Identification of *Aspergillus fumigatus* multidrug transporter genes and their potential involvement in antifungal resistance

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Abstract

*Aspergillus fumigatus* can cause severe fatal invasive aspergillosis in immunocompromised patients but is also found in the environment. *A. fumigatus* infections can be treated with antifungals agents among which azole and echinocandins. Resistance to the class of azoles has been reported not only from patient samples but also from environmental samples. Azole resistance mechanisms involve for most isolates alterations at the site of the azole target (*cyp51A*); however, a substantial number of isolates can also exhibit non-*cyp51A*-mediated mechanisms.

We aimed here to identify novel *A. fumigatus* genes involved in azole resistance. For this purpose, we designed a functional complementation system of *A. fumigatus* cDNAs expressed in a *Saccharomyces cerevisiae* isolate lacking the ATP Binding Cassette (ABC) transporter *PDR5* and that was therefore more azole-susceptible than the parent wild type. Several genes were recovered including two distinct ABC transporters (*atrF, atrI*) and a Major Facilitator transporter (*mdrA*), from which *atrI* (*Afu3g07300*) and *mdrA* (*Afu1g13800*) were not yet described. *atrI* mediated resistance to itraconazole and voriconazole, while *atrF* only to voriconazole in *S. cerevisiae*. Gene inactivation of each transporter in *A. fumigatus* indicated that the transporters were involved in the basal level of azole susceptibility. The expression of the transporters was addressed in clinical and environmental isolates with several azole resistance profiles. Our results show that *atrI* and *mdrA* tended to be expressed at higher levels than *atrF* in normal growth conditions. *atrF* was upregulated in 2/4 of azole-resistant environmental isolates and was the only gene with a significant association between transporter expression and azole resistance. In conclusion, this work showed the potential of complementation to identify functional transporters. The identified transporters were suggested to participate in azole...
resistance of *A. fumigatus*; however, this hypothesis will need further approaches to be verified.

**Key words:** *Aspergillus*, azole resistance, multidrug transporters, Cyp51A.

**Introduction**

*Aspergillus fumigatus* is one of the most prevalent fungal pathogen causing severe fatal invasive aspergillosis (IA) in immunocompromised patients. Incidence of IA due to *Aspergillus* spp. has raised over the last years and has overtaken infections due to *Candida* spp. as the most frequent fungal disease found in European hospitals.

Several antifungal agents for the treatment of *A. fumigatus* infections are available. Azoles (itraconazole (ITC), voriconazole (VRC), and posaconazole) directly target the ergosterol pathway by inhibiting lanosterol 14α-demethylase (*cyp51A*), which is an enzyme involved in the important step of ergosterol biosynthesis. Polyenes (amphotericin B) form complexes with ergosterol as the final product of this pathway. The third class of antifungal agents that were recently introduced are echinocandins (caspofungin, micafungin, anidulafungin) and inhibit β-1,3-glucan synthase, which is a fungus-specific target critical for the formation of fungal cell walls.

*A. fumigatus* can be found in the environment in compost sites, vineyards and soil samples. The class of azoles (imazalil, prochloraz or propiconazole) is also widely used for crop protection. Since this fungus is exposed to azoles in agricultural and medical environments, development of resistance can be expected. As a matter of fact, a large number of *A. fumigatus* azole-resistant isolates from environmental as well as clinical origin have been reported until now.[4–11] Mechanisms of azole resistance have been investigated in filamentous fungi and particularly in *A. fumigatus*. Either point mutation(s) or overexpression of the target gene of azoles (*cyp51A*) can be involved in azole resistance. A variety of amino acid mutations have been now reported.[12–14] A remarkable combination of 2 mutations in *cyp51A* (L98H substitution and a 34 bp tandem repeat in the promoter, TR34) is enriched in azole-resistant isolates from environmental origin but is also found in patient isolates. The presence of the environmental *cyp51A* mutation signature in *A. fumigatus* isolates of clinical origin has led to the conclusion that these patient isolates may originate from the environment. The use of azole in the agriculture has probably seeded the emergence of the environmental *cyp51A* mutation signature in *A. fumigatus*.15–17 Another important resistance mechanism is the ability of various families of membrane proteins to mediate efflux of toxic compounds. This process is common for several azole-resistant fungal pathogens such as *Candida albicans*, *Candida glabrata* or *Cryptococcus neoformans* and involves ATP Binding Cassette (ABC) transporters and Major Facilitators Superfamily (MFS) transporters. These transporters are in general upregulated in azole-resistant isolates. One may expect to observe similar mechanisms operating in *A. fumigatus*; however, only a few reports have until now addressed this question. So far, Nascimento et al.[18] reported upregulation of the ABC-transporters *AfuMDR3* and *AfuMDR4* in laboratory mutants resistant to ITC. da Silva Ferreira et al.[19] showed induction of five ABC-transporters, *abcA* to *abcE* and of the MFS transporters *mfsA* to *mfsC* in response to VRC in *A. fumigatus*. Increased expression of the ABC-transporter *atrF* has been observed in some *A. fumigatus* isolates.[20] More recently, Fraczek et al.[21] addressed the expression of several ABC-transporters and MFS transporters identified on the basis of their similarity with *C. albicans* ABC-transporters (*CDR1, CDR2, CDR4*) and MFS transporters (*MDR1*). The authors found an association between expression of *cdr1B* (*Afua1g14330*) and ITC resistance in non-*cyp51A*-mediated *A. fumigatus* resistant isolates. Another study from Paul et al.[22] discovered that the deletion of *cdr1B* (named *abcB* in their work) and *abcA* (a close homolog of *abcB*) increased ITC susceptibility in *A. fumigatus*. This work was therefore consistent with the data of Fraczek et al.[21] and with the idea that *cdr1B* has a potentially important role in azole resistance of *A. fumigatus*. Even if no *abcA* expression data are available for *A. fumigatus*, Paul et al.[23] expressed *abcA* heterogeneously in *Saccharomyces cerevisiae* and could demonstrate that this transporter mediated rhodamine efflux, thus indicating its functionality. Taken together, the existing data on the implication of multidrug transporters in azole resistance of clinical *A. fumigatus* isolates cannot still firmly attribute the development of azole resistance to the expression of specific transporters.

In this work, we proposed to isolate other *A. fumigatus* genes conferring azole resistance by functional complementation in a *S. cerevisiae* multidrug transporter mutant (*pdr5Δ*) that is hypersusceptible to azoles.[24] A cDNA library from *A. fumigatus* was introduced in *S. cerevisiae*, thus permitting the identification of four genes able to complement azole hypersusceptibility of the yeast mutant. The involvement of these genes in azole resistance was next...
investigated with the help of their inactivation in *A. fumigatus* and by addressing their expression in diverse *A. fumigatus* isolates.

### Material and methods

#### Strains and media

A total of 7 *Aspergillus fumigatus* clinical isolates were selected on the basis of their ITC susceptibility (Table 1). *Saccharomyces cerevisiae* strains used in this study are listed in Table 2. *Escherichia coli* DH5α was used as a host in plasmid propagation and was grown in liquid Luria-Bertani (LB, Difco) broth or on LB plates supplemented with ampicillin (100 μg/ml, Sigma) when required.

*A. fumigatus* isolates were maintained on 2% malt extract agar for one week at 37°C. Conidia were harvested from one week-old cultures grown at 25°C on malt agar extract (2% malt extract, 2% bacto agar) and were used as an inoculum for all cultures. In parallel, all *Aspergillus* isolates were plated to obtain individual colonies and then were propagated on slopes of Sabouraud agar (Oxoid, LTD, Hampshire, England) for a permanent culture collection in our laboratory (storage at +4°C). The fungi were grown at 37°C in either Sabouraud or standard RPMI 1640 medium (Angus, USA), which contained 0.2% glucose, 0.165 M morpholine propane sulfonic acid (MOPS, Sigma-Eldrich, St Louis, USA) buffered to pH 7.0. Minimal medium (MM) contained 10 g/l dextrose, 0.92 g/l ammonium tartrate, 0.52 g/l MgSO4, 0.52 g/l KCl, 1.52 g/l KH2PO4, 1.1 mg/l H3BO3, 2.2 mg/l ZnSO4, 7 H2O, 0.5 mg/l MnCl2, 4H2O, 0.5 mg/l FeSO4, 7H2O, 0.16 mg/l CoCl2, 2H2O, 0.16 mg/l CuSO4, 5 H2O, 0.11 mg/l (NH4)6Mo7O24, 4 H2O, 0.5 mg/l Na2EDTA, pH adjusted to 6.8 with 1.0 N NaOH.

*S. cerevisiae* isolates were grown and maintained on Yeast Extract-Peptone-Dextrose (YEPD containing 2% (w/v) peptone (Difco, Laboratories, Detroit, MI, USA), 1% (w/v) yeast extract, 2% (w/v) glucose). Agar was supplemented to 2% (w/v) when required. Selection for all the auxotrophic markers was performed on a minimal medium (Yeast Nitrogen Basis, YNB) added with a Complete Supplement Mixture (CSM) lacking the appropriate supplement(s) (Bio 101® Systems, Q-Biogene, CA, USA) and with or without agar at 2% final.

#### Plasmids and primers

The different plasmids used in this work are as following: pFL61 is an episomal yeast · *E. coli* shuttle vector with *URA3* marker; pBluescript SK+ was from Stratagene (Amsterdam, The Netherlands); pAN7.1 is an *E. coli*

### Table 1. *A. fumigatus* strains used in this study.

<table>
<thead>
<tr>
<th>Clinical Isolates</th>
<th>Genotypea</th>
<th>MIC ITC (μg/ml)</th>
<th>MIC VRC (μg/ml)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS 144–89</td>
<td>Wild type</td>
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<td>0.5</td>
<td>d’Enfert et al.59</td>
</tr>
<tr>
<td>Af10</td>
<td>Wild type</td>
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<td>0.25</td>
<td>Denning et al.60</td>
</tr>
<tr>
<td>Af72</td>
<td>G54E</td>
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<td>0.25</td>
<td>Denning et al.61</td>
</tr>
<tr>
<td>Br130</td>
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<td>Mosquera et al.9</td>
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<tr>
<td>Br181</td>
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<tr>
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<td>0.5</td>
<td>Diaz-Guerra et al.62</td>
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<tr>
<td>CM2164 (SO/3829)</td>
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<td>0.5</td>
<td>0.5</td>
<td>Diaz-Guerra et al.62</td>
</tr>
</tbody>
</table>

Environmental Isolates

<table>
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<tr>
<th>Strain name</th>
<th>Genotypea</th>
<th>MIC ITC (μg/ml)</th>
<th>MIC VRC (μg/ml)</th>
<th>References</th>
</tr>
</thead>
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<td>AFenv200</td>
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<td>0.25</td>
<td>Meneau and Sanglard58</td>
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<tr>
<td>AFenv51</td>
<td>F46Y, M172V, E427K</td>
<td>&gt;16</td>
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<td>Meneau and Sanglard58</td>
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<td>AFenv119</td>
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<td>AFenv135</td>
<td>Wild type</td>
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<td>0.25</td>
<td>Meneau and Sanglard58</td>
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<tr>
<td>AFenv155</td>
<td>Wild type</td>
<td>2</td>
<td>16</td>
<td>Meneau and Sanglard58</td>
</tr>
</tbody>
</table>

aNomenclature: AFenv x: *A. fumigatus* from Environment, isolate x.

Abbreviations: ITC, itraconazole; VRC, voriconazole; MIC: minimal inhibitory concentration.

### Table 2. *S. cerevisiae* isolates used in this study.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
<th>Reference</th>
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<td>YKKBa-7</td>
<td>MATa, ura3-52, lys2-801amber, ade2-101ocbre, trp1-Δ63, his3-Δ200, leu2-Δ1</td>
<td>Mahe et al.31</td>
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<td>YKKB-13</td>
<td>MATa, ura3-52, lys2-801amber, ade2-101ocbre, trp1-Δ63, his3-Δ200, leu2-Δ1, Δpdr5::TRP1</td>
<td>Mahe et al.31</td>
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plasmid vector with the hygromycin B phosphotransferase gene (HygR).27

Construction of an A. fumigatus cDNA library and use in S. cerevisiae

A cDNA library from the A. fumigatus isolate CBS144-89 was constructed in the pFL61-multicopy S. cerevisiae vector containing a strong constitutive PGK1 promoter (phosphoglycerate kinase), thus allowing strong constitutive expression of A. fumigatus cloned cDNAs in standard growth conditions.26 A. fumigatus cDNAs were cloned into the unique NotI site. The cDNA library was introduced in the S. cerevisiae strain YKKB-13 (Table 2). Transformants (1 to 5 × 10⁴ individual colonies) were pooled and grown overnight at 30°C with shaking in selective YNB liquid medium. YNB (CSM -uracil -tryptophan) medium was used for the YKKB-13 complemented transformants. The culture of each pool was diluted at cell density of 1.5 × 10⁷ cells/ml and five µl of a serial 10-fold dilution were spotted on selective YNB medium containing azoles. Yeast colonies growing on selective media were recovered and plasmids rescued in E. coli using the RPM Yeast Plasmid Isolation Kit (Bio 101R Systems). After rescue of recombinant plasmids in E. coli, the presence of inserts was confirmed by NotI digestion. Inserts (cDNA) were next sequenced at their 5′- and 3′-extremities using primers matching the 5′ and 3′-region of the A. fumigatus cloning site (primers pFL61 sens and pFL61 asens, see supplementary Table S1). Sequencing reactions were carried out in an ABI PRISM 3100 automated sequencer and a Big Dye Terminator DNA sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA). The entire cDNA sequence of the insert was obtained and blasted against the Aspergillus Genome Database in order to obtain the identity of the inserted cDNA.

Positive plasmids were transformed back into YKKB-13, and three colonies of each transformed strain were chosen and replated on the same appropriate selective plate. Each strain was then cultivated overnight in selective liquid medium at 30°C. The ability of the different cDNAs inserted to complement the hypersusceptibility of YKKB-13 was confirmed in YNB selective medium containing the drug against which the plasmid was selected.

Construction of gene disruption cassettes for deletion of corresponding genes in A. fumigatus

Constructions for the disruption of the genes isolated in this study were designed by a similar method. First, the complete open reading frame (ORF) of the genes was amplified from A. fumigatus genomic DNA with their 5′- and 3′-untranslated regions (UTR) using the pair of primers (listed as “gene.A” and “gene.B” in supplementary Table S1) and cloned into SK+ with compatible restriction sites A and B. Next, an inverse PCR creating new restriction sites C and D was performed on this subcloned plasmid to maintain the 5′- and 3′-UTRs from each gene but by omitting each ORF (pair of primers “gene.C” and “gene.D”). PCR products were digested with restriction enzymes C and D and were ligated to a C-D fragment from pAN7.1 containing HygR.27 Finally, each construction was linearized by A and B digestions and linear fragments were used for protoplast transformation of A. fumigatus CBS144-89.

A. fumigatus protoplast transformation

The transformation procedure was based on a method previously described by Malardier et al.28 After protoplast transformation and overnight growth, A. fumigatus transformants were selected on MM with 100 μg/ml hygromycin for 10 days at 21°C. Positive transformants were replated on malt agar medium with hygromycin selection. Genomic DNA from transformants able to grow on this medium were prepared and digested with appropriate restriction enzymes and tested by Southern blot analysis to verify the correct recombination events at the expected genomic loci (see Supplementary Material and Methods).

RNA extraction and real-time quantitative RT-PCR

Total RNA from A. fumigatus strains was prepared by the following steps. An overnight culture in RPMI 1640 at 37°C was filtered with Whatman filter paper (N° 54, Whatman International Ltd Maidstone, England) in order to harvest mycelia that were immediately frozen in liquid nitrogen. RNA was extracted using the Qiagen RNeasy Plant Mini Kit according to manufacturer’s instructions and followed by an RNase-free DNase I treatment for 1 h at room temperature. Reverse transcription was carried out with the Invitrogen Reverse Transcription System kit. Real-time PCR experiments were performed on an ABI Prism 7000 Sequence Detection System kit using intercalation of SYBR green as fluorescence reporter. Reagents from the Quantitect SYBR® Green PCR kit (Qiagen AG) were used for all reactions. PCR were performed in triplicates in microtiter plates (thermo-fast 48 wells, VWR AG) and carried out with the following parameters: one cycle at 94°C for 15 min, 40 temperature cycles consisting of steps at 94°C for 30 s, at 55°C for 30 s and at 72°C for 30 s. In all experiments, negative controls were included to exclude possible contaminations. Quantification was based on the Cycle Threshold (CT)
values, which are inversely proportional to the amount of detected transcripts. Results were first normalized using the CT values obtained for the housekeeping gene β-tubulin as internal control. Second, CT values obtained for each tested cDNA were compared to the CT values obtained for the cDNA corresponding to the wild type A. fumigatus isolate. For analysis, the amplification efficiency of the tested genes and internal control was first determined. Serial dilutions of cDNA (from 1/10 to 1/1000) were amplified by real-time PCR using each set of gene-specific primers. The data from the amplification plots were used for the construction of the standard curves. In order to achieve this, the cDNA dilutions were plotted as a function of the corresponding CT value of each gene. From the resulting linear relationships, standard curve equations were obtained and $R^2$ values were calculated for each data set to estimate the efficiency of the real time PCR. Finally, the amounts of target copies contained in an unknown sample were determined by extrapolation from the linear regression of standard curves obtained for each primer set. qPCR Primers for each gene are listed in Table S1 and named as primers “gene.E” and “gene.F”.

Rapid DNA extraction from A. fumigatus strains

Genomic DNA extractions of A. fumigatus strains were performed according to Girardin et al.29 Strains were cultured in 6 ml of Sabouraud medium for 20 h at 37°C in absence of agitation. The mycelial mat was collected, frozen in liquid nitrogen and pound with a mortar and pestle. The mycelial powder was resuspended in 800 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 250 mM NaCl, 1% SDS, 50 μg/ml proteinase K) and incubated for 10 min at 65°C. The slurry was then extracted with 1 volume of phenol-chloroform-isooamyl alcohol (25:24:1, Fluka, Buchs, Switzerland) and centrifuged for 1 h at 13,000 rpm. The aqueous phase was treated with RNase A (6 μg/ml) for 3 h at 37°C and extracted with 1 volume of chloroform-isooamyl alcohol (24:1). DNA was precipitated by adding 1 volume of isopropanol. The pellet was washed with 70% ethanol and dried at room temperature. The DNA was resuspended in 100 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and conserved at −20°C.

Sequencing of cyp51A alleles

Sequencing of cyp51A from A. fumigatus strains was performed by first amplification of cyp51A from cDNAs or genomic DNA with primers Cyp51A-pGal and Cyp51A-tTet (Supplementary Table S1). Sequencing was carried out with primers Cyp51A 1–4 (sens and asens) (Supplementary Table S1) to help the identification of cyp51A mutations. Sequencing reactions were carried out as indicated above.

Determination of the A. fumigatus mutant phenotypes

Conidial suspensions (five μl of serial ten-fold dilution from $10^5$ to $10^6$ conidia/ml) were spotted on MM containing ITC or VRC. Plates were incubated at 37°C for 48 h.

Drug susceptibility assays in A. fumigatus

The microdilution broth method was used according to CSLI (M38-A2) guidelines for the in vitro susceptibility testing.30 Conidia were obtained by growth of isolates on Sabouraud or malt agar at 37°C for 3–4 days. Conidia were collected by flooding the agar surfaces with phosphate-buffered saline and 0.05% Tween 80 (Fluka) and viability was tested for each isolate. The number of CFU per ml was determined by plating different volumes of an appropriate diluted cell suspension ($10^{-6}$) on Sabouraud agar. The standard medium RPMI 1640 (Sigma) was used for the in vitro susceptibility testing of all the drugs used in this study). One hundred fifty μl of drug free medium was dispensed into each well of a 96-well plate (Costar) and 50 μl of drug-containing medium at the appropriate concentration was added in the first wells of the plate. The starting solutions were prepared in RPMI. A serial two-fold dilution was next performed in order to obtain concentrations ranging from 16 to 0.016 μg/ml for each antifungal tested. Drug-free controls were included in each 96-well plate. Plates were stored at −80°C until use. Aliquots of 50 μl of a stock conidial suspension (at $7 \times 10^7$ viable conidia/ml) were added in each well of a plate. The final volume in each well was 200 μl and the final concentration of the inoculum was $1.8 \times 10^4$ conidia/ml. Plates were incubated for 2 days at 35°C after which visual readings were performed. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the drug that showed complete growth inhibition as compared to the growth of drug-free medium.

S. cerevisiae serial dilution drug susceptibility assays

The susceptibility of S. cerevisiae strains to a variety of compounds was tested qualitatively by spotting serial dilutions of yeast cultures on YNB medium agar plates containing different drugs. This allows an easy visualization of growth differences between the different yeast strains tested. The spotting test was accomplished by growing cells overnight in YNB. The cells were first diluted to $2 \times 10^7$
cells/ml in H₂O. This initial concentration was further diluted to yield concentrations ranging from 2 × 10⁷ to 2 × 10⁴ cells/ml. Aliquots of 5 μl of each cell suspension were spotted on each of the different plates and incubated for 48 h at 30°C. To enable the isolation of drug resistance genes from A. fumigatus cDNAs expressed in S. cerevisiae YKKB-13, drug concentrations were optimized to allow the visualization of growth differences between the hypersusceptible mutant strain (YKKB-13) and their parental wild type strains (YKKBa-7).

Chemicals and reagents

The antifungal powders were obtained from their respective manufacturers. ITC was obtained from Cilag AG (Shaffhausen, Switzerland), and FLC and VRC were obtained from Pfizer (Zurich, Switzerland).

Results

Isolation of A. fumigatus genes potentially involved in azole resistance

To isolate candidate drug resistance genes from A. fumigatus, a functional complementation assay with an A. fumigatus cDNA library was performed into a S. cerevisiae recipient strain. It lacks the major ABC transporter PDR5 (YKKB-13). This mutant possesses an ideal background to test drug resistance generated by an heterologously expressed protein, since it displays hypersusceptibility to a wide range of substrates including antifungal agents.

Transformation of the hypersusceptible S. cerevisiae strain with an A. fumigatus cDNA library yielded sufficient number of transformants (approx. 50000 arranged in several pools) for further screening. A screening for drug resistance was performed using fluconazole (FLC) and VRC at appropriate concentrations. Some individual colonies were able to grow in presence of antifungals and were further selected. Out of 24 FLC-resistant colonies obtained with the screening of the cDNA library in YKKB-13, 8 colonies were recovered after the second transformation step and allowed isolation of four distinct resistance genes. One sequenced cDNA corresponded to yap1 (Afu6g09930) and belonged to the bZIP transcription factor family. The second cDNA showed similarity with ABC transporter genes from fungal species but was not yet described in A. fumigatus. This gene is known as Afu3g07300 in the Aspergillus Genome Database. We designated this gene atrI in agreement with current gene nomenclature, since atrI (Afu3g03670) is already assigned to A. fumigatus. The third cDNA exhibited similarity to MFS transporters and the corresponding gene was named mdrA (Afu1g13800).

Finally, an ABC transporter previously isolated from A. fumigatus (atrI, Afu6g04360) was recovered.

Characteristics of the transporter atrI and mdrA

The ABC transporter atrI (Afu3g07300) isolated from the azole resistance screening in S. cerevisiae encodes for a protein of 1472 amino acids residues. It belongs to the group of ABC-G proteins according to the ABC transporter classification adopted by Kovalchuk and Driessen, in which transporters known to play a role in azole resistance are also present, including PDR5 and CDR1. ABC-G members have a typical structural organization of ABC transporters characterized by two homologous halves (NBD-TMD). The hydrophilic nucleotide-binding domain (NBD) is linked in three successive domains: two short peptide motifs involved in ATP binding and coupled with ATP hydrolysis for the transport process, i) a glycine-rich (Walker A) and ii) a hydrophobic motif (Walker B) intercalated by iii) an ABC signature found in these transporters. In addition to the NBD, the transporters contain hydrophobic transmembrane domains (TMD) composed of six α-helices. Among non-Aspergillus spp., the closest ABC transporter is PMG11_07584 from Penicillium brasiliannum with 78% identity. Comparison of the atrI cDNA and with genome data (Aspergillus Genome Database) revealed the presence of an intron of 44 nucleotides and contained typical consensus splice sites (GT and AG for the 5′- and 3′-splice sites, respectively).

The MFS transporter mdrA (Afu1g13800) encodes for a protein of 668 amino acids. This gene was previously reported by Pain et al. and was shown to be located in a region encompassing the nitrate assimilation gene cluster (AfA35g10.20c). mdrA belongs to the drug/H+ antiporter family DHA1 with 12 trans-membrane domains according to Costa et al. mdrA is closely related to the Tpo-related MFS transporters such as the C. albicans FLU1 gene, which was shown to be involved in azole efflux. Excluding Aspergillus spp., mdrA is most closely related to Pcam013g080690 from Penicillium camemberti (78% identity), which is a sucrose/H+ symporter. Three introns of 58-, 52- and 84 bp were identified along the corresponding ORF.

Pattern of antifungal cross-resistance conferred by the A. fumigatus genes overexpressed in S. cerevisiae

To assess whether the above-mentioned genes were able to induce resistance to a wide range of azoles, YKKB-13 expressing atrI, atrr, mdrA or yap1 were plated on different media containing FLC, ITC and VRC. The parental strain,
YKKBa-7, was used as growth control for azole susceptibility testing. As shown in Figure 1, the expression of atr1 in S. cerevisiae could revert the defective growth phenotype of the parent isolate YKKB-13 by restoring resistance to all azoles tested. Expression of atrF in YKKB-13 yielded resistance to FLC and VRC, whereas expression of mdrA conferred a more specific resistance profile by restoring growth mainly to VRC and weakly to FLC. This feature is consistent with the narrow specificity expected for this kind of MFS transporter. Expression of yap1 conferred strong resistance to FLC and VRC to S. cerevisiae. This result will be further discussed (see Discussion). Given that yap1 was previously described by Lessing et al.39, this gene was not further investigated here.

In conclusion, the above-presented experiments showed that multidrug transporters from the ABC and MFS families and the transcription factor yap1 expressed in S. cerevisiae can confer resistance to a large spectrum of azole derivatives but with specific differences in their resistance patterns. In addition, the experiments showed that the use of S. cerevisiae expressing heterologous transporters allowed the evaluation of their substrate specificity.

Expression analysis of atrF, atrl, mdrA in A. fumigatus isolates from clinical origin

In several fungal species, upregulation of multidrug transporters is a mechanism of azole resistance. Many studies have revealed that drug efflux mediated by increased expression of transporters is an important mechanism of resistance in fungi.40–42 We analyzed several A. fumigatus isolates that were divided as azole-susceptible and azole-resistant (Fig. 3). The azole-resistant clinical isolates contained mutations in cyp51A (Table 1). Taking isolate CBS144-89 as a reference for expression of atrF, atrl and mdrA, the results of Figure 3 indicate that atrl and mdrA are expressed at higher levels than atrF (P < .01) in resistant isolates as compared to susceptible isolates. When grouping isolates as azole-resistant and azole-susceptible, only the expression of atrl significantly differs (P < .05) between the two groups. Expression of atrl differs from 3.9 to 8.5-fold as compared to CBS144-89 in azole-resistant isolates, while it varies from 1 to 3.7-fold in azole-susceptible isolates (Fig. 3). Although only a restricted number of isolates were investigated here, these results suggest that atrl could contribute to azole resistance to some extent.

Expression analysis of atrF, atrl, mdrA in A. fumigatus isolates from environmental origin

We next addressed the expression of the different transporters in A. fumigatus originating from the environment.

Figure 1. Serial dilution assays of S. cerevisiae cells (YKKB-13) expressing A. fumigatus genes conferring resistance to azole antifungals. Strains were spotted on minimal medium containing various antifungal drugs. Concentrations are given and were optimized to compare growth of both positive (wild type YKKBa-7) and negative controls (YKKB-13). Plates were incubated for 24 h at 30°C. FLC, fluconazole; ITC, itraconazole; VRC, voriconazole.

Figure 2. Antifungal susceptibility pattern of A. fumigatus mutant strains. Cultures at 10⁶ spores/ml were diluted and spotted on Minimal Medium (MM) with various concentrations of antifungals (ITC and VRC) at the given concentrations. Plates were incubated at 37°C for 48 h.
Our interest was to probe their expression primarily in isolates with decreased azole susceptibility or azole resistance. For this purpose, 184 isolates from several environmental sites around Switzerland (compost sites, vineyards and crop areas) were first collected between 2001 and 2003 and their susceptibility to azoles tested.43 Our results showed that 3.8% of environmental isolates tested possessed decreased susceptibility to ITC (MIC ≥ 4 μg/ml; 7/184) and only one was VRC-resistant (MIC ≥ 4 μg/ml). Selected isolates from this collection are shown in Table 1. Isolate AFenv200 was azole-susceptible and was used for comparisons with other isolates. One isolate (AFenv51) exhibits the highest ITC MIC (16 μg/ml) and was isolated from a compost site. This isolate was particularly interesting because of its VRC MIC (1 μg/ml) that is higher than most of susceptible isolates (0.25 μg/ml). Another type of antifungal susceptibility profile was observed for the environmental isolate AFenv119, in which almost all obtained MICs were high. This isolate showed a VRC MIC of 2 μg/ml. Similarly, AFenv155 showed a same pattern of susceptibility than AFenv119, except for VRC (MIC = 16 μg/ml). AFenv119 was the only VRC-resistant in the environmental collection.

Since azole resistance in A. fumigatus from environmental sources is often mediated by alterations of the target cyp51A, we determined cyp51A polymorphisms in the selected environmental isolates. As summarized in Table 1, cyp51A polymorphisms were only observed in AFenv51 (F46Y, M172V, E427K). Strikingly, these polymorphisms had already been reported in azole-resistant isolates from clinical origins.10,13,44–46 Thus, the F46Y, M172V, and E427K substitutions probably contribute to the azole MIC patterns of this isolate. A parallel experiment that explored this question was performed by heterologous expression in S. cerevisiae and showed that expression of the cyp51A1 cDNA resulted in azole resistance (see supplementary Fig. S2). On the opposite, the other environmental isolates did not exhibit cyp51A polymorphisms, thus suggesting that other azole resistance mechanisms may operate.

We therefore tested the expression of atrF, atrl, mdrA in the environmental isolates summarized in Table 1. The expression values were normalized to those of the azole-susceptible isolate AFenv200. As shown in Figure 4, atrl and mdrA showed little variation of expression as compared to AFenv200. However, atrF expression was about 3-fold higher in AFenv51 and AFenv135 as compared to AFenv200. Interestingly, these isolates had ITC MIC ≥ 8 μg/ml. Thus, it is possible that atrF contributes to some extent to increase ITC MICs in A. fumigatus.

Discussion

ABC and MFS transporters have a highly relevant function in multidrug resistance in pathogenic fungi. Many studies have revealed that drug efflux mediated by increased expression of ABC or MFS transporters could be an important mechanism of fungicide resistance in fungi.40–42 In A. flavus, resistance to ciclofungin was attributed to the overexpression of MFS genes, among which AflMDR1...
and AflMDR2. Overexpression of ABC transporter genes atrA, atrB, atrC, and atrD in A. nidulans was shown to be correlated with azole resistance. The grey mould fungus Botrytis cinerea that causes losses of commercially important fruits, vegetables and ornamentals upregulates ABC and MFS transporters to drive fungicide resistance in the fields.

In A. fumigatus, mechanisms of antifungal resistance have deserved more intensive investigations in the past ten years. Alteration of theazole target cyp51A is a major mechanism in clinical and environmental isolates. However, there are now also several non-cyp51A-mediated azole-resistant isolates for which the mechanisms remain partially unsolved. Recent whole genome approaches have been undertaken to resolve azole resistance mechanisms. While a study confirmed a cyp51A mutation in clinical and environmental isolates, another identified additional SNPs in other genes associated with azole resistance (e.g., the transcriptional regulator ganA, the HMG-CoA reductase homolog, ABC-transporters Afu4g14760 and Afu2g10530). Azole resistance was however induced under laboratory conditions in this study.

Isolation of A. fumigatus genes involved in antifungal resistance was addressed here by functional expression in S. cerevisiae. For this purpose, a cDNA library made from A. fumigatus mRNA was constructed in a yeast expression vector and was transformed in a S. cerevisiae strain lacking the ABC transporter gene PDR5. Three transporters, atrF, atrI, and mdrA, were recovered. atrF was already described by Slaven et al. as an ABC transporter gene upregulated in A. fumigatus after ITC treatment. The function of atrF was however not clearly determined in their study. Here, the expression of atrF in S. cerevisiae conferred resistance to FLC and VRC, and therefore demonstrated that this gene is functional. In S. cerevisiae, atrI yielded resistance to all azoles tested, while expression of mdrA resulted in strong VRC resistance only. The restricted resistance profile of mdrA is consistent with the idea that MFS transporters have a narrower substrate capacity than ABC transporters. In addition to these transporters, yap1 was also recovered from the S. cerevisiae complementation system. Complementation in S. cerevisiae of such a transcriptional regulator (C. albicans YAP1) was already reported and was known to control the expression of the MFS transporter FLR1. It is therefore likely that yap1 controls a similar gene in S. cerevisiae. yap1 in A. fumigatus controls the response to oxidative stress.

We addressed the potential involvement of atrF, atrI and mdrA in the development of A. fumigatus antifungal resistance by two different approaches including drug resistance phenotypic characteristics of mutants and expression analysis in several A. fumigatus isolates. Given that atrF−, atrI− and mdrA− mutants exhibited enhanced susceptibility to ITC and that mdrA− strain showed enhanced susceptibility to VRC only, it can be assumed that these transporters play a role in basal level of azole susceptibility in A. fumigatus. Similar conclusions were drawn by Paul et al. where abcA and abcB (cdr1B) were inactivated, thus resulting in increased ITC susceptibility. It remains to be established whether or not these transporters play a role in azole resistance in A. fumigatus from clinical and/or environmental origins. Gene deletions of these genes may be performed in these strain backgrounds in the future to answer this question.

Expression levels of atrF, atrI and mdrA were tested here in several A. fumigatus isolates. atrI and mdrA were in general more expressed in clinical isolates than atrF. The later was upregulated at higher levels than the two others in environmental isolates. Our results highlight complex transcriptional regulation of these transporters. At this stage, they cannot firmly reveal their participation in azole resistance development. Fraczek et al. have probed this question more extensively with larger strain and transporter panels. They observed some correlation between upregulation of cdr1B (abcB) and occurrence of azole resistance, however without paralleled genetic evidence. A systematic transcriptional analysis remains to be performed in azole-resistant isolates to give a better overview on the possible involvement of multidrug transporters in azole resistance. This is a challenging question, given the high number of these transporters (45 ABC transporters; 275 MFS transporters; http://www.membranetransport.org/) in the A. fumigatus genome and since several transporters may be participating simultaneously to azole resistance.

Environmental isolates from this study were collected between 2001 and 2003. The proportion of isolates with elevated azole MICs was low (up to 3.8%) and the only obvious resistance mechanism was associated with simultaneous mutations in cyp51A (F46Y, M172V, E427K). The known environmental cyp51A mutation signature (TR43/L98H) was not detected in this isolate collection and thus does not reflect the current trends of A. fumigatus azole resistance and associated mechanisms prevailing in several countries. The environmental strain collection of the present study was of small size and azole resistance selection was not performed positively as usually carried out nowadays. Even if it is possible that the typical cyp51A environmental mutation was not present in environmental isolates sampled at this period, both the small size collection and the azole resistance selection method might have contributed to the absence of the typical cyp51A environmental mutation signature in our isolates.

In conclusion, the functional complementation system used here had this interesting capacity to select for
functional systems that would transport azoles. This functional selection directs the choice of transporters to be further tested. This system could however be used with other azoles to select for more specific transporters. On the other hand, the cDNA library that was designed here was from normal growth conditions and not from conditions (in vitro azole treatment) in which azole resistance genes could be better identified. Lastly, the functional complementation system used here could be applied for selecting other functional A. fumigatus genes, however providing that a functional selection strategy can be used. Future experiments are planned to address these issues.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary Material

Supplementary material is available at Medical Mycology online (http://www.mmy.oxfordjournals.org/).

References


