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Functional analysis of an ATP-binding cassette transporter protein from *Aspergillus fumigatus* by heterologous expression in *Saccharomyces cerevisiae*

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Abstract

Aspergillus fumigatus is the major filamentous fungal pathogen in humans. Although *A. fumigatus* can be treated with many of the available antifungal drugs, including azole compounds, drug resistant isolates are being recovered at an increasing rate. In other fungal pathogens such as the *Candida* species, ATP-binding cassette (ABC) transporter proteins play important roles in development of clinically-significant azole resistance phenotypes. Central among these ABC transporter proteins are homologues of the *Saccharomyces cerevisiae* Pdr5 multidrug transporter. In this work, we test the two *A. fumigatus* genes encoding proteins sharing the highest degree of sequence similarity to *S. cerevisiae* Pdr5 for their ability to function in a heterologous *pdr5Δ* strain of *S. cerevisiae*. Expression of full-length cDNAs for these two Afu proteins failed to suppress the drug sensitive phenotype of a *pdr5Δ* strain and no evidence could be obtained for their expression as green fluorescent protein (GFP) fusions. To improve the expression of one of these Afu ABC transporters (XP_755847), we changed the sequence of the cDNA to use codons corresponding to the major tRNA species in *S. cerevisiae*. This codon-optimized (CO Afu abcA) cDNA was efficiently expressed in *pdr5Δ* cells and able to be detected as a GFP fusion protein. The CO Afu abcA did not correct the drug sensitivity of the *pdr5Δ* strain and exhibited a high degree of perinuclear fluorescence suggesting that this fusion protein was localized to the *S. cerevisiae* ER. Interestingly, when these experiments were repeated at 37 °C, the CO Afu abcA was able to complement the drug sensitive phenotype of *pdr5Δ* cells and exhibited less intracellular fluorescence. Additionally, we found that the CO Afu abcA was able to reduce resistance to drugs like phytosphingosine that act via causing mislocalization of amino acid permeases in fungi. These data suggest that the Afu abcA protein can carry out two different functions of Pdr5: drug transport and regulation of protein internalization from the plasma membrane.

Keywords

Aspergillus fumigatus; ABC transporter; Heterologous expression; *Saccharomyces cerevisiae*; Functional complementation

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fgb.2013.06.004>.

1. Introduction

As the population of immunocompromised patients continues to increase, *A. fumigatus*-based fungal disease has increased in a similar fashion. Although existing antifungal drugs are efficacious in treating *A. fumigatus* infections, the inevitable rise in resistant isolates has begun (reviewed in Pfaller, 2012). The primary antifungal drug treatment involves azole compounds such as voriconazole. Most resistant *A. fumigatus* isolates contain changes in the target of these azole drugs, the lanosterol α 14 demethylase encoded by the *cyp51A* gene. However, increasing numbers of azole resistant organisms are being recovered that possess no detectable change at their *cyp51A* locus, suggesting the possibility that alternative mechanisms of azole resistance are at work (reviewed in Bowyer et al., 2011; Escibano et al., 2012; Tashiro et al., 2012).

A likely contributor to drug resistance in *A. fumigatus* are membrane transporters of the ABC family (Dean et al., 2001). These proteins have been extensively studied in other fungal pathogens, especially in *Candida albicans* and *Candida glabrata*, where azole resistance occurs as a result of the overexpression of particular ABC transporter proteins (Morschhauser, 2010; Shahi and Moye-Rowley, 2009; Klein et al., 2011; Prasad and Goffeau, 2012). One of the most important of these *Candida* ABC transporters is the Cdr1 protein, first discovered on the basis of its ability to complement the drug sensitive phenotype of a *pdr5Δ* strain of *S. cerevisiae* (Prasad et al., 1995). Pdr5 is the founding member of these ABCG-type ABC transporters in fungi that act as strong drug resistance determinants (Balzi et al., 1994; Bissinger and Kuchler, 1994; Hirata et al., 1994). Pdr5 and its relatives are thought to act as broad specificity, ATP-dependent drug efflux pumps (recently reviewed in (Prasad and Goffeau, 2012). Overexpression of Pdr5 in *S. cerevisiae* results in a strain that exhibits resistance to a large number of different drugs.

The *A. fumigatus* genome encodes 50 different genes classified as ABC transporter proteins (Kovalchuk and Driessen, 2010). To facilitate the analysis of these *A. fumigatus* proteins, we employed the approach of heterologous expression in a *pdr5Δ* strain of *S. cerevisiae*. This strategy has been used extensively in the analysis of ABC transporter proteins from the *Candida* species (Moran et al., 1998; Nakamura et al., 2001; Sanglard et al., 1999; Wada et al., 2002) and allows a relatively simple evaluation of the function of foreign transporters in the well-characterized *S. cerevisiae* background.

Initial experiments aimed at producing the two ABC transporters from *A. fumigatus* that shared the highest degree of sequence similarity were unsuccessful likely owing to the presence of unfavorable codons in the *A. fumigatus* sequences. We selected the Afu ABC transporter sharing the highest degree of sequence similarity with Sc Pdr5 (XP_755847), which we designated abcA, and had this gene chemically synthesized in a form that was codon-optimized for strong expression in the *S. cerevisiae* background. When we combined this codon-optimized cDNA with a higher growth temperature than is typically employed in *S. cerevisiae* experiments, we found that the Afu abcA protein localized to the plasma membrane and complemented *pdr5Δ* drug sensitivity. From these experiments, we conclude that Afu abcA is capable of fulfilling at least two different functions of Pdr5 and that its efficient folding/trafficking requires expression at an elevated temperature. These data suggest that abcA and Pdr5 are likely to be carrying out similar roles in *A. fumigatus* and *S. cerevisiae*, respectively.

2. Materials and methods

2.1. Strain and media

The two *S. cerevisiae* strains used in this study were *pdr5Δ* (Katzmann et al., 1994) and *pdr5Δyor1* (Johnson et al., 2010) strains in the SEY6210 background. Untransformed strains were maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) medium. Upon plasmid transformation by standard lithium acetate protocol (Ito et al., 1983), cells were maintained in selective SC-ura [6.7 g/L Difco yeast nitrogen base w/o amino acids, 2% glucose medium, 1.92 g/L Ura drop out mix (Sigma)], which was also used for spot testing of drugs on solid plates with 1.5% agar.

2.2. Plasmids

The list of oligonucleotides for PCR amplification and plasmids used in this study is given in Tables 1 and 2, respectively. The intronless *abcA* gene was PCR amplified from genomic DNA isolated from *A. fumigatus* Af293 strain as 5 exonic DNA fragments. Exons 1, 2, 3, 4 and 5 were PCR amplified using the following primer pairs: Exon 1 – CUP1pr-*abcA*-Ex1-F and *abcA*-Ex1-R; Exon 2 – *abcA*-Ex2-F and *abcA*-Ex2-R; Exon 3 – *abcA*-Ex3-F and *abcA*-Ex3-R; Exon 4 – *abcA*-Ex4-F and *abcA*-Ex4-R and Exon 5 – *abcA*-Ex5-F and *abcA*-Ex5-GFP-R. The primers were designed such that each exonic DNA fragment had a 50 bp overlap with the adjacent exon. 5' termini of exon 1 DNA had a 50 bp overlap with the 3' end of the *S. cerevisiae* CUP1 promoter (CUP1pr) DNA sequence while the 3' termini of exon 5 DNA had a 50 bp overlap with the 5' end of the GFP DNA sequence. In all cases, high fidelity Phusion (New England Biolabs) Pfu polymerase was used for PCR amplification. These PCR amplified exonic DNA fragments were co-transformed along with the pRS316-CUP1-FUR4-GFP gapped within FUR4 by HindIII/BamHI digestion, into the SEY6210 *pdr5Δ* strain for the generation of pRS316-CUP1-Afu *abcA*-GFP by gap repair recombination in *S. cerevisiae*. Plasmids were isolated from *S. cerevisiae*, and recovered in *E. coli* DH10B cells by electroporation. Plasmids isolated from DH10B cells were used for restriction mapping, sequencing, and for further use.

The *abcA* gene adapted for optimal expression in *S. cerevisiae* was chemically synthesized (Genscript, Inc) as 2 DNA fragments that overlapped with each other by 200 bp. The 2.4 kb N-terminal *abcA* DNA fragment was cloned in the low copy pJET2.1/blunt vector in between the XhoI and XbaI sites within the polylinker. The 2.4 kb C-terminal *abcA* DNA fragment was cloned in the pUC57 vector in the EcoRV site. The N-terminal and C-terminal *abcA* DNA fragments were released from the respective vectors by XhoI/HindIII and KpnI/PstI digestions and were co-transformed along with the pRS316-CUP1-FUR4-GFP gapped within FUR4 by HindIII/BamHI digestion, into the SEY6210 *pdr5Δ* strain for the generation of pRS316-CUP1-Sc Afu *abcA*-GFP by gap repair recombination in *S. cerevisiae*. Appropriate recombinants were identified on the basis of high level production of membrane-associated fluorescence and functional assays.

While the use of the codon-optimized Afu *abcA* provided strong expression in *S. cerevisiae*, we encountered serious problems when attempting to recover the correctly recombined fusion gene in *E. coli*. Bacterial transformants were found to contain only incorrect versions of the pRS316-CUP1-Sc Afu *abcA*-GFP fusion that appeared to represent deletion derivatives of this plasmid. We were concerned that the inability to retrieve this plasmid was due to inappropriate expression of the extremely hydrophobic *abcA* in bacteria as was previously described for the cystic fibrosis trans-membrane conductance regulator (Gregory et al., 1990). To reduce this expression, we replaced the high-copy-number bacterial replication origin of the pRS316 plasmid with a lower copy origin from the pACYC177 plasmid. The p15A origin of replication and KanR marker gene were PCR amplified using

primers pRS316-KpnI-p15A-F and pRS316-PmlI-KanR-R from pACYC177 DNA. The above primers were designed such that the 5' terminus of the PCR product had a 48 bp overlap with pRS316 sequence upstream of KpnI site and the 3' terminus had a 55 bp overlap with pRS316 sequence downstream of PmlI site. This PCR product was co-transformed with pRS316-*CUP1-FUR4*-GFP digested with KpnI/PmlI (to remove the pBluescript origin and AmpR gene) into the *S. cerevisiae* SEY6210 strain to generate pRS316-p15A ori-kanR-*CUP1-FUR4*-GFP plasmid by gap repair recombination under uracil selection. The plasmid recovered from *S. cerevisiae* was then transformed into *E. coli* DH10B strain by electroporation and selected for kanamycin resistance. The structure of this plasmid was confirmed by restriction mapping. Using this reduced copy number plasmid, the gap repair recombination described above for the codon-optimized Afu *abcA* clone was repeated. The use of this lower copy number origin permitted the isolation of the desired recombinant.

2.3. Spot assays

Overnight cultures grown in SC-ura at 30 °C were re-inoculated in fresh media at an A_{600} of 0.05 and grown to mid-log phase cells (A_{600} of around 0.5) at either 30 °C or 37 °C, corresponding to the temperature at which the spotted drug plates were incubated. 500 cells were spotted onto drug plates at indicated drug concentrations and incubated at either 30 °C or 37 °C. When gradient drug plates were used for spot assays, the concentration of drug indicated correspond to maximum drug concentration on the plate.

2.4. Fluorescence microscopy

SEY6210 derivatives lacking either Pdr5 (*pdr5Δ*) or Pdr5 and Yor1 (*pdr5Δ yor1*) transformed with GFP-tagged *FUR4* or *abcA* plasmids were grown overnight in SC-ura at 30 °C and subcultured from an A_{600} of 0.05–0.5 in SC-ura at either 30 °C or 37 °C. Cells were then washed and resuspended in sterile water and visualized for GFP fluorescence and Nomarski optics using an Olympus (Tokyo, Japan) BX-60 microscope with a 100× oil immersion objective. Images were captured using a Hamamatsu (Shizuoka, Japan) ORCA charge-coupled device camera.

2.5. Western blotting

For Western blot analysis of the GFP-tagged *abcA* or *FUR4* transformants, cells were grown in SC-ura to an A_{600} of 0.8 in SC-ura, and whole cell extracts were prepared using the TWIRL buffer (8 M urea, 5% SDS, 10% glycerol, 50 mM Tris, pH 6.8, 5% β-mercaptoethanol) extraction method. Briefly, 2 A_{600} units of cells were resuspended in 100 μl of TWIRL buffer and vortexed for 3 min with glass beads at 4 °C and centrifuged for 5 min at 12,000 rpm in an Eppendorf microcentrifuge at 4 °C. 20 μl of supernatant were electrophoresed on 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% nonfat dry milk in phosphate-buffered saline, and then probed with polyclonal anti-GFP antiserum (1:200). Horseradish peroxidase conjugated secondary antibody and an ECL kit (Pierce) were used to visualize immunoreactive protein.

2.6. Rhodamine 6G assay

The rhodamine efflux study was essentially carried out as described by Kolaczowski et al. (1996) with a few modifications. The strains were pregrown in SC-ura broth overnight at 30 °C and then reinoculated into fresh media to an initial A_{600} of 0.1. After incubation for 3 h at 37 °C, equal numbers of cells from the exponential-phase cultures were harvested, washed twice with water and resuspended in 0.5 ml HEPES buffer (50 mM HEPES-NaOH, pH 7.0). 2-Deoxy-β-glucose and rhodamine 6G were added to a final concentration of 5 mM and 5 μM, respectively, and incubated at 37 °C for 2 h. After incubation, rhodamine-6G-loaded

cells were spun down, washed twice with HEPES buffer and finally resuspended in 4 ml HEPES buffer. 1 mM glucose was added to initiate the energy dependent efflux of rhodamine 6G. The cell suspension was incubated at 37 °C. At time zero, i.e. immediately after the addition of glucose, and after 15, 30 and 45 min of further incubation, 0.5 ml of sample was withdrawn, cells were removed by centrifugation and the fluorescence of rhodamine 6G extruded in the supernatant (100 µl) was measured at an excitation and emission wavelength of 529 nm and 553 nm, respectively, on a SpectraMAX GeminiXS microplate fluorimeter (Molecular Devices).

3. Results

3.1. Generation of *S. cerevisiae* adapted *A. fumigatus* *abcA* expressing plasmid in *S. cerevisiae*

Since *A. fumigatus* contains 15 different ABCG class ABC transporter-encoding genes (Kovalchuk and Driessen, 2010), we first aligned these with Sc Pdr5 in order to select Afu proteins with the highest sequence similarity. The homologues closest to Sc Pdr5 are shown in Fig. 1A. If the stringency of the alignment is further increased, only two Afu proteins share adequate sequence similarity to satisfy this criterion: XP_755847 and XP_752803. We designated these two ABC transporters as *abcA* and *abcB*.

To express these Afu genes in Sc, we first constructed full-length cDNAs based on the predicted structures of each gene. These were prepared by PCR amplification across all predicted intron-exon boundaries directly from genomic DNA. Efforts to construct full-length cDNAs from the respective mRNAs were complicated by the large size of these transcripts (data not shown). The different amplification products were linked together by homologous recombination in *S. cerevisiae* cells (see Supplementary Fig. 1 for a description of this strategy for *abcA*). Both *abcA* and *abcB* were inserted into a pRS316 plasmid backbone. This insertion placed both cDNAs under control of the Sc *CUP1* promoter and fused GFP to their C-terminal amino acid. These expression clones were introduced into *pdr5Δ* cells. Transformants tested for their ability to correct the drug hypersensitive phenotype caused by loss of Pdr5 as well as for the expected fluorescence signal from the GFP fusion proteins. Neither cDNA produced any detectable change in these two parameters (data not shown).

The failure of these cDNAs to function in the *S. cerevisiae* strain prompted us to more closely examine the sequences of the two *A. fumigatus* clones. In each case, a large number (19 in the case of *abcA*) of inefficiently translated codons were present in the native *A. fumigatus* coding sequence that could contribute to undetectable production of the polypeptide in the heterologous *S. cerevisiae* background (data not shown). In order to address this possibility, we purchased a chemically synthesized cDNA that was codon-optimized (CO) in order to use the most abundant *S. cerevisiae* tRNAs. This CO Afu *abcA* cDNA clone was constructed by recombination in *S. cerevisiae* (strategy shown in Supplementary Fig. 1). This cDNA was again placed under control of the Sc *CUP1* promoter and expressed as a GFP fusion protein. The proper construction of this clone was verified by DNA sequencing and then transformed into *pdr5Δ* cells to assess its function.

3.2. Confirmation of expression of heterologous *abcA* in *S. cerevisiae*

We transformed low-copy-number plasmids expressing either Fur4-GFP or the CO Afu *abcA*-GFP from the Sc *CUP1* promoter into strains lacking the *PDR5* gene. *FUR4* encodes the Sc uracil permease and has no known influence on drug resistance. Transformants were grown to mid-log phase at 30 °C, protein extracts prepared and then subjected to western blotting analysis using anti-GFP antiserum (Fig. 1B).

Detection of the CO Afu abcA-GFP protein was easily accomplished under these conditions. This fusion protein accumulates at the appropriate molecular weight. Expression of the CO Afu abcA-GFP is much stronger than Fur4-GFP under these experimental conditions.

3.3. Functional status of heterologous abcA in *S. cerevisiae*

Having confirmed that our CO Afu abcA-eGFP construct is expressed in *S. cerevisiae*, we wanted to examine the functional status of this protein. The transformants described above were grown to mid-log phase and then placed as spots of 500 cells on rich medium containing fluconazole. These plates were then incubated at 30 °C for 48 h and then photographed (Fig. 2A).

We were surprised to see that there was no significant difference in fluconazole resistance between *pdr5Δ* cells expressing either abcA-GFP or Fur4-GFP. We wondered if the very different standard growth conditions of *A. fumigatus* (37 °C) and *S. cerevisiae* (30 °C) might have an impact on the relative lack of function of the abcA-GFP protein expressed in this heterologous environment. We repeated this fluconazole resistance assay but allowed the plates to develop at 37 °C.

As can be seen (Fig. 2A), increasing the incubation temperature led to a readily discernable increase in fluconazole resistance supported by abcA-GFP expressed in *S. cerevisiae*. It is important to note that the level of fluconazole resistance provided by abcA-GFP is still quite low compared to native Pdr5. Wild-type cells are capable of growth in the presence of 20 µg/ml fluconazole while these transformed cells grow very poorly at concentrations as low as 1 µg/ml (data not shown). However, these findings support the view that abcA is capable of complementing the fluconazole sensitivity caused by loss of Pdr5.

While the drug efflux activity of Pdr5 is well-described, more recent findings have implicated this transporter in other event at the membrane. The presence of Pdr5 appears to down-regulate other drug transporters (Kolaczowska et al., 2008) and amino acid permeases (Johnson et al., 2010). Resistance to the DNA damaging agent 4-nitroquinoline-N-oxide (4-NQO) is mediated in large part by the ABCG homologue Snq2 (Decottignies et al., 1995) and enhanced upon loss of Pdr5 (Kolaczowska et al., 2008). To determine if abcA was capable of correcting this other Pdr5-dependent phenotypes, the transformants described above were placed on media containing either 4-NQO or PHS (Fig. 2B). Expression of abcA was able to lower the enhanced 4-NQO resistance while expression of Fur4 was not. Similarly, loss of Pdr5 has been shown to increase resistance to the sphingolipid intermediate phytosphingosine (PHS) (Kihara and Igarashi, 2004). PHS causes mistargeting of amino acid permeases, including the tryptophan-specific permease Tat2, to the vacuole where these proteins are degraded (Chung et al., 2001; Skrzypek et al., 1998). We found that loss of Pdr5 also inhibits the vacuolar turnover of Tat2, likely due to a lowered rate of endocytosis (Johnson et al., 2010). As seen for 4-NQO, expression of abcA was able to reduce PHS tolerance while the presence of the *CUP1*-driven *FUR4* gene had no effect. These data indicate that abcA can carry out functions of Pdr5 related to drug transport and also control of permease function.

3.4. Efflux activity supported by heterologous abcA

To examine the quantitative function of Afu abcA expressed in *S. cerevisiae*, we compared the ability of this *A. fumigatus* transporter to efflux the fluorescent dye rhodamine 6G. Transport of this dye is carried out by Pdr5 and provides a convenient assay for function of this transporter (Kolaczowski et al., 1996).

Wild-type cells were used as a control for normal levels of Pdr5 function. The expression plasmids producing either Fur4- or abcA-GFP were introduced into a *pdr5Δ* strain as above. These three different strains were grown to mid-log phase at 37 °C and assayed for their relative ability to extrude rhodamine 6G into the culture supernatant as described (Kolaczowski et al., 1996). Wild-type cells rapidly exported rhodamine 6G while *pdr5Δ* transformants expressing abcA-GFP produced a lower but easily detectable rate of rhodamine 6G transport (Fig. 3). The Fur4-GFP transformants exhibited a rate of transport that was barely measurable over the entire time course of the experiment. As the plate assays indicated, these biochemical measurements confirm that abcA-GFP is capable of restoring Pdr5-like function to cells lacking their normal copy of *PDR5*.

3.5. Differential steady-state localization of heterologous abcA in response to growth temperature

Since our functional assays described above indicate the importance of the growth temperature in the function of the abcA, we wanted to determine if the *A. fumigatus* protein was localized similarly at 30 °C and 37 °C. We visualized the location of both the abcA- and Fur4-GFP fusion proteins using their fused GFP moieties. Transformants expressing one of these fusion proteins were grown to mid-log phase at either 30 °C or 37 °C and then photographed under visible or fluorescent light (Fig. 4).

Growth at 37 °C led to a clear reduction in the level of intracellular fluorescence seen in abcA-GFP-expressing cells. The strong perinuclear fluorescence pattern produced by abcA-GFP at 30 °C is likely caused by much of this protein accumulating at the endoplasmic reticulum while this fluorescence signature was reduced when cells were grown at 37 °C. Pdr5 functions at the plasma membrane and the increased function of abcA-GFP seen at 37 °C correlates with this observed reduction in intracellular localization of the transporter. Together, these data suggest that trafficking of abcA-GFP to the plasma membrane is enhanced at the higher temperature. Fur4-GFP accumulated in intracellular compartments; a behavior that did not change in response to temperature (data not shown).

4. Discussion

The role of ABC transporters in drug resistance in fungal pathogens is best established in the *Candida* species (reviewed in Cannon et al., 2009; Morschhauser, 2010). Azole-resistant patient isolates of *Candida albicans* and *Candida glabrata* are commonly found that overproduce their respective Pdr5 homologue called Cdr1 (recently reviewed in Prasad and Goffeau, 2012). To date, the role of ABC transporters in azole tolerance in *A. fumigatus* has not assumed the importance seen in *Candida*. The vast majority of azole tolerant *A. fumigatus* isolates contain mutations in the promoter and/or coding sequence of the *cyp51A* gene encoding the target of this drug, lanosterol α 14 demethylase (Denning et al., 1997; Diaz-Guerra et al., 2003). However, other data suggest that mechanisms with no involvement of *cyp51A* are at work in the population of azole resistance *A. fumigatus* (Nascimento et al., 2003). Based on the presence of Cdr1 homologues with high sequence similarity in *A. fumigatus*, we believe it is only a matter of time before azole resistance mechanisms involving these transporters are discovered. Very recently, patient isolates with enhanced azole resistance dependent on overproduction of a Cdr1 homologue have been identified (Fraczek et al., 2013) strongly supporting this prediction. This places a premium on understanding the shared and unique features of the *A. fumigatus* transporter proteins.

To begin the systematic analysis of the Afu Pdr5/Cdr1 homologues, we wanted to develop a facile system in which to analyze the function of these transporters. The *pdr5Δ* background in *S. cerevisiae* has been used to analyze a number of different transporters, ranging from *Candida* to human (Kiser et al., 2001; Nakamura et al., 2001). We were surprised that our

initial attempts to produce the Afu transporter sharing the highest degree of sequence similarity with Pdr5, *abcA*, were unsuccessful. We resolved the initial expression problems by using a chemically-synthesized form of *abcA* cDNA that employed codons corresponding to the major *S. cerevisiae* tRNAs. Even these measures were not sufficient to produce functional *abcA* in *S. cerevisiae* until we grew the cultures at higher temperatures. Indeed, *A. fumigatus* thrives at elevated temperatures, suggesting that proper folding and localization may require higher temperatures for certain proteins derived from this organism. These experiences are useful guides for future experiments involving production of *A. fumigatus* proteins in the foreign environment of *S. cerevisiae*.

These experiments confirm that *abcA* is capable of correcting several defects caused by loss of Pdr5 in *S. cerevisiae*. Extensive literature links Pdr5 in particular and ABC transporters in general to drug resistance through their action as efflux pumps (reviewed in Prasad and Goffeau, 2012). Based on the high degree of sequence similarity between *abcA* and Pdr5, we anticipated that the *A. fumigatus* transporter would correct the azole sensitivity of the *pdr5Δ* strain. The putative function of Pdr5 as a regulator of the activity of other plasma membrane proteins is a more recently discovered function and much less well documented. PHS resistance of strains lacking *pdr5Δ* correlates with defects in the removal of the high-affinity tryptophan permease Tat2 from the plasma membrane (Johnson et al., 2010). Additionally, expression of the *SNQ2* gene encoding an ABC transporter that confers 4-NQO resistance is elevated in *pdr5Δ* cells (Kolaczowska et al., 2008). Although the understanding of the molecular mechanisms underlying these Pdr5-dependent functions is incomplete, *abcA* expressed heterologously can reverse both of these different phenotypes. We speculate that the regulatory function of Pdr5 is due to the action of this protein as a regulator of phospholipid asymmetry that in turn influences homeostasis of membrane proteins embedded in this bilayer (Kean et al., 1997; Pomorski et al., 2003). This suggests that in its native *A. fumigatus* environment, *abcA* can also provide similar functions. We are currently analyzing the behavior of *A. fumigatus* strains both lacking and overproducing *abcA*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ABC	ATP-binding cassette
ABCG	class G ABC transporter
Afu	<i>Aspergillus fumigatus</i>
CO	codon-optimized
Sc	<i>Saccharomyces cerevisiae</i>
SC-ura	synthetic complete medium lacking uracil
YPD	yeast-peptone-dextrose growth medium

Fig. 1.

Relationship of *A. fumigatus* ABCG homologues to *S. cerevisiae* Pdr5. (A) The relatedness of the 6 ABCG homologues from *A. fumigatus* sharing the most sequence similarity to *S. cerevisiae* Pdr5 is shown. This alignment was generated using Blast Tree View at the NCBI Database (Maximum Sequence Difference = 0.6). The two ABCG proteins with the highest degree of sequence similarity are XP_755847.1 (abcA) and XP_752803.1 (abcB). (B) Western blot analysis of codon-optimized (CO) cDNA encoding *A. fumigatus* abcA expressed in *S. cerevisiae*. Strains with the indicated relevant genotypes listed at the bottom were transformed with low-copy-number vectors expressing either CO abcA or Sc Fur4 from the *CUP1* promoter as GFP fusion proteins. Transformants were grown to mid-log phase, protein extracts prepared and subjected to western blotting using anti-GFP antibodies. At this level of exposure, Fur4-GFP is difficult to visualize.

Fig. 2.

Function of *abcA* expressed in *S. cerevisiae*. Plasmids directing the expression of either *abcA*-GFP or *Fur4*-GFP from the *CUP1* promoter were introduced into the indicated *S. cerevisiae* strains. (A) Transformants were grown to mid-log phase and then placed in duplicate on YPD medium containing 0.5 µg/ml fluconazole. Two identical plates were prepared and then placed at either 30 °C or 37 °C as noted. These plates were incubated for 48 h and then photographed. (B) Reversal of resistance phenotypes caused by loss of Pdr5. A *pdr5Δ yor1* strain was used to enhance the relevant phenotypes as described (Kolaczowska et al., 2008; Johnson et al., 2010). Transformants from above were placed on YPD medium containing the indicated concentrations of 4-nitroquinoline-N-oxide (4-NQO) or phytosphingosine (PHS). Resistance to these compounds is elevated in the absence of Pdr5 (Kolaczowska et al., 2008). Transformants were incubated at 37 °C and the plates photographed.

Fig. 3.

Rhodamine 6G transport by *abcA*. Either wild-type or *pdr5Δ* cells carrying the indicated expression plasmids were grown to mid-log phase. Cells were deenergized, loaded with rhodamine 6G and then washed. Cells were then resupplied with glucose and aliquots removed at various times. Samples were centrifuged and the level of rhodamine 6G in the supernatant determined using a fluorimeter.

Fig. 4. Temperature-regulated trafficking of *abcA* in *S. cerevisiae*. A. Transformants expressing either *abcA*- or *Fur4*-GFP were grown in selective medium at the indicated temperatures to mid-log phase. Cells were then visualized using Nomarski optics (DIC) or under fluorescent light (GFP). B. The indicated fields in the *abcA*-GFP panels above (Left = 30°, Right = 37°) were magnified 4× in order to see the fluorescence signal more clearly.

Table 1

Primers.

Primer	Sequence
CUPpro-abcA-Ex1-F	ATATAGAAGTCATCGAAATAGATATTAAGAAAAACAACTGTAACGctagaatggcgaatgcaaccatccta
abcA-Ex1-R	ACAAACCATGAGTTTCTCCCGCAATCGTCTTGAGGAAGGTTGAACATCCACTGCCTGGCCGGCCAAGTAC
abcA-Ex2-F	GGATTGTCCGCAGCGGCGAAATGCTTGTTGTAAGTGGCCGGCCAGGCAGTGGATGTTCAACCTTCCTCA
abcA-Ex2-R	TGATCATGAGAGACTCGAAAGCATAACCGATCGGATTCAAATAGTTCAGC
abcA-Ex3-F	GCTGAACATTTGAATCCGATCGTTATGCTTTTCGAGTCTCTCATGATCA
abcA-Ex3-R	AAAATGTGAAGCCAATGAACAGAGGCGGAATGATACTCGTTGCAGCCTTT
abcA-Ex4-F	AAAGGCTGCAACGAGTATCATTCCGCCTCTGTTTCATTGGCTTCACATTTT
abcA-Ex4-R	AAGAGCCGATGGACTTGCCAGCACACCATTGAAAATGAGACAAAGTGAGA
abcA-Ex5-F	TCTCACTTTGTCTCATTTTCAATGGTGTGCTGGCAAGTCCATCGGCTCTT
abcA-Ex5-GFP-R	AGACAGAAAATTTGTGACCATTAACATCACCATCTAATTCAACCAAggatccctcctcttgatcttgag
pRS316-Kpn1-p15A-F	CCATGCAAATGAGAAGATGCTTGTATATCGGAAAAATTTAGCGCTGGAttaataagatgatctcttgag
pRS316-PmlI-Kan-R	TATTTTAAATTTATATATTTATATTAATAAAATTTAAATTATAATTATTTTATAGccgtcccgtaagtc

Table 2

Plamid.

Plasmid	Description	Source
pRS316-CUP1 pro-FUR4-GFP		Robert Piper
pSP1	pRS316-Af abcA-GFP	This study
pSP4A	pJET2.1-Sc Af abcA N terminus	This study
pSP4B	pUC57-Sc Af abcA C terminus	This study
pSP13	pRS316-p15A ori-kanR-CUP1 pro-FUR4-GFP	This study
pSP14	pRS316-p15A ori-kanR-CUP1 pro-Sc Af abcA-GFP	This study