Contributions of Aspergillus fumigatus ATP-Binding Cassette Transporter Proteins to Drug Resistance and Virulence

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In yeast cells such as those of Saccharomyces cerevisiae, expression of ATP-binding cassette (ABC) transporter proteins has been found to be increased and correlates with a concomitant elevation in azole drug resistance. In this study, we investigated the roles of two Aspergillus fumigatus proteins that share high sequence similarity with S. cerevisiae Pdr5, an ABC transporter protein that is commonly overproduced in azole-resistant isolates in this yeast. The two A. fumigatus genes encoding the ABC transporters sharing the highest sequence similarity to S. cerevisiae Pdr5 are called abcA and abcB here. We constructed deletion alleles of these two different ABC transporter-encoding genes in three different strains of A. fumigatus. Loss of abcB invariably elicited increased azole susceptibility, while abcA disruption alleles had variable phenotypes. Specific antibodies were raised to both AbcA and AbcB proteins. These antisera allowed detection of AbcB in wild-type cells, while AbcA could be visualized only when overproduced from the hspA promoter in A. fumigatus. Overproduction of AbcA also yielded increased azole resistance. Green fluorescent protein fusions were used to provide evidence that both AbcA and AbcB are localized to the plasma membrane in A. fumigatus. Promoter fusions to firefly luciferase suggested that expression of both ABC transporter-encoding genes is inducible by azole challenge. Virulence assays implicated AbcB as a possible factor required for normal pathogenesis. This work provides important new insights into the physiological roles of ABC transporters in this major fungal pathogen.

The filamentous fungal pathogen Aspergillus fumigatus is the most common cause of invasive mold infection in humans, and it is associated with an alarmingly high mortality rate. Some triazole antifungal drugs (voriconazole and itraconazole) inhibit the growth of A. fumigatus in vitro and are effective in treatment of A. fumigatus infections; however, development of resistance to these chemotherapeutics is a growing concern (1). While alterations in the cyp51A gene, which encodes the enzymatic target of azole drugs, are commonly found, recent studies have provided evidence that other mechanisms of resistance are also present. One of the most common routes ofazole tolerance in other fungal pathogens involves the overproduction of a drug efflux pump, often of the ATP-binding cassette (ABC) transporter family (reviewed in reference 2). These azole resistance transporters are of the ABCG class of ABC transporters and are found in pathogenic yeasts like Candida albicans and Candida glabrata, in addition to Saccharomyces cerevisiae.

The major S. cerevisiae ABCG azole transporter is the Pdr5 protein (3–5). This plasma membrane-localized ABC transporter protein is overproduced in multidrug-resistant cells as a result of transcriptional activation by the related Pdr1 and/or Pdr3 zinc cluster-containing transactivator proteins (reviewed in references 6 and 7). Pdr5 is thought to act as a broad-specificity ATP-dependent drug efflux transporter (8). More recent evidence suggests that Pdr5 acts via control of phospholipid asymmetry in the plasma membrane in cooperation with another plasma membrane-localized ABC transporter called Yor1. The YOR1 gene is also controlled by Pdr1 and Pdr3 but produces an ABCB class transporter (9–11).

Extensive analyses with the pathogenic yeast species C. albicans and C. glabrata have demonstrated that these organisms, like S. cerevisiae, also express ABCG class transporters that are highly transcriptionally upregulated to produce an azole-tolerant phenotype (12–14). These transporters share a high degree of sequence similarity with S. cerevisiae Pdr5 (ScPdr5) and are referred to as C. albicans Cdr1 (CaCdr1) or C. glabrata Cdr1 (CgCdr1).

The role of ABC transporters in azole resistance in A. fumigatus is less clear-cut. A large body of evidence has accumulated demonstrating the occurrence of genetic alterations in the cyp51A gene encoding lanosterol 14α-demethylase, the target enzyme for azole drugs (15). Early analyses of azole-resistant A. fumigatus isolates indicated that the majority of these organisms contained alterations in the cyp51A coding sequence and often in the transcriptional control region (16). However, other experiments determined that changes in ABC transporter gene expression could be linked to increased azole resistance (17, 18, 19). More recent surveys of azole-resistant clinical isolates found that a large fraction of these organisms contained no detectable change at their cyp51A locus (1, 20). Importantly, overexpression of a gene encoding a Pdr5 homologue was found to be required for azole resistance in a strain with a normal cyp51A gene (21). Together, these findings support the view that, as in other fungal pathogens, transcriptional upregulation of ABC transporter gene expression is an important contributor to this clinically key phenotype.

We set out to systematically explore the contributions of various ABC transporters to drug resistance in A. fumigatus. A challenge to this study is the presence of a generally large number of related ABC transporters (50 total) and a specifically increased number of ABCG-type proteins (15 ABCG transporters) (22). To focus our analysis, we restricted the initial experiments to the two

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proteins sharing the greatest sequence similarity to ScPdr5 and the single protein in *A. fumigatus* with highest sequence similarity to ScYor1.

**MATERIALS AND METHODS**

*A. fumigatus* strains, growth conditions, and transformation. Three strains were used in this study: the *A. fumigatus* Af293 strain, for which the entire genome is available (25); the *alkA* (A1151) strain (24); and the *alkAΔ* (AFS35/strain (25)). These strains were typically grown at 37°C in rich medium (YG; 0.5% yeast extract, 2% glucose) or in minimal medium described elsewhere (28). For regeneration of protoplasts upon transformation into the Af293 background (only 1 was obtained). We also generated three independent isolates (typically 3) were generated, with the exception of the gene. These were cloned by homologous recombination in *Scerevisiae*.

TABLE 1 *A. fumigatus* strains used in this study

<table>
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<th>Strain</th>
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<td>Wild type</td>
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</tr>
<tr>
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<td><em>alkAΔ::pyrG</em></td>
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</tr>
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<td><em>alkAΔ::loxP</em></td>
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<td><em>alkAΔ::hph</em></td>
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</tr>
<tr>
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<td><em>yorAΔ::hph</em></td>
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<td><em>alkBΔ::hph</em></td>
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<td><em>alkBΔ::hph</em></td>
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<td><em>alkBΔ::hph</em></td>
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</tr>
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<td><em>alkBΔ::hph</em></td>
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</tr>
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<td>SPF66</td>
<td>AFS35</td>
<td><em>alkBΔ::hph</em></td>
<td>This study</td>
</tr>
<tr>
<td>SPF44</td>
<td>AFS35</td>
<td><em>pyrG::hph::alkA-luc::pyrG</em></td>
<td>This study</td>
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<tr>
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<td>AFS35</td>
<td><em>pyrG::hph::alkB-luc::pyrG</em></td>
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Plasmids. DNA manipulations were done using standard procedures (29) or according to the manufacturer’s instructions. A list of plasmids used in this study is provided in Table 2. The *alkA*, *alkB*, and *yorA* knock-out constructs were made taking advantage of recombination cloning in *S. cerevisiae* in pSP19 (30), amenable to propagation in *S. cerevisiae* and *A. fumigatus* transformation mediated by *Agrobacterium tumefaciens*. The disruption constructs were generated in pSP19, as described below. Sequences of all primers are available on request. Each cassette had the following components: *loxP-hph-loxP* selection cassette, flanked by a 1-kb region immediately upstream and downstream of the coding sequence of the gene to be deleted. The primers used for amplifying DNA upstream and downstream to the targeted gene were designed in pairs such that one had a 40-bp overlapping sequence with the selection cassette at one end and a 40-bp overlap sequence with the termini of pSP19, gapped by XbaI and Xhol digestion, at the other end. These primer pairs were designed to also contain 20 nucleotides to permit amplification of the desired segment of the *A. fumigatus* genome. The *loxP-hph-loxP* selection cassette was released from the plasmid pSKB57 (provided by Stacey Klutts, University of Iowa) by SpeI/EcoRV cleavage and gel purification. The *yorA*, *alkA*, and *alkB* deletion plasmids were named pSP36, pSP44, and pSP45, respectively. These plasmids were transformed into *A. tumefaciens* EHA10 for subsequent transformation into *A. fumigatus* Af293 strain. The *alkA* and *alkB* deletion alleles in the A1151 and AFS35 strains were generated as follows. Plasmid pSP44 was digested with SmalI. Upon digest with EcoRV cleavage and gel purification. The *yorA*, *alkA*, and *alkB* deletion plasmids were named pSP36, pSP44, and pSP45, respectively. These plasmids were transformed into *A. tumefaciens* EHA10 for subsequent transformation into *A. fumigatus* Af293 strain. The *alkA* and *alkB* deletion alleles in the A1151 and AFS35 strains were generated as follows. Plasmid pSP44 was digested with SmalI. The digestion product was purified by agarose gel electrophoresis. The purified DNA was ligated into pRS316 (31) by recombinational cloning in *S. cerevisiae*. These were cloned by homologous recombination in *S. cerevisiae*. The *loxP-hph-loxP* selection cassette was generated as described above, the flanking regions for homologous integration into the *A. fumigatus* genome and the *hspA* promoter were generated by PCR amplification using primers that had a 40-bp overlap sequence with pRS316. The plasmids carrying the *hspA-alkA* and *hspA-alkB* promoter fusions were named pSP46 and pSP47, respectively. The plasmids were released from their respective vector backbones by NotI and SpeI digestion for protoplast transformation.

The *alkA* gene was tagged using a green fluorescent protein (GFP)-*loxP-pyrG-loxP* fragment generated from PHL83 (Fungal Genetic Stock Center) digested with XbaI and SpeI. The above-mentioned DNA was flanked with 1 kb corresponding to the 3′ end of *alkA* and 1.5 kb of DNA immediately downstream of the *alkA* gene. These two segments of DNA were generated by primer pairs that had a 40-bp overlap with the XbaI-linearized pRS316 and 20 nucleotides of the *alkA* gene or 40 nucleotides of the *GFP-loxP-pyrG-loxP* fragment and 20 nucleotides of the *alkA* gene. These were cloned by homologous recombination in *S. cerevisiae* to generate pSP38. Digestion of pSP38 by Nhel and MluI allowed the release of the *alkA-GFP* tagging construct for protoplast transformation into the *yorA* A1151 strain with selection for uracil prototrophy. The *alkB* gene was tagged with GFP using the *loxP-hph-loxP* selection cassette, flanked by GFP and a 1.5-kb region corresponding to DNA immediately downstream of the *alkB* gene. The GFP gene was PCR amplified from pHL83 and was preceded by 1 kb of PCR-amplified DNA corresponding to the 3′ end of the *alkB* gene.

**TABLE 2** Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<td>pHLL83</td>
<td>pCR2.1 TOPO GA5-GFP::loxP-pyrG-loxP</td>
<td>FGSC</td>
</tr>
<tr>
<td>SKB57</td>
<td>pCR2.1 TOPO loxP-hph-loxP</td>
<td>S. Klutts</td>
</tr>
<tr>
<td>pSP19</td>
<td>pDHT ScCEN6 SCR1F4 ScURA3</td>
<td>30</td>
</tr>
<tr>
<td>pSP36</td>
<td>pSP19 yorAΔ::loxP-hph-loxP</td>
<td>This study</td>
</tr>
<tr>
<td>pSP44</td>
<td>pSP19 alkΔ::loxP-hph-loxP</td>
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</tr>
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<td>pSP45</td>
<td>pSP19 alkBΔ::loxP-hph-loxP</td>
<td>This study</td>
</tr>
<tr>
<td>pSP46</td>
<td>pRS316 loxP-hph-loxP::hspA-alkB</td>
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</tr>
<tr>
<td>pSP47</td>
<td>pRS316 loxP-hph-loxP::hspB-alkB</td>
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</tr>
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<td>pSP48</td>
<td>pRS316 alkA-GFP::loxP-hph-loxP</td>
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<td>pSP49</td>
<td>pET42A GST-alkA</td>
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<tr>
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<td>pET42A GST-alkB</td>
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To put *alkA* and *alkB* under the control of strong *hspA* promoter, the *loxP-hph-loxP* selection cassette was placed between a 1.5-kb region immediately upstream of the target gene and 1-kb *hspA* promoter (30), followed by a 1.5-kb region corresponding to the 5′ end of the target gene and inserted into pRS316 (31) by recombinational cloning in *S. cerevisiae*. While the *loxP-hph-loxP* selection cassette was generated as described above, the flanking regions for homologous integration into the *A. fumigatus* genome and the *hspA* promoter were generated by PCR amplification using primers that had a 40-bp overlap sequence with pRS316 linearized by XbaI and the *hph-loxP* selection cassette. The plasmids carrying the *hspA-alkA* and *hspA-alkB* promoter fusions were named pSP46 and pSP47, respectively. The plasmids were released from their respective vector backbones by NotI and SpeI digestion for protoplast transformation.

During the *A. fumigatus* disruption mutant, multiple independent isolates (typically 3) were generated, with the exception of the *alkAΔ* disruption in the Af293 background (only 1 was obtained).
end of the abcB gene in the tagging construct. All the PCR products had a 40-bp overlap with the adjoining DNA fragments to generate the abcB-GFP-loxP-hph-loxP-abcB tagging construct in XbaI-linearized pRS316 by homologous recombination in S. cerevisiae as described above for abcA. The resulting plasmid was named pSP58. The tagging construct was released from this plasmid by SpeI and XhoI digestion for protoplast transformation into the AS535 strain with selection for hygromycin resistance.

**Generation of polyclonal antibodies against AbcA and AbcB.** Fragments of 360 and 330 bp (corresponding to the N-terminal, non-transmembrane protein regions spanning 120 and 110 amino acids [aa]) from AbcA and AbcB, respectively, were PCR amplified and cloned in frame as BamHI/SalI fragments downstream of the glutathione (GST) tag in PET42a (EMD Millipore, Inc.) to form pSP40 and pSP41 and then expressed in E. coli BL21(DE3). The resulting plasmids were used to transform E. coli BL21(DE3). Two liters of transformed bacteria were grown to log phase and induced with 1 mM IPTG (isopropyl-D-thiogalactopyranoside) for 90 min. Cell lysates were prepared and used to determine the spore concentration. The spores were then appropriately diluted and plated on YM agar plates and incubated at 37°C for 48 h. The MIC endpoint was defined as the lowest concentration that produced complete inhibition of growth. Quality control was ensured by testing the following strains recommended in CLSI standard M38-A2: Candida parapsilosis ATCC 204304, Aspergillus flavus ATCC 204304.

**Spot assay.** Fresh spores of A. fumigatus were suspended in 1× phosphate-buffered saline (PBS) supplemented with 0.01% Tween 20 (1× PBST). The spore suspension was counted using a hemocytometer to determine the spore concentration. The spores were then appropriately diluted in 1× PBST so that ~20 spores (in 5 μl) were spotted on YG medium with or without the drug. The plates were incubated at 37°C and inspected for growth every 12 h.

**GFP localization.** Firefly luciferase assays were done using a firefly luciferase assay kit (obtained from Biotechn, Inc.) as described in reference 30. Luminescence was measured using an IVIS 100 imaging system (Caliper Life Sciences). The number of photons emitted per second (luminescence) was then normalized to the amount of total protein present in the cell lysate per ml using a Bradford assay. To estimate fold induction of promoters in the presence of voriconazole, one relative luminescent unit was calculated as luminescence emitted by the promoter-reporter gene immediately (0 h) after treatment with drug. A sublethal dose of voriconazole was used to minimize any effects of cell death on the observed luciferase activity measured.

**Western blotting.** A. fumigatus spores were grown overnight at 37°C in rich medium. The mycelia were harvested by filtration, washed in deionized water, and used to prepare crude cell lysates. The mycelia (5 mg [wet weight]) were ground in liquid nitrogen using a mortar and pestle and then resuspended in 1 ml of A. fumigatus extraction buffer (50 mM HEPES [pH 7.7], 1 M sucrose, 50 mM NaCl, 1 mM dithiothreitol [DTT], 1 mM EDTA). The cell suspension was vortexed in the presence of 0.5-mm glass beads for 5 min and subjected to low-speed centrifugation (850 × g for 5 min) at 4°C. The supernatant was taken and subjected to high-speed centrifugation (16,000 × g for 15 min) at 4°C. The pellet was taken and resuspended in 250 μl of 1× Laemmli buffer and incubated at 37°C for 15 min. Twenty-five microliters of this suspension was electrophoresed by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% nonfat dry milk in phosphate-buffered saline, and then probed with appropriate polyclonal rabbit antisera (1:500). Horseradish peroxidase-conjugated secondary antibody and an ECL kit (Pierce) were used to visualize immunoreactive protein.

**RESULTS**

**A. fumigatus contains a large family of ABCG and ABCC class ABC transporters.** While circumstantial evidence exists that ABC transporters are involved in azole resistance in *A. fumigatus*, no systematic investigation of this question has previously been undertaken. Given that the Cdr1 ABC transporter plays a major role in azole susceptibility in *Candida* species, we set out to analyze the ABCG class transporters in *A. fumigatus* that shared the most sequence similarity with this protein. We used the sequence of *S. cerevisiae* Pdr5 as the prototype fungal ABCG transporter since this intensively studied protein represents the best-understood and founding member of these membrane proteins. The characteristic structure of ABCG transporters resembling Pdr5 is shown in Fig. 1A.

A BLAST search comparing the ScPdr5 sequence against the *A. fumigatus* genome was performed (Fig. 1B). This compari-
son identified 15 proteins showing significant sequence conservation to Pdr5. The two proteins with the highest degree of sequence similarity were those with GenBank accession numbers XP_755847 and XP_752803. We selected these for further analyses and designated them AbcA and AbcB, respectively. The \textit{abcB} gene has also been designated \textit{cdr1B} by Fraczek and colleagues (21).

Along with these ABCG-type transporters, we also characterized the \textit{A. fumigatus} homologue of \textit{S. cerevisiae} Yor1. ScYor1 is the only known ABCC class transporter in this yeast that is localized to the plasma membrane (33). The other ABCC transporters are found in the vacuolar membrane (34, 35). A BLAST search using the ScYor1 protein sequence identified 10 ABCC class transporters in \textit{A. fumigatus} but, interestingly, only a single protein that shared the unique structure of ScYor1. Typically, ABCC proteins precede this duplicated structure with a set of 5 transmembrane domains, first described for the mammalian Mrp1 protein (36). ScYor1 and the \textit{A. fumigatus} protein with GenBank accession number XP_751910 both possess the duplicated ABCC transmembrane domains and ABC domains but lack this amino-terminal set of transmembrane segments. Since the \textit{A. fumigatus} protein mirrored this unusual structure, this was taken as evidence that it represents an authentic homologue of ScYor1, and we designated the gene \textit{yorA}.

\textbf{abcB and abcA knockout strains are sensitive to azole drugs.}

In order to assess the physiological roles of the three selected ABC transporter proteins, our first goal was to construct a series of isogenic mutant strains lacking the corresponding coding sequences in the genome. For each gene, a disruption allele was prepared in which 1 kb of 5' and 3' noncoding DNA was present flanking a hygromycin resistance determinant. We first constructed null alleles of \textit{abcA}, \textit{abcB}, and \textit{yorA} in the \textit{A. fumigatus} strain Af293. These null alleles were introduced into Af293 using \textit{Agrobacterium tumefaciens}-mediated transformation, and hygromycin-resistant isolates were recovered. We confirmed the correct integration of these disruption cassettes by PCR. Representative isolates were grown and tested for the ability to tolerate a series of drug challenges by placing spores on a plate containing a gradient of drug as described previously (37, 38). These plates were incubated at 37°C and then photographed (Fig. 2).

\textbf{Loss of either abcA or abcB led to an increase in azole susceptibility in Af293 cells.} A null allele of \textit{yorA} had no significant effect.
on azole tolerance. However, the data for \( \text{abcB} \) were weakened by the difficulty in obtaining disruption mutants of this gene in the \( \text{Af293} \) background. Since this raised the concern that the behavior we observed was not representative of a typical \( \text{abcB} \) null allele, we constructed multiple \( \text{abcB}/\text{H9004} \)::HYG alleles in two other strain backgrounds to address this issue.

Loss of the Ku70/80 heterodimeric protein has been found to strongly stimulate homologous recombination in several organisms, including \( \text{A. fumigatus} \) (24, 39, 40). We employed two different strains with different defects in the Ku80 complex to increase the frequency of obtaining homologously targeted integration events. These strains were \( \text{A1151} \) (\( \text{akuB}/\text{H9004} \)) and \( \text{AfS35} \) (\( \text{akuA}/\text{H9004} \)). Homologous integration was found to be high enough in these genetic backgrounds that the use of the laborious and time-intensive \( \text{A. tumefaciens} \)-mediated transformation was replaced with a more facile protoplast transformation technique. We were able to obtain at least two isolates of all disruption alleles (including \( \text{abcB}/\text{H9004} \)::HYG) using these backgrounds. We compared the abilities of these disruption mutants to grow in the presence of azole drugs using the gradient plate assay described above (Fig. 2B and C).

Loss of \( \text{abcB} \) caused a large decrease in azole sensitivity in all backgrounds that were tested, consistent with this gene being an important participant in tolerance to these drugs. The absence of \( \text{abcA} \) had more variable effects on voriconazole resistance, as its removal from \( \text{A1151} \) caused a negligible increase in susceptibility. However, an \( \text{abcA}/\text{H9004} \)::HYG allele in \( \text{AfS35} \) did elicit a modest increase in voriconazole sensitivity.

We also assayed the relative azole tolerance of these strains using a liquid growth assay. Spores of the relevant strains prepared in the \( \text{Af293} \) genetic background were grown in liquid media containing 2-fold serial dilutions of either voriconazole or itraconazole. Plates were observed every 24 h, and the lowest drug concentration at which no growth could be seen was noted (Fig. 3).

As in the radial growth assay on solid media, strains lacking \( \text{abcB} \) were the most sensitive to either azole challenge. The presence of the \( \text{abcA}/\text{HYG} \) allele produced a more subtle increase in azole susceptibility, while the effect of the \( \text{yorA}/\text{HYG} \) mutation could be detected only after extended incubations. These data confirm that the drug phenotypes indicated by our radial growth experiments are accurately assigned.

While these manipulations tested the effect of the loss of these transporters, we also wanted to explore the consequence of elevated expression of \( \text{abcA} \) and \( \text{abcB} \). To accomplish this, we prepared constructs in which the strong \( \text{hspa}^{\text{STAT}} \) promoter was placed upstream of the ATG for either \( \text{abcA} \) or \( \text{abcB} \). S' to this
promoter, we placed the hygromycin resistance gene. This cassette was flanked by 1 kb of DNA from the amino-terminal coding sequence and the promoters of abcA and abcB. Both of these constructs were introduced into As35 cells and hygromycin-resistant transformants selected. We recovered only hspA^{SSA1}-driven abcA clones and tested these for their resistance to voriconazole (Fig. 2C).

The presence of the hspA^{SSA1}-abcA fusion gene consistently increased voriconazole resistance. The inability to recover similar constructs for abcB is not understood presently.

**Analysis of abcA and abcB expression.** The results of the genetic analyses indicated that AbcB was an important contributor to azole tolerance, while AbcA played a minor role that could be uncovered when a strong promoter drove its expression. To correlate the level of expression of these two different proteins with their azole resistance phenotypes, we generated rabbit polyclonal antibodies against recombinant AbcB.

Detection of AbcA protein produced from the endogenous gene was not possible using our anti-AbcA antiserum. However, in strains containing the hspA-abcB fusion gene, the correctly sized protein was readily apparent. This finding is consistent with the ability of this fusion gene to increase voriconazole resistance shown in Fig. 2C. Treatment of these cells with ethanol, a stress known to increase hspA expression, led to a modest further elevation of AbcA expression.

Western blot comparison of extracts from wild-type or abcΔ cells using the anti-AbcB antiserum detected a polypeptide species of 170 kDa. A similar experiment using proteins derived from abcBΔ cells failed to detect this protein. This antiserum was capable of recognizing AbcB protein produced from the wild-type gene.

Having established that full-length AbcA and AbcB can be detected in cells, we wanted to determine where these membrane transporter proteins might be localized. To begin to examine this issue, we prepared fusions of each ABC transporter to the green fluorescent protein (GFP). In both cases, GFP was placed at the C terminus of the ABC transporter with expression controlled by the native A. fumigatus setting, AbcA-GFP (and AbcB-GFP) seemed to efficiently reach the plasma membrane.

There were differences observed in the fluorescence from these two membrane proteins. The AbcB-GFP fusion generated a more punctate appearance, while the AbcA-GFP fusion was more evenly distributed. Both of these patterns were clearly above the

FIG 4 Expression of AbcA and AbcB. (A) Whole-cell protein extracts were prepared from A. fumigatus strains of the indicated genotypes. The hspA-abcB-containing fusion strain was either unchallenged (−) or induced with 5% ethanol addition (+). Equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S dye to visualize total protein (top) or with a polyclonal antiserum directed against AbcA (bottom). Molecular mass markers, in kilodaltons, are shown in kilodaltons. (B) Whole-cell protein extracts prepared from the A. fumigatus strains of the indicated genotypes were processed as for panel A, except that the Western analyses were carried out using an antibody directed against recombinant AbcB.

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Western blot comparison of extracts from wild-type or abcΔ cells using the anti-AbcB antiserum detected a polypeptide species of 170 kDa. A similar experiment using proteins derived from abcBΔ cells failed to detect this protein. This antiserum was capable of recognizing AbcB protein produced from the wild-type gene.

Having established that full-length AbcA and AbcB can be detected in cells, we wanted to determine where these membrane transporter proteins might be localized. To begin to examine this issue, we prepared fusions of each ABC transporter to the green fluorescent protein (GFP). In both cases, GFP was placed at the C terminus of the ABC transporter with expression controlled by the wild-type promoter. Transformants were obtained and mycelia visualized using Nomarski optics and fluorescence microscopy (Fig. 5).

Both GFP fusion proteins were found to be associated with the cell surface. Notably, we found no detectable intracellular fluorescence, which contrasted with our previous studies of heterologously expressed AbcA-GFP in S. cerevisiae (38). In this foreign environment, we could readily detect perinuclear AbcA-GFP. In the native A. fumigatus setting, AbcA-GFP (and AbcB-GFP) seemed to efficiently reach the plasma membrane.

There were differences observed in the fluorescence from these two membrane proteins. The AbcB-GFP fusion generated a more punctate appearance, while the AbcA-GFP fusion was more evenly distributed. Both of these patterns were clearly above the
background seen in cells lacking a GFP fusion and consistent with both ABC transporters exhibiting steady-state localization to the plasma membrane. Further experiments are required to confirm this suggestion.

Transcriptional induction during voriconazole stress. We have previously described development of a firefly luciferase reporter gene system that works in *A. fumigatus* (30) and use of this system to produce fusion genes to several fungal promoters. Our finding of the roles of AbcA and AbcB in voriconazole resistance prompted us to test the ability of this azole drug to influence expression levels of these transporters using the luciferase fusion genes already generated. The promoters for *abcA*, *abcB*, and the glycolytic gene *gpdA2* were fused to firefly luciferase to make transcriptional reporters for each gene. These were integrated into the *A. fumigatus* genome at the *pyrG* locus. Appropriate integrants were grown to log phase and then treated with voriconazole for 45 or 90 min. Whole-cell protein extracts were prepared and assayed for their luciferase enzyme activities (Fig. 6).

Both the *abcA* and *abcB* promoters were found to be inducible upon voriconazole treatment. Importantly, the *gpdA2* promoter was unresponsive to voriconazole, consistent with azole drug induction being restricted to genes involved in its detoxification. These data suggest that control of *abcA* and *abcB* expression by voriconazole occurs via induction of elements within the promoters of these genes.

**Role of AbcA and AbcB in virulence during *Galleria* infection.** To analyze the role of AbcA and AbcB during pathogenesis, we utilized the *Galleria mellonella* (greater wax moth) infection model. The *G. mellonella* caterpillar has been established to be a successful model in identifying genes crucial for virulence in many pathogenic fungi, such as the yeasts *C. albicans* and *Cryptococcus neoformans* as well as filamentous fungi such as different species of *Aspergillus*, including *A. fumigatus*. Briefly, equal numbers of spores from various *A. fumigatus* strains were injected into *G. mellonella* larvae along with a buffer control. The outcome of this infection was determined by monitoring killing of the larvae, in both the presence and absence of drug (voriconazole) administration. The percentages of larvae that survived infection were plotted with time (Fig. 7).

In the absence of drug, spores lacking the *abcB* gene were found to be hypovirulent (*P* < 0.001). No significant differences were seen between the other strains, including strains lacking or overproducing AbcA. This finding suggests the possibility that AbcB is required for normal pathogenesis of *A. fumigatus*, irrespective of its role in azole resistance.

To assess the modification of the course of infection by azole drugs, voriconazole was injected 1 h after the initial exposure to *A. fumigatus* spores. Treatment with voriconazole strongly improved larval survival. Interestingly, the presence of the *hspA-abcA* overexpression cassette strain showed a trend toward enhanced virulence of cells in this assay. The significance of this effect was modest (*P* values of 0.052 for *hspA-abcA* versus *abcBΔ* and 0.1 for *hspA-abcA* versus wild type). Taken together, these data support the view that AbcB is required for normal virulence and that overexpression of AbcA may enhance azole resistance during infection.

**DISCUSSION**

Azole resistance in *A. fumigatus* appears to be entering a dynamic phase in its development. Most azole-resistant mutants previously described appeared to be associated with alterations in the *cyp51A*...
of drug resistance in resistant isolates of transporters in acquisition of drug resistance in patients. Azole-implicate increased azole resistance (21). The earlier work described in refer-
tion of an ABC transporter-encoding gene called cdr1B (referred to in this work as abcB) was elevated and likely the cause of increased azole resistance (21). The earlier work described in reference 21 provided the first evidence for the role of cdr1B (abcB) in azole resistance in wild-type A. fumigatus. In this study, we have extended our understanding of this ABC transporter protein with the finding that Cdr1B/AbcB is critical for azole resistance in several common laboratory strains, detecting the polypeptide chain for the first time, localizing the protein to the plasma membrane, and providing evidence that this protein may be required in pathogenesis. Combined with the previous study (21), these data implicate Cdr1B/AbcB as an important participant in the biology of drug resistance in A. fumigatus.

Other fungal pathogens have clearly established roles for ABC transporters in acquisition of drug resistance in patients. Azole-resistant isolates of C. albicans and C. glabrata are readily obtained that can be shown to exhibit elevated expression of ABC transporter-encoding genes. Extensive analyses of these azole-tolerant organisms have implicated alterations in the transcriptional con-

FIG 7 Virulence effects of AbcA and AbcB. G. mellonella larvae were injected with the indicated A. fumigatus strains (2.5 x 10^5 spores) or a buffer control (PBST). All these experiments employed the AFS35 background. These injec-
tions were done either without (A) or with (B) an initial injection with voriconazole to assess the ability of this azole drug to protect the larvae from infection. Survival of injected larvae was assessed every 24 h after injection.

trol of the relevant ABC transporter genes leading to large elevations in transcript and protein level (13, 14). In both Candida species, single amino acid substitutions in a zinc cluster-containing transcription factor leads to this constitutive induction of ABC transporter expression (42, 43). The increased azole tolerance in these strains is genetically quite stable. The more recent identification (21) of strains overproducing cdr1B (also called abcB or abcC) in a wild-type cyp51A background will be interesting to examine for the nature of the transcriptional activation of this key azole resistance-conferring ABC transporter-encoding gene.

We also provide evidence that the promoters of both abcA and abcB can drive azole-inducible gene expression. Previous microarray experiments indicated that a number of different azole-inducible genes are present in A. fumigatus (19). Several ABC transporter-encoding genes were profiled in this work, and abcB (also called abcC or cdr1B) was found to be highly inducible by voriconazole challenge. The voriconazole induction seen in the microarray experiments was much greater than we report here, but this could be due to differences in strain backgrounds or experimental manipulations. Irrespective of the magnitude of induction, some fraction of this is supported by the promoter regions of both these ABC transporter-encoding loci. Additionally, this inducibility may reduce the generation of transcription factor mutations, such as those seen in Candida species and S. cerevisiae, and instead allow an acute and reversible response to azole challenge. Once the drug concentration has dropped sufficiently, the inducing stimulus may be removed and expression of drug resistance genes returned to baseline.

The data reported here also provide the first insight into the subcellular localization of both AbcA and AbcB. Both of these proteins were found on the A. fumigatus plasma membrane but did not exhibit a uniform distribution. The punctate fluorescence pattern of each transporter suggests that these may be enriched in discrete subdomains of the A. fumigatus plasma membrane. Since very little is known of the cell biology of membrane trafficking in this organism, an important subsequent goal will be to further analyze localization of AbcA and AbcB to the plasma membrane in comparison with other protein to establish the specificity of this observed localization. It was surprising to see no evidence for intracellular fluorescence of either ABC transporter protein. While the yeast Pdr5 homologues, it is typical to see the protein localized to the plasma membrane but also to the intracellular vacuole, the site of degradation of this and other proteins (44). Using our antibodies and GFP fusion proteins, we will address the itinerary of the A. fumigatus ABC transporters.

While it is common for ABC transporters to play significant roles in drug resistance, we also found that loss of AbcB led to a decrease in virulence in the G. mellonella infection model. This defect was seen irrespective of the presence of azole drug and suggests that AbcB is required for normal pathogenesis of the fungus. This effect is not seen with AbcA; instead, AbcA appears to work in a more typical fashion by increasing tolerance toazole drugs (certainly when overproduced). Another striking difference between abcA and abcB was the relative ease of genetically modifying either locus. We could easily obtain strains overproducing or lacking AbcA but had to go to some length to recover adequate numbers of isolates of abcB null alleles and were not able to recover a strain that overproduced AbcB from the hspA promoter. These findings are consistent with AbcB playing a fundamentally important role in A. fumigatus physiology behind drug resistance that is dosage
sensitive. Our future experiments will be aimed at testing this idea for the unique role of AbCB in this important filamentous fungal pathogen.

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