Interrogation of Related Clinical Pan-Azole-Resistant *Aspergillus fumigatus* Strains: G138C, Y431C, and G434C Single Nucleotide Polymorphisms in *cyp51A*, Upregulation of *cyp51A*, and Integration and Activation of Transposon Atf1 in the *cyp51A* Promoter††

Ahmed M. Albarrag,1,2 Michael J. Anderson,2 Susan J. Howard,2 Geoff D. Robson,3 Peter A. Warn,2,4 Dominique Sanglard,5 and David W. Denning1,2,3,4,*

Department of Medical Microbiology, College of Medicine and University Hospital, King Saud University, Riyadh, Saudi Arabia; School of Translational Medicine,† Faculty of Life Sciences,‡ and University Hospital of South Manchester,§ The University of Manchester, Manchester Academic Health Science Centre, Manchester, United Kingdom and Institute of Microbiology, University of Lausanne, and University Hospital Center, Rue de Bugnon 48, CH-1011, Lausanne, Switzerland

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Multiple *Aspergillus fumigatus* isolates from a patient with two aspergillomas complicating chronic pulmonary aspergillosis were pan-azole resistant. Microsatellite typing was identical for all isolates despite major phenotypic and some growth rate differences. Three different *cyp51A* mutations were found (G138C, Y431C, and G434C), of which the first two were demonstrated by heterologous expression in a hypersusceptible *Saccharomyces cerevisiae* strain to be at least partly responsible for elevated MICs. *cyp51A* and *cyp51B* gene duplication was excluded, but increased expression of *cyp51A* was demonstrated in three isolates selected for additional study (7- to 13-fold increases). In the isolate with the greatest *cyp51A* expression, an *Atf1* transposon was found inserted 370 bp upstream of the start codon of the *cyp51A* gene, an integration location never previously demonstrated in *Aspergillus*. Two transcription start sites were identified at 49 and 136 bp upstream of the start codon. The role of the *Atf1* transposon, if any, in modulating *cyp51A* expression remains to be established. Increased mRNA expression of the transporters *AfuMDR1* and *AfuMDR4* also was demonstrated in some isolates, which could contribute to azole resistance or simply represent a stress response. The diversity of confirmed and possible azole resistance mechanisms demonstrated in a single series of isogenic isolates is remarkable, indicating the ability of *A. fumigatus* to adapt in the clinical setting.

*Aspergillus fumigatus* is a common saprophytic filamentous fungus with a worldwide distribution (33), and it is the most common etiological agent of aspergillosis. Invasive aspergillosis is associated with high morbidity and mortality (19), and in a recent case series chronic pulmonary aspergillosis was shown to have a 50% mortality rate during an 18-month period (26). It is estimated that there are over 3 million patients worldwide with allergic bronchopulmonary aspergillosis (8) and more than 1 million have chronic pulmonary aspergillosis (CPA) following tuberculosis (9). Azoles represent first-line therapy for all forms of aspergillosis and comprise the only class of fungicide approved for all forms of aspergillosis and comprise the only class of first-line therapy for mycotic infections (9), with great variation in different localities. The prevalence of resistance is now >8% (5, 16), predominantly in CPA cases undergoing therapy. Both centers reported a continuing rise in azole resistance frequency.

Several mechanisms of azole resistance have been described for *Aspergillus* species. Triazoles are demethylase inhibitors and target the ergosterol biosynthetic pathway, an essential component of the fungal cell membrane, by binding to the Cyp51 family of cytochrome P450s, (sterol 14α-demethylases), causing the depletion of ergosterol and the accumulation of lanosterol or eburicol and other toxic 14α-methyl sterols (18). The most commonly reported azole resistance mechanism in clinical isolates of *A. fumigatus* is due to mutations in the target gene resulting in amino acid substitutions in sterol 14α-de-methylase that presumably reduce drug binding. There are two Cyp51 enzymes in *A. fumigatus* encoded by two genes, *cyp51A* (*pdmAerg11*) and *cyp51B* (*pdmBerg11*) (23). A number of missense mutations have been described in clinical isolates and in laboratory mutants, although almost all alterations linked with resistance are in *cyp51A* (15). Mutations in four codons (N22, G54, L98, and M220) have been shown to be the direct cause of resistance by replacing the wild-type copy of *cyp51A* in an *A. fumigatus* strain with a copy of the gene containing the putative resistance mutation (7, 11, 22, 25). Decreased intracellular azole accumulation has been demonstrated in one itraconazole-resistant clinical isolate and two laboratory mutants (10, 21), and several efflux pumps of the ABC transporter and major facilitator protein superfamilies have been described in *A. fumigatus*, although none have been proven to date to play a
direct role in resistance by pumping out azoles (6, 7, 27, 36, 40). Finally, an amino acid substitution at codon 98 (L98H) has been shown to cause pan-azole resistance, but it does so only when linked with a duplication of a 34-bp sequence in the 5′ upstream region of cyp51A, resulting in the upregulation of the gene (24). Neither mutation on its own was shown to be sufficient to cause resistance to azoles in transformants alone.

Having identified a sequential series of pan-azole-resistant isolates from a single patient, we investigated the mechanism of resistance in selected isolates and extended our investigation to determine the mechanistic causality of the mutations found, and we identified a transposon insertion upstream of cyp51A. These novel findings are highly significant in understanding azole resistance in *A. fumigatus*.

**MATERIALS AND METHODS**

**Media.** The following solid media containing 1.5% agar (Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid), 1% (wt/vol) glucose with Vogel’s salts (VB [broth]), and Sabouraud dextrose broth (SDB), 1% (wt/vol) glucose with Vogel’s salts (VB [broth]). RPMI is a defined medium routinely used for susceptibility testing. Our aud dextrose is a complex medium, Vogel’s is a defined minimal medium, and RPMI-1640 Auto-Mod buffered and supplemented as described above. Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, United Kingdom) were used: Sabouraud dextrose agar (SDA; 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ence detection system (Roche) was used with one PCR-derived A64 probe amplified from F12776 genomic DNA, using primers shown in Table 2, with the same conditions as those for cyp51A and cyp51B amplification.

Expression and gene copy number analysis using real-time PCR. Nephelometer shake flasks containing 40 ml of VB (defined medium) and inoculated with 1 × 10^6 spores per ml (final) were incubated at 35°C until the cultures reached an optical density of 580 nm (OD580) of 6.0 to 6.5, at around 16 h of growth. For expression in the presence of itraconazole, cultures were grown to an OD580 of 3.0 to 3.5 (mid-logarithmic phase) before adding 4 mg/liter of itraconazole in 20% (wt/vol) cycloheximide. Control flasks with cycloheximide only also were analyzed. Two-milliliter samples were removed at 0, 1, 2, 4, and 8 h. Cultures were repeated on another day so that four flasks in total were grown for each isolate. Two RNA extractions were carried out from each culture from 50 to 100 mg (wet wt) of mycelium, snap-frozen in liquid nitrogen, using a Qiagen RNaseasy Plant Midi Kit (Qiagen, West Sussex, United Kingdom). RNase-free DNase I (Qiagen) was used to ensure the complete removal of all genomic DNA. RNA concentrations and purity were determined using a NanoDrop ND-1000 spectrophotometer. Primers used for real-time PCR are listed in Table 2. Finally, all reverse transcription-PCRs (RT-PCRs) were designed so that one of the primers spanned an exon-exon junction in the target cDNA. Primer specificities were confirmed by the presence of single bands only on agarose gels and by sequencing PCR products. The primer curve analyzers were formed after all reverse-PCRs to confirm the presence of single peaks only over the entire temperature range. Real-time PCR was performed with the Mx3000P Real-Time PCR system (Stratagene) with Brilliant SYBR green QPCR master mix or a Brilliant SYBR green QRT-PCR master mix one-step kit (Stratagene). Each RNA and DNA sample was analyzed twice. Twenty-microliter reaction mixtures were set up with 100 ng of total RNA (or 200 ng of genomic DNA) in 20 μl of 1× SDB, VB, and RPMI. A final concentration of 5 × 10^6 CFU/ml in 15 ml of medium was incubated at 35°C with shaking. Aliquots (0.1 ml) were removed at hourly intervals, and the proportion of spores that had germinated was determined. Spores were considered to have germinated when the length of the germ tube was at least half the diameter of the conidium. A total of 200 spores were counted for each determination. Experiments were carried out in duplicate.

Functional complementation of erg11 in Saccharomyces cerevisiae. Information on the functional complementation of erg11 in Saccharomyces cerevisiae is provided in the supplemental material.

Quantitative and statistical analyses. (i) Real-time PCR. Eight and replicate samples were analyzed for the expression studies and the gene copy number study, respectively. Each expression experiment was repeated once with fresh spores, with two RNA extractions performed per culture and two PCRs carried out per RNA sample. The benA gene encoding β-tubulin, as described by Pfaff et al. (17), was used for normalizing both gene copy number and transcript levels, and final results were given relative to the appropriate reference gene and AF10. This method combines the normalization and amplification efficiencies of the target gene with the calibrator, an untreated control, and is represented as the expression at time zero. The benA gene is known to be a single-copy gene, and it has been found to be expressed constitutively and is not affected by experimental conditions, such as itraconazole treatment (Paul Bowyer, personal communication). For all PCRs, the square regression correlation coefficients (R^2) ranged between 0.989 and 1.000. PCR amplification efficiency was calculated using the following formula: efficiency (E) = 10^-1(1/R^2^). The efficiency of each replicate varied from 1.9 to 2.1, which was acceptable. The optimal threshold was chosen automatically and was used to calculate the threshold cycle (Ct) for samples. Relative values of the target genes were normalized to the control gene, benA, as described by Pfaff et al. (17), and final results are given relative to the appropriate reference gene and AF10. This method combines the normalization and amplification efficiencies of the target and reference genes to correct for differences between the two assays using the relative expression software tool (REST). For the calibrators, the change in Ct is set to equal 0, and therefore the fold change in gene expression for the control is equal to 1. In the time course study, the calibrator, an untreated control, represented expression at time zero. (ii) Fitness. All calculations were performed using SPSS v13.0. All experiments were carried out at least twice on different days with at least quadruplicate measurements taken for each time point. Statistical analyses involving two groups were made by Student’s t test, whereas analysis of variance (ANOVA) multiple-comparison tests were used to compare more than two groups. For growth rates, each replicate was analyzed separately before the rates were combined to give the means ± standard errors of the means (SEM). The range of the partial growth curves were used for statistical analysis. The colony radial growth rate was calculated as described previously (35). The specific growth rate was analyzed as described previously (30). For the conidiation and germination experiments, the replicates from both experiments were expressed as the means before the two means were combined to give the overall value ± SEM. Hyphal growth unit length, in μm, was calculated as the ratio between the total length of the mycelium and the number of hyphal tips (41).

RESULTS

The susceptibilities of the tested isolates are shown in Table 1. Applying the proposed interpretative breakpoints (32, 34, 42), all eight isolates were considered to be pan-azole resistant. The isolates were susceptible to amphotericin B and caspofungin (data not shown) (17).

Phenotypic characterization of the isolates. Six of the isolates produced typical colony morphologies for A. fumigatus, where rapid conidiation results in gray-green colonies (Fig. 1). In contrast, isolate F12776 produced smaller colonies (Fig. 1), and F13747 produced poorly sporulating colonies. Growth rate, conidial yield, and percent germination data are summarized in Table 3. The quantitative experiments confirmed our initial observations, where F12776 had a reduced colony radial growth rate relative to those of the other isolates (e.g., on RPMI, 50 versus 349 μm h^-1; P < 0.01), and F13747 and F12776 produced fewer spores per mm² (e.g., on RPMI, 0.4 × 10^6 and 0.6 × 10^6 versus 8 × 10^6 to 10 × 10^6 for the other
strains). Interestingly, F12776 had a significantly faster specific growth rate than all of the other isolates, whereas F13747 had a significantly slower one (0.16 for F12776, 0.142 for F12760, and 0.119 for F13747 in SDB; *P < 0.01*). Finally, it was noted that F13747 germinated more slowly than the other isolates (Fig. 2); in SDB, 32% of F13747's conidia had germinated at 8 h, whereas an average of 74% of conidia had germinated for the other seven isolates.

The majority of the isolates had a typical microscopic morphology for *A. fumigatus*. The germlings of F12776, on the other hand, were more compact and swollen, and the hyphae showed increased branching. A quantitative determination of branching confirmed this observation.

**Molecular characterization of the isolates.** Molecular typing showed that all eight isolates were the same type [A, (CA)₉(GA)₁₀; B, (CA)₉(CA)₁₀; C, (CA)₁₃; and D, (CA)₁₃](17). The G138C substitution identified in the cyp51A gene was found in all isolates except F12776 and F13747 (Table 1). Two novel amino acid substitutions were found: Y431C in F12776 and G434C in F13747. Two silent mutations in codons S216S and G434C in F13747. Two silent mutations in codons S216S and P394P (T1182G) were identified in the cyp51B gene of all eight isolates (data not shown). Real-time PCR was carried out on single copies of *cyp51A*, *AfuMDR2*, and *AfuMDR4* genes (Fig. 3). F12776 had a higher level of *cyp51A* expression (2.9×) than AF41, as did the other two isolates (2.1× for F12760 and 1.6× for F13747), whereas *AfuMDR2* and *AfuMDR4* had similar expression profiles, with the highest expression in F12760 followed by that in F12776 and then F13747. It is particularly noteworthy that the expression of the *cyp51A* gene in all three isolates was considerably higher than that in other isolates that we have studied, including three unrelated azole-susceptible isolates and the isogenic azole-resistant pair of two of these (9.6-, 13.4-, and 7.2-fold higher expression for F12760, F12776, and F13747, respectively, than the expression of susceptible isolate AF41). The effect of itraconazole on gene expression also was studied during a 4-h time course. A concentration of itraconazole was used that had no effect on the growth of these three pan-azole-resistant isolates but that had been shown to reduce the growth of an azole-susceptible isolate by 50%. Values were expressed relative to that of a cyclodextrin control (used to eliminate any potential impact on expression from cyclodextrin, which is used to solubilize itraconazole clinically), and only those genes where a statistically significant increase in expression during the time course was observed are shown in Fig. 4. The expression of *cyp51A* and *cyp51B* increased only in F13747, and the largest increase was only 2-fold for *cyp51A* at 4 h. Far larger increases were observed in an azole-susceptible isolate (AF41), as might be expected when ergosterol production has been increased.

**TABLE 3. Determinations of growth rates, conidial yields, and percent germination at 8 h**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colony radial growth rate(a) in (μm·h⁻¹):</th>
<th>SDB specific growth rate (h⁻¹):</th>
<th>Conidial yield (×10⁶ conidia/mm²):</th>
<th>% Germination at 8 h(b)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SDA</td>
<td>RPMI</td>
<td>1% GLU</td>
<td></td>
</tr>
<tr>
<td>F11628</td>
<td>431</td>
<td>372</td>
<td>334</td>
<td>0.142</td>
</tr>
<tr>
<td>F12041</td>
<td>425</td>
<td>352</td>
<td>324</td>
<td>0.142</td>
</tr>
<tr>
<td>F12760</td>
<td>395</td>
<td>338</td>
<td>289</td>
<td>0.147</td>
</tr>
<tr>
<td>F12776</td>
<td>127*</td>
<td>50*</td>
<td>127*</td>
<td>0.160*</td>
</tr>
<tr>
<td>F12865</td>
<td>418</td>
<td>348</td>
<td>319</td>
<td>0.138</td>
</tr>
<tr>
<td>F13535</td>
<td>384</td>
<td>360</td>
<td>292</td>
<td>0.142</td>
</tr>
<tr>
<td>F13746</td>
<td>407</td>
<td>331</td>
<td>288</td>
<td>0.144</td>
</tr>
<tr>
<td>F13747</td>
<td>411</td>
<td>343</td>
<td>294</td>
<td>0.119*</td>
</tr>
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</table>

*All experiments were repeated once. *, *P < 0.01.*

*The growth rates of each replicate (8 or 16) were calculated before being averaged.

*Averages from 16 replicates.

*Averages from 8 replicates.*

FIG. 1. Colony morphology of three of the isolates on SDA medium after incubation at 35°C for 96 h. The colony diameter of F12776 was 30 to 35 mm and was 60 to 70 mm for the other two isolates. F13535 is an example of the morphology of the majority of the isolates.
restricted and a positive feedback mechanism is activated (up to 7.5-fold relative to the level for the untreated control at 4 h for \textit{cyp51A} [data not shown]). Increasing amounts of mRNA were seen for \textit{AfuMDR1} in F12760, for \textit{AfuMDR3} in F12776, and for \textit{AfuMDR4} in F12776 and F13747, although the increases generally were significantly different only at 4 h and were less than 2-fold higher than those in controls.

As the expression level of \textit{cyp51A} in the three isolates studied was considerably higher than in those of other isolates that we have measured, the region upstream of the start codon was sequenced as a starting point for a molecular explanation. No nucleotide substitutions were identified in 1,719 bp upstream of the ATG start in F12760 and F13747 compared to the sequence of two azole-susceptible isolates. In contrast, a 1,822-bp insertion was found in F12776 370 bp upstream of the start codon in the same orientation as \textit{cyp51A}. The insertion has all the features of a type II transposon with a 100% conserved 45-bp terminal inverted repeat and a single open reading frame (ORF) encoding a transposase of 556 amino acids. The transcriptional start sites (TSS) were determined using 5' RACE.

FIG. 2. Germination of conidia from all eight isolates. Counts were measured at hourly intervals in SDB at 35°C. Each point represents the means from eight replicates ± standard deviations. Typical apical hyphae grow singly or with a bifurcate structure, lateral branches that tend to grow out perpendicularly, and hyphae that grow relatively straight. F12776, however, showed atypical swollen hyphae that did not grow in straight lines.

FIG. 3. Relative expression levels of the five \textit{cyp51A}, \textit{cyp51B}, \textit{AfuMDR1}, \textit{AfuMDR3}, and \textit{AfuMDR4} genes in three of the isolates. Expression was normalized to β-tubulin and is the means from four experiments.
RACE for F12760 and F12776, as well as for two unrelated azole-susceptible clinical isolates (AF41 and AF10) and the azole-resistant isogenic pair of one of them (AF72). Two TSSs were identified lying 49 and 136 bp upstream of the start codon. As judged by the intensity of the amplified RACE products on agarose gels, F12760 and F12776 utilized only the −136 TSS, whereas the other three isolates utilized both TSSs to a similar extent (data not shown). Based on this evidence, the insertion of a transposon 234 bp upstream of the TSS did not have any effect on the initiation site for transcription.

Since both the sequencing data and the multilocus microsatellite typing indicate that Aft1 had transposed into the promoter of cyp51A while this strain was growing in the patient, Southern hybridizations were carried out using four restriction nucleases to identify the overall distribution of this transposon in five out of the eight isolates. The earliest available isolate (F11628), as well as the two obtained at the same time as F12776 (F12760) and F13747 (F13746), were analyzed with restriction enzymes where the expected size of the cyp51A fragment was of a reasonable size to resolve on an agarose gel. The Southern hybridizations using internal Aft1 probes of EcoRI and BglII digestions are shown in Fig. 5. Similar results were obtained with BamHI and KpnI (data not shown). Twenty hybridizing bands were distinguishable on three of the gels in four of the isolates with identical banding patterns being observed. Only 19 bands could be resolved on the KpnI gel where, in general, fragment sizes were larger. In contrast, differences in the pattern of hybridizing bands were observed in F12776 compared to those of the other four isolates. The same 19 or 20 bands were observed in the KpnI, BamHI, and BglII digestions, whereas 1 band of approximately 12 kb was missing and another of ~7.5 kb was present (Fig. 5). More noteworthy, however, was the observation of a unique band only present in F12776 for each of the four digestions. These four bands were of the expected sizes to represent the copy of Aft1 upstream of the cyp51A gene.

Functional complementation in S. cerevisiae of cyp51A alleles associated with azole resistance. The three cyp51A mutations associated with pan-azole resistance in the isolates from the CPA patient were transformed into S. cerevisiae to address their involvement in azole resistance. Alleles of cyp51A with the G138C, Y431C, or G434C mutations were amplified from total cDNA, cloned into a yeast shuttle vector (YEp51), and transformed into an S. cerevisiae mutant lacking the en-
Markers are indicated on the left side of the gels. Hyperladder I (Bioline) a band (indicated by an oval) of approximately 12 kb is missing from the present only in the F12776 lane; the expected size is 7,522 bp. In addition, F12776 DNA. A band (indicated by the arrow) of approximately 7.5 kb is

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to include azoles among its hybridized with a 282-bp 

Aft1 282-bp DNA from five of the isolates. (A) A BglI digestion hybridized with a 282-bp Aft1 probe derived from PCR carried out on F12776 DNA. A band (indicated by the arrow) of approximately 7.5 kb is present only in the F12776 lane; the expected size is 7,522 bp. In addition, a band (indicated by an oval) of approximately 12 kb is missing from the F12776 lane, and two bands (indicated by asterisks) have not hybridized with the same intensity as in the other four lanes. The positions of the size markers are indicated on the left side of the gels. Hyperladder I (BioLine) and λ DNA monocut mix (New England Biolabs) were used.

dogenous ERG11, the target gene for azoles, and PDR5, a gene encoding an efflux pump known to include azoles among its substrates.

The susceptibilities of S. cerevisiae transformants containing individual cyp51A cDNAs were tested by spotting 10-fold serial dilutions containing various concentrations of itraconazole, voriconazole, and posaconazole (see Fig. S1 in the supplemental material). These mutants were compared to a haploid mutant containing a cyp51A allele from a susceptible strain of A. fumigatus. The transformants carrying the G138C and Y431C mutations were approximately 10-fold less susceptible to all three azoles than the control transformant (see Fig. S1). In contrast, and unexpectedly, the G434C mutant was hypersusceptible, with no growth occurring at any dilution of cells and for all drug concentrations (see Fig. S1). Similar results were obtained for the other three duplicate mutants (data not shown). Immunodetection by Western blotting with anti-A. fumigatus Cyp51A antibody carried out on the same cultures as those used for the susceptibility assay confirmed that similar amounts of protein had been expressed during this experiment for all six transformants (data not shown). In addition, the G138C form of the protein had a slightly reduced mobility relative to that of the other Cyp51A proteins.

DISCUSSION

All eight isolates from a single patient case were pan-azole cross-resistant. Two isolates (F12776 and F13747) had different mutations in the cyp51A gene (Y431C and G434C) from the rest (G138C). Alteration G138C has been identified previously in an azole-resistant laboratory mutant (20). However, substitutions Y431C and G434C have not been reported in clinical isolates or laboratory mutants to our knowledge (16). The heterologous expression of cyp51A in S. cerevisiae confirmed that two of the missense mutations (G138C and Y431C) were at least partly responsible forazole resistance. The heterologous expression of the G434C cyp51A, however, resulted in an unexpected hypersusceptible phenotype, raising questions about the impact of this substitution on cyp51A and why this strain was azole resistant. Our supposition is that heterologous expression in yeast is not a perfect model for single nucleotide polymorphisms at the carboxy end of the protein. Perhaps there is second-order confounding of protein-protein interactions not existing in Aspergillus and the possible loss of the anchoring of the protein. Additional work using an Aspergillus-based heterologous system is probably required to dissect out how G434C confers azole resistance, because our other mechanistic studies do not fully explain this.

In addition to the cyp51A sequence, these isolates showed further divergence despite having the same microsatellite type. Phenotypically F12776 (Y431C) was significantly slower growing, and both it and F13747 (G434C) sporulated poorly. In filamentous fungi, hyphal extension and branching frequency are interdependent, and the following relationship has been established: E = Gμ, where E is the mean rate of hyphal extension, G is the hyphal growth unit length (mean hyphal length per hyphal tip in a mycelium), and μ is the specific growth rate (38). The hyphal growth unit length for F12776 was significantly smaller (163 μm for F12776, 243 μm for F12760, and 259 μm for F13747 on SDA; P < 0.01). So even though F12776 has a higher specific growth rate than all other isolates, its mean rate of hyphal extension is less than those of the other isolates (26 for F12776, 31 for F13747 and 36 for F12760) and would be expected to produce smaller, more compact colonies.

Previously the primary mechanism of resistance reported in azole-resistant A. fumigatus has been alterations to the target gene cyp51A. However, there is mounting evidence of the contribution of other mechanisms (5). In addition to mutations in cyp51A, the expression of this gene was found to be higher in these isolates than in others tested. The increased expression of the homologous gene in cyp51A in Candida (erg11) has been linked with resistance (13). In A. fumigatus, the duplication of cyp51A resulted in a rise in MIC (29) and a tandem repeat in the promoter region of the cyp51A gene that leads to upregulation (accompanied with mutations at codon 98) also conferred resistance (24). The increase in cyp51A expression in the case described here may be the result of the translocation and activation of a single copy of a multicopy transposon into the 5’ upstream region of cyp51A, probably into the promoter region. This A. fumigatus transposon has been characterized in a previous study, where it was clustered with other fungal elements of the Fot1/Pogo group and labeled Aft1 (14). Hey et al. (14) demonstrated that all 10 clinical isolates studied had copies of this transposon variably distributed around their genomes, although one of them contained a truncated form of ~1.7 kb. They further showed that its target site is TA, which is duplicated after insertion. This duplication was found in the upstream region of F12776.

Of potential importance in the induction of resistance is the finding that a susceptible isolate, AF41, had a remarkable increase in cyp51A expression with itraconazole, unlike the
other susceptible isolate, AF10. AF41 is the same molecular type as AF72, which was one of the first two resistant isolates reported, and it was isolated from the same patient (10). AF72 has a cyp51A G54 mutation. If some isolates are able to survive azole therapy by upregulating cyp51A, it may be that subsequent mutations could emerge. The upstream tandem repeat associated with L98H could contribute to the emergence of resistance using this mechanism.

The molecular typing of the isolates confirmed that they all were identical. Because of the high discriminatory power of this typing technique, the most parsimonious explanation for the isolates having the same type is that the patient was originally infected by a single strain, which developed resistance and further evolved in situ within the lung. Azole resistance must have evolved independently more than once within this patient, as cyp51A mutations were not accumulated. We have additional evidence of in situ evolution from other patients who have developed azole resistance in aspergillosis but through different mechanisms (16).

In conclusion, this study on isolates obtained during 2 years from a single patient with bilateral aspergillosis has generated a number of unique findings. Most notable are a novel SNP (Y431C) conferring high-level azole cross-resistance and confirmation that another SNP (G138C) we previously reported is the cause of azole resistance. Also novel for Aspergillus is the concomitant and variable upregulation of cyp51A and the variable inducibility of cyp51A in different strains with itraconazole. Some alteration in transporter expression is in-

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REFERENCES


evidence for the involvement of the choline transport system and acetylcholinesterase. Microbiology 141:1309–1314.


