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Transporters in the *Paracoccidioides brasiliensis* transcriptome: insights on drug resistance

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ABSTRACT. In the struggle for life, the capacity of microorganisms to synthesize and secrete toxic compounds (inhibiting competitors) plays an important role in successful survival of these species. This ability must come together with the capability of being unaffected by these same compounds. Several mechanisms are thought to avoid the toxic effects. One of them is toxin extrusion from the intracellular environment to the outside vicinity, using special transmembrane proteins, referred to as transporters. These proteins are also important for other reasons, since most of them are involved in nutrient uptake and cellular excretion. In cancer cells and in pathogens, and particularly in fungi, some of these proteins have been pointed out as responsible for an important phenotype known as multidrug resistance (MDR). In the present study, we tried to identify in the *Paracoccidioides brasiliensis* transcriptome, transporter-ortholog genes from the two major classes: ATP binding cassette and major facilitator superfamily transporter. We found 22 groups with good similarity with other fungal ATP binding cassette

transporters, and four *Paracoccidioides brasilienses* assembled expressed sequence tags that probably code for major facilitator superfamily proteins. We also focused on fungicide resistance orthologs already characterized in other pathogenic fungi. We were able to find homologs to *C. albicans* CDR1, CDR2, and MDR1, *Saccharomyces cerevisiae* PDR5 and *Aspergillus* AtrF genes, all of them related to azole resistance. As current treatment for paracoccidioidomycosis mainly uses azole derivatives, the presence of these genes can be postulated to play a similar role in *P. brasiliensis*, warning us for the possibility of resistant isolate emergence.

Key words: Drug resistance, *Paracoccidioides brasiliensis*, ATP binding cassette transporters, Major facilitator superfamily

INTRODUCTION

Paracoccidioides brasiliensis is a fungus which shows a natural dimorphism that can be induced *in vitro* by changing the growth temperature: at 26°C it is found as mycelium form, and at 36-37°C, it presents yeast cells (San Blas et al., 1982). This fungus is a human pathogen, being the etiologic agent of paracoccidioidomycosis (PCM), a serious systemic human disease. This is the most prevalent systemic mycosis in Latin America, where it mainly affects rural workers and immunocompromised patients (San Blas, 1993). This disease is acquired by spore (or conidia) or mycelium fragment inhalation; infection is facilitated by host epithelial lesions (Restrepo and Jimenez, 1988).

The pathogenesis degree varies according to host features and infecting lineage virulence. The immune response of the hosts against *P. brasiliensis* depends on factors such as sex, age, nutritional state, and genetic inheritance. PCM can be restricted to the respiratory tract or become disseminated throughout the organism, becoming lethal (Franco et al., 1987). Thus, the forms of PCM can be divided into two groups: PCM infection, which is generally self-limited and restricted to the site of contact with fungi fragments or to a single organ, affecting both sexes indistinctly, and PCM disease, which preferentially attacks males and can evolve benignly to a PCM infection or disseminate systemically, causing severe damage to the host.

PCM treatment lasts up to five years and basically uses sulfonamides, azoles and amphotericin B, with good cure rates (Hahn et al., 2002). Although not yet described for *P. brasiliensis*, the isolation of fungi resistant to these fungicides is becoming frequent (Del Sorbo et al., 2000). Especially against azoles, transmembrane proteins that can function as efflux pumps, avoiding intracellular drug concentration, give this resistant phenotype. As these are mainly microorganisms closely related to *P. brasiliensis*, in this study we searched the *P. brasiliensis* transcriptome for orthologs related to this function. The assigned PbAESTs, which are the *P. brasilienses* assembled ESTs (expressed sequence tags), singlets and contigs are listed all over this study. The search was done focusing on gene orthologs to those already described as related to resistance, to look for targets for controlling fungus growth.

TRANSPORTERS

Transport is one of the most important and fascinating aspects of life and is an essential requirement for all organisms. Transport systems serve the cell in many ways, allowing the entry of all essential nutrients and ions, providing a means to regulate metabolic concentrations by catalyzing excretion products and deleterious substances of metabolic pathways, from organelles to cells. This permits the communication between cells, making it an important component of cell-environment relationships (Pao et al., 1998).

Biological membranes probably appeared very early during evolution, in order to isolate hydrophilic compounds from the surrounding medium, facilitating catalyzed reactions in an efficient manner. Biomembranes constitute efficient barriers against hydrophilic molecules, most of which can penetrate cells only by specific inward transport systems, or their entry is restricted to the endocytic pathway (Van Bambeke et al., 2000).

Transport by diffusion is possible only down a concentration gradient, and it is limited to solutes that are able to bypass hydrophobic membranes. Therefore, membrane-bound proteins with specialized transport functions mediate the transport of most compounds through the membrane. Ion channels are membrane complexes mediating the movement of ions across plasma membranes as well as membranes of cell organelles. These channels form a pore, allowing the passive flux of ions down an electrochemical gradient. Opening of these channels is generally gated. This means that the opening is regulated by changes in membrane potential or membrane stretching, or through the binding of a ligand. Ion channels play a role in diverse functions, such as osmoregulation, cell growth, development, and nutrient uptake (Andrade et al., 1999). In contrast to ion channels, facilitators or carriers bind molecules that are to be transported, and they undergo a reversible change in conformation during transport. The transporter must couple the carrier process to another energy-producing process. If energy expenditure is coupled to transmembrane solute translocation, this catalytic system can become an active transporter that facilitates the diffusion.

Active transporters (or porters) can function by uniport, symport, or antiport mechanisms (Figure 1). Uniporters, also called single-species transporters, or facilitated diffusion carriers, catalyze the transport of a single molecular species and transport therefore occurs independently of the movement of other molecular species (Figure 1C). Symporters, also classically called co-transporters, catalyze the transport of two or more molecular species in the same direction (Figure 1B). Antiporters, also called countertransporters, catalyze the exchange of one or more molecular species for another (Figure 1A). Antiport processes can be subdivided into two categories: antiport of solute-solute transport and antiport of solute-cation transport (Dahl et al., 2004).

These transport proteins play important physiological roles in different molecules, transporting such as amino acids, ions, sugars, lipids (pheromones, alarmones, hormones), co-factors, and other substrates that are essential for biological systems, across biological membrane structures.

On the basis of energy source and structural relationships, these allover spread active transport systems can be divided into two major classes: primary active transporters and electrochemical potential-driven transporters - secondary active transporters (Figure 2).

Transporters that use various forms of energy compose the primary active transport system. Transporters belonging to this system usually couple with ATP hydrolysis as they trans-

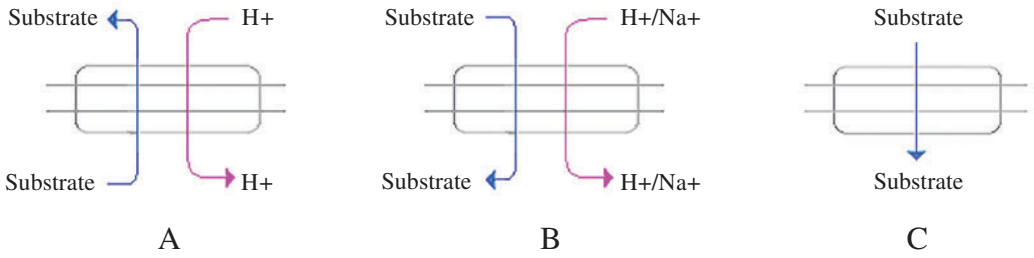

Figure 1. Mechanisms of translocation across the cytoplasmatic membrane. **A**, Antiport; **B**, symport and **C**, uniport. The translocation systems are represented by squares.

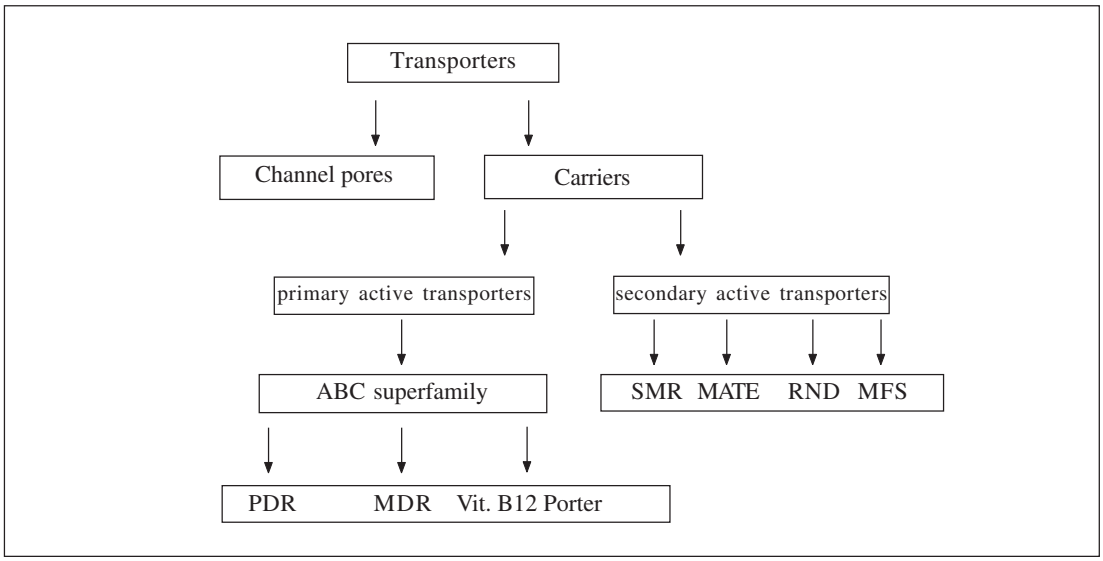

Figure 2. The scheme illustrates the currently classification of transporters, according to Saier Jr. (2000). The transporters are agruped in so-called superfamilies, families and subfamilies. ABC = ATP binding cassette; PDR = pleiotropic drug resistance; MDR = multidrug resistance; SMR = small multidrug resistance; MATE = multidrug and toxic compound extrusion; RND = resistance-nodulation-cell division; MFS = major facilitator superfamily.

locate metabolites across the membranes. The hydrolysis provides the energy to transport solutes against an electrochemical gradient. Two families of ATP-utilizing transporters have been described: the P-type ATPases, which make up a large superfamily of ATP-driven pumps involved in transmembrane transport of charged substrates, and the ATP binding cassette (ABC) transporters (Andre, 1995). We examined this latter group in detailed, since they usually play the most important role in drug efflux in eukaryotic cells (Lage, 2003).

ABC transporters

The diversity in substrate specificity is reflected in the diversity of physiological roles played by ABC transporters in the cell. ABC transporters are found in all taxa and form one of the largest transporter superfamilies, which contains both uptake and efflux transport systems (Saier Jr., 2000). The chemical nature of the substrates handled by ABC transporters is extremely diverse, ranging from inorganic ions to sugars and large polypeptides.

The best characterized S*accharomyces cerevisiae* ABC transporters are those involved in multidrug (MDR) or pleiotropic drug resistance (PDR), which results in simultaneous resistance to a number of unrelated compounds. The overexpression of certain ABC transporters is the most frequent cause of resistance to cytotoxic agents, including antibiotics, antifungals, herbicides, and anticancer drugs (Higgins, 2001). Several ABC transporter genes have been detected and characterized in filamentous fungi, such as *Penicillium digitatum* (Nakaune et al., 1998), *Magnaporthe grisea* (Urban et al., 1999), *Mycosphaerella graminicola* (Zwiers and De Waard, 2000), *Botrytis cinerea* (Schoonbeek et al., 2001), other phytopathogenic fungi (Lee et al., 2001) and *Aspergillus* species (Del Sorbo et al., 1997; Tobin et al., 1997; Andrade et al., 2000a,b).

All ABC transporters have the same basic molecular architecture (Higgins, 1995). It is important to distinguish ABC transporter proteins from ABC proteins. Both types of proteins are defined by the presence of a highly conserved approximately 215-amino acid consensus sequence, designated as ABC, ABC domain, ABC-ATPase domain, or nucleotide binding domain (NBD). This domain contains two short peptide motifs, a glycine-rich (Walker A) and a hydrophobic motif (Walker B), both involved in ATP binding, and it is commonly present in all nucleotide-binding proteins (Walker et al., 1982). A third consensus sequence is named ABC signature and is unique in ABC domains, being found in transporters (Hyde et al., 1990; Croop, 1993).

ABC-containing proteins couple the phosphate bond energy of ATP hydrolysis to many cellular processes and are not necessarily restricted to transport functions. However, the proper meaning of the term ABC transporter protein is also designated as traffic ATPase or permease for import systems. This is satisfied when the ABC proteins are also associated with a hydrophobic, membrane-embedded transmembrane domain (TMD), usually composed of at least six transmembrane α-helixes. TMDs are also designated as membrane-spanning domains that are believed to determine the ABC transporter protein substrate specificity. Some of the predicted membrane-spanning α-helixes may not be crucial to transport, but rather can be engaged in auxiliary functions, such as membrane insertion or regulation (Higgins, 2001).

The minimal structural requirement for a biologically active ABC transporter seems to be two TMDs and two ABCs $[TMD\text{-}NBD]_2$ (Ambudkar et al., 1992). In mammals, the functionally active ABC proteins consist of at least four such domains, $[TMD\text{-}NBD]_2$. In some ABC transporter encoding genes, the different domains may be present within one polypeptide chain (full-transporters), or within two separate proteins (half-transporters) that are assembled in the membrane as structural subunits (Sarkadi et al., 2004). Some subfamilies of fungal ABC transporters are described as "half-sized" transporters; these are thought to be functional after assembly as homodimers or heterodimers (Del Sorbo et al., 2000).

We found 22 groups (including contigs and singlets) annotated as ABC transporters in the *P. brasiliensis* transcriptome. Their predicted proteins were also analyzed, by searching for INTERPRO ABC classical motifs (www.ebi.ac.uk/InterProScan). The results showed that 11 of the 22 ABC annotated *P. brasiliensis* groups have one of those motifs: ABC transporter domain (INTERPRO - IPR003439) or P-loop ATP/GTP-binding site motif A (INTERPRO - IPR001687) (Table 1).

We also used the *S. cerevisiae* MIPS database to search for any transporters related to fungal defense mechanisms (Table 2). This table includes major facilitator superfamily (MFS), PDR and other ABC transporters. Among these, we have highlighted multidrug phenotype ortholog genes, based on MIPS description. The listed PbAESTs represent paralogs of those

Table 1. ABC transporters found in the *Paracoccidioides brasiliensis* transcriptome.

PbAEST	Best hit organism	e-value	INTERPRO ABC transporter domain	INTERPRO P-loop ATP/GTP-binding site motif A	Other INTERPRO domains	Remarks
1454	*L. tropica*	1e-35	IPR003439	IPR001687		ABC-type MDR system, ATPase component
255	*S. cerevisiae*	e-164	IPR003439	IPR001687		Killer toxin resistant; Kre30p
2585	*A. fumigatus*	0	IPR003439	IPR001687		MDR protein 1
393	*A. fumigatus*	2e-36				ABC transporter PDR12
687	*S. pombe*	2e-59				ABC transporter possibly mitochondrial ATM1
689	*A. fumigatus*	1e-80				ABC transporter Pdr5p
841	*S. pombe*	1e-60				ATP-dependent transporter CAF16
2741	*S. cerevisiae*	4e-40				ATP-dependent permease ADP1
2883	*V. inaequalis*	1e-73	IPR003439			ABC transporter (ABC1) PDR15
2906	*S. pombe*	3e-64	IPR003439	IPR001687		ATPase components of ABC transporters [Spo]
3123	*E. nidulans*	1e-29		IPR001687		ABC multidrug transport protein ATRC, MDL2
3428	*M. musculus*	3e-14				ABC transporter MDL1
4517	*A. thaliana*	8e-31				ABC, subfamily G
4798	*S. pombe*	6e-44	IPR003439			Heavy metal tolerance protein precursor
4680	*E. nidulans*	1e-79	IPR003439			MDR protein
4933	*S. cerevisiae*	1e-13		IPR001687	IPR006209	Homolog to ATP-dependent permeases; Adp1p, contains EGF-like domain
4861	*N. crassa*	1e-77		IPR001687		ABC transporter, peroxisomal, long-chain fatty acid import
5041	*T. rubrum*	4e-41				MDR protein STE6
5285	*B. fuckeliana*	3e-58				ABC transporter-like protein
5352	*A. fumigatus*	7e-98	IPR003439			MDR protein 2
5902	*E. coli*	2e-86				ATP-binding transport protein homologous to *S. cerevisiae* MDL1p

ABC = ATP binding site; PbAEST = *P. brasiliensis* assembled expressed sequence tag; MDR = multidrug resistance.

Table 2. MIPS transporter-related genes found in the *Paracoccidioides brasiliensis* transcriptome.

PbAEST	*Saccharomyces cerevisiae*		*Candida albicans*		*Neurospora crassa*		*Homo sapiens*		Remarks
	MIPS (gene name)[Φ]	e-value	ORF code*	e-value	AC number	e-value	AC number	e-value	
PbAEST 2763	YMR088c[•]	2e-06	CA1322	1e-06	1nc550_030	2.9e-09	NP_004769.1	6e-04	MDR similar ATPase, MFS (*S. cerevisiae*); related MDR protein fnx1 (*N. crassa*); related to putative multidrug transporter Mfs1.1 (*N. crassa*)
PbAEST 5813		3e-18	CA3253	6e-16	b11e5_230	9.3e-18	-	-	
PbAEST 3553	YHR048w[•]	4e-21	CA4882	2e-27	6nc360_420	1.1e-32	-	-	
PbAEST 393	YDR011w (SNQ2)	4e-21	CA0608	2e-19	1nc100_090	1.9e-34	AAG52982.1	2e-08	ABC transporter involved MDR, PDR (*S. cerevisiae*); *C. albicans* CDR1 homologue
PbAEST 1454	YOL075c[•]	8e-09	CA3828	9e-11	CAD79694.1	1.2e-82	AAC05632.1	2e-37	ABC protein (*S. cerevisiae*); *C. albicans* CaSNQ2
PbAEST 2883	YOR153w (PDR5)	1e-61	CA3892	4e-66	6nc360_520	6.9e-60	AAC97367.1	8e-16	ABC transporter involved in MDR, PDR (*S. cerevisiae*); *C. albicans* CDR4; *H. sapiens* breast cancer resistance protein
PbAEST 689		1e-53	CA3892	3e-54	6nc360_520	5.6e-55	AAL06598.1	6e-20	
PbAEST 3123	YLR188w (MDL1)	4e-20	CA2382	9e-16	xnc081_270	6.7e-25	O95342lAB11	2e-21	Mitochondrial ABC transporter (*S. cerevisiae*); CaMDL1; MDR protein 1 (*Aspergillus fumigatus*); multidrug transport protein ATRC (*Emericella nidulans*); MDR protein 1 (*A. nidulans*)
PbAEST 2585		2e-57	CA2384	6e-58	xnc081_270	4.0e-159	NP_000918.1	e-100	
PbAEST 687	YMR301c (ATM1)	8e-61	CA0931	1e-52	2nc610_370	1e-69	NP_004290.1	5e-51	Mitochondrial ABC transporter (*S. cerevisiae*)
PbAEST 4798		7e-30	CA0931	1e-20	3nc400_080	4.0e-68	AAG33617.1	1e-42	
PbAEST 4680	YPL270w (MDL2)	9e-29	CA2382	2e-26	xnc081_270	7.2e-68	BAA92038.1	7e-51	
PbAEST 5352		3e-59	CA2384	2e-51	94c8_130	7.0e-82	NP_036221.1	2e-54	
PbAEST 4861	YKL188w (PXA2)	5e-30	CA4990	2e-37	b17c10_260	5.4e-75	JC5712	6e-51	ABC transporter of long-chain fatty-acids import into peroxisomes
PbAEST 5041	YKL209C (STE6)	0.016	CA5727	0.54	xnc081_270	1.5e-36	AAA59575.1	1e-18	ABC transporter (*S. cerevisiae*), *Trichophyton rubrum* MDR phenotype (nr); *H. sapiens* P-glycoprotein
PbAEST 255	YER036c (KRE30)	1e-167	CA3733	1e-171	b24g20_030	1.5e-168	AAH06323.1	5e-51	ABC transporter
PbAEST 4517	YCR011c (ADP1)	6e-11	CA4390	5e-86	3nc442_040	5.8e-87	G02068	3e-14	ABC transporter - similarity to Pdr12p, Cdr1p, Pdr10p, Pdr15p, and Pdr5p (*S. cerevisiae*)
PbAEST 4176	YIL048W (NEO1)	5e-56	CA2277	1e-54	NE7566	8e-67	NM_198531	8e-61	ATPase that leads to neomycin-resistance (*S. cerevisiae*, *N. crassa*); CaDRS21 membrane-spanning Ca-ATPase (*C. albicans*)

[Φ]Entry and gene names based on MIPS nomenclature; [•]No gene names on MIPS site; MIPS [mips.gsf.de/genre/proj/yeast/index.jsp].
PbAEST = *P. brasiliensis* assembled expressed sequence tag; MDR = multidrug resistance; MFS = major facilitador superfamily; ABC = ATP binding cassette; PDR = pleiotropic drug resistance.

genes. We also assigned PbAEST orthologs for sequences found in *C. albicans*, *N. crassa* and *Homo sapiens.*

Multidrug resistance transporters

Microorganisms, as well as cancer cells, may also exhibit a cross-resistant phenotype against several unrelated drugs that differ widely in molecular structure and target specificity. This phenotype has been termed MDR. Different types of MDR phenotypes have been described, and most drug efflux pumps confer an MDR phenotype, corresponding to the large variety of substrates that they may recognize, including several classes of antibiotics, as well as non-antibiotic drugs.

The first described multidrug efflux pump was mammalian P-glycoprotein, an ATP-driven pump that provides resistance to a broad spectrum of compounds, including anticancer chemotherapeutic agents (Ling, 1997; Ambudkar et al., 1999). In many cancers, P-glycoprotein is overexpressed, contributing to resistance to clinically important chemotherapeutic drugs that are P-glycoprotein substrates.

Pleiotropic drug resistance transporters

The PDR transporters share several biochemical features with the human P-glycoprotein (Kolaczkowski et al., 1996, 1998), and they constitute the main class of ABC-drug-efflux pumps in yeasts and fungi. The first and, until now, best-characterized yeast PDR transporter is the product of the PDR5 gene. Its promoter region presents a pleiotropic drug-responsive element, the binding site of the transcriptional activators Pdr1p and Pdr3p that control transcription of PDR5 and other drug-resistance-related genes (Balzi and Goffeau, 1995). We were able to identify two PbAESTs (a singlet and a contig) with a high degree of homology with the *S. cerevisiae* PDR5 gene (Table 2); however, no PDR1 or 3 was found. The protein Pdr5p has been shown to share nucleotide triphosphatase activities, as well as substrates and modulators, with the human MDR1-P-glycoprotein (Kolaczkowski et al., 1998; Conseil et al., 2001; Rogers et al., 2001). The predicted topography of Pdr5p comprises two hydrophobic domains, each composed of six transmembrane segments (TMS_6), and two cytoplasmic NBD, showing the structure named $(TMS_6\text{-NBD})_2$ "full-transporter" (Klein et al., 1999; Dassa and Bouige, 2001). Each half-Pdr5p starts with an NH_2-terminal NBD, followed by the first TMS_6 tract, whereas in P-glycoprotein the TMS_6 tracts precede the NBD. Thus, despite similar mechanisms of substrate recognition and transport, the significance of such domain inversion in yeast ABC transporters is unknown. This is mainly related to the lack of structural information on yeast ABC transporters, when compared with mammalian full-transporters (Ferreira-Pereira et al., 2003).

Non-ATP binding cassette transporters

The secondary-active transport systems mediate the drug efflux reaction in a coupled exchange with protons or sodium ions along a concentration gradient, as symport or antiport translocation systems. Members of the secondary transporters are the small multidrug resistance family (SMR), the multidrug and toxic compound extrusion family (MATE), the resistance-nodulation-cell division family (RND), and the MFS (Figure 2). Among these, SMR, MATE

and RND are widespread in bacteria and barely represented in the fungi. The opposite situation is observed to MFS, which is rarely found in bacteria and is often present among fungi (Murakami and Yamaguchi, 2003).

The SMR transporters are normally composed of around 100 amino acids that appear as four helixes (Paulsen et al., 1996). Some SMR family members have not been found to exhibit an MDR phenotype, in spite of extensive studies (Mordoch et al., 1999). The MATE transporters are typically composed of approximately 450 amino acids, arranged into 12 helixes. This novel family was identified only quite recently, with a characterization of NorM, a multidrug Na^+-antiporter from *Vibrio parahaemolyticus*, which confers resistance to dyes, fluoroquinolones and aminoglycosides (Borges-Walmsley et al., 2003). The RND family is composed of approximately 1000-amino acid residues (Tseng et al., 2003). They are predicted to adopt a 12-helical structure and possess large periplasmatic or extracytoplasmatic domains between helixes 1 and 2 and between helixes 7 and 8 (Murakami et al., 2002; Mao et al., 2002; Elkins and Nikaido, 2003).

Major facilitador superfamily transporters

MFS includes more than 1,000 evolutionarily related proteins, and it is implicated in the transport of a variety of solutes and metabolites across the membranes of organisms, ranging from bacteria to humans (Busch and Saier Jr., 2002). MFS-motivated transport accross membranes is driven by the proton-motive force, which is composed of membrane potential and electrochemical proton gradients; consequently, MFS transporters (Figure 2) are termed as secondary active transport systems (Lewis, 1994). Unlike ABC transporters, MFS transporters have no characteristic signature. They are around 500-amino acid residues in length and show an RND-like 12-helix structure, although with smaller extra- or intra-cellular domains. More than 350 uniporters, symporters, and antiporters of sugars, peptides, drugs, organic, and inorganic ions fall within this superfamily (Pao et al*.,* 1998). These authors have also identified a 13-residue consensus motif between the transmembrane spans 2 and 3. Meaningful sequence homology occurs among all members of this superfamily. Toxin export by MFS is also associated with virulence in plant pathogens (Del Sorbo et al, 2000). In our transcriptome study, we were able to annotate four PbAEST as MFS. They are homologs to two well-characterized MFS in fungi (Table 2). PbAESTs 2763 and 5813 correspond to the YMR088c MIPS gene, which is postulated to code an *S. cerevisiae* MFS, and are also homologous to a similar *N. crassa* protein. PbAESTs 3000 and 3553 seem to be homologous to YHR048w from *S. cerevisiae* and *Schizosaccharomyces pombe* MFS genes.

ANTIFUNGAL RESISTANCE

Systemic fungal infections are a big problem for clinicians; mucosal and invasive opportunistic fungal infections have increased during the past two decades. This is a consequence of the rising number of immunocompromised hosts, such as HIV-infected individuals, transplant recipients, and patients submitted to immunosuppressive therapies or broad-spectrum antibiotics. Another factor that contributes to the severity of opportunistic infections is the development of resistance to antifungal agents (Lupetti et al., 2002). Over the past decade, we have seen the emergence of resistant isolates from different pathogenic fungi, including *Candida* spp, *Cryp-*

tococcus neoformans, and from some invasive molds, like *Aspergillus* spp and *Histoplasma capsulatum* (Alexander and Perfect, 1997; Wheat et al., 1997; Kontoyiannis and Lewis, 2002).

Two major classes of xenobiotic transporters are involved in drug resistance in fungi: ABC transporters and MFS transporters. In contrast to prokaryotic microorganisms, in fungi the ABC transporters comprise the largest number of membrane-spanning efflux pumps (Higgins, 1992). Thus, it was not surprising that the first described ABC transporter homolog to human P-glycoprotein from a non-mammalian system was that from *S. cerevisiae*. This transporter, consisting of 1290-amino acid residues, is designated as Ste6p and is encoded by the STE6 gene (McGrath and Varshavsky, 1989). Ste6p, exhibiting a $[TMD\text{-}NBD]_2$ configuration, is physiologically involved in the transport of the mating α-factor pheromone, but it has no role in drug resistance. However, among at least 29 putative ABC transporter encoding genes in *S. cerevisiae*, five of them encode ABC transporters - Pdr5p, Pdr12p, Snq2p, Ycf1p, and Yor1p. They mediate MDR phenotype when present in multiple copies (Decottignies and Goffeau, 1997). In our transcriptome we found an *S. cerevisiae* STE6-related PbAEST (5041) and although this group exhibits low homology with its *S. cerevisiae* counterpart, it shows a high degree of similarity with other fungi, STE 6 homologs (Table 2).

The drug resistance phenomenon in fungi is characterized by a failure of antifungal therapy to control a fungal infection (Alexander and Perfect, 1997), which is measured as an increase in minimum inhibitory concentration, when compared to values obtained for susceptible reference organisms (Sanglard and Odds, 2002). In a clinical context, whenever antifungal agents are used to combat fungal infections, the exposure of fungal pathogens to these drugs is therefore expected to give rise to resistant isolates from an initially susceptible population (Sanglard, 2002). The increasing number of fungal infections documented in hospitals around the world could favor the occurrence of this phenomenon, as the number of antifungal treatments becomes higher. The increased use of antifungal agents in recent years has led to the development of resistance to these drugs (Beck-Sague and Jarwis, 1993). Resistance of microbes to antimicrobial agents has potentially serious implications for the management of infections (Sanglard and Odds, 2002). Antifungals are grouped mainly into five groups, on the basis of their site of action: polyenes, azoles, flucytosine, candins, and allylamines (Balkis et al., 2002; Sanglard, 2002), but current treatment of systemic mycoses, including PCM, is mainly based on the use of polyenes (e.g., amphotericin B) and azoles, such as triazoles - e.g., itraconazole and fluconazole (Lupetti et al., 2002).

Molecular basis of drug resistance in fungi

Resistance to chemotherapy is a common clinical problem in patients with infectious diseases as well as in patients with cancer, since it causes a decrease in the chance for successful treatment. From bacterial cells, fungi, and protozoa, to the complexity of human cancer cells, resistance has become a challenge (White et al., 1998). Furthermore, antifungal resistance can be classified as primary or intrinsic, when it is present before exposure to antifungal agents, and secondary or acquired, which develops after exposure to antifungals (Kontoyiannis and Lewis, 2002). However, it will be dependent on the type of fungal pathogen to be treated and the type of antifungal agents applied (Sanglard, 2002).

The emergence of an MDR phenotype is still more problematic, and it can occur during treatment of infections or malignant tumors. This happens when the prokaryotic or eukaryotic

microorganism and neoplasic cell drug targets are non-reactive to a variety of drugs that have different structures and functions (Lage, 2003). Although this phenotype has not been documented for PCM, we found at least five PbAESTs in the *P. brasiliensis* transcriptome that show similarity with other fungal orthologs and, one that is similar to a protozoan ortholog thought to code for MDR-related proteins. Table 1 shows these PbAESTs and another (16) identified in the *P. brasiliensis* Genome Project database as ABC transporters.

Several types of molecular mechanisms contribute to drug resistance phenotype in eukaryotic cells. In general, the most frequent mechanisms that originate resistance include the increase of cellular target levels, the decrease of these cellular targets affinity for the drug, enzymatic drug inactivation or degradation, and upregulation of drug efflux control genes. Therefore, more than one of these mechanisms can happen simultaneously, causing different cellular alterations (White et al., 1998). The main fungicide drugs used in fungal infection therapy are listed in Table 3.

Flucytosine

Flucytosine (5-fluorocytosine) is a pyrimidine analog working as an antifungal agent through conversion to 5-fluorouracil, which can be so on incorporated into RNA, causing premature chain termination in addition to an inhibition of DNA synthesis due to its effects on thymidilate synthase. Target cells must possess the machinery necessary to make this conversion; this includes a cytosine permease to permit internalization of the drug, cytosine deaminase to convert it to 5-fluorouracil, and uracil phosphoribosyl transferase to convert 5-fluorouracil into a substrate for nucleic acid synthesis. Because most filamentous fungi do not produce the enzymes needed to metabolize flucytosine and are consequently not responsive to drug (Odds et al., 2003), the spectrum is restricted to pathogenic yeasts. Primary mechanisms, such as poor uptake of the drug by cytosine permease alterations, or secondary mechanisms, such as a decrease in the metabolism of flucytosine to toxic metabolites, are commonly found in resistance to flucytosine in *Candida* spp and *C. neoformans* (Whelan, 1987). We have not found a cytosine permease-related group in the *P. brasiliensis* transcriptome, but we have annotated two groups, PbAEST 20 and 4726 that are similar to cytosine deaminase. Another two groups, PbAESTs 466 and 1502, were identified as being uracil phosphorybosil transferase similar sequences.

Polyenes

This class of drugs plays important role in PCM treatments. Amphotericin B targets ergosterol, the major component in fungal membranes, which is also very important for a variety of cellular functions, such as the fluidity and integrity of the membrane. Ergosterol is also the main component of secretory vesicles in *S. cerevisiae*, and it has an important role in mitochondrial respiration. Alterations in membrane ergosterol content due to alterations in the ergosterol biosynthetic pathway seem to be the main mechanism involved in amphotericin B-induced resistance in *C. albicans* (Dick et al., 1980), *C. neoformans* (Perfect and Cox, 1999), and other emerging filamentous fungi and yeasts (Kontoyiannis and Lewis, 2002). Such modifications in sterol content are also found in *P. brasiliensis* strains exposed to amphotericin B (Hahn and Hamdan, 2000). Since resistance is relatively rare, and diminished ergosterol content seems to

Table 3. Mechanisms and action spectrum of major antifungal agents.

Antifungal	Mechanism of action	Spectrum/comments
Polyenes Amphotericin B Nystatin	Interaction with ergosterol and destabilization of cell membrane functions; cell death	Broad activity against *Candida* spp, *Cryptococcus neoformans* and filamentous fungi (except, of the *Aspergillus* spp, *A. terreus* and *A. flavus).* Used to control PCM
Pyrimidine analogue 5-fluorocytosine	Interferes with DNA/RNA synthesis	Activity against *Candida* spp, *Cryptococcus* spp. Rapid emergence of resistance when used as monotherapy
Azoles Ketoconazole Fluconazole Itraconazole Voriconazole Posaconazole	Inhibition of cytochrome P450 14α-lanosterol demethylase	Fluconazole is active against most *Candida* spp and *Cryptococcus* spp but has no activity against invasive molds. Other azoles such as itraconazole, voriconazole and posaconazole have improved activity against invasive molds. Itraconazole is frequently used in PCM patients
Echinocandins	Inhibition of cell-wall glucan synthesis, leading to susceptibility of fungal cell to osmotic lysis	Rapidly fungicidal against *Candida* spp and moderate against *Aspergillus* spp, including azole-resistant species. Poor activity against *C. neoformans*
Allylamines	Inhibition of squalene epoxidase	Activity against most dermatophytes, poor activity against *Candida* spp

PCM = paracoccidioidomycosis.

be evolutionary disfavored (Lupetti et al., 2002), problems arise mainly because of toxicity to mammalian cells more than because of drug resistance phenomena. Hence it is a fungicidal agent that is preferentially used in short-term treatments (Odds et al., 2003).

Azoles

Within different categories of antifungal drugs, the development of azole resistance is the most relevant medical problem. Treatment failures have been observed following the extensive use of fluconazole for *Candida* infection, frequently associated with relapses of oropharyngeal candidiasis in AIDS patients (Kelly et al., 1997). The major cellular target of azoles in yeasts and fungi is a cytochrome P450 (Erg11p), C14α-demethylase, involved in the ergosterol pathway. The resulting ergosterol depletion and accumulation of C14α-demethyl-sterols, such as lanosterol, interfere with ergosterol functions (Lupetti et al., 2002). Both effects result in growth arrest in most species, although, in some other species (*C. neoformans* and *A. fumicatus*), specific azoles, such as itraconazole, have a fungicidal effect (Klepser et al., 1998; Manavathu et al., 1999; Lass-Florl et al., 2001). In the *P. brasiliensis* transcriptome, we were able to find PbAEST 1797, whose predicted product shares 80% similar amino acids with *Aspergillus fumigatus* Erg11p.

Three major mechanisms are associated with secondary resistance in *C. albicans* by reducing azole accumulation: 1) over expressing the target Erg11p or altering the C14α-demethylase binding site, 2) mutations downstream of the ergosterol pathway (Erg5p and Erg3p) leading to the accumulation of less toxic sterols, and 3) increased drug efflux (Sanglard and Odds, 2002; Odds et al., 2003). Genes involved with efflux have been identified in *C. albicans*: *CDR1* (Prasad et al., 1995) and *CDR2* (Sanglard et al., 1997), which code for ABC transporters. A third gene is *MDR1* (Ben-Yaacov et al., 1994), which codes for a protein belonging to the MFS of transporters. This gene has also been assigned as a virulence gene fitting Falkow's postulate, which means that its disruption generates an attenuation phenotype that is reversed by its complementation. All of these gene orthologs could be assigned in the *P. brasiliensis* genome (Table 4).

Do Nascimento et al. (2002) have identified genes encoding ABC transporters that confer pleiotropic drug resistance, designated *abcA-D*, in *Aspergillus* isolates. One of these genes, *abcD*, was cloned and characterized. In our transcriptome analysis, we found an *abcA* homolog (Table 4). Besides the detection of upregulated ABC expression, other features contribute to the fungicide resistant phenotype in *C. albicans*, like the ability to form biofilms, which is associated with high fluconazole resistance (Mukherjee et al., 2003). These authors used a set of isogenic *Candida* strains lacking one or more of the drug efflux pumps, Cdr1p, Cdr2p, and Mdr1p, to determine their role in fluconazole resistance within the biofilm context. Additionally, variation in sterol profile as a possible mechanism of drug resistance was investigated. They concluded that parental and MDR mutant strains formed similar biofilms, indicating a lack of involvement of efflux pumps in resistance at late stages of biofilm formation. Indeed, biofilms formed by double and triple mutants were more susceptible to fluconazole than was the wild-type strain. Sterol analysis showed that ergosterol levels were significantly decreased at biofilm intermediate and mature phases, compared to those in early-phase. These studies suggest that other features, such as sterol content and phase-specific mechanisms, control fungal azole resistance. Other mechanisms that do not include transporter usage are described for various

Table 4. PbAEST-related transporter genes found in fungi other than *Saccharomyces cerevisiae* associated with drug resistance.

Gene	GenBank (accession number)	PbAEST	e-value	% Similarity	Remarks	References
Candida albicans						
CDR1	X77589	PbAEST 2883	5e-62	146/197 (74%)	ABC transporter; strongly involved in azole resistance	Prasad et al., 1995
CDR2	U63812	PbAEST 2883	3e-62	146/197 (74%)	ABC transporter; strongly involved in azole resistance	Sanglard et al., 1997
MDR1	CAA76194	PbAEST 3000	4e-24	111/195 (56%)	MFS multidrug efflux; related to cycloheximide resistance protein	Ben-Yaacov et al., 1994
		PbAEST 3553	2e-11	62/125 (49%)		
FLU1	AF188621	PbAEST 3553	4e-19	70/126 (55%)	MFS multidrug efflux transporter. Other names: CaMDR1 and Ben^r	Calabrese et al., 2000
Aspergillus nidulans						
AbcD	AF173826	PbAEST 2883	1e-59	102/142 (71%)	ABC transporters; pleiotropic drug resistance	Do Nascimento et al., 2002
AtrA	Z68904	PbAEST 2883	2e-76	102/127 (80%)	ABC transporters; pleiotropic drug resistance	Del Sorbo et al., 1997
AtrB	Z68905	PbAEST 689	6e-57	85/123 (69%)	ABC transporters; pleiotropic drug resistance	Del Sorbo et al., 1997
AtrC	AF082072	PbAEST 2585	1e-80	103/132 (78%)	ABC transporter protein; multidrug resistance protein	Angermayr et al., 1999
AtrD	AF071411	PbAEST 4680	4e-96	164/180 (91%)	ABC transporter protein; multidrug resistance protein	Andrade et al., 2000
AtrF	AJ309281	PbAEST 2883	1e-59	102/142 (71%)	ABC-type multidrug transport system	
Aspergillus fumigatus						
MDR1	U62934	PbAEST 4680	2e-92	158/177 (89%)	ABC-type multidrug transport system; multiple drug resistance	Tobin et al., 1997
MDR2	U62936	PbAEST 5352	e-110	108/114 (94%)	ABC-type multidrug transport system; multiple drug resistance	Tobin et al., 1997
MDR3	AF503774	PbAEST 5232	0.020	36/78 (46%)	Multiple resistance to itraconazole	Do Nascimento et al., 2003
MDR4	AF503773	PbAEST 2585	1e-82	249/441 (56%)	Multiple resistance to itraconazole	Do Nascimento et al., 2003
		PbAEST 5352	1e-39	242/467 (51%)		
AbcA	AJ417501	PbAEST 2883	2e-62	153/219 (69%)	ABC-type multidrug transport system, ATPase	Do Nascimiento et al., 1999
		PbAEST 689	3e-44	86/176 (48%)		
AtrF	AJ311940	PbAEST 2883	4e-67	86/185 (46%)	ABC-type multidrug transport system, ATPase	Slaven et al., 2002
		PbAEST 689	7e-53	175/302 (57%)		
Paracoccidioides brasiliensis						
Pfr1	AJ558104	PbAEST 5352	0.0	607/608 (99%)	ABC-type multidrug protein lipid transport system, induction up regulated as response to azoles in *P. brasiliensis*	Gray et al., 2003

PbAEST = *Paracoccidioides brasilienses* assembled expressed sequence tag; ABC = ATP binding cassette; MFS = major facilitador superfamily.

organisms and fungi, such as *C. neoformans* and *A. fumigatus* azole-resistant isolates, which overexpress P450 C14α-demethylase (Tobin et al., 1997; Perfect and Cox, 1999), and *H. capsulatum* isolates that show a decrease of ergosterol biosynthesis after exposure to fluconazole, which probably reduces the accumulation of toxic sterols (Wheat et al., 1997). Increasing content of Erg11p by gene amplification or upregulation of the corresponding gene was best investigated in *C. glabrata* (Marichal et al., 1997), and reduced affinity for azole is also described in *C. kruzei* (Sanglard, 2002). Because sequence alterations may be simply due to allelic variations, the involvement of mutations in azole resistance is difficult to be evaluated, although some reported mutations influencing bond interference with fluconazole have been investigated since Podust et al. (2001) obtained a C14α-demethylase crystal. Other mechanisms that not include transporter participation, such as overexpression of P450 C14α-demethylase (Tobin et al., 1997; Perfect and Cox, 1999) are described for *C. neoformans* and *A. fumigatus* azole-resistant isolates and for some *H. capsulatum* isolates that show a decrease in ergosterol biosynthesis after exposure to fluconazole, which probably reduces the accumulation of toxic sterols (Wheat et al., 1997).

Gray et al. (2003) described a gene in *P. brasiliensis* that encodes a half-ABC transporter, designated PFR1, an ABC-superfamily member, involved in multidrug resistance. The PFR1 gene is predicted to encode an 827-amino acid protein that, in common with mammalian MDR1, has a transmembane-NBD topology and seems to be a mitochondrial protein. The transcription of the PFR1 gene is induced by the triazole drug fluconazole, but not by amphotericin B, suggesting a role in transport-mediating azole resistance, and an ABC transporter is induced as part of the cellular response to drug treatment. We found a PFR1 singlet in our transcriptome (PbAEST 5352). This singlet is 616 nucleotides long, and it is identical to the yet described gene (Table 4). The promoter region of PFR1 contains a pleiotropic drug-responsive element-like consensus sequence, which could be the element responsible for up-regulation of PFR1 transcripts in response to fluconazole. The NBD of PFR1 was expressed and purified from *Escherichia coli*, and was shown to retain ATPase activity, consistent with PFR1 functioning as a homodimeric transport ATPase.

Paracoccidioidomycosis treatment

Paracoccidioides brasiliensis is a very sensitive organism when exposed to antifungal drugs, in contrast with other pathogenic fungi. Based on the *P. brasiliensis* sensibility profile, different therapeutic schedules are available for PCM treatment. Several classes of antifungal drugs have been widely employed for this disease, including the sulfonamides (sulfadiazine, sulfadoxine, sulfamethoxypyridazine, cotrimazine, and cotrimoxazole) as a maintenance treatment, amphotericin B, azole compounds (ketoconazole, fluconazole and itraconazole) and terbinafine. The cure rates achieved with these various drugs have ranged between 69 and 100%. Although comparative clinical trials in PCM are missing, it is believed nowadays that itraconazole is the drug of choice for treatment of most clinical forms of the disease (Telles, 2002).

Hahn and colleagues (2002) reported terbinafine activity against *P. brasiliensis in vitro*, thus suggesting that this allylamine can be considered a new option for PCM therapy. Currently, there are six new antifungal drugs in advanced stages of clinical investigation, including three new triazoles and three compounds belonging to a new class of antifungal drugs, the echinocandins that act by inhibiting cell wall synthesis. The second-generation triazoles include

ravuconazole, posaconazole and voriconazole. These compounds are very active *in vitro* and in animal models against *Cryptococcus* spp, *Aspergillus* spp, *Trichosporon* spp, and other endemic mycosis agents, including *P. brasiliensis*. Among the echinocandins, caspofungin and micafungin do not show *in vitro* activity against *P. brasiliensis*; therefore, they are not useful for treating PCM (Telles, 2002).

CONCLUDING REMARKS

In this study we were able to identify 22 PbAESTs related to ABC transporters and three other groups that code for two MFS. Although rarely reported for *P. brasiliensis*, emergence of resistant isolates to the usual antifungals is becoming frequent. Especially for azoles, efflux pumps play a major role in resistant phenotypes. A recent study showed that ketoconazole-resistant isolates could be found in PCM patients (Hanh et al., 2003). This result, taken together with the finding of drug resistance in closely related microorganisms and our findings concerning the *P. brasiliensis* transcriptome, lead us to suggest that there is a need to make efforts towards the control of potential drug resistance *P. brasiliensis*. This can be assumed based on the same principles for other pathogenic fungi, since similar resistance genes can be involved.

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