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The homeostasis of iron, copper, and zinc in *Paracoccidioides brasiliensis*, *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 brasiliensis*, *Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii*. Although prior studies have identified certain aspects of metal regulation in *Cryptococcus* species, little is known regarding the regulation of these elements in *P. brasiliensis*. We also present amino acid sequences analyses of deduced proteins in order to examine possible conserved domains. The genomic data reveals, for the first time, genes associated to iron, copper, and zinc assimilation and homeostasis in *P. brasiliensis*. Furthermore, analyses of the three fungal species identified homologs to genes associated with high-affinity uptake systems, vacuolar and mitochondrial iron storage, copper uptake and reduction, and zinc assimilation. However, homologs to genes involved in siderophore production were only found in *P. brasiliensis*. Interestingly, *in silico* analysis of the genomes of *P. brasiliensis Pb*01, *Pb*03, and *Pb*18 revealed significant differences in the presence and/or number of genes involved in metal homeostasis, such as in genes related to iron reduction and oxidation. The broad analyses of the genomes of *P. brasiliensis, C. neoformans* var. *grubii*, and *C. gattii* for genes involved in metal homeostasis provide important groundwork for numerous interesting future areas of investigation that are required in order to validate and explore the function of the identified genes and gene pathways.

Keywords: micronutrient homeostasis, pathogenic fungi, infection

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

RESULTS AND DISCUSSION

IRON

Uptake of iron at the cell surface by the reductive system

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

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

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

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

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

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

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

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

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

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

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

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

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

(Continued)

Table 1 | Continued

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

(Continued)

Table 1 | Continued

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

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

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

Conserved domains in proteins related to the reductive iron metabolism

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

Siderophore production

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

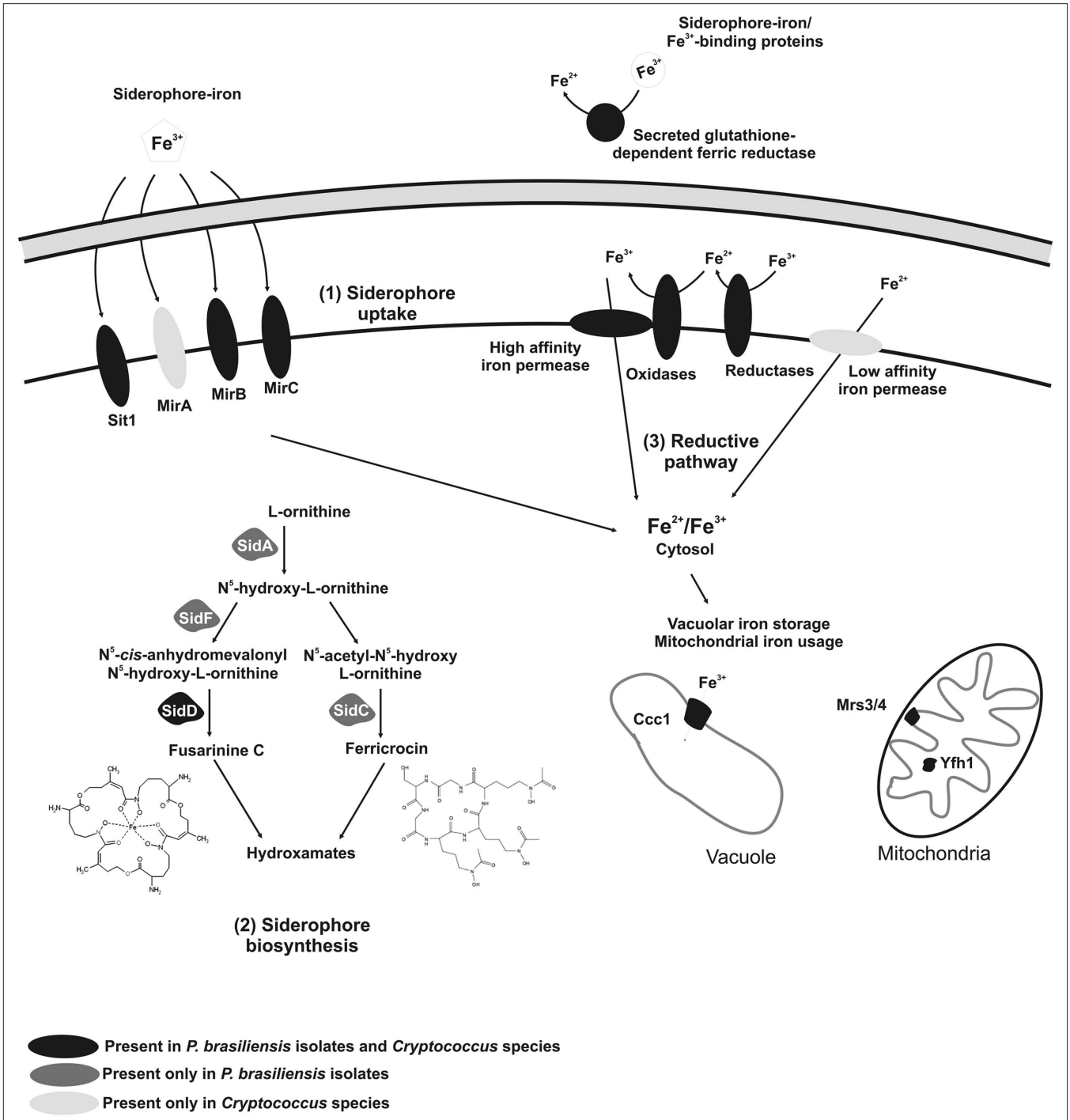

FIGURE 1 | Schematic comparison of iron metabolism in *P. brasiliensis* isolates and *Cryptococcus* species. Sit1p, MirAp, MirBp and MirCp are membrane transporters that traffic siderophores bound to ferric iron into the intracellular environment. SidAp, SidFp, SidCp and SidDp are enzymes from the biosynthetic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

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

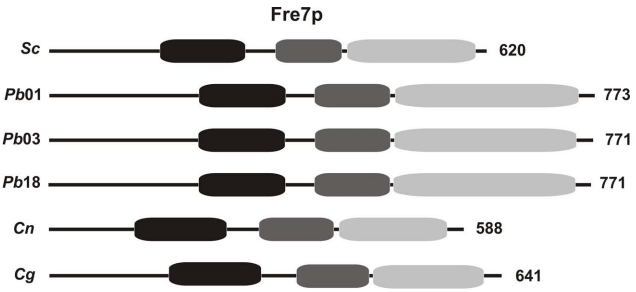

FIGURE 2 | Domains found in a Frep related to iron reductive uptake in *P. brasiliensis* isolates and *Cryptococcus* species. The found domains are: ferric reductase domain (black boxes), FAD-binding domain (dark gray boxes) and NAD-binding domain (light gray boxes). The length of each protein, in amino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

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

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

Conserved domains in proteins related to siderophore biosynthesis

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

Siderophore uptake

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

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

Analysis of transmembrane domains in siderophore–iron transporters

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

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

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

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

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

Iron source preferences

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

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

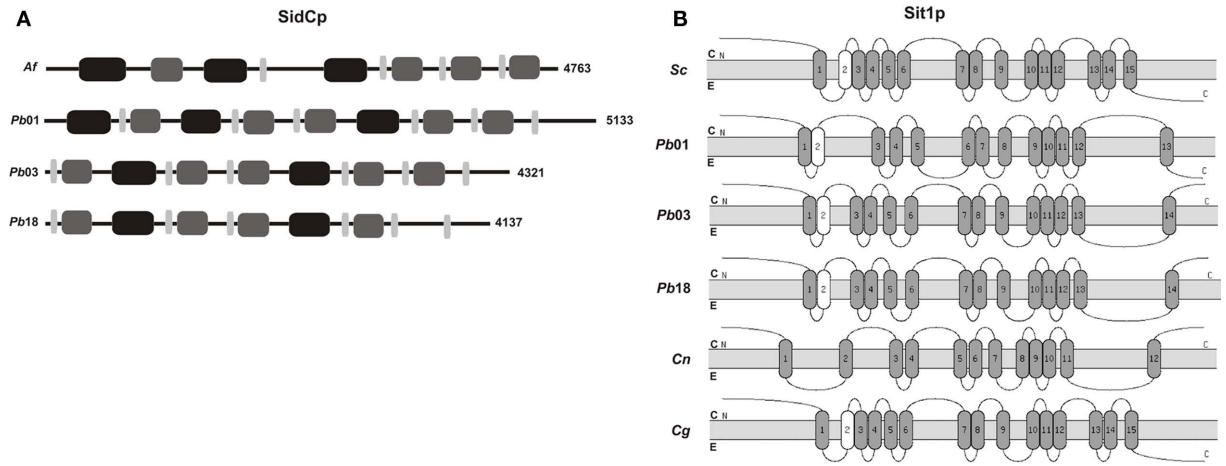

FIGURE 3 | Conserved features in proteins involved in biosynthesis and uptake of siderophores. (A) Modular organization of SidCp in *P. brasiliensis* isolates. Black boxes represent adenylation (A) domain, dark gray boxes illustrate the condensation (C) domain and light gray boxes represent the peptidyl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

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

COPPER

Copper uptake by the reductive system

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

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

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

phage, sensitivity to copper starvation and defects in polysaccharide capsule formation and melanization (Chun and Madhani, 2010). The gene *ctr1* for the high-affinity copper transporter of the plasma membrane and the genes *cup1* and *cup2* for metallothioneins were not found in *Cryptococcus* species. These analyses suggest that the high-affinity copper transport in cryptococcal cells is primarily performed by the protein encoded by *ctr3*.

Analysis of conserved motifs present in copper transporters

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

ZINC

Zinc uptake

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

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

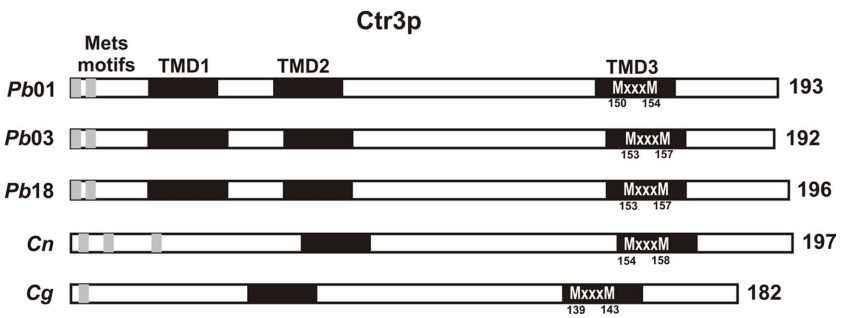

FIGURE 4 | Conserved features found in the primary structure of Ctr3p of *P. brasiliensis* isolates and *Cryptococcus* species. Ctr3p from *P. brasiliensis* isolates contains three putative transmembrane domains (TMD1-3, shown in black) while Ctr3p from *Cryptococcus* species presents only two TMDs.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

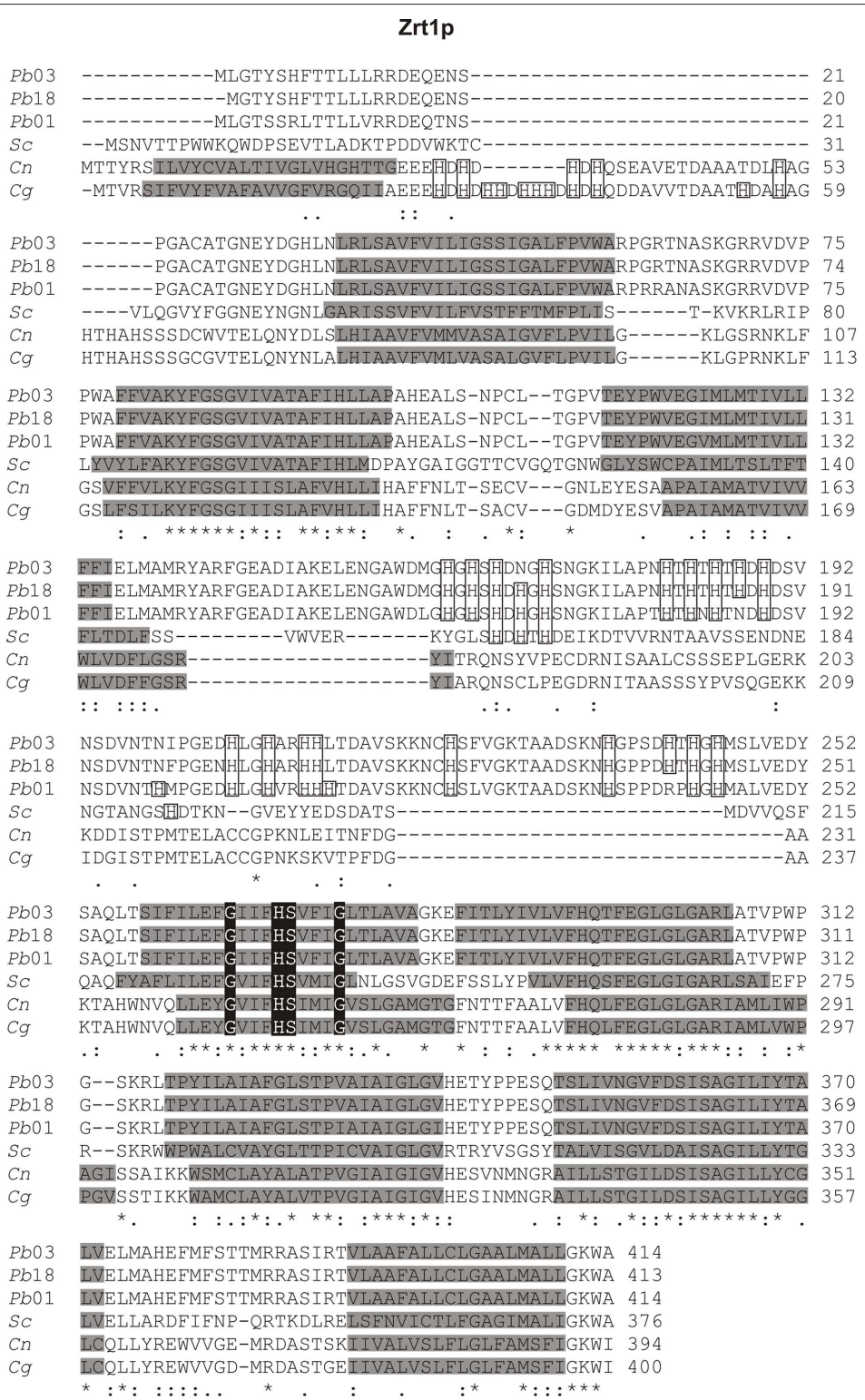

FIGURE 5 | Alignment of amino acid sequences of Zrt1p from *S. cerevisiae*, *P. brasiliensis* isolates and *Cryptococcus* species. The predicted transmembrane domains are shown in gray boxes. The black boxes inside the transmembrane segment contain conserved histidine-serine and glycine residues. The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

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

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

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

CONCLUSION

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

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APPENDIX

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

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

(Continued)

Table A1 | Continued

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

(Continued)

Table A1 | Continued

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

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

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

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

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

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

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