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Fungal PDR transporters: phylogeny, topology, motifs and function

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Abstract

The overexpression of pleiotropic drug resistance (PDR) efflux pumps of the ATP-binding cassette (ABC) transporter superfamily frequently correlates with multidrug resistance. Phylogenetic analysis of 349 full-size (~160 kDa) PDR proteins (Pdrps) from 55 fungal species, including major fungal pathogens, identified 9 separate protein clusters (A, B, C, D, E, F, G, H1a/H1b and H2). Fungal, plant and human ABCG-family Pdrps possess a nucleotide-binding domain [NBD] and a transmembrane domain [TMD] in a family-defining 'reverse' ABC transporter topology [NBD-TMD] that is duplicated [NBD-TMD]₂ in full-size fungal and plant Pdrps [NBD-TMD]₂. Although full-size Pdrps have similar halves indicating early gene duplication/fusion, they show asymmetry of their NBDs and extracellular loops (ELs). Members of cluster F are most symmetric and may be closely related to the evolutionary ancestor of Pdrps. Unique structural elements are predicted, new PDR-specific motifs identified, and the significance of these and other structural features discussed.

Keywords

ATP-binding cassette; ABC; pleiotropic drug resistance; PDR; multidrug resistance; MDR; azoles; PDR motif A; PDR motif B; antifungal

Introduction

Fungi cause significant morbidity and mortality in humans, particularly in the immunocompromised (Pfeller and Diekema 2007). They cause superficial infections of skin or mucosal surfaces and they can disseminate hematogenously. The azole antifungals are generally well tolerated and the second generation triazoles such as voriconazole (VCZ) are effective against a wider range of fungi than their predecessors (Odds et al. 2003; Shao et al. 2007; Pasqualotto and Denning 2008). Some fungi, such as *Candida glabrata* and *Candida krusei*, often show reduced susceptibility to azoles and resistance can be induced in *Candida albicans*, *Candida glabrata* and *Cryptococcus neoformans* (Kanafani and Perfect 2008). An important mechanism causing azole resistance is energy dependent drug efflux catalysed by

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membrane-located efflux pumps (Sanglard and Bille 2002) that usually belong to the PDR sub-family of ABC transporters. While the expression of Pdrps strongly correlates with azole resistance, the mechanisms of substrate binding and translocation, and the identity of the 'natural' substrates of these pumps are not known. Recent advances in whole genome sequencing have provided open reading frame sequences for entire repertoires of Pdrps from increasing numbers of fungi of industrial, agricultural and medical importance. This information permits the use of sequence comparison to identify residues and motifs conserved across species, and establishes evolutionary relationships among Pdrps. This review uses these approaches to identify features of Pdrps that may be important for function, including antifungal drug resistance.

Clinical context

Candida species cause superficial infections of the mucous membranes in a significant proportion of the population (Odds 1979; Cannon et al. 1995; Sobel 2007). Denture wearers often suffer from *Candida*-associated denture stomatitis (Wilson 1998), and many women have recurrent vulvovaginal candidiasis (Sobel 2007) while their premature babies often contract thrush during birth. Severe oropharyngeal candidiasis (OPC) is common in AIDS patients who do not have access to highly active anti-retroviral therapy (HAART) (de Repentigny et al. 2004), while oral candidiasis often affects cancer patients undergoing chemotherapy and/or radiotherapy (Davies et al. 2006). When fungi penetrate the epithelial surfaces of immunocompromised hosts they can cause invasive fungal infections (IFIs) that are associated with high morbidity and mortality. The fungal genera most often associated with IFIs are *Candida*, *Aspergillus* and *Cryptococcus* (Pfaller et al. 2006).

Candida species are the most common cause of opportunistic mycoses worldwide, with 72-228 infections per million population (Pfaller et al. 2006). They are also the fourth leading cause of nosocomial bloodstream infections in the USA (Wisplinghoff et al. 2004). In Europe and the USA the majority of invasive infections are caused by *C. albicans*, followed by *C. glabrata*, *C. tropicalis* and then *C. parapsilosis* (Pfaller and Diekema 2007). *C. tropicalis* is often associated with fungaemia and invasive candidiasis in patients with cancer, especially leukemics and hematopoietic stem cell (bone marrow) transplant recipients (Wingard 1995; Kontoyiannis et al. 2001). *C. parapsilosis* is frequently found on the hands of health care workers (Strausbaugh et al. 1994), forms biofilms on catheters (Kuhn et al. 2002), and has been implicated in nosocomial outbreaks of catheter-associated fungemia (Sarvikivi et al. 2005). *C. krusei* is the fifth most common cause of candidiasis (Pfaller et al. 2008) and accounts for 2% - 4% of all *Candida* blood stream infections (Pfaller and Diekema 2007).

Aspergillus species are commonly encountered in the environment, often in rotting vegetation. Clinical aspergillosis includes both an allergic reaction to the fungus and an invasive lung infection that can disseminate to other organs in the severely immunocompromised (Pfaller et al. 2006). Invasive aspergillosis (IA) accounts for 60-80% of IFIs and has an associated mortality rate of more than 50% (Kontoyiannis et al. 2005; Lai et al. 2008). The most common cause of IA is *A. fumigatus* (85%) followed by *A. flavus* (5-10%) and *A. terreus* (2-10%) (Kontoyiannis and Bodey 2002; Singh and Paterson 2005; Pfaller et al. 2006). *A. niger*, *A. nidulans* and *A. ustus* are isolated rarely. IA affects a narrower range of patients than invasive candidiasis, with severe neutropenia a major risk factor (Pfaller et al. 2006; Shao et al. 2007).

Cryptococcosis is an invasive fungal infection caused predominantly by the yeasts *Cryptococcus neoformans* and *C. gattii*. Pulmonary cryptococcosis results from inhalation of the yeast. Hematogenous dissemination leads to cryptococcal meningitis (Lin and Heitman 2006) in severely immunocompromised patients. In AIDS patients without HAART, cryptococcosis has an attributable mortality of up to 44% (Corbett et al. 2002). Cryptococcosis

is a significant opportunistic infection in solid organ transplant recipients, with a mortality of 42% (Silveira and Husain 2007). The standard treatment for cryptococcal meningitis is a combination therapy; two weeks with amphotericin B (AMB) and 5-fluorocytosine (5-FC) followed by fluconazole (FLC) for a minimum of ten weeks. Lifetime treatment with FLC is recommended to prevent relapse should the patient become immunocompromised (Saag et al. 2000).

There are few classes of antifungal agents with which to treat patients with IFIs. The target of the widely used azole antifungals is the cytochrome P₄₅₀ enzyme 14 α -lanosterol demethylase, which is an essential enzyme of ergosterol biosynthesis (Sanglard and Bille 2002; Odds et al. 2003). The enzyme, encoded by *ERG11* (also referred to as *CYP51*), is anchored in the endoplasmic reticulum by a single transmembrane segment. Inhibition of Erg11p depletes membranes of ergosterol and results in the accumulation of toxic sterols that inhibit growth (Sanglard and Bille 2002; Akins 2005). The fact that azole antifungals are often fungistatic rather than fungicidal facilitates the development of drug resistance.

Azole drug resistance

The mechanisms responsible for azole antifungal drug resistance in fungi have been reviewed extensively elsewhere (Kontoyiannis and Lewis 2002; Sanglard and Bille 2002; Akins 2005) and will be noted briefly. The drug target Erg11p can be overexpressed or develop point mutations that reduce azole binding (White et al. 1998; Sanglard and Bille 2002). Some filamentous fungi possess two *CYP51* genes (*CYP51A* or *CYP51B*) that are found in two distinct gene clusters (Mellado et al. 2001; Ferreira et al. 2005). *A. fumigatus* is innately resistant to FLC and ketoconazole (KTC). Gene knock-out experiments show that AfuCyp51Ap, but not AfuCyp51Bp, is responsible for the innate resistance (Mellado et al. 2005). Although the prevalence of itraconazole (ITC)- or VCZ-resistant *A. fumigatus* isolates is low, resistance is usually due to point mutations in AfuCyp51Ap (Nascimento et al. 2003; da Silva Ferreira et al. 2004; Chen et al. 2005).

A common cause of high-level azole resistance in fungi is overexpression of plasma membrane transport proteins that pump the azoles out of cells, thus reducing intracellular azole concentrations below the levels at which Erg11p is inhibited (White et al. 1998; Perea et al. 2001). The expression of *C. albicans* genes encoding drug efflux pumps often correlates with the increased azole resistance of clinical isolates (White 1997; Maebashi et al. 2001; Rogers and Barker 2003). This is also the case for *C. glabrata* (Sanglard et al. 1999; Bennett et al. 2004), and *C. neoformans* (Posteraro et al. 2003; Sanguinetti et al. 2006). In *C. krusei* an insensitivity of Erg11p to azoles combined with the expression of PDR efflux pumps such as CkAbc1p is thought to be responsible for its innate azole resistance phenotype (Katiyar and Edlind 2001; Lamping et al. 2009).

Efflux-mediated antifungal resistance

There are two main classes of efflux pumps responsible for fungal drug resistance, each with a different pumping mechanism and source of energy. ATP-binding cassette (ABC) proteins are primary transporters that use energy from the hydrolysis of ATP. Major facilitator superfamily (MFS) pumps are secondary transporters that utilize the electrochemical gradient across the plasma membrane to translocate substrates. Both classes of pumps are integral membrane proteins with distinctive functional domains: ABC pumps contain nucleotide-binding domains (NBDs) while both ABC and MFS pumps contain transmembrane domains (TMDs) that confer substrate specificity.

In *C. albicans*, the expression of two ABC pumps Cdr1p and Cdr2p and the MFS transporter Mdr1p (Ben^rp) have been implicated in the resistance of clinical isolates to azoles (Sanglard

et al. 1995; White 1997; Maebashi et al. 2001; Perea et al. 2001). Expression of the ABC pumps is more often associated with resistance than expression of Mdr1p, and the expression of Cdr1p appears to be the predominant contributor to clinically significant azole resistance (Holmes et al. 2008; Tsao et al. 2009). In *C. glabrata*, azole resistance can be caused by expression of the ABC proteins Cdr1p and Cdr2p (also called Pdh1p) (Miyazaki et al. 1998; Sanglard et al. 2001; Izumikawa et al. 2003). In *C. neoformans* the ABC proteins CneAfr1p and CneMdr1p are the only efflux pumps that have been linked to antifungal drug resistance (Thornewell et al. 1997; Sanguinetti et al. 2006). There is no convincing evidence for a contribution by MFS transporters to azole resistance in *C. glabrata* or *C. neoformans* although the *A. fumigatus* Mdr3p MFS pump may be involved in resistance (Nascimento et al. 2003).

Fungal ABC transporters

ABC transporters are found in all living organisms. Most ABC proteins are membrane proteins and many are thought to transport a variety of substances across membranes. The functional unit of ABC transporters consists of a cytoplasmic NBD and a membrane associated TMD (Taglicht and Michaelis 1998; Dassa and Bouige 2001; Higgins 2001; Bouige et al. 2002; Dean 2002; Rees et al. 2009). There is one such unit in half-size transporters and two units in full-size transporters (Fig. 1). The NBDs (NBD1 and NBD2) in full-size transporters are involved in cooperative ATP binding and hydrolysis. The TMDs (TMD1 and TMD2) usually operate in pairs and each usually includes 6 putative α -helical transmembrane segments (TMS1-12). The number and arrangement of the NBDs and TMDs within the pump polypeptide varies according to the type of ABC protein (Table 1). The four domain fungal ABC proteins typically have a molecular mass of approximately ~160 kDa. The high level of homology usually found between the amino-terminal and carboxy-terminal halves of the protein suggests gene duplication and fusion, but subtle differences between each half of the protein may have functional implications. Many organisms, including fungi, have half-size transporters consisting of one NBD and one TMD (Gbelska et al. 2006). Biochemical and crystallographic evidence indicates that the half-size transporters are likely to function as either hetero- or homodimers (Higgins 2007).

Sequencing of entire fungal genomes has revealed the repertoire of ABC proteins in a range of species (Dassa and Bouige 2001; Bouige et al. 2002; Verrier et al. 2008). The first fungal genome to be sequenced was that of *Saccharomyces cerevisiae* (Goffeau et al. 1996), and it was found to encode 29-30 ABC proteins (Decottignies and Goffeau 1997; Taglicht and Michaelis 1998). Each ABC protein contains at least one NBD but a subset lacks a predicted TMD. This subset comprises proteins involved in DNA repair or other ATP-requiring functions. *C. albicans* is predicted to have a similar number (27) of ABC proteins as *S. cerevisiae* (Gaur et al. 2005) and *C. glabrata* approximately two-thirds that number (18) (Dujon et al. 2004). Much larger numbers of ABC proteins are found in *A. fumigatus* (49) and *C. neoformans* (54) (Loftus et al. 2005; Nierman et al. 2005).

ABC proteins can be divided into sub-classes according to sequence homology and domain topology (Dassa and Bouige 2001; Bouige et al. 2002; Dean 2002; Verrier et al. 2008). Fungal ABC proteins have been most extensively studied in the model yeast *S. cerevisiae* (Decottignies and Goffeau 1997; Taglicht and Michaelis 1998; Bauer et al. 1999; Gbelska et al. 2006). The ABC proteins of *S. cerevisiae* have been classified into six classes: PDR, MDR (multidrug resistance), MRP (multidrug resistance-associated protein), RLI (RNase L inhibitor), ALDP (adrenoleukodystrophy protein), and YEF3 (yeast elongation factor EF-3) (Taglicht and Michaelis 1998) (Table 1). Of these, the PDR, MDR, and MRP are most often associated with antifungal resistance. The major pumps involved in the azole resistance of *C. albicans*, *C. glabrata*, *C. krusei* and *C. neoformans* are Pdrps.

Domain arrangements of ABC transporters

The domain arrangement of most eukaryotic ABC transporters is [TMD-NBD]₂ (Bauer et al. 1999; Bouige et al. 2002; Dean 2002; Gaur et al. 2005; Tusnady et al. 2006; Verrier et al. 2008). This is achieved either by the formation of homo- or heterodimers of two half-size transporters (e.g. human TAP1/2 also known as ABCB2/3 according to the nomenclature of the Human Genome Organization (HUGO)) or, as is the case for most eukaryotic ABC transporters, by a single polypeptide encoding a full-size transporter (e.g. human ABCB1 and ABCC1). Fungal PDR transporters have a reverse topology [NBD-TMD]₂ (Fig. 1 and Table 1). The plant PDR transporters are the only other class of full-size ABC transporters with the same reverse [NBD-TMD]₂ arrangement (van den Brule and Smart 2002; Jasinski et al. 2003; Crouzet et al. 2006; Verrier et al. 2008). Although full-size PDR transporters have yet to be found in bacteria or any animal species, numerous half-size ABC transporters with a [NBD-TMD] topology have been identified in plants, fungi, and animals. For example, the white, brown and scarlet (WBC) transporters of *Drosophila melanogaster* are believed to transport the red and brown eye color pigment precursors guanine and tryptophan (Bouige et al. 2002). WBC homologs are found in many species and are involved in both the trafficking of lipids and in multidrug resistance (e.g. human ABCG2; (Dean 2002)). Plants such as *Arabidopsis thaliana*, rice or tobacco possess numerous WBC homologs, but their functions are poorly understood (Verrier et al. 2008). Homologs of these half-size PDR or WBC transporters have also been detected in some fungi (e.g. *ADPI* in *S. cerevisiae* (Purnelle et al. 1991)). Verrier et al., (2008), proposed a unified nomenclature for plant ABC proteins that groups both half-size WBC and full-size PDR transporters into the ABCG family, consistent with the HUGO nomenclature for human and mouse ABC transporters (Dean 2002).

The NBDs of ABC proteins usually contain seven conserved motifs: 1) The Walker A motif or P-loop GX₄GK[S/T] (X is any amino acid) connects a conserved β-sheet with an adjacent α-helix; 2) The Walker B motif hhhhD (h is any aliphatic amino acid) that forms a β-sheet; 3) The ABC signature motif or C-loop LSGGQ that connects two α-helices just upstream of the Walker B motif; 4) The Q-loop located between the Walker A and ABC signature motif that is N-terminal of a helical structure that makes close contact with the intracellular loop (IL) regions of MDR transporters (Dawson and Locher 2006; Aller et al. 2009); 5) The pro-loop that connects the C-terminal α-helix of the ABC signature motif with the Walker B β-sheet; 6) The H-loop (or switch region) located ~30 amino acids C-terminal of the Walker B motif that connects a conserved β-sheet with an adjacent α-helix (Decottignies and Goffeau 1997; Linton and Higgins 1998); and 7) The D-loop that connects the Walker B β-sheet with a structurally conserved α-helix. In fully functional PDR transporters the H-, P-, Q-, and D-loops, together with the ABC signature motif from the opposite NBD, form one of two ‘composite’ nucleotide-binding pockets (CNBP1 and CNBP2) (Taglicht and Michaelis 1998; Jones and George 2004). A useful illustration of the essential motifs within the NBD structure is given by Jones and George, (2004).

Domain interactions and substrate transport in ABC transporters

It is widely accepted that substrate transport by ABC transporters involves interactions between the membrane bound and cytosolic domains. These interactions are normally tightly coupled with substrate binding, ATP binding and ATP hydrolysis. Bacterial ABC transporters usually fully couple substrate transport with ATP hydrolysis despite being multiunit entities made up of separate NBDs and TMDs. The ‘ATP switch model’ proposed by Higgins and Linton (Higgins and Linton 2004) reflects current thinking on the efflux mechanism of ABC transporters. The first step is the binding of substrate to a high-affinity binding site in the TMD that is usually considered to be open to the cytosol or the inner leaflet of the membrane. Binding of efflux substrate triggers a conformational change in the NBDs via formation of new contacts

between the IL regions and the 'helical domain' of the NBDs. These allow NBD dimerization in the presence of ATP. The resultant conformational change opens the substrate-binding pocket to the extracellular space and the substrate is released. After substrate release, hydrolysis of the bound ATP returns the transporter to its internally open conformation and the transport cycle can be repeated. Thus, binding of ATP, and not its hydrolysis, is considered the power stroke of substrate transport. Despite significant biochemical efforts, the molecular details of this model remain to be determined (Jones and George 2004; Jones et al. 2009).

Both human ABCB1 and ABCC1, the prototype MDR- and MRP-type transporters, respectively, are associated with multidrug resistance of cancer cells (Dean 2002). Their innate basal level of ATPase activity is stimulated several-fold in the presence of substrate. Fungal ABC transporters are not stimulated significantly by the presence of xenobiotic substrates and a fully uncoupled transport mechanism for the archetypal fungal PDR transporter Pdr5p has been suggested (Ernst et al. 2008). In contrast, Sauna and colleagues (Sauna et al. 2008) proposed coupling between TMS2 and NBD1 of Pdr5p via contact between the Q-loop of NBD1 and IL1 (Fig. 1). They reported restoration of substrate transport in a transport-deficient, but 'ATPase active', S558Y mutant of Pdr5p by a N242K mutation located 2 amino acids N-terminal to the invariant glutamate of the Q-loop in NBD1 (Sauna et al. 2008). As discussed below, S558 is close to the extracellular boundary in TMS2 and part of a stretch of amino acids conserved in fungal Pdrps.

The importance of contact between the Q-loop and IL regions in coupling substrate transport to ATP hydrolysis is illustrated by the human peptide transporters TAP1 and TAP2 (Herget et al. 2007). These two half-size MDR-type transporters are members of the human ABCB family of ABC transporters. TAP1/2 form a functional heterodimer that is involved in the adaptive immune response. TAP1/2 transports small peptide antigens into the ER lumen for presentation on MHC class I molecules. The binding of small peptides by TAP1/2 involves IL1 and leads to a conformational change and new contacts between IL1 and the Q-loop of TAP1. This triggers dimerization of the two NBDs in the presence of ATP, followed by substrate transport and ATP hydrolysis (Herget et al. 2007). The amino acid sequence of the IL1 peptide sensor and transmission interface of TAP1 (Herget et al. 2007) is conserved in the IL regions of human ABCB1 (Aller et al. 2009), the *Staphylococcus aureus* ABCB1 homolog Sav1866 (Dawson and Locher 2006), the bacterial lipid A exporter MsbA (Chang 2003) and even the *E. coli* vitamin B12 importer BtuCD (Locher et al. 2002). Despite obvious differences between the MDR, MRP, and PDR families of ABC transporters, the use of contact points between the 'helical domain' of NBDs and IL regions that couple ATP hydrolysis to substrate transport in both directions appears conserved (Schneider and Hunke 1998; Holland and Blight 1999; Dawson and Locher 2006; Higgins 2007; Hollenstein et al. 2007; Rees et al. 2009).

Although fungal Pdrps such as *S. cerevisiae* Pdr5p and *C. albicans* Cdr1p are obviously associated with multidrug resistance, their biological functions are probably related to membrane biogenesis and lipid homeostasis (Shahi and Moye-Rowley 2009). This could mean that the 'uncoupled' ATPase activity claimed for Pdr5p (Ernst et al. 2008) may actually reflect ATPase activity that is coupled to the transport of endogenous pump substrate(s) such as phospholipids, sphingolipids or sterols.

Crystal structures of the *S. aureus* multidrug transporter Sav1866 (Dawson and Locher 2006) and the mouse homolog of human ABCB1 (Aller et al. 2009) confirm features of existing models for the transport mechanism. Both transporters have a central binding cavity large enough to bind many different substrates, and sometimes more than one substrate simultaneously. An intriguing question is how can multidrug transporters sequester, with sufficient affinity, a diversity of chemically unrelated hydrophobic and charged compounds from the cytoplasm or lipid bilayer? Useful insight comes from the crystal structures of the *S.*

aureus multidrug binding transcription regulator QacR with six different structurally diverse drugs bound (reviewed by (Schumacher and Brennan 2003)). Similar to ABCB1 (Aller et al. 2009) these structures reveal a sizeable drug-binding pocket that includes multiple drug-binding mini-pockets. The drug-binding pocket is exceptionally rich in aromatic residues and contains negatively charged amino acids that can neutralize positively charged substrates such as rhodamine 6G.

Phylogenetic characterization of fungal PDR transporters

Phylogenetic and biochemical studies suggest that ABC transporters are evolutionarily ancient proteins (Dassa and Bouige 2001). Individual families are thought to have emerged before the separation of the three kingdoms of living organisms and functional constraints are probably responsible for the conservation of individual families (Saurin et al. 1999; Dassa and Bouige 2001; Bouige et al. 2002).

Despite their importance in the response of fungi to their environment, little is known about the structure and transport mechanism of Pdrps. We have recently data-mined the available fungal genome sequences to identify full-size PDR-type open reading frames (ORF) in fungal pathogens of humans. Alignments of the predicted protein sequences and phylogenetic analysis allowed the subdivision of fungal PDR transporters from 14 fungal species into 8 distinct clusters (clusters A to H), (Cannon et al. 2009). This review extends that analysis by considering Pdrps from 55 fungal species belonging to the Ascomycota and the Basidiomycota phyla that include pathogens of both humans and plants (Table 2, Figs. 2 and 3).

The following criteria were used to identify Pdrps from these species: 1) Access to the protein sequence from a reputable database (where possible we used the UniProt sequences because of their superior annotation); 2) An e-value $< 10^{-35}$ when compared by BlastP with the *S. cerevisiae* Pdr5p query (this high level of similarity is identified only in full-size ABC transporters of the typical [NBD-TMD]₂ Pdrp topology (Balzi et al. 1994)); 3) A polypeptide length > 1000 amino acid residues to eliminate partial or half-size sequences; and 4) Sequences suspected to originate from redundant submissions to databases were eliminated manually. The protein sequences and other information for the 349 Pdrps identified are compiled in Supplementary Table 1. The number of Pdrps per species varies greatly. For example, only two Pdrps were identified in *S. pombe* compared with 20 in *Aspergillus terreus* (Table 2). This differential gene multiplication may reflect the environmental niches occupied by each species and the range of xenobiotics they produce or encounter.

A phylogenetic tree of 78 Pdrps from 12 representative species was generated (Fig. 3). These species comprise the two reference-species *S. cerevisiae* and *C. albicans*, plus one species from each of the Ascomycota and Basidiomycota classes (Table 2 and Fig. 2 black dots). Clustering of the resultant 78 full-size Pdrps confirmed the identification of the A to H clusters described previously (Cannon et al. 2009) and split the H cluster into clusters H1 and H2 (Fig. 3). As previously reported, clusters A (Pdr5p/10p/15p-like), D (Snq2p/Pdr12p-like) and E (Aus1p/Pdr11p-like) contain only Saccharomycotina members (Cannon et al. 2009). The relative position of cluster E is variable and it associates with cluster D in some tree configurations. It is striking that the *S. cerevisiae* Pdr5p-like cluster A shares a common ancestor with cluster B that comprises members from the Pezizomycotina and Basidiomycota species only. Similarly, the *S. cerevisiae* Snq2p-like cluster D shares a common ancestor with cluster H1 that contains Pezizomycotina and Basidiomycota members. In some phylogenetic reconstructions, cluster H1 is split into two by the presence of some cluster D Pdrps. As a consequence, we distinguish two sub-clusters H1a and H1b. The H1b Pdrps share a more recent common ancestor with cluster D than cluster H1a Pdrps (Table 2 and Fig. 3). *S. cerevisiae* Pdr5p and Snq2p are multidrug efflux pumps with partially overlapping substrate specificities (Kolaczowski et al.

1998). Our analysis indicates that their differentiation may have occurred during or after speciation of the Basidiomycota.

Cluster F (YOL075c-like) is the only cluster with members from each of the three phyla analyzed: Saccharomycotina, Pezizomycotina and Basidiomycota (Cannon et al. 2009). Multiple cluster F Pdrps within individual species are rare and F is the only cluster where the Walker A1 motif contains the GXGK sequence found in most other ABC transporters instead of the GXGC motif that characterizes almost all other Pdrps (Table 3 and Supplementary Table 1) (Balzi et al. 1994). Furthermore, in contrast to all other fungal Pdrps, cluster F members exhibit symmetric NBDs more typical of mammalian ABC transporters (Table 3). Members of the F cluster are therefore considered, in a broad sense, to be Pdrps and may be close living relatives of a common ancestor. Cluster E (Aus1p/Pdr11p-like) Pdrps contain neither the GXGC nor the GXGK core motif in NBD1 and lack an ABC signature motif in NBD2, but are otherwise similar to other fungal Pdrps (Table 3 and Supplementary Table 1). Notably, the cysteine of the core Walker A1 motif is replaced by a valine or serine in five Pdrps (Table 3 and Supplementary Table 1). This suggests that the consensus fungal Pdrp Walker A1 motif lacks the lysine found in most other ABC transporters rather than contains a cysteine. Members of clusters E and F are in a strict sense not Pdrps as they do not possess this typical GXGC core sequence in Walker A1.

Characterization of PDR clusters

After classifying the 349 Pdrps into the 9 clusters A-H2 (Table 2, Fig. 3), individual clusters were investigated in greater detail. This analysis used a subset of 263 Pdrps remaining after removing 85 (~25%) gene sequences (marked with an asterisk in Supplementary Table 1) that appeared to have incorrect intron/exon boundary predictions or possible sequencing errors (these minor nucleotide changes are unlikely to have affected the phylogenetic cluster analysis). The Pdrps within the 9 clusters were aligned with the ClustalW(accurate) program (Supplementary Figs. 1-12). The PredictProtein server (Rost et al. 2004) was then used to predict TMSs and the secondary structures of 5-10 selected Pdrps within each cluster. Important motifs (highly conserved primary sequences in the NBDs and TMDs) plus the predicted topology of each PDR cluster are summarized in Table 3. ClustalW alignments for each PDR cluster are presented in Supplementary Figs. 1-10 with subcluster H1a and H1b split into two (Supplementary Figs. 8 and 9). The topology predictions of the 5-10 Pdrps selected for each cluster correlated well with the TMS boundaries predicted for ScPdr5p, CaCdr1p and CkAbc1p (Saini et al. 2005; Tutulan-Cunita et al. 2005; Holmes et al. 2006; Prasad et al. 2006; Lamping et al. 2009). They consistently identified 12 TMSs for each Pdrp with a maximum 2-residue variation in the localization of individually predicted TMSs in the Pdrp alignments shown in Supplementary Figs. 1-12. Some PDR cluster G and F proteins were exceptions. Their TMS numbers and boundaries, especially for TMS1, 2, 7 and 8, were not recognized easily because their primary sequences were less hydrophobic. It is emphasized that although the predicted sub-domains of the Pdrps appear consistent with available biochemical data, PDR transporter topology awaits experimental verification, especially in the TMDs. Gaps appeared in the alignments within the TMDs. These usually occurred within predicted ELs (e.g. EL1 of cluster D shown in Supplementary Figs. 4 and 11). Our observations are consistent with significant TMS and IL size and primary sequence conservation but greater variation in EL size.

Closer inspection of individual PDR clusters indicated that cluster B PDR transporters can be divided into six orthologous families (Supplementary Fig. 2), cluster D proteins into two orthologous families (SNQ2- and PDR12-type; Supplementary Fig. 4), cluster F proteins into three different protein families (Supplementary Fig. 6), cluster H1a into four (Supplementary Fig. 8), and cluster H2 also into four protein families (Supplementary Fig. 10), while no division

into sub-families or orthologs was obvious for PDR cluster A, C, E, G and H1b transporters (Supplementary Figs. 1, 3, 5, 7 and 9).

Fungal PDR transporters have a PDR-specific ‘composite’ nucleotide binding pocket 1 (CNBP1) and a typical CNBP2

A characteristic of fungal PDR transporters is asymmetry between their NBDs (Gauthier et al. 2003; Prasad et al. 2006; Ernst et al. 2008).

NBD1s of fungal PDR clusters

The NBD1s of most fungal PDR transporters (members of PDR clusters A, B, C, D, G, H1 and H2) contain a typical ABC signature motif (ABC1) [V/I/L/C]SGGE but have degenerate, fungal PDR-specific Walker A1 and B1 motifs (Fig. 1 and Table 3). Walker A1 contains a C instead of the K required for ATP hydrolysis in other ABC transporters ie GX₂GXGC[S/T] vs GX₄GK[S/T] (Jha et al. 2003b; Jha et al. 2003a). The fungal PDR Walker B1 X₃WD shares only its last two amino acids with the consensus Walker B motif hhhhD (Rai et al. 2005). Furthermore, the essential E at the beginning of the D-loop is replaced with a D, the highly conserved Q of a typical ABC transporter NBD1 Q-loop is replaced with an E, the pro-loop P with any amino acid X, and the H-loop H with a Y (Fig. 1 and Table 3).

In contrast, NBD1 of cluster F proteins is comparable to the typical NBD of ABC transporters i.e. Walker A1 is GX₂GXGK, ABC1 is (L/C)SGGE, Walker B1 is hhhhD, Q is conserved in the Q-loop, P in the pro-loop, E is found at the beginning of the D-loop and H is conserved in the H-loop (Table 3 and Supplementary Fig. 6).

The three cluster E proteins are thought to be cholesterol importers and may be the only fungal Pdrps that import, rather than export, substrates (Li and Prinz 2004; Nakayama et al. 2007). They have typical fungal PDR-specific ABC1 and Walker B1 motifs, an E in the Q-loop and no conserved P in the pro-loop, but they lack the Walker A1 motif. Unlike all other PDR transporters, except cluster F Pdrps that have the typical H, cluster E proteins have a S instead of a Y in the H-loop of NBD1 (Table 3 and Supplementary Fig. 5).

NBD2s of fungal PDR clusters

The fungal PDR transporters, except those in clusters E and F, have typical ABC transporter Walker A2, Walker B2, pro-, Q-, and H-loop motifs in NBD2 (Fig. 1 and Table 3). However, the ABC signature motif (ABC2) of the full-size Pdrps in NBD2 (except clusters E and F) is a degenerate consensus LNVEQ sequence (Fig. 1 and Table 3). The NBD2s of cluster E and F Pdrps also have the typical Walker A2 and Walker B2 motifs and the typical pro-, Q-, and H-loops (Table 3). However, there are detectable differences in their ‘helical domain’ organization and in ABC2. Cluster E proteins have insertions and deletions in the ‘helical domain’ and, most significantly, lack 5 amino acids in ABC2 (Table 3 and Supplementary Figs. 5 and 11). Cluster F proteins have a slightly larger ‘helical domain’ with 6 amino acids inserted near Walker A2, but their typical ISGGE ABC2 signature motif is consistent with the symmetry of their NBDs (Table 3 and Supplementary Figs. 6 and 11). A summary of these features is given in Fig. 1 and Table 3 and individual features are highlighted in Supplementary Figs. 1-12.

Fungal PDR transporters form fungal PDR-specific composite NBD1/NBD2 ATP-binding pockets

Crystal structures of bacterial and human ABC transporter NBDs show two nucleotide-binding pockets mediate functional NBD dimerization. These are located at the NBD1 and NBD2 interface, with each NBD monomer contributing to both binding pockets. If the catalytic

activity of fungal Pdrps involves similar intramolecular interactions between the NBDs, their transport activity will require the contribution from a composite nucleotide binding pocket 2 (CNBP2; comprising Walker A2, Walker B2 and ABC1) with the hallmarks of a typical ABC transporter ATP-binding pocket and a composite nucleotide binding pocket 1 (CNBP1; comprising Walker A1, Walker B1 and ABC2) specific for Pdrps (see Fig. 4 and (Ernst et al. 2008)). It is not clear whether the atypical CNBP1 of PDRs can bind and hydrolyse ATP or perhaps performs a regulatory function unique to PDRs (Wada et al. 2005). Although such functions may be possible in PDR monomers the isolation of *S. cerevisiae* Pdr5p as dimers (Ferreira-Pereira et al. 2003) may indicate that intermolecular interactions are also important for regulation and function of Pdrps.

Additional characteristics of the cytosolic domains of fungal Pdrps

PDR cluster A is the best-studied family of medically important Pdrps. It includes the prototypic multidrug efflux pumps *S. cerevisiae* Pdr5p and *C. albicans* Cdr1p, *C. glabrata* Cdr1p and Cdr2p (also known as Pdh1p), and *C. krusei* Abc1p. In PDR cluster A the helical domain of NBD1 is ~6 amino acids larger than in NBD2 (63-4 compared to 58; Table 3). This size difference between the 'helical-domains' of NBD1 and NBD2 is conserved in all PDR clusters except cluster F proteins that have 'helical domains' of similar size (Table 3). In human ABCC1 a change of helical domain symmetry affects substrate transport and ATP hydrolysis in a fashion similar to the K684M mutation of the essential K of Walker A1 (Gao et al. 2000). Furthermore, the linker region between the H-loop of NBD1 and TMS1 in fungal PDRs is ~155 amino acids while the equivalent linker region of NBD2 is ~15 amino acids shorter (Table 3). Finally, all fungal Pdrps apart from E and F members have N-terminal extensions of ~100-200 amino acids (Table 3 and Fig. 4). The size and amino acid sequences of these extensions are variable but seem cluster specific. These yet to be explored cytosolic N-terminal extensions are rich in polar (S/T) and charged amino acids that could provide contact points for accessory proteins or post-translational modifications that modulate the function, localization, and/or turnover of Pdrps.

PDR-specific topology of TMDs including ELs and ILs

Extracellular loops

The small EL1 (11-16 amino acids with most Pdrps containing 12 amino acids; Figs. 1 and 4 and Table 3) appears quite conserved within groups of Pdrps i.e. distinct sub-clusters or families of orthologous proteins of possibly similar function. The size of EL2 is also quite conserved ie 7-16 amino acids, with most comprising 10 amino acids (Table 3). Consistent with the apparent two-fold symmetry of ABC transporters, EL4 (usually 12 amino acids) is of identical size to EL1. The size of EL5 is less conserved and ranges from 8 (cluster F) to 23 (clusters A and B) amino acids (Table 3). All PDR transporters contain two large, partially homologous, extracellular loops, EL3 and EL6 (Fig. 4 and Table 3).

ELs may contribute to substrate transport

Overexpression of PDR transporters often confers resistance to xenobiotics, and this resistance can be ablated (chemosensitization) with pump inhibitors such as FK506 (Nakamura et al. 1993; Kralli and Yamamoto 1996) and milbemycins (Lamping et al. 2007; Lamping et al. 2009). Fungi expressing Pdrps can also overcome the chemosensitization of pump inhibitors, by acquiring suppressor mutations in the pump protein. We have identified suppressor mutations in *S. cerevisiae* Pdr5p and *C. albicans* Cdr1p that overcome pump inhibition by FK506, milbemycins and an in-house D-octapeptide derivative (Monk BC et al. and Tanabe K et al., NIID Tokyo; unpublished results). The mutations were located predominantly in EL3 and EL6 but also in EL1, EL4 and occasionally within individual TMSs. Further evidence for

a role of ELs in substrate transport comes from random mutagenesis of *S. cerevisiae* Pdr5p (Tutulan-Cunita et al. 2005). Two amino acid mutations in EL5 and EL6 were found to affect the transport of a subset of efflux pump substrates. Other mutations affecting substrate specificity were located in TMS4 and TMS9, the 'helical domain' close to the Q-loop of NBD1, and near the D-loop. Thus drug interactions within Pdrps depend on structural elements located in different parts of the protein, but often in the ELs. The functional importance of ELs in other ABC transporters is illustrated by the uncharacteristically large EL1 of human ABCA1 that is essential for ApoA-I mediated cholesterol transport (Wang et al. 2000). Mutations in NBD1, NBD2, EL1, the similarly large EL4 and the much smaller EL6 of ABCA1 cause Tangier disease and familial HDL deficiency but few mutations associated with Tangier disease are located in any of the 12 TMS of ABCA1 (Tanaka et al. 2001).

PDR-specific motifs in EL3 and EL6

Alignment of the EL3 and EL6 sequences of 263 fungal Pdrps reveals four major characteristics: 1) EL3 and EL6 are similar but have distinct features e.g. EL3 is shorter (~90 amino acids) than EL6 (~110 amino acids). Only PDR cluster F proteins have similar sized but smaller (~70 amino acids) EL3 and EL6 (Table 3); 2) The Pdrps contain two invariant Cs and one invariant E in EL3. They also contain four invariant Cs and a highly conserved E located 4 amino acids C-terminal of the first invariant C in EL6 (only ZYRF08888 of cluster D and CNAG-06338, CNAG-06348 and B6H8M2 of cluster G have a D instead; Fig. 4, Table 4 and Supplementary Tables 2 and 3). Cluster F proteins are the exception. They have only 2 conserved Cs in each of EL3 and EL6; 3) The N-terminal portions of EL3 and EL6 reveal new conserved hydrophobic, PDR-specific motifs named PDR motif A and PDR motif B in EL3 (Fig. 4 and Table 4), and a hydrophobic motif equivalent to PDR motif A in EL6 (Fig. 4 and Table 5); and 4) Predicted secondary structures in EL3 and EL6 appear strongly conserved (Fig. 4).

EL3. Four highly conserved amino acids G[Y/F]X[I/L/V] are predicted to form a short β -sheet after the α -helix of TMS5 leading into EL3 (Fig. 5). This is followed by a short loop of 4 variably charged hydrophilic amino acids, then two highly conserved α -helices linked by two conserved amino acids [N/D]P (Fig. 4). While some residues in these two α -helices are specific for individual orthologs (e.g. the *SNQ2*, *PDR12* or *AFR* family) other residues, mostly on one face of a helical wheel projection, are strongly conserved between all clusters. Both helices contain conserved aliphatic and aromatic amino acids: α -helix1 = MX₂WX₂W[h/F]X[Y/W]; α -helix2 = [h/F]YXX[F/Y]EX[h/V][h/V]XNEF. The only invariant amino acid is the E at the C-terminal end of helix 2 (Table 4). These two motifs are also conserved in other PDRs (full-size and half-size plant, fungal and human PDRs; data not shown). We have therefore denoted these two conserved α -helices PDR motif A and PDR motif B (Fig. 4 and Table 4). They are not to be confused with the plant PDR-specific motifs PDR sig1, PDR sig2 and PDR sig3 described by van den Brule and Smart, (2002). The C-terminus of EL3 is predicted to contain three short β -sheets with a few highly conserved amino acids that may be important for its structure (Fig. 4) ie two invariant Cs, three highly conserved Gs and three aromatic amino acids CX_{~20}CX₃GX₃GX₅GX₂[Y/F]X₄[Y/F]X₁Y (Supplementary Table 2). Located five amino acids downstream is the conserved sequence WRNFG[I/V] that connects the C-terminus of EL3 with TMS6 (Fig. 5). The ~150 amino acid 'PDR_CDR' protein family motif that is recognized in Pdrps by Blastp searches (pfam06422 (Gauthier et al. 2003; Finn et al. 2008)) includes this C-terminal half of EL3 from the second conserved C, TMS6, the conserved β -sheet mentioned in the next section, and the linker region of Pdrps (Fig. 4).

EL6. Three conserved amino acids G[I/V][M/L/V] begin EL6 as part of a short predicted β -sheet at the end of the TMS11 α -helix (Fig. 5). This conserved β -sheet is followed by 8 variably charged hydrophilic amino acids and then a conserved stretch of 10-11 amino acids rich in

aromatic residues (FWX₍₁₋₂₎[F/W]hYX₃P). This arrangement and the hydrophobic stretch of conserved amino acids is similar to EL3, however, unlike PDR motif A, the conserved hydrophobic motif of EL6 is often predicted to form a β -sheet (Fig. 4). Similar to the highly conserved P that separates PDR motifs A and B in EL3, an invariant P separates the hydrophobic motif of EL6 from an adjacent α -helix (Fig. 4, Table 5 and Supplementary Figs. 1-12). However, unlike PDR motif B, the primary sequence of this α -helix is only conserved within Pdrp orthologs (e.g. compare the four protein families of cluster H1a shown in Supplementary Fig. 8). Cluster F proteins also have two predicted α -helices separated by an invariant P. However, their primary sequences are less well conserved and can be readily divided into three distinct groups, or perhaps orthologs (marked with red, green and purple sidebars in Supplementary Fig. 6). Like EL3, the remainder of EL6 CX₃[E/D]X₃[F/L/V/I]X₆₋₉CX₂[Y/F]X₃[Y/F]X₄₋₁₆GX₁₀₋₁₂CX[F/Y/V]C (Supplementary Table 3) contains a few conserved amino acids, including four invariant Cs, an invariant acidic E/D and a few aromatic amino acids. There are 20 amino acids between the most C-terminal of the invariant Cs and a conserved exit motif WR[N/D][F/Y/W/V/L/I]G[I/L/F/V] that is very similar to the C-terminal portion of EL3 (Fig. 5).

EL3 and EL6 of Pdrps have conserved structural features likely to be important for their transport mechanism

We hypothesize that the Pdrps have evolved conserved structural features in their large ectodomains to fulfill unique functions that differentiate them from other ABC transporter families. Human half-size PDR transporters ABCG1, 2, 4, 5 and 8 (Dean 2002) share their TMD topology with fungal and plant Pdrps (small EL1 and EL2 and large EL3; Supplementary Tables 4 and 5). Their large EL3 (~70 amino acids) also contains a predicted hydrophobic α -helix rich in aromatic amino acids and a conserved α -helix 2, very similar to fungal PDR motifs A and B, with the caveat that the five ABCG proteins fall into two subgroups. The PDR motifs A and B of ABCG2 and ABCG5 are very similar to those of fungal PDRs but they are more degenerate in ABCG1, 4 and 8 (data not shown).

The six highly conserved Cs of EL3 and EL6 of fungal Pdrps seem likely to have structural, and possibly functional, importance. The second conserved C in EL6 was mutated in a functional characterization of *C. albicans* Cdr1p (C1418Y). The mutation increased the susceptibility of the Cdr1p-expressing host to some xenobiotics (cycloheximide and miconazole) but not others (e.g. FLC) (Shukla et al. 2003). Studies with ABCG2 also highlight the significance of EL Cs (Henriksen et al. 2005). C592 and C608 of ABCG2 EL3 form intramolecular disulfide bonds that appear important for substrate specificity as reported for *C. albicans* Cdr1p and consistent with the hypothesis that ELs play important roles for substrate transport. Furthermore, C603 contributes to the homodimer formation required for ABCG2 function through the formation of intermolecular disulfide bonds (Henriksen et al. 2005).

Although there were no conserved Cs in EL3 or EL6 of the plant PDR transporters that we examined, each EL3 and EL6 contains an invariant negatively charged amino acid like all fungal PDR transporters (i.e. the E of PDR motif B in EL3 and a D in a position similar to the invariant E in EL6 of fungal PDRs). The importance of conserved negatively charged amino acids in TMSs for the binding and transport of positively charged hydrophobic drugs has been demonstrated in bacterial multidrug transport systems such as EmrE (Muth and Schuldiner 2000) or MdfA (Edgar and Bibi 1999). We postulate that these conserved E and D residues in EL3 and EL6 of Pdrps may be important for the binding/transport of hydrophobic and/or positively charged efflux pump substrates and that EL3 and EL6 contribute to substrate binding during the transport cycle.

IL1 and IL3 are highly conserved in fungal PDR transporters

Pdrps appear to possess unique IL arrangements. Crystal structures show that MDR-type ABC transporters have two large ILs in each TMD that are required for the cross-talk between the NBDs and TMDs (Dawson and Locher 2006; Aller et al. 2009). In contrast, plant, fungal, and human PDR transporters are predicted to possess one large and one small IL in each TMD (Figs. 1 and 4, Table 3 and Supplementary Tables 4 and 5). The TMD topology of Pdrps may indicate a unique TMD arrangement that is distinct from MDR transporters. According to Rees et al., (2009), other yet to be discovered TMD folds for ABC transporters almost certainly exist in addition to the three TMD folds identified so far (type I and II importers and type III exporters such as Sav1866 or ABCB1; (Rees et al. 2009)). While the Pdrp IL2 and IL4 are very short and have no obvious conserved motifs, IL1 (34 amino acids) and IL3 (31 amino acids) are highly conserved within Pdrp clusters and/or families and are similar in size to the large ILs of MDR type transporters. IL1 and IL3 are rich in conserved charged and hydrophobic amino acids, especially near the center of each loop (Tables 6 and 7). Secondary structure predictions indicate that the TMS2 α -helix extends ~6 amino acids into IL1 while the equivalent TMS8 α -helix of TMD2 breaks at the beginning of IL3 followed by a short (~5 amino acid) β -sheet. Both IL1 and IL3 then share a predicted centrally located ~6 amino acid α -helix reminiscent of the 'coupling helices' of IL1 and IL3 of MDRs (Dawson and Locher 2006; Aller et al. 2009). The C-terminal half of IL1 is invariably predicted to be an α -helix that is broken at the entrance to TMS3 by three highly conserved amino acids [E/D]hP (Table 6; h = I, V, L or M). The equivalent α -helix in the C-terminal half of IL3 reaches 3 amino acids into the predicted TMS9 region and is broken by the same conserved amino acid triplet EhP (Table 7). IL1 has a negatively charged amino acid at each end with a core of highly conserved amino acids that includes two invariable positively charged amino acids centered around the predicted 'coupling helix' (Table 6). The C-terminal α -helix of IL1 (X_{10} between P and [E/D] in Table 6) is highly conserved within individual PDR clusters, perhaps reflecting a selective functionality (Supplementary Figs. 1-12). IL3 possesses highly conserved amino acids across the whole region including the C-terminal α -helix (Table 7).

TMDs of fungal PDR transporters have positively charged N-terminal α -helices and a highly conserved β -sheet just after TMS6

Individual TMS are thought to determine the broad substrate specificity of ABC transporters by contributing to a large, centrally located, binding pocket that is open to either the cytosolic or the extracellular space during the reaction cycle (Egner et al. 2000; Shukla et al. 2003; Saini et al. 2005; Tutulan-Cunita et al. 2005). There are conserved features flanking the TMDs (Fig. 4). All Pdrps have a ~15 amino acid α -helix located ~4 amino acids N-terminal of the TMS1 and TMS7 α -helices (Fig. 4). Both α -helices are rich in positively charged amino acids (Fig. 5 and Supplementary Figs. 1-12) and these may be important for determining the insertion of the first TMS of each half of the transporter in the membrane (Hartmann et al. 1989; von Heijne 1995; Gafvelin et al. 1997). As a general rule, the net charge difference between the 15 amino acids N-terminal and the 15 residues C-terminal of the first hydrophobic TMS of an integral membrane protein determines its orientation, with the more positively charged part remaining in the cytosol (Gafvelin et al. 1997). The orientation of the first TMS then automatically determines the orientation of the following TMSs. The topology of fungal Pdrps proposed in Figs. 1 and 4 complies with this rule. Both helices prior to TMS1 and TMS7 have a similar core of conserved amino acids. They include an invariant Q (except one PDR G cluster protein) and one or two invariant Rs. The consensus for the α -helix N-terminal of TMS1 is QX_6RX_{12} and the consensus for the α -helix N-terminal to TMS7 is $QX_6RX_{5-6}RX_3YX_2$ (Fig. 5).

A predicted ~6 amino acid β -sheet located ~9 amino acids C-terminal to TMS6 (Fig. 4) is conserved in PDR cluster A, B, C and D proteins (Supplementary Figs. 1, 2, 3, 4, 11 and 12)

while cluster H proteins have a unique β -sheet motif (Supplementary Figs. 8-12). The ~9 amino acids separating TMS6 and this β -sheet are highly conserved within Pdrp orthologs (Supplementary Figs. 11 and 12). These sequences may contribute to the transport mechanism or the substrate specificity within groups of PDR transporters.

TMS boundaries of fungal PDR transporters are highly conserved

Conservation of primary sequence within any given TMS is higher within, than between, clusters. Highly conserved amino acids present in all Pdrps are summarized in Fig. 5 (the percent conservation of individual amino acids is also shown) and circled in blue in the 90% consensus sequence lines shown at the bottom of Supplementary Figs. 1-12. These conserved sequences are likely to be important for the overall structure and function of Pdrps but are less likely to be involved in their substrate specificity. Overall it appears that most TMS boundaries are highly conserved containing either hydrophobic or charged amino acids (for details see Fig. 5). Each TMS is predicted to form a long (~18-30 amino acids) α -helix except TMS3 and TMS9 that consist of two shorter α -helices broken by the highly conserved amino acid triplet EhP at the end of IL1 and IL3, as discussed above. Similar to the invariant negatively charged amino acids in EL3 and EL6 described above the highly conserved Es in TMS3 and TMS9 may also be involved in the binding and/or transport of positively charged, hydrophobic amino acids. It is striking that full-size plant PDR transporters also contain conserved E or D residues in similar positions in their respective TMS3 and TMS9 (data not shown). Interestingly, both TMS1 and TMS7 have conserved polar amino acids S (TMS1) or [S/T] (TMS7) close to EL1 and EL4, respectively. The conserved TMS1 S residue is predicted to be located near the S558 of ScPdr5p TMS2 that has been implicated in coupling ATP hydrolysis and substrate transport (Sauna et al. 2008). TMS10 and the cytosolic side of TMS11, including IL4, show no conservation of primary sequence. Finally, most Pdrps are predicted to have a very short C-terminal tail (~10-20 amino acids) located in the cytosol. This tail is extremely rich in positively charged amino acids with an average net positive charge of ~5-6 (Fig. 4). This may be important for its association with the inner leaflet of the plasma membrane lipid bilayer.

Concluding remarks

Fungal Pdrps are a large family of ABC transporters involved in the import and export of a diversity of compounds across biological membranes. Although the expression of some members has been correlated with clinically significant antifungal drug resistance, the physiological functions of these and other family members are poorly understood. The availability of Pdrp sequences in well-curated databases of whole fungal genomes permits phylogenetic analysis, searches for Pdrp-specific primary sequence motifs and the ability to ascribe some putative functions. We have categorized Pdrps from 55 fungal species into 9 separate clusters with one group (H1b) overlapping two clusters (D and H1a). Pdrps possess a unique set of features that may have evolved from an ancient common ancestor that appears to be most closely related to PDR cluster F proteins. It is also possible that Pdrps arose independently of PDR cluster F proteins through gene fusion of two different half-size Pdrps (Bouige et al. 2002; Crouzet et al. 2006). Typical fungal Pdrps have uniquely conserved 'asymmetric' NBDs. They have PDR-specific arrangements of ILs and ELs, they contain PDR-specific motifs in EL3 (the hydrophobic PDR motif A and PDR motif B), and they possess a highly conserved PDR-specific hydrophobic motif in EL6. PDR motif A and PDR motif B are separated by a conserved [N/D]P sequence and have an invariant E at the C-terminus of PDR motif B. EL6 is slightly larger. It also contains an invariant P separating its hydrophobic motif from an adjacent, structurally conserved, α -helix and an invariant E three amino acids C-terminal of the first invariant C (Fig. 4).

Although we have focused on fungal Pdrps, plants possess a large number of Pdrps and the human ABCG family of transporters can be categorized as Pdrps as well. Unlike plants and fungi, humans appear to have only half-size PDR transporters (Dean 2002). All half-size PDR transporters have NBDs that resemble NBD2s of full-size fungal and plant PDRs, but their EL3 size can vary significantly (~51-101 amino acids) (Supplementary Table 5). We queried databases for the PDR-specific motifs, PDR motif A and PDR motif B, and found these two motifs are present in all Pdrps including those from plants and humans but not in any other ABC transporters. Despite obvious similarities between fungal and plant PDRs (all half-size and full-size Pdrps from *Arabidopsis thaliana*, rice and tobacco were tested) the following differences were found: 1) Plant Pdrps have a plant specific ABC signature motif in NBD2 (van den Brule and Smart 2002); 2) Plant Pdrps have a ~30 amino acids larger NBD1 'helical domain' than fungal Pdrps (compare Tables 3 with Supplementary Table 4); and 3) Plant Pdrps lack the invariant Cs found in EL3 and EL6 of fungal Pdrps. We suggest that while fungal and plant Pdrps share a common ancestor and have important PDR-specific features in common and are therefore likely to share a similar transport mechanism, adaptive evolution has minimized their functional homology. In short, plant and fungal Pdrps do not appear to share any orthologs (Crouzet et al. 2006).

Our analysis of the literature also indicates that a universal nomenclature of half-size and full-size PDR transporters is required. We recommend that the HUGO nomenclature for ABC transporters be adopted but in a way that both the relatedness of genes (e.g. SNQ2, PDR12 or AFR orthologs of different fungal species) and their classification as half-size or full-size PDR transporters can still be recognized.

While the function of most PDR transporters discussed in this review remains to be elucidated it seems likely that different Pdrp orthologs share different subsets of substrates. The phylogenetic analysis presented here permits this hypothesis to be tested by comparing the properties of individual cluster members heterologously expressed in a suitable host such as *S. cerevisiae*. This approach could help elucidate how cluster members within this important family of ABC proteins bind and translocate their range of substrates. Another important outcome of the present study has been the discovery of orthologs with possibly similar function, in different fungal species. Thus, the analysis of the function of a Pdrp in one species may give important clues to the function of orthologs in other species. Since ABC pump function depends upon coordinated interaction between different parts of the molecule, the discovery of motifs specific to individual Pdrp clusters or sub-clusters should provide information about their biochemical properties. In the light of our growing understanding of possible structure-function relationships, the use of site-directed mutagenesis to modify individual motifs, the analysis of single nucleotide polymorphisms and the creation of chimeric transporters that contain different combinations of domains, sub-domains and motifs can all provide practical methods to explore PDR transporter structure and function.

However, most critical will be the validation of the topological map of Pdrps proposed in this study and the structural resolution of at least one member of this important protein family. The ability to express affinity tagged versions of many of these pumps in *S. cerevisiae* and the availability of inhibitors of these enzymes will be a major advantage in obtaining structural information at high resolution. Greater understanding of Pdrp structure and function will ultimately help elucidate Pdrp-mediated drug resistance and facilitate structure directed design of 'smart' drugs that inhibit multiple targets simultaneously and thus reduce the likelihood of drug resistance (Monk and Goffeau 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Predicted topology of a typical full-size fungal PDR transporter showing the major topological features and highlighting the asymmetry of consensus motifs in the NBDs (see text for details; N = N-terminus; C = C-terminus; NBD = nucleotide binding domain; TMD = transmembrane domain; EL = extracellular loop; IL = intracellular loop). The transmembrane segments are numbered 1-12. Conserved amino acids in brackets are given in the one letter code with X representing any amino acid, and h any aliphatic amino acid.

Fig. 2.

Phylogenetic tree of the 55 fungal species used for the analysis of fungal PDR transporters based on (James et al. 2006). Black dots indicate the fungal species that were used for the cluster analysis of fungal PDR transporters presented in Fig. 3. P indicates a pathogenic species (animal pathogens are shown in black and plant pathogens in gray). The Ascomycota species used in this analysis belong to three different subphyla: 1) The Pezizomycotina from four classes (Dothiomycetes including *Pyrenophora tritici-repentis*, Eurotiomycetes such as *Aspergillus niger*, Leotiomycetes such as *Botryotinia fuckeliana*, and Sordariomycetes including *Neurospora crassa* and *Magnaphorthe grisea*); 2) The Saccharomycotina (including *S. cerevisiae* and *C. albicans*); and 3) The Taphrinomycotina (such as *Schizosaccharomyces pombe*). The Basidiomycotina species analysed here belong to two subphyla: the Agaricomycotina (including *C. neoformans*, *Coprinopsis cinerea* and *Laccaria bicolor*) and the Ustilaginomycotina (such as *Ustilago maydis*).

Fig. 3. Phylogenetic tree of 78 PDR transporters from 12 fungal species representing the different phylogenetic classes of Dykarya. The tree is based on a Parsimony method with 100 bootstraps. Only branches with at least 50 % support are presented (extended Majority rule in Phylip PROTPARS). Blue arrows indicate PDR transporters from fungal species belonging to the Basidiomycetae. The relative phylogenetic position of the SCHPO347 sequence from *Schizosaccharomyces pombe* at the border of clusters F and H2 is very unstable from one bootstrap run to the next and thus difficult to assign unequivocally to cluster F or H2.

Fig. 4.

Analysis of the primary and secondary structure predictions of fungal PDR transporters revealed highly conserved ELs, particularly EL3 and EL6. These have unique structural features and highly conserved motifs indicating possible involvement in the substrate transport mechanism (the hydrophobic PDR motif A and PDR motif B in EL3 separated by a conserved linker and a highly conserved hydrophobic EL6 motif). EL3 and EL6 possess six invariant Cs, two in EL3 and four in EL6. They also contain two invariant E, one each in EL3 and EL6. Predicted interactions between essential components of the NBDs and the intracellular loop regions are also shown ie the 'composite' nucleotide binding pockets 1 (CNBP1) and 2 (CNBP2) that consist of conserved motifs from each of the two NBDs are circled and the predicted interactions between the Q-loops and IL1 and IL3 are highlighted with open squares. Also highlighted are predicted positively charged α -helices close to TMS1 and TMS7 that may be involved in membrane insertion/protein folding, and a predicted short, intracellular β -sheet close to TMS6 that is conserved between individual clusters of PDR transporters and possibly involved in aspects of the transport mechanism.

Fig. 5.

Conservation of amino acids in the TMDs of fungal PDR transporters. 244 PDR transporters were used for this analysis (all cluster A, B, C, D, E, G, H1a, H1b and H2 proteins but without cluster F transporters). Only conserved amino acids of TMS1-12 and some highly conserved amino acids adjacent to these TMSs are shown. The conserved amino acids are shown using the one letter code with the percent conservation given underneath each individual residue. Non-conserved amino acids are depicted as X and the number of these amino acids are indicated as subscript and in italics. Individual TMS (1-12) are shown as grey boxes. Each TMS is 18 amino acids long. A dot represents any amino acid between conserved residues. EL1-6 are presented as thick black lines connecting individual TMSs and IL1-4 are shown as thick grey lines. The conserved amino acids N-terminal to TMS1 and TMS7 are part of the two predicted positively charged α -helices (~20 amino acids in size) shown in Fig. 4.

Table 1

Classes of fungal ABC transporters.

Transporter class	Topology	Example	Putative function
Half-size PDR	[NBD-TMD] ₁	<i>S. cerevisiae</i> Adp1p	Membrane transport
PDR	[NBD-TMD] ₂	<i>S. cerevisiae</i> Pdr5p	Multidrug resistance
		<i>C. albicans</i> Cdr1p	Azole drug resistance
		<i>C. neoformans</i> Afr1p	Azole drug resistance
MDR	[TMD-NBD] ₁	<i>A. fumigatus</i> Mdr1p	Cilofungin resistance
		<i>C. neoformans</i> Mdr1p	Azole drug resistance
	[TMD-NBD] ₂	<i>S. cerevisiae</i> Ste6p	α -factor export
MRP	[TMD-NBD] ₂	<i>S. cerevisiae</i> Yor1p	Multidrug resistance
Others			
RLI/ALDP	[TMD-NBD] ₁	<i>S. cerevisiae</i> Pxa1p	Fatty acid transport
YEF3	[NBD] ₂	<i>S. cerevisiae</i> Yef3p	Translation
	[NBD]	<i>S. cerevisiae</i> Caf16p	Transcription

Table 2 Number of PDR genes present in fungal species^a and their cluster distribution (A-H2).

Ascomycota													
Pezizomycotina													
Dothideomycetes	N/A ^b	A	B	C	D	E	F	G	H1a	H1b	H2	Total	
<i>Alternaria brassicicola</i> (Ab)											1	1	
<i>Leptosphaeria maculans</i> (Lm)									1		1	2	
<i>Mycosphaerella graminicola</i> (Mycg)	1		1	2					1		1	6	
<i>Phaeosphaeria nodorum</i> (Pn)			1	1					4		1	7	
<i>Pyrenophora tritici-repentis</i> (Pt)			2	1					3		2	8	
<i>Venturia inaequalis</i> (Vi)				1					1			2	
Total	1		4	5					10		6	26	
Eurotiomycetes	N/A	A	B	C	D	E	F	G	H1a	H1b	H2	Total	
<i>Aspergillus clavatus</i> (Ac)			4	2			1	1	4		2	14	
<i>Aspergillus flavus</i> (Af)			3	2			1	1	2		3	12	
<i>Aspergillus fumigatus</i> (Afu)			4	2			1		4		2	13	
<i>Aspergillus niger</i> (Anig; CBS 513.88/FGSC A1513)			5	6					2		2	15	
<i>Aspergillus oryzae</i> (Ao)			4	3			1	1	3		6	18	
<i>Aspergillus terreus</i> (At)			8	2			1		5		4	20	
<i>Coccidioides immitis</i> (Ci)			2	1			1		2			6	
<i>Emericella nidulans</i> (An; <i>Aspergillus nidulans</i> ^c)			5	2					4		3	14	
<i>Neosartorya fischeri</i> (Nf)			7	2			1		4		3	17	
<i>Penicillium chrysogenum</i> (Pc)			5	2			1	2	3		3	16	
<i>Penicillium digitatum</i> (Pd)			3								1	4	
<i>Penicillium expansum</i> (Pe)			2	1			1	1	3		2	10	
<i>Talaromyces stipitatus</i> (Ts)			2	1			1	2	4		1	11	
<i>Trichophyton rubrum</i> (Tr)				1								1	
Total			54	27			10	8	40		32	171	
Leotiomycetes	N/A	A	B	C	D	E	F	G	H1a	H1b	H2	Total	
<i>Botryotinia fuckeliana</i> (Bf)			1	1					2		2	6	
<i>Monilinia fructicola</i> (Mf)											1	1	
<i>Sclerotinia sclerotiorum</i> (Ss)			1	1					2		1	5	
Total			2	2					4		4	12	
Sordariomycetes	N/A	A	B	C	D	E	F	G	H1a	H1b	H2	Total	
<i>Chaetomium globosum</i> (Cglo)				1					1		2	4	
<i>Gibberella moniliformis</i> (Gm; <i>Fusarium verticillioides</i> ^c)			1									1	
<i>Gibberella pulicaris</i> (Gp)			1									1	
<i>Magnaporthe grisea</i> (Mg)			4				1		2			7	
<i>Neurospora crassa</i> (Nc)									2			2	
<i>Podospira anserina</i> (Pa)			1	1					2		1	5	
<i>Trichoderma atroviride</i> (Ta; <i>Hypocrea atroviridis</i> ^c)											1	1	
Total			7	2			1		7		4	21	
Total (Pezizomycotina)													
	N/A	A	B	C	D	E	F	G	H1a	H1b	H2	Total	
	1		67	36			11	8	61		46	230	

Saccharomycotina

Saccharomycetes	N/A	A	B	C	D	E	F	G	H1a	H1b	H2	Total
<i>Ashbya gossypii</i> (Eg; <i>Eremothecium gossypii</i> ^d)		2			2							4
<i>Candida albicans</i> (Ca)		4			1		1					6
<i>Candida dubliniensis</i> (Cd)		3			1							4
<i>Candida glabrata</i> (Cg)		2			2	1	1					6
<i>Candida guilliermondii</i> (Cgu)		1					1					2
<i>Candida lusitanae</i> (Cl)		3					1					4
<i>Candida tropicalis</i> (Ct)		6			2		1					9
<i>Debaryomyces hansenii</i> (Dh)		4			1							5
<i>Candida krusei</i> (Ck)		1										1
<i>Kluyveromyces lactis</i> (Kl)		2			2		1					5
<i>Kluyveromyces thermotolerans</i> (Kth)		1			1		1					3
<i>Lodderomyces elongisporus</i> (Le)		3			1							4
<i>Pichia stipitis</i> (Ps)		4			1		2					7
<i>Saccharomyces cerevisiae</i> (Sc)		3			3	2	1					9
<i>Saccharomyces kluyveri</i> (Sk)		3			5		1					9
<i>Vanderwaltozyma polyspora</i> (Vp)					3							3
<i>Yarrowia lipolytica</i> (Yl)			4		1		1					6
<i>Zygosaccharomyces rouxii</i> (Zr)		4			4		1					9
Total		46	4		30	3	13					96

Total (Saccharomycotina)

46	4			30	3	13						96

Taphrinomycotina

Schizosaccharomycetes	N/A	A	B	C	D	E	F	G	H1a	H1b	H2	Total
<i>Schizosaccharomyces japonicus</i> (Sj)										1		1
<i>Schizosaccharomyces pombe</i> (Sp)		1								1		2
Total		1								2		3

Total (Taphrinomycotina)

1										2		3

Total (**Ascomycota**)

2	46	71	36	30	3	24	8	61	2	46		329

Basidiomycota

Agaricomycotina

Tremellomycetes	N/A	A	B	C	D	E	F	G	H1a	H1b	H2	Total
<i>Cryptococcus neoformans</i> (Cne)			2				1	3	1			7
Total			2				1	3	1			7

Agaricomycetes	N/A	A	B	C	D	E	F	G	H1a	H1b	H2	Total
<i>Coprinopsis cinerea</i> (Cc)									1	2		3
<i>Laccaria bicolor</i> (Lb)									1	4		5
Total									2	6		8

Total (Agaricomycotina)

2							1	3	3	6		15

Ustilaginomycotina

Ustilaginomycetes	N/A	A	B	C	D	E	F	G	H1a	H1b	H2	Total
<i>Ustilago maydis</i> (<i>Usm</i>)									2	1		3
<i>Malassezia globosa</i> (<i>Malg</i>)										2		2
Total									2	3		5

Total (Ustilaginomycotina)									2	3		5

Total (Basidiomycota)		2					1	3	5	9		20

Total PDRs for all species	2	46	73	36	30	3	25	11	66	11	46	349

^a Abbreviations of species names used for Supplementary Figs. 1-12 are shown in brackets

^b N/A : not assigned to cluster

^c Anamorph/Teleomorph (MycoBank, <http://www.mycobank.org>)

^d Synonym (MycoBank, <http://www.mycobank.org>)

Table 3

ul (top) and C-terminal halves (bottom) of individual clusters of full-size fungal PDR transporters.

↔	Q-loop	helical domain	ABC1 (C-loop)	↔	Walker B1	D-loop	↔	H-loop	H-TMS1	EL1	IL1	EL2	IL2	EL3
43	E	63-64	[V/I]SGGE	15	[L/F/V/L][Q/H]CWD	N	32	Y	152-156	12-14	34	10	12	88-91
43-(45)	E	64	VSGGE	15	[L/I/F]Q[C/A]WD	N	32	Y	153-156	12	34	10	12	89-92
(42)-43	E	64	V[S/P]GGE	15	[L/I/M/V/F][A/G/C/Q][A/S/C]WD	N	32	Y	155-157	12	34	10	12	87-91
42-43	E	64	VSGGE	15	[L/V/L/F][Y/F][C/S/A]WD	N	32	Y	154-157	12-16	32-34	10	12	93-95
44	E	57	VSGGE	15	VYLWD	N	32	S	155-165	12	34	10	12	92
37-58	Q	57-61	[L/C]SGGE	15	[V/I/L/F][L/M/F][C/L]D	E	31-33	H	128-145	15-16	37-38	7-16	8	66-82
42	E	59-64	VSGG[E/Q]	15	[L/V][I/T/F/A/Q][C/S/M][W/L/F/Y]D	N	32	Y	139-156	12	34	10	12	91-92
41-42	E	58-66	[V/I]SGGE	15	[V/I/L/T][V/L/Q/M/CT/A/S][C/A/S/G]WD	N	32	Y	145-166	12	34	10	12	88-99
42	E	63-72	VSGGE	15	[V/I][A/G/V/L][C/A/L/M][W/F/Y]D	N	32	Y	154-175	12	34	10	12	87-92
41-42	E	65	VSGGE	15	[V/I][F/Q/Y/V/M/A][C/F/T/V]WD	N	32	Y	150-154	12	34	10	12	90-93

Q-loop	helical domain	ABC2 (C-loop)	↔	Walker B2	D-loop	↔	H-loop	H-TMS7	EL4	IL3	EL5	IL4	EL6
Q	58	LN[V/I]EQ	16	L[L/V/I]FLD	E	31	H	132-140	12	31	22	9	104-110
Q	58	LNVE[Q/R]	16	L[L/C/F/I][F/L/F/V/I]D	E	31	H	138-148	12	31	22	9	106-119
Q	58	LNVE[C/Q]	16	L[L/I/V]FLD	E	31	H	136-144	12	31	15-(22)	9	107-111
Q	58	LNVEQ	16	LLF[L/V]D	E	31	H	138-146	12	(30)-31	(15)-16	9	107-108
Q	48	----Q	16	LLFLD	E	31	H	146-158	12	31	16	8	107
Q	59-60	ISGGE	15	[V/I][L/L/F]LD	E	31-32	H	129-142	11	34	8	11	64-72
Q	58-59	L[N/G][L/V]E[Q/E]***	15-16	[L/V][L/F/L]LD	E	31	H	131-148	12	30-31	16	9	107-110
Q	54-58	L[N/S/A/G/D/T][V/Q//P/T/E][E/G][Q/E/K/D/A]	16	[L/V][L/M]FLD	E	31	H	132-144	12	30-31	14-18	9	107-111
Q	(54)-58	LNVEQ***	16	LLFLD	E	31	H	138-142	12-14	31	14-16	9	96-110
Q	58-(59)	L[S/N][V/I]EQ	15-16	L[L/L]FLD	E	31	H	133-137	12	31	15-(16)	9	108-114

orders; H-TMS1 = the region between the H-loop and TMS1; TMS6-A2 = the region between TMS6 and Walker A2; H-TMS7 = the region

are ordered from left to right in order of abundance.

ordering conserved amino acids or motifs (↔) or found within the indicated structures.

two cluster G, and all cluster E PDR transporters.

Table 4
Conservation of amino acids in the PDR motifs A and B of full-size fungal PDR transporters.

	PDR motif A										linker		PDR motif B													
aa *	M	x	x	W	x	x	W	h	x	Y	x	N	P	h	x	Y	x	F	E	x	h	h	x	N	E	F
							F			W		D									V	V				
% **	81	-	-	99	-	-	98	100	-	96	-	94	95	95	-	89	-	97	87	-	94	89	-	88	100	94

* Amino acids are shown in single letter code with h = I, L or M, and X = any amino acid. Amino acids are ordered, from the top, in descending order of abundance.

** Numbers represent the percent conservation of the primary sequence positions in 244 full-size fungal PDR transporters (PDR cluster F proteins excluded).

Table 5

Conservation of the hydrophobic motif of EL6* in full-size fungal PDR transporters.

EL6 motif										
aa **,**	F	W	X	(X)	F	h	Y	X	X	P
					W					
% ****	95	98	-	(-)	95	96	87	-	-	100

* This motif is equivalent to the PDR motif A and the linker of EL3 shown in Table 4.

** Amino acids are shown in single letter code with h = I, L or M and **X** = any amino acid.

Amino acids are ordered, from the top, in descending order of abundance.

*** (X) indicates that this amino acid is present in some, but not other, Pdrps (see text for detail).

**** Numbers represent the percent conservation of the primary sequence positions in 244 full-size fungal PDR transporters (PDR cluster F proteins excluded).

Table 6

Conservation of amino acids in the IL1 of full-size fungal PDR transporters.

IL1																			TMS3		
aa *, **	E	I	X ₃	Y	X ₂	R	X	I	X ₂	R	X ₃	Y	A	X	Y	X	P	X ₁₀	E	I	P
		L		F				V		K		F	S		F				D	V	
				W				L					G							L	
								M												M	
% ***	96	91	-	84	-	99	-	100	-	100	-	95	91	-	85	-	94	-	93	95	96

* Amino acids are shown in single letter code with x = any amino acid. Amino acids are ordered, from the top, in descending order of abundance.

** Subscripts next to X denote the number of amino acids.

*** Numbers represent the percent conservation of the primary sequence positions in 244 full-size fungal PDR transporters (PDR cluster F proteins excluded).

Table 7
Conservation of amino acids in the IL3 of full-size fungal PDR transporters.

IL3																												TMS9			
aa *, **	x	p	x	F	x ₃	R	x	I	Y	E	x	R	E	x ₂	S	K	x	Y	x	W	x ₂	F	I	x ₃	I	x ₂	E	I	P		
				Y				V	F	D						R		F					L		V			L			
								L	W							N							V				V				
								M														M					M				
% ***	-	86	-	90	-	97	-	98	97	84	-	100	99	-	92	96	-	99	-	93	-	85	84	-	81	-	99	90	91		

* Amino acids are shown in single letter code with X = any amino acid. Amino acids are ordered, from the top, in descending order of abundance.
** Subscripts next to X denote the number of amino acids.
*** Numbers represent the percent conservation of the primary sequence positions in 244 full-size fungal PDR transporters (PDR cluster F proteins excluded).