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A New *Aspergillus fumigatus* Resistance Mechanism Conferring In Vitro Cross-Resistance to Azole Antifungals Involves a Combination of *cyp51A* Alterations

E. Mellado,1* G. Garcia-Effron,1 L. Alcázar-Fuoli,1 W. J. G. Melchers,2 P. E. Verweij,2 M. Cuenca-Estrella,1 and J. L. Rodríguez-Tudela1

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Fourteen *Aspergillus fumigatus* clinical isolates that exhibited a pattern of reduced susceptibility to triazole drugs were analyzed. The sequences of the *cyp51A* gene from all isolates showed the presence of a point mutation at t364a, which led to the substitution of leucine 98 for histidine (L98H), together with the presence of two copies of a 34-bp sequence in tandem in the promoter of the *cyp51A* gene. Quantitative expression analysis (real-time PCR) showed up to an eightfold increase in the level of expression of the *cyp51A* gene compared to that by the susceptible strain. Three PCR fragments of one azole-resistant strain (strain CM2627) that included the promoter with the tandem repeat and part of *cyp51A* with the t364a mutation or PCR fragments with only one of the modifications were used to replace the *cyp51A* gene of an azole drug-susceptible *A. fumigatus* wild-type strain (strain CM237). Only transformants which had incorporated the tandem repeat in the promoter of the *cyp51A* gene and the L98H amino acid substitution exhibited similarly reduced patterns of susceptibility to all triazole agents and similarly increased levels of *cyp51A* expression, confirming that the combination of both alterations was responsible for the azole-resistant phenotype.

Infections with *Aspergillus fumigatus*, one of the most prevalent airborne fungal pathogens causing invasive aspergillosis worldwide, result in high rates of mortality and morbidity in immunocompromised hosts (20, 21). *A. fumigatus* is intrinsically resistant to fluconazole, but other triazole drugs like itraconazole and voriconazole are active both in vitro and in vivo against this species. Also, the new triazoles, such as posaconazole and ravuconazole, have been shown to have good in vitro activities against this species (5, 7, 17, 37, 45). In many centers voriconazole has become a common choice for the primary therapy of invasive aspergillosis, as the drug has been shown to have superior efficacy compared with those of other licensed antifungal drugs, confirming that Cyp51 is the target of these antifungal agents (30). In itraconazole-resistant *A. fumigatus* strains, two molecular mechanisms of resistance toazole drugs have been described: first,azole drug resistance in *A. fumigatus* seems to be very different from that in *Candida* spp. In *A. fumigatus*, there are two different but related Cyp51 proteins, and these are encoded by *cyp51A* and *cyp51B* (28). Targeted disruption of the *cyp51A* gene in different itraconazole-resistant *A. fumigatus* strains resulted in strains with similar patterns of decreased susceptibility to azole drugs, confirming that Cyp51A is the target of these antifungal agents (30). In itraconazole-resistant *A. fumigatus* strains, two molecular mechanisms of resistance toazole drugs have been described: first,azole drug resistance in *A. fumigatus* seems to be mostly related to point mutations in Cyp51A (2, 15, 25, 29, 32); and second, reduced intracellular accumulation, due to either increased expression of efflux pumps (13, 46) or reduced penetration of the drug (24), have also been proposed.

In filamentous fungi,azole drugs inhibit ergosterol biosynthesis by targeting the enzyme 14α-sterol demethylase (Cyp51). So far, the prevalence of mechanisms forazole drug resistance in *A. fumigatus* seems to be very different from that in *Candida* spp. In *A. fumigatus*, there are two different but related Cyp51 proteins, and these are encoded by *cyp51A* and *cyp51B* (28). Targeted disruption of the *cyp51A* gene in different itraconazole-resistant *A. fumigatus* strains resulted in strains with similar patterns of decreased susceptibility to azole drugs, confirming that Cyp51A is the target of these antifungal agents (30). In itraconazole-resistant *A. fumigatus* strains, two molecular mechanisms of resistance toazole drugs have been described: first,azole drug resistance in *A. fumigatus* seems to be mostly related to point mutations in Cyp51A (2, 15, 25, 29, 32); and second, reduced intracellular accumulation, due to either increased expression of efflux pumps (13, 46) or reduced penetration of the drug (24), have also been proposed.

Regarding the modification of *A. fumigatus* Cyp51, specific mutations in *cyp51A* have been associated with two different susceptibility profiles: (i) cross-resistance to itraconazole and posaconazole has been associated with amino acid substitutions at glycine 54 (G54) (15, 25, 32); and (ii) a pattern of itraconazole resistance, characterized by different patterns of elevated MICs forazole drugs, has been linked to different amino acid substitutions at methionine 220 (M220) (2, 29).

Here we describe the analysis of a new molecular mechanism responsible for a phenotype of *A. fumigatus* cross-resistance toazole drugs. We have determined that a base change causing an amino acid substitution in Cyp51A (L98H) in combination with the duplication in tandem of a 34-bp sequence in the *cyp51A* promoter, which is responsible for the increased
level of \textit{cyp51A} gene expression, accounted for the resistant phenotype.

(Part of this study was presented at the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, December 2005.)

\section*{MATERIALS AND METHODS}

\textbf{Fungal strains and media.} The fungal strains used in the study included (i) \textit{A. fumigatus} strain CM237 (CM refers to the fungal collection of the Mycology Reference Laboratory at the Centro Nacional de Microbiologia, ISCIII) was used to describe the \textit{A. fumigatus} isolate and \textit{cyp51B} gene sequences and was also the control strain (28); (ii) \textit{A. fumigatus} strain \textit{akuB} \textit{KU80} (10) was used as the recipient strain for transformation by electroporation by using genomic DNAs from \textit{A. fumigatus} strain CM2627 and the following primer sets: (i) primer set P450A1-P450A2, (ii) primer set A7-A5, and (iii) primer set A7-A2 (28). First, the \textit{cyp51A} and \textit{cyp51B} genes were amplified linear fragments of \textit{A. fumigatus} (Fig. 1). Linear fragments were obtained by PCR amplification of \textit{cyp51A} by using "TR," "L98H," or "TRL98H," followed by a number, depending on the DNA transformation protocol (28). The full coding sequences of \textit{A. fumigatus} \textit{cyp51A} and \textit{cyp51B} genes were amplified by PCR and both strands were sequenced, as described previously (15).

To rule out the possibility that any sequence changes identified were due to PCR-induced errors, each isolate was independently fully analyzed twice. The nucleotide sequences of the \textit{cyp51A} and \textit{cyp51B} genes in strains CM237 and \textit{akuB} \textit{KU80} are identical.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
Sex (age) & Underlying disease & Aspergillus diagnosis (time) & \textit{A. fumigatus} isolate & Date of isolation (day-mo-yr) & Genotype & MIC (\textmu g/ml) & \\
\hline
F & Unknown & Unknown & CM2627 & 21-10-2003 & A & 0.25 & 8–4–8–4 & 0.5 & \\
M (15) & X-linked CGD & ITC (6 yr) & V13/02 - CM3271 & 04-04-2002 & B & 0.25 & 8–4 & 8–1 & \\
M (73) & No & V27/28 - CM3273 & 03-12-2003 & C & 0.25 & 8–4 & 4–8–1 & \\
F (77) & AML & V28/78 - CM3275 & 02-03-2004 & D & 0.25 & 8–4 & 8–0.5–1 & \\
M (16) & Hyper-IgE syndrome & V34/75 - CM3276 & 19-11-2004 & E & 0.25 & 8–4 & 4–0.5 & \\
M (76) & Pulmonary fibrosis & No & V41/26 - CM3820 & 26-06-2005 & F & 0.25 & 8–4–8–4 & 0.5–1 & \\
M (31) & CGD & ITC (>10 yr) & V45/07 - CM3819 & 01-11-2005 & G & 0.25 & 8–8 & 8–4 & \\
F (68) & AML & V48/27 - CM3936 & 14-02-2006 & H & 0.25 & 8–4 & 8–4 & \\
M (62) & COPD & No & V49/09 - CM4023 & 05-04-2006 & I & 0.25 & 8–4 & 8–4 & \\
M (45) & AML & ITC (>2 yr) & V49/77 - CM4050 & 11-05-2006 & J & 0.25 & 8–4 & 4–8–4 & \\
\hline
\end{tabular}
\caption{Clinical data for patients infected or colonized with azole-resistant \textit{A. fumigatus}, genotypes, and ranges of MICs of different antifungals for the isolates$^a$}
\end{table}

\textbf{Antifungal susceptibility testing: MICs.} Broth microdilution susceptibility testing was performed as described in CLSI (formerly NCCLS) document M38-A (33), with modifications (6, 35, 40). Itraconazole (Janssen Pharmaceutical S.A., Madrid, Spain), voriconazole (Pfizer S.A., Madrid, Spain), ravenaconazole (Bristol-Myers Squibb, Madrid, Spain), posaconazole (Schering Plough, Madrid, Spain), and amphotericin B (Sigma, Madrid, Spain) were tested. Susceptibility tests were performed at least three times with each strain on different days. \textit{Aspergillus flavus} ATCC 204304 and \textit{A. fumigatus} ATCC 204305 were used as quality control strains for susceptibility testing.

\textbf{PCR amplification and sequence analysis of \textit{cyp51A} and \textit{cyp51B} genes.} Conidia from each strain were inoculated in 3 ml GYE broth and grown overnight at 37°C. Mycelial mats were recovered and subjected to a DNA extraction protocol (28). The full coding sequences of \textit{cyp51A} and \textit{cyp51B} were amplified by PCR and both strands were sequenced, as described previously (15). To rule out the possibility that any sequence changes identified were due to PCR-induced errors, each isolate was independently fully analyzed twice. The nucleotide sequences of the \textit{cyp51A} and \textit{cyp51B} genes in strains CM237 and \textit{akuB} \textit{KU80} are identical.

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\hline
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M (15) & X-linked CGD & ITC (6 yr) & V13/02 - CM3271 & 04-04-2002 & B & \\
M (73) & No & V27/28 - CM3273 & 03-12-2003 & C & \\
F (77) & AML & V28/78 - CM3275 & 02-03-2004 & D & \\
M (16) & Hyper-IgE syndrome & V34/75 - CM3276 & 19-11-2004 & E & \\
F (76) & Pulmonary fibrosis & No & V41/26 - CM3820 & 26-06-2005 & F & \\
M (31) & CGD & ITC (>10 yr) & V45/07 - CM3819 & 01-11-2005 & G & \\
F (68) & AML & V48/27 - CM3936 & 14-02-2006 & H & \\
M (62) & COPD & No & V49/09 - CM4023 & 05-04-2006 & I & \\
M (45) & AML & ITC (>2 yr) & V49/77 - CM4050 & 11-05-2006 & J & \\
\hline
\end{tabular}
\caption{Clinical data for patients infected or colonized with azole-resistant \textit{A. fumigatus}, genotypes, and ranges of MICs of different antifungals for the isolates$^a$}
\end{table}

\textit{Aspergillus fumigatus} transformations. Three linear DNA fragments were used throughout this work for azole-susceptible \textit{A. fumigatus} (CM237 and \textit{akuB} \textit{KU80}) strain transformations: (i) a fragment containing the coding sequence of the \textit{cyp51A} gene with a single nucleotide change (364a) that substitutes leucine for histidine (named the L98H fragment), (ii) a fragment that includes the \textit{cyp51A} promoter sequence of the gene with the 34-bp tandem repeat (named the TR fragment), and (iii) a fragment containing the coding sequence of \textit{cyp51A} gene with a 564a change that results in the L98H substitution in combination with the 34-bp TR in the \textit{cyp51A} promoter (named the TR-L98H fragment) (Fig. 1). Linear fragments were obtained by PCR amplification of \textit{cyp51A} by using RNA isolation and reverse transcription-PCR. Total RNA was extracted from \textit{A. fumigatus} through out this work for azole-susceptible \textit{A. fumigatus} (CM237 and \textit{akuB} \textit{KU80}) strain transformations.

\textbf{Electroporation.} The transformants were labeled with \textit{"TR," "L98H," or "TRL98H," followed by a number, depending on the DNA fragment used for the electroporation, and preceded by the identification number of the recipient strain (CM237 or \textit{akuB} \textit{KU80}).

\textbf{RNA isolation and reverse transcription-PCR.} Total RNA was extracted from the \textit{A. fumigatus} azole-susceptible strains (strains CM237 and \textit{akuB} \textit{KU80}), two of the clinical isolates (isolates CM2627 and CM3275), the transformants obtained from strain CM237 (transformants CM237-TRL98H1 and CM237-TRL7), and the transformants obtained from strain \textit{akuB} \textit{KU80} (transformants \textit{akuB} \textit{KU80}-TR2, \textit{akuB} \textit{KU80}-TR3, \textit{akuB} \textit{KU80}-L98H1, and \textit{akuB} \textit{KU80}-L98H3). The reverse transcription reactions were performed as described before (1). First, we evaluated the \textit{cyp51A} transcription level differences between azole-susceptible \textit{A. fumigatus}}
NEW AZOLE CROSS-RESISTANCE MECHANISM IN *A. FUMIGATUS* 1899

**RESULTS**

**Antifungal susceptibility testing: MICs.** All 14 strains were obtained from patients under different clinical conditions, and only half of the patients had a history of exposure to azole drugs (Table 1). To investigate whether these mutations originated from a clonal *Aspergillus* strain, the different isolates were genotyped by using a panel of microsatellites or STRs. STR analyses yield highly reproducible, exact typing results. Each multiplex reaction amplified three trinucleotide repeats. For each different STR, between 6 and 85 alleles were found. The isolates from all patients showed unique genetic profiles, indicating no genetic relatedness between the different *A. fumigatus* isolates (Table 1). The 14 strains exhibited a common pattern of reduced susceptibility to all four triazoles tested with various MICs, depending on the antifungal drugs, with posaconazole being the most active compound in vitro. The MICs for amphotericin B showed no variations between any strains (Table 1).

**PCR amplification and sequence analysis of cyp51A and cyp51B genes.** Sequence analysis of *cyp51A* showed the same 3464a base change in all strains, resulting in an amino acid substitution with histidine (L98H) at the leucine 98 codon. Two isolates (both from the same patient) had two extra base changes: these were t960a and t1554a, which were responsible for amino acid changes of serine for threonine at codon 297 (S297T) and phenylalanine for isoleucine at codon 495 (F495I), respectively (Table 2). All strains also contained three different point mutations in the *cyp51B* gene that were not conserved across the 14 isolates, although all of them were silent (Table 2). The sequence alignments of the region encompassing L98 revealed that this amino acid residue is not totally conserved in all yeasts and molds. However, the fact that all azole-resistant strains carried the same mutation

---

**Genotyping of *Aspergillus* isolates.** The *A. fumigatus* isolates were genotyped by using a panel of short tandem repeats (STRs). Two sets of three STRs (M3 and M4) consisting of tri- and tetranucleotide motifs were used in separate multicolor multiplex PCRs to analyze both *cyp51A* and *cyp51B* gene fragments: (A) Wild-type strain CM237; (B) azole-resistant strain CM2627. The primer sets were as follows: (i) primer set A7-A2, which contains the TR and the t3464a base change; (ii) primer set A7-A2, which contains only the 34-bp TR; and (iii) primer set P450A1-P450A2, which amplifies the full *cyp51A* gene, including only the t3464a base change (L98H).

**Antifungal susceptibility testing: MICs.** All 14 strains were obtained from patients under different clinical conditions, and only half of the patients had a history of exposure to azole drugs (Table 1). To investigate whether these mutations originated from a clonal *Aspergillus* strain, the different isolates were genotyped by using a panel of microsatellites or STRs. STR analyses yield highly reproducible, exact typing results. Each multiplex reaction amplified three trinucleotide repeats. For each different STR, between 6 and 85 alleles were found. The isolates from all patients showed unique genetic profiles, indicating no genetic relatedness between the different *A. fumigatus* isolates (Table 1). The 14 strains exhibited a common pattern of reduced susceptibility to all four triazoles tested with various MICs, depending on the antifungal drugs, with posaconazole being the most active compound in vitro. The MICs for amphotericin B showed no variations between any strains (Table 1).

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strongly suggested that the substitution was associated with azole drug resistance. Moreover, all strains had a duplication in tandem of a 34-bp sequence in theazole drug resistance. Moreover, all strains had a duplication strongly suggested that the substitution was associated with azole drug resistance.

The region encompassing the mutation and the repeat in the promoter were sequenced in 25 A. fumigatus deposited in the GenBank database (accession numbers AF338659 and AF222068).

The region encompassing the mutation and the repeat in the promoter were sequenced in 25 A. fumigatus azole-susceptible clinical strains, as well as in several A. fumigatus azole-resistant strains with different known resistance mechanisms. None of the strains had either the variation at codon L98 or the 34-bp tandem duplication.

**Levels of Cyp51A expression by A. fumigatus azole-resistant strains.** The levels of accumulation of cyp51A mRNA in two azole-resistant clinical strains (strains CM2627 and CM3275) in comparison with those in the A. fumigatus azole-susceptible CM237 control strain were analyzed. The values presented in Table 3 are the differences in the levels of transcription of cyp51A between the azole-resistant strains and azole-susceptible strain CM237 normalized to the levels of transcription of the reference β-tubulin gene for each strain included in the assay.

**Replacement of wild-type cyp51A gene with different gene modifications.** Therefore, the cyp51A gene fragments which had been amplified from strain CM2627 by PCR and which carried the different cyp51A modifications were electroporated into wild-type (azole-susceptible) A. fumigatus strain CM237. The different PCR fragments (L98H, TR, and TRL98H) were individually electroporated into wild-type A. fumigatus strain CM237 (Fig. 1). When itraconazole (5 μg/ml) was used for selection, mutants (n = 15) were obtained only when TRL98H

---

**TABLE 2. Nucleotide and amino acid substitutions in cyp51A and cyp51B genes from A. fumigatus clinical isolates**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>TR promoter</th>
<th>Codon 54</th>
<th>Codon 98</th>
<th>Codon 220</th>
<th>Codon 297</th>
<th>Codon 495</th>
<th>Codon 35</th>
<th>Codon 394</th>
<th>Codon 464</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM237 (wild type)</td>
<td>(−)</td>
<td>GGG</td>
<td>CTC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>CCT</td>
<td>ATT</td>
</tr>
<tr>
<td>akub^KU80</td>
<td>(−)</td>
<td>GGG</td>
<td>CTC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>CCT</td>
<td>ATT</td>
</tr>
<tr>
<td>M03/669 - CM2627</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>CCT</td>
<td>ATT</td>
</tr>
<tr>
<td>V13/02 - CM3271</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG_96CCG</td>
<td>TCG_1554AT</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V13/03 - CM3272</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG_96CCG</td>
<td>TCG_1554AT</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>1495ATA</td>
</tr>
<tr>
<td>V27/28 - CM3273</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V28/78 - CM3275</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V34/75 - CM3276</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V34/76 - CM3277</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V34/77 - CM3278</td>
<td>(+)</td>
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<td>364CAC</td>
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<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V34/78 - CM3279</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V41/26 - CM3820</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V45/07 - CM3819</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V48/27 - CM3936</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V49/09 - CM4023</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
<tr>
<td>V49/77 - CM4050</td>
<td>(+)</td>
<td>GGG</td>
<td>364CAC</td>
<td>ATG</td>
<td>TCG</td>
<td>TTT</td>
<td>TCT</td>
<td>1283CGG</td>
<td>ATT</td>
</tr>
</tbody>
</table>

Amino acids (G54) (L98H) (M220) (S297T) (F4951) (S358S) (P394P) (I464I)

---

**TABLE 3. A. fumigatus cyp51A mRNA transcription levels of different clinical and mutant strains with respect to that for wild-type strain CM237**

<table>
<thead>
<tr>
<th>Strain</th>
<th>cyp51A mRNA transcription levela</th>
<th>VC (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geometric mean</td>
<td>SD</td>
</tr>
<tr>
<td>CM2627</td>
<td>7.70</td>
<td>1.45</td>
</tr>
<tr>
<td>CM3275</td>
<td>8.05</td>
<td>1.25</td>
</tr>
<tr>
<td>CM237-L98HTR1</td>
<td>8.10</td>
<td>1.36</td>
</tr>
<tr>
<td>CM237-TR7</td>
<td>4.23</td>
<td>1.02</td>
</tr>
<tr>
<td>akub^KU80 recipient</td>
<td>1.48</td>
<td>0.10</td>
</tr>
<tr>
<td>akub^KU80-TR2</td>
<td>4.74</td>
<td>0.91</td>
</tr>
<tr>
<td>akub^KU80-TR3</td>
<td>8.23</td>
<td>1.96</td>
</tr>
<tr>
<td>akub^KU80-L98H1</td>
<td>0.65</td>
<td>0.15</td>
</tr>
<tr>
<td>akub^KU80-L98H3</td>
<td>1.32</td>
<td>0.14</td>
</tr>
</tbody>
</table>

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*a Geometric means for at least two different RNAs and six cDNAs. SD, standard deviation.

*b VC, coefficient of variation
TR3) were obtained when the PCR-generated TR fragment was used for transformation and five mutants (strains akuB<sup>KL300</sup>-L98H1 to akuB<sup>KL300</sup>-L98H5) were obtained when the PCR-generated L98H fragment was used for transformation.

** Mutant analysis. Several different aspects of the *A. fumigatus* transformants were analyzed: (i) antifungal drug susceptibility; (ii) the DNA sequence, to verify the incorporation of sequence changes; and (iii) cyp51A mRNA transcription levels.

In order to rule out the possibility of the integration of extra copies of cyp51A in the transformation procedure, DNAs from every transformant were digested with a restriction enzyme, blotted, and hybridized (29) with a labeled fragment of every transformant were digested with a restriction enzyme, and hybridized (29) with a labeled fragment of cyp51A.

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The cyp51A gene including the promoter area and the cyp51B genes from all transformants were sequenced. All mutants analyzed (nine) had incorporated the mutated cyp51A allele corresponding to the fragment that was introduced (Table 4). With the exception of the L98H mutation and/or duplication of the repeat in tandem, none of the transformants had any other mutation in either cyp51A or cyp51B compared to the sequence of wild-type strain CM237. The *A. fumigatus* transformants' susceptibilities to triazole drugs were determined as described above. All the transformants with both cyp51A alterations (TR and L98H) exhibited susceptibility profiles similar to those of the clinical isolates (Table 4). However, transformant CM237-TR7 had a slight but not significant increase in azole drug MICs (*P* > 0.01) that did not correspond to an azole-resistant phenotype. Similarly, transformants obtained with the akuB<sup>KL300</sup> strain (containing either the TR or the L98H modification independently) had a slight but not significant increase in azole drug MICs (*P* > 0.01) that did not correspond to an azole-resistant phenotype (Table 4).

The levels of cyp51A mRNA expression in two transformants (strains CM237-TRL98H1 and CM237-TR7) in comparison with those in *A. fumigatus* azole-susceptible recipient strain CM237 and two azole-resistant clinical strains (strains CM2627 and CM3275) were analyzed. The relative increases in the levels of cyp51A expression obtained in CM237-TRL98H1 were eightfold compared to the level of expression in the wild-type strain, whereas the levels of cyp51A transcription in CM237-TR7 were increased only fourfold compared to the level of transcription in the wild type (Table 3). Also, the levels of cyp51A mRNA expression in four mutants (strains akuB<sup>KL300</sup>-TR2, akuB<sup>KL300</sup>-TR3, akuB<sup>KL300</sup>-L98H1, and akuB<sup>KL300</sup>-L98H3) in comparison with that in their *A. fumigatus* recipient akuB<sup>KL300</sup> strain were analyzed. The relative increases in the levels of cyp51A expression obtained in strains akuB<sup>KL300</sup>-TR2 and akuB<sup>KL300</sup>-TR3 were between five- and eightfold those in the akuB<sup>KL300</sup> parental strain, whereas the levels of cyp51A transcription in strains akuB<sup>KL300</sup>-L98H1 and akuB<sup>KL300</sup>-L98H3 were similar to that in the parental akuB<sup>KL300</sup> strain (Table 3).

** DISCUSSION**

In *Candida* spp., the majority of reports on azole drug resistance are related to the increased efflux of azole drugs due to the overexpression of efflux pumps of different types (4, 39, 42). In addition, a number of reports have identified polymorphisms in the erg11 gene (which is homologous to cyp51A) from clinical *Candida albicans* isolates that are responsible for and/or that are associated with fluconazole resistance (16, 26, 43). On the other hand, the resistance of filamentous fungi to different demethylase inhibitors (DMIs) used for agricultural purposes has mainly been correlated with one amino acid change (Y136F) in the azole target, Cyp51 (11, 12, 53), or with its overexpression (18, 23, 44, 46). In *A. fumigatus*, azole drug resistance has been described for both laboratory mutants and
clinical strains and has mainly been attributed to alterations in the target enzyme (Cyp51A) (2, 15, 25, 28, 32). Even though analysis of the A. fumigatus genome has shown the existence of more than 40 ATP binding cassette transporter (ABC) homologs and more than 100 major facilitator transporter genes (five times more than the number in yeast) (49), isolates that overexpress efflux pumps as an azole resistance mechanism have rarely been described. Nevertheless, there are a number of reports of efflux pump overexpression related to azole resistance in A. fumigatus, although these have been A. fumigatus mutant strains generated in the laboratory and not clinical isolates (9, 32). Only one clinical strain (strain AF72) has been found to overexpress (by five times) the atrF gene (which encodes an ABC transporter efflux pump), but only when it is grown in the presence of itraconazole (46).

Up to now, decreases in the susceptibility of A. fumigatus to itraconazole and posaconazole only (15, 25, 32) or to all azole drugs (2, 29) have exclusively been linked to specific mutations in cyp51A. The 14 A. fumigatus clinical strains described in this study present a similar triazole cross-resistance phenotype but have none of the known Cyp51A amino acid substitutions. A new resistance mechanism that consisted of the presence of a duplication of a TR in the cyp51A promoter and a L98H substitution in the cyp51A-coding sequence was found in all isolates. This fact strongly suggests that both alterations were related to the azole-resistant phenotype. Nevertheless, we conducted experiments to analyze each of the cyp51A modifications individually.

The results obtained with the mutants (strains CM237-TR7, akuBcyc50-TR2, and akubcyc50-TR3) suggest that the duplication of the 34-bp TR by itself seems to be insufficient for reproduction of the resistant phenotype, even though there was a four- to eightfold increase in the level of cyp51A expression which corresponded to a slight increase in azole MICs. Previous reports have related azole resistance to the high level of cyp51/erg11 expression in both yeast and filamentous fungi. The mechanisms that have been associated with increased expression include gene or chromosome duplication and promoter modification. The first mechanism, known in mammals as gene amplification, was described in Candida glabrata (27) and in an engineered laboratory strain of Aspergillus niger (50).

The latter mechanism has been seen in an A. nidulans triazole-resistant strain obtained by amplification or overexpression of the A. nidulans cyp51 gene expressed in a high-copy-number plasmid (34). An increased level of cyp51 expression has been suggested as a possible mechanism for sterol DMI resistance in the plant pathogen Mycosphaerella graminicola (48). Similarly, a DMI resistance mechanism has been described for another plant pathogenic mold, Venturia inaequalis. The high level of cyp51 expression was correlated with the presence of 553-bp insertion located in the gene promoter (44). Also, just recently, the analysis of 59 DMI-resistant isolates of Bumilleria jaapii has shown 5- to 10-fold increased levels of cyp51 expression related to various forms of a truncated non-long terminal direct repeat, long interspersed nuclear retrotransposon element (23).

In Penicillium digitatum a mechanism similar to the one that we describe has been reported for strains that have a 126-bp sequence tandemly repeated in the cyp51 promoter and that is directly related to a pattern of resistance to different DMIs. Nevertheless, the number of tandem repeats proved to be essential for the production of a high level of DMI resistance (18). Therefore, the results obtained in this study suggest that the 34-bp sequence in the promoter region of cyp51A might be a transcriptional enhancer and that its duplication is responsible for the increased level of expression of cyp51A and the higher A. fumigatus MICs for azole drugs. However, these results also suggest that the TR duplication by itself is not enough to confer the azole cross-resistant phenotype shown by these strains. On the other hand, the introduction of the L98H amino acid substitution at Cyp51A alone seems to be responsible for only a slight increase in the A. fumigatus MICs for azole drugs without influencing the cyp51A expression levels. In the A. fumigatus Cyp51A protein, the leucine at position 98 is close to the first substrate recognition site (SR51). This residue is conserved throughout yeast and filamentous fungi but not in plant or human Cyp51 proteins. An homology model based on the X-ray crystal structure of Cyp51 from Mycobacterium tuberculosis was used to predict the specific Cyp51A residues within A. fumigatus Cyp51A in combination withazole drugs (54). However, the specific residue L98 has not been implicated in any azole drug interactions with the protein (54). The search for Cyp51A mutations at L98 in A. fumigatus strains with intermediate MICs of azole drugs will help to clarify whether this amino acid substitution is present in strains isolated in the clinical setting and/or in environmental isolates (these experiments are already under way in our laboratory).

Finally, the results obtained with the mutants incorporating both cyp51A alterations (TR and L98H) confirmed that the combination of both modifications is needed to reproduce the azole-resistant phenotype shown by the clinical strains.

It is quite remarkable that since itraconazole-resistant A. fumigatus strains were first described, A. fumigatus strains with phenotypes of resistance to single or multiple azoles have emerged in subsequent years. The recent isolation (2002 to 2006) of a number of A. fumigatus strains, all unrelated, with a novel mechanism of azole resistance suggests that these molds might be exposed to the selective pressure of azole compounds either in the patient or in the environment. This aspect requires further studies, especially in areas where 14α-demethylase inhibitors are extensively used in agriculture.

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REFERENCES


