            |
|             | peptide synthetase | *P. brasiliensis* 03/PABG_03726.1 | Peptidyl carrier domain | –                      | No             |
|             |                   | *P. brasiliensis* 18/PADG_00102.1 | –                 | –                      | No             |
|             |                   | *C. gattii/CNBG_2041.2 | –                 | –                      | No             |
|             |                   | *C. neoformans/CNAG_03588.2 | Condensation domain | –                      | No             |
| SidG         | N^5-transacylase   | *C. neoformans/CNAG_04355.2 | MYND-type zinc finger domains | –                      | No             |
|             |                   | *C. gattii/CNBG_2703.2 | Acetyltransferase domain | –                      | No             |
| Sit1/Am3     | Siderophore        | *P. brasiliensis* 01/PAAG_06516.1 | MFS1 domain       | 12                     | No             |
|             | transporter        | *P. brasiliensis* 03/PABG_02063.1 | –                 | 14                     | No             |
|             |                   | *P. brasiliensis* 18/PADG_00462.1 | –                 | 14                     | No             |
|             |                   | *C. neoformans/CNAG_00815.2 | –                 | 13                     | No             |
|             |                   | *C. gattii/CNBG_1123.2 | –                 | 13                     | No             |
| MirA         | Siderophore        | *C. neoformans/CNAG_02083.2 | MFS1 domain       | 12                     | No             |
|             | transporter        | *C. gattii/CNBG_5232.2 | –                 | 11                     | No             |
| MirB         | Siderophore        | *P. brasiliensis* 01/PAAG_01685.1 | MFS1 domain       | 14                     | No             |
|             | transporter        | *P. brasiliensis* 03/PABG_03732.1 | –                 | 14                     | No             |
|             |                   | *P. brasiliensis* 18/PADG_00095.1 | –                 | 14                     | No             |
|             |                   | *C. neoformans/CNAG_07751.2 | –                 | 14                     | No             |
|             |                   | *C. gattii/CNBG_2036.2 | –                 | 14                     | No             |
| MirC         | Siderophore        | *P. brasiliensis* 01/PAAG_02233.1 | MFS1 domain       | 8                      | No             |
|             | transporter        | *P. brasiliensis* 03/PABG_04747.1 | –                 | 12                     | No             |
|             |                   | *P. brasiliensis* 18/PADG_05373.1 | –                 | 12                     | No             |
|             |                   | *C. neoformans/CNAG_07519.2 | –                 | 10                     | No             |
|             |                   | *C. gattii/CNBG_10872 | –                 | 14                     | Yes            |

*Amino acid sequence analysis was performed using the online software SMART.
†Accession numbers: PAAG refers to Pb01; PABG refers to Pb03; PADG refers to Pb18; CNAG refers to C. neoformans var. grubii and CNBG refers to C. gattii.