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Aspergillus fumigatus Intrinsic Fluconazole Resistance Is Due to the Naturally Occurring T301I Substitution in Cyp51Ap

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Aspergillus fumigatus intrinsic fluconazole resistance has been demonstrated to be linked to the *CYP51A* gene, although the precise molecular mechanism has not been elucidated yet. Comparisons between *A. fumigatus* Cyp51Ap and *Candida albicans* Erg11p sequences showed differences in amino acid residues already associated with fluconazole resistance in *C. albicans*. The aim of this study was to analyze the role of the natural polymorphism I301 in *Aspergillus fumigatus* Cyp51Ap in the intrinsic fluconazole resistance phenotype of this pathogen. The I301 residue in *A. fumigatus* Cyp51Ap was replaced with a threonine (analogue to T315 at *Candida albicans* fluconazole-susceptible Erg11p) by changing one single nucleotide in the *CYP51A* gene. Also, a *CYP51A* knockout strain was obtained using the same parental strain. Both mutants' antifungal susceptibilities were tested. The I301T mutant exhibited a lower level of resistance to fluconazole (MIC, 20 μg/ml) than the parental strain (MIC, 640 μg/ml), while no changes in MIC were observed for other azole- and non-azole-based drugs. These data strongly implicate the *A. fumigatus* Cyp51Ap I301 residue in the intrinsic resistance to fluconazole.

A*spergillus fumigatus* is the most common hyphomycete to cause disease in humans (1–3). It is intrinsically resistant to ketoconazole and fluconazole but normally susceptible to the other available azole antifungal agents (itraconazole, posaconazole, voriconazole, and isavuconazole) (4–8). The molecular mechanism for fluconazole intrinsic resistance has not been described yet. However, a hypothetical molecular mechanism has been proposed by Edlind et al., who linked *A. fumigatus* fluconazole intrinsic resistance with a naturally occurring amino acid substitution in Cyp51Ap (14-α sterol demethylase A) (9). These authors carried out an *in silico* comparison of the *Candida albicans* Erg11p and *A. fumigatus* Cyp51Ap sequences and found that among the residues most commonly implicated in fluconazole resistance in *C. albicans* (Y132, T315, S405, G464, and R467) (10, 11), only the T315 residue is not conserved in *A. fumigatus* Cyp51Ap and is naturally replaced by a nonpolar isoleucine (I301). In *C. albicans*, the replacement of the polar T315 residue by the nonpolar alanine (T315A) is enough to confer fluconazole resistance on the yeast (10).

The aim of this study was to molecularly confirm that the natural polymorphism I301 in the Cyp51Ap is necessary and sufficient to explain the intrinsic reduced fluconazole susceptibility of *A. fumigatus*. An *A. fumigatus* mutant harboring the I301T substitution was generated, and susceptibilities to fluconazole and other antifungals were tested. Also, a *CYP51A*-defective mutant was obtained using the same parental strain in order to compare their antifungal susceptibility patterns.

MATERIALS AND METHODS

Strains. *Aspergillus fumigatus* akuB[KU80] (12) was considered the wild-type strain, and its DNA was used as the template for all PCRs. It was the recipient strain for electroporation assays. *Escherichia coli* TOP10 (Promega) was used to propagate all plasmids.

Genetic constructs. A transformation plasmid named LMDM-P87 was generated. It contains a mutated T973C *CYP51A* gene (that leads to the amino acid substitution I301T in Cyp51Ap) with its intact 5′ flanking region. The *CYP51A* 3′ untranslated region (UTR) is interrupted by a hygromycin B resistance cassette (*hph*) between nucleotides 126 and 127

upstream of the *CYP51A* stop codon. Plasmid LMDM-P87 was obtained in two sets of three PCRs each (Fig. 1). The first set was aimed to introduce the mutation T973C into *CYP51A*. In the first reaction of this set, primers A7 and A19 were used to amplify a 1,452-bp fragment which contained a 462 bp of the *CYP51A* promoter region plus 990 bp of the first portion of its coding sequence (5′ UTR and 5′ of the open reading frame [ORF]). The second PCR employed oligonucleotides A18 and A17, which amplify a 790-bp fragment including 664 bp of the 3′ portion of the *CYP51A* ORF and 126 bp upstream of the *CYP51A* stop codon (3′ UTR). Primer A18 carries the mutation T973C in the middle of its sequence. Primer A19 is reverse complementary with A18, and both generate an overlapping region of 35 bp which was used in the final fusion PCR, performed as follows. The two previously generated fragments (1,452 bp and 790 bp) were used as templates together with the primers A7 and A17. The resulting 2.2-kb product was cloned into the pGEM-T Easy vector (Promega) to obtain plasmid LMDM-P75. Both strands of the complete 2.2-kb construct were sequenced to confirm the presence of the mutation, as described previously (13). In parallel, a second set of PCRs was performed. The *hph* cassette was fused to a 0.9-kb fragment of the 3′ UTR region of *CYP51A* starting 127 bp upstream of the *CYP51A* stop codon. The resistance cassette was used as a selection marker for recombinants, while the 0.9-kb fragment was used later as a flanking region for homologous integration together with the 5′ UTR-*CYP51A*. In the first PCR amplification of this set, the 1.4-kb *hph* cassette was obtained from plasmid pUM102 (*A. fumigatus CYP51A*Δ*hph*) (a kind gift of Emilia Mellado) (14) using primers H1 and HF2. The second PCR was performed using primers HF1 and H2 in order to obtain the 0.9-kb fragment of the 3′ UTR of the *CYP51A* gene described before. HF1 and HF2 are reverse complementary primers
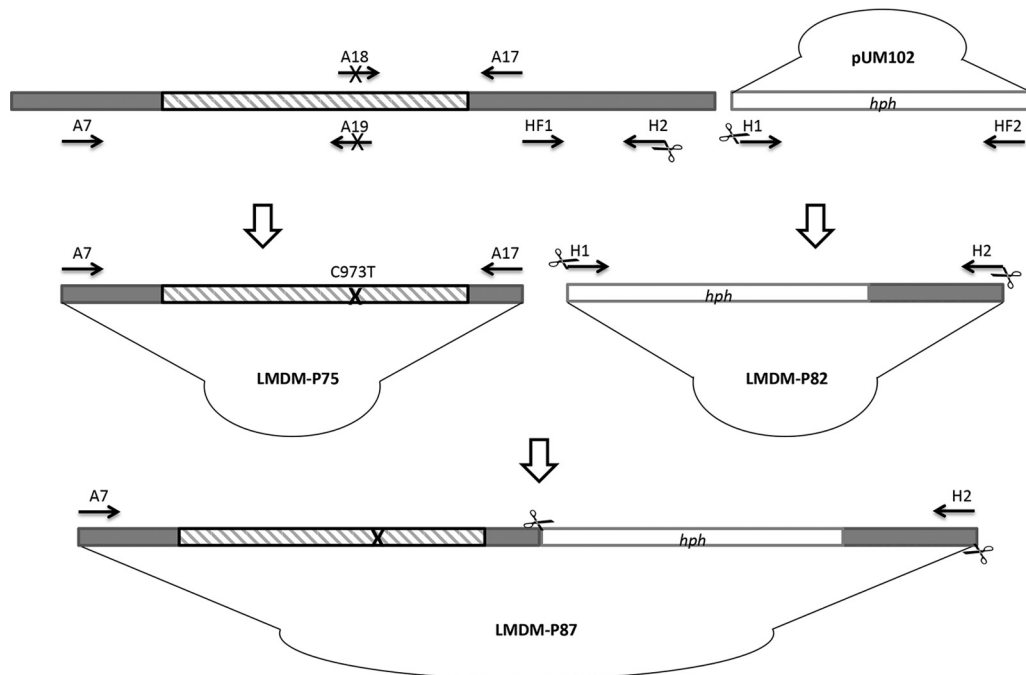
FIG 1 Construction of the LMDM-P87 plasmid employed to generate the *A. fumigatus CYP51A* T973C mutant strain. Striped and gray boxes represent the *CYP51A* coding and UTR sequences, respectively. Unfilled boxes indicate the hygromycin resistance cassette (*hph*) obtained from the pUM102 plasmid. Black arrows symbolize oligonucleotide primers. The cross symbol represents the introduced T973C mutation. SacI restriction sites are indicated with scissors (primers H1 and H2).

designed to allow the fusion of the described fragments in the next reaction. The last PCR of this set was done using the 1.4-kb (*hph*) and the 0.9-kb fragments as templates and primers H1 and H2. The last oligonucleotides include a SacI site on both ends. The resulting construct (2.3 kb) was cloned into a pGEM-T Easy vector to obtain the plasmid LMDM-P82.

To generate the complete transformation vector, the 2.3-kb fragment with an *hph* cassette was released from the LMDM-P82 by SacI digestion by following the manufacturer's instructions (Promega). Simultaneously,

LMDM-P75 was linearized with SacI and dephosphorylated using calf intestinal alkaline phosphatase (CIAP; Promega) according to the manufacturer's protocol. Afterwards, the 2.3-kb fragment was ligated with T4 DNA ligase (Promega) to the linearized LMDM-P75 to create LMDM-P87. The complete vector sketch can be seen in Fig. 1. Primer sequences are described in Table 1.

Transformations. Two linear PCR fragments were used for *A. fumigatus* akuB^KU80 transformation: (i) the cassette containing the mutated

TABLE 1 Oligonucleotide primers used in this work

Primer	Sequence (5′–3′)[a]	Orientation	Use
A7[b]	TCATATGTTGCTCAGCGG	Sense	LMDM-P87 construction and evaluation of the transforming vector integration
A19	GCATAATCCAGGCGCTG**G**TGGACGAAGACGAATGC	Antisense	LMDM-P87 construction
A18	GCATTCGTCTTCGTCCA**CC**AGCGCCTGGATTATGC	Sense	LMDM-P87 construction
A17	GGCCAGTAAGGTCTGAATAAG	Antisense	LMDM-P87 construction
H1	TTT<u>GAGCTC</u>GTTAACTGATATTGAAGGAGCATTTTTTGGGC	Sense	LMDM-P87 construction
H2	ACG<u>GAGCTC</u>CATCGAACCTCTCGTGTGACTATG	Antisense	LMDM-P87 construction
HF1	AGAGTAGATGCCGACCGGGAACCAGTTAACCCTGAAGTGTTGTTGCCTATACTGAG	Sense	LMDM-P87 construction
HF2	CTCAGTATAGGCAACAACACTTCAGGGTTAACTGGTTCCCGGTCGGCATCTACTCT	Antisense	LMDM-P87 construction
P450-1[c]	ATGGTGCCGATGCTATGG	Sense	*CYP51A* knockout cassette amplification
P450-2[c]	CTGTCTCACTTGGATGTG	Antisense	*CYP51A* knockout cassette amplification
A10	ATTGCCGCAGAGATGTCC	Antisense	Evaluation of transforming vector integration and *CYP51A* expression
HS3	ACATGGCGTGATTTCATATGCGCG	Sense	Evaluation of transforming vector integration
HS4	TGGTCAAGACCAATGCGGAGCATA	Antisense	Evaluation of transforming vector integration
A14	CCAGAGAGACTTTGACACAG	Sense	Evaluation of transforming vector integration
A1[b]	CTTCTTTGCGTGCAGAGA	Sense	Evaluation of *CYP51A* expression

[a] Letters in bold indicate the mutated nucleotide. Underlining indicates a SacI restriction site.
[b] From the work of Mellado et al. (28).
[c] From the work of Diaz-Guerra et al. (13).

CYP51A gene together with the *hph* selection marker (LMDM-P87) and (ii) the *CYP51A* knockout cassette (14). Fragments were PCR amplified with the primer pairs A7/H2 and P450.1/P450.2, respectively. Transformation experiments by electroporation were carried out as described before (13) using 0.3 μg of the PCR fragments. Transformants were selected with 350 μg/ml of hygromycin B (HygB; InvivoGen) in minimal medium (MM) (15) and subcultured for further analysis. The incorporation of the *hph* cassette was phenotypically confirmed by plating the strains in duplicate in MM with 350 μg/ml of HygB.

Integration confirmation. Genomic DNA from HygB-resistant transformants and the parental strain were obtained (14). Two multiplex PCRs were performed to confirm the homologous recombination of the mutated *CYP51A/hph* cassette and the knockout cassette (Fig. 2A). The first multiplex reaction was performed with two primer pairs, with the aim of verifying the integration of the *hph* cassette in the *A. fumigatus* genome. Primers A7 and A10 were used as a PCR control, as they hybridize the 5′ UTR and the ORF of the *CYP51A*, respectively. Primers HS3 and HS4 were used to amplify a fragment of the *hph* cassette. Thus, two PCR fragments were expected when the *hph* cassette was integrated (1,066 bp and 386 bp). In contrast, only the 1,066-bp fragment would be amplified in nontransformant strains. The second multiplex PCR was meant to confirm the homologous recombination of the mutated *CYP51A*, using the primers A14, HS3, and HS4. Primer A14 was designed to hybridize the *CYP51A* 5′ flanking region 603 bp downstream the *CYP51A* start codon, which is outside the construction cloned into LMDM-P87. Homologous or ectopic recombination would be confirmed by the amplification of two fragments (3.5 kb and 386 bp) or one (386 bp) band, respectively. No bands were expected for nontransformant strains.

PCRs. PCR amplifications were performed in a 25-μl volume by following the Pegasus DNA polymerase (PBL, Buenos Aires, Argentina) manufacturer's instructions in an Applied Biosystems thermocycler (Tecnolab-AB, Buenos Aires, Argentina). The thermocycler was programed for one initial step of 2 min at 94°C followed by 30 cycles of 30 s at 95°C, 30 s at the primer pair's melting temperature (T_m), and 1 min per kilobase of the expected PCR product at 72°C and then a final cycle of 10 min at 72°C.

Confirmation of the expression of the mutated *CYP51A*. Total RNA was extracted with RNAzol (RNAzolRT; MRC Inc.) from mutant strains, and reverse transcription (RT) was performed with avian myeloblastosis virus (AMV) reverse transcriptase enzyme (Promega, Argentina) according to the manufacturer's protocol. The obtained cDNA was used as the template for a PCR performed with primers A1 and A10 (flanking the 70-bp intron of *CYP51A*).

Antifungal susceptibility testing. Susceptibility testing was performed by following the broth microdilution reference method published in document M38-A2 of the Clinical and Laboratory Standards Institute (CLSI) (16). Itraconazole, posaconazole, voriconazole, fluconazole, amphotericin B, and caspofungin (all purchased from Sigma-Aldrich, Argentina) were tested. Concentration ranges of fluconazole were modified from what is standardized to 640 to 1.25 μg/ml to establish differences in fluconazole susceptibilities between the wild-type and mutant strains. Moreover, fluconazole and voriconazole susceptibility were also evaluated by disk diffusion following CLSI document M51-A (17) using commercial disks (Oxoid, Argentina) and by agar diffusion using fluconazole MIC test strips (fluconazole 256-μg MIC test strips; Liofilchem SRL). Susceptibility tests were performed in triplicate on three different days.

RESULTS

To establish the role of the I301 residue of Cyp51Ap in the intrinsic fluconazole resistance of *A. fumigatus*, two mutant strains were generated. One harbors the mutation T793C in *CYP51A*, which leads to the I301T amino acid substitution, while the other is a *CYP51A*-defective strain. These mutants were named LMDM-1030 and LMDM-32, respectively. The homologous recombination was confirmed by multiplex PCR using the primers described in Materials and Methods. The mutant strains LMDM-1030 and LMDM-32 showed the genomic integration of the *hph* cassette (Fig. 2B, lanes 4 and 5, respectively). For both mutants, two PCR bands were observed (386 bp and 1,066 bp) corresponding to the amplification of the DNA region between primers HS3/HS4 and A7/A10, respectively. Similar results were obtained with LMDM-P87 and pUM-102 DNAs, which were used as reaction controls. On the other hand, parental strain akuB^KU80 showed only one 1,066-bp band. The homologous recombination of both constructions in LMDM-1030 and LMDM-32 was confirmed by a second multiplex PCR. Two PCR bands were obtained when DNAs from both mutants were used (Fig. 2B, lanes 8 and 9). LMDM-1030 showed 3.5-kb and 386-bp bands which correspond to the amplification using primers A14 and HS4 and the pair HS4 and HS3, respectively. The smaller band shows the presence of *hph*. The 3.5-kb band demonstrates that the construction was integrated, replacing the wild-type *CYP51A* gene, since the A14 primer hybridizes the *CYP51A* 5′ UTR but in a region not included in the construction (A14 hybridizes 603 bp upstream of the start codon). Moreover, the size of the amplicon demonstrates that the *hph* cassette was integrated in the 3′ UTR, 126 bp upstream of the *CYP51A* stop codon. For LMDM-32, the multiplex PCR also showed two bands but with different sizes (2.4 kb and 386 bp). The smaller band demonstrates the *hph* cassette integration as described before, while the 2.4-kb band shows that the *hph* cassette was integrated inside the *CYP51A* ORF region. As expected, when *A. fumigatus* akuB^KU80 DNA was used, no amplification was obtained (Fig. 2B, lane 6). The incorporation of the T973C mutation in the CYP51A gene of the LMDM-1030 mutant strain was confirmed by sequencing (Fig. 2C).

The naturally occurring polymorphism T301I at *A. fumigatus* Cyp51Ap is responsible for the fluconazole resistance phenotype. The *CYP51A*-defective strain LMDM-32 and the T973C mutant LMDM-1030 were morphologically indistinguishable from the parental strain, *A. fumigatus* akuB^KU80. However, azole MICs were substantially different. It was clear that the deletion of *CYP51A* in LMDM-32 decreased the azole MICs 32- to 4-fold for fluconazole and the other azole drugs tested, respectively. In contrast, LMDM-1030 MICs were significantly lower only for fluconazole (32-fold) (Table 2). As expected, there were no susceptibility differences to nonazole antifungals between mutants and parental strains (Table 2). The fluconazole and voriconazole susceptibility differences between the wild-type and mutant strains were confirmed by disk diffusion susceptibility testing. The parental strain showed no inhibition zone when fluconazole disks were used, while the inhibition zone diameter for both mutant strains was 19 mm. Turning to voriconazole, the parental and LMDM-1030 strains showed the same inhibition diameter (32 mm), whereas the knockout mutant showed an inhibition zone of 48 mm (Table 2 and Fig. 3). Moreover, fluconazole MIC differences between akuB^KU80 and both mutants were also verified by agar diffusion using Liofilchem MIC test strips. Using this methodology, the akuB^KU80 strain showed a MIC of >256 μg/ml, while both mutants exhibited a MIC of 8 μg/ml (64-fold lower) (Table 2 and Fig. 4).

***CYP51A* gene expression in LMDM-1030.** The construction transformed into LMDM-1030 carried a mutated *CYP51A* ORF with a 3′ UTR modification (*hph* insertion). The genomic integration of the construction could alter the *CYP51A* gene transcription into mRNA, producing a decreased azole MIC phenotype as observed in the *CYP51A* knockout strains (14). LMDM-1030 and LMDM-32 strains showed low fluconazole MICs. Thus, we de-
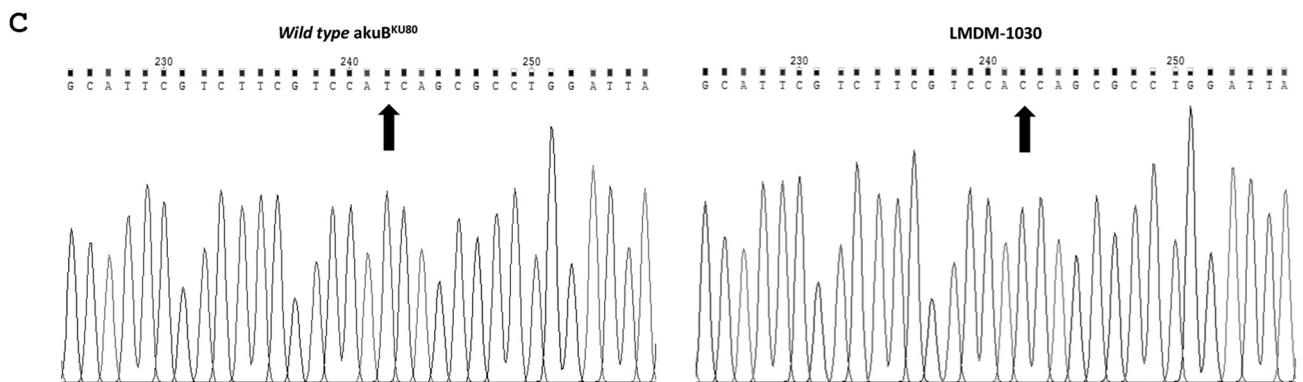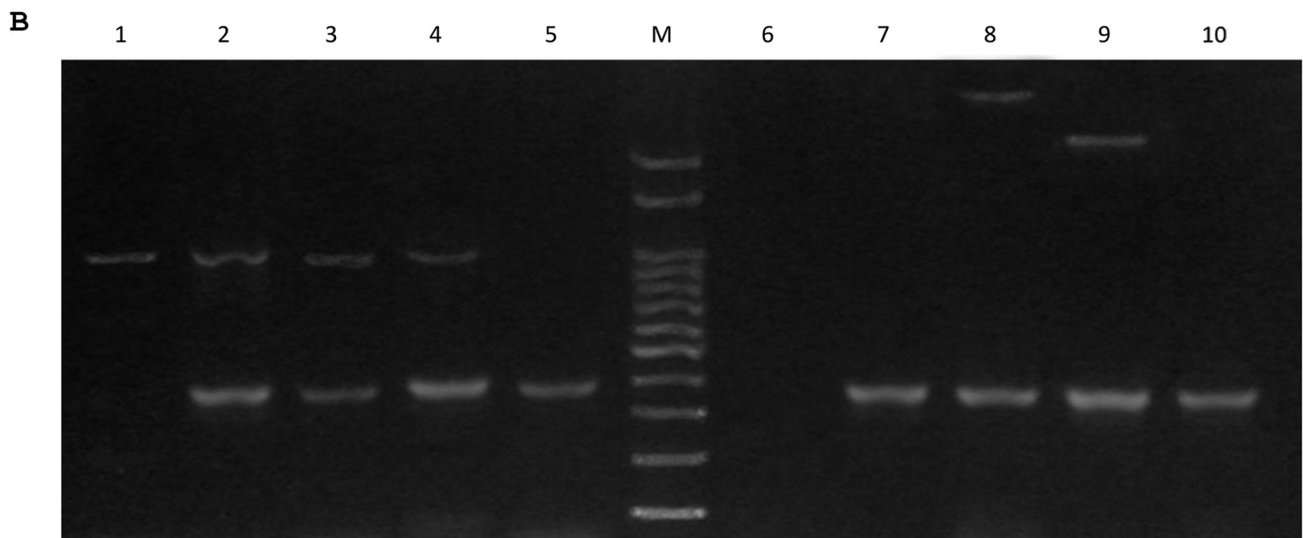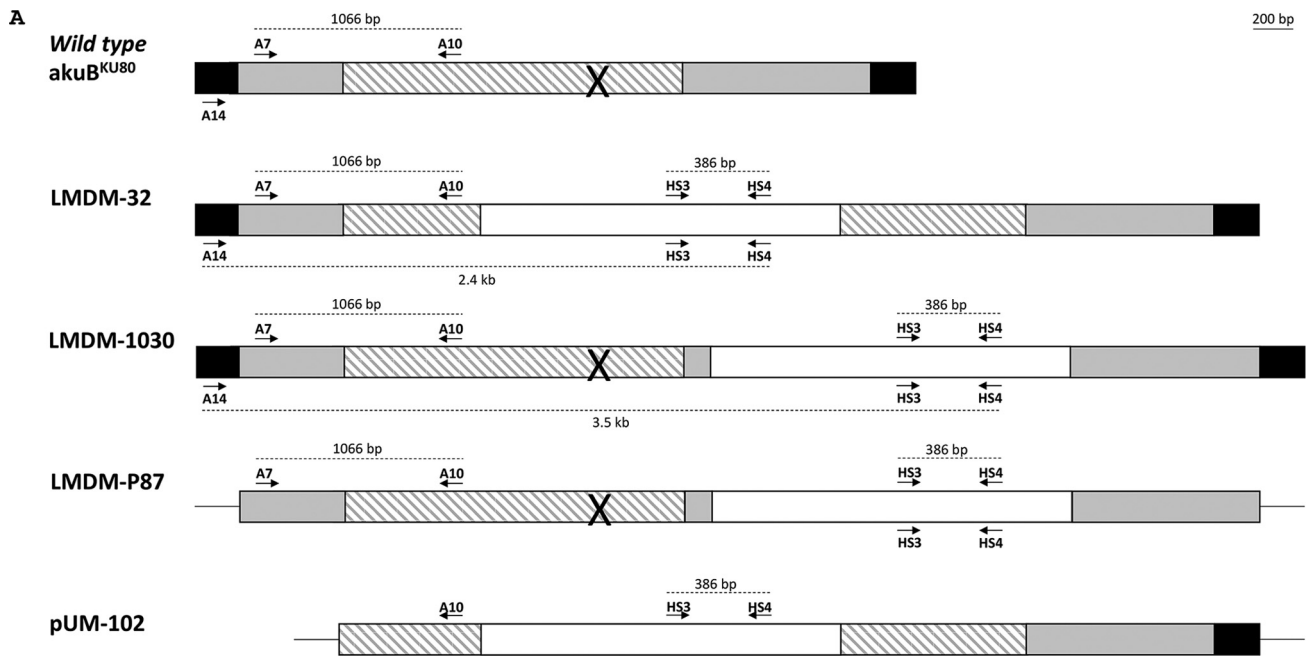
FIG 2 Recombination confirmation. (A) Schematic representation of the gene constructions and primer relative positions. Striped and gray boxes represent the *CYP51A* coding and UTR sequences, respectively. Unfilled boxes indicate the hygromycin resistance cassette (*hph*). Black boxes show the *CYP51A* UTRs not included in the constructions. Lines (in LMDM-P87 and pUM-102) represent the pGEM-T Easy vector. Arrows symbolize oligonucleotide primers. The cross symbol represents the introduced T973C mutation. Dotted lines represent the sizes of the PCR fragments. (B) Multiplex PCRs aimed to confirm homologous recombination events in the studied mutants. Lanes 1 to 5 show the results of the multiplex PCR designed to verify the *hph* cassette integration (primers A7/A10 and HS3/HS4). Lanes 6 to 10 show the amplification products of the multiplex PCR meant to confirm the homologous recombination of the mutated *CYP51A* (primers A14, HS3, and HS4). M, 100-bp ladder. Lanes 1 and 6, wild-type akuB[KU80]; lanes 2 and 7, LMDM-P87; lanes 3 and 8, LMDM-1030; lanes 4 and 9, LMDM-32; lanes 5 and 10, pUM-102. (C) Sequencing chromatograms showing the mutation T973C in the *CYP51A* gene of the LMDM-1030 strain.

TABLE 2 Susceptibility testing results of the *Aspergillus fumigatus* strains used in this study

Strain	MIC (μg/ml) of antifungal agent[a]					
	FLC	ITC	PCZ	VRC	AMB	CSF
A. fumigatus akuB[KU80]	640.00 (>256.00/0)	0.12	0.25	0.12 (ND/32)	0.50	0.06
LMDM-1030	20.00 (8.00/19)	0.06	0.25	0.12 (ND/32)	0.50	0.06
LMDM-32	20.00 (8.00/19)	0.03	0.06	0.03 (ND/48)	0.50	0.06

[a] Geometric means of at least 3 repetitions performed on different days. In parentheses are the MICs and diameters obtained by agar diffusion for fluconazole and voriconazole (Liofilchem MIC test strips/inhibition diameter, in millimeters). FLC, fluconazole; ITC, itraconazole; PCZ, posaconazole; VRC, voriconazole; AMB, amphotericin B; CSF, caspofungin; ND, not done.

cided to evaluate the *CYP51A* expression during hyphal growth in order to determine whether a loss of *CYP51A* expression might cause or contribute to the fluconazole MIC change. Total RNAs from the akuB[KU80] and LMDM-1030 strains were extracted, and reverse transcription reactions were carried out. Afterwards, genomic DNA and cDNA from both strains were used as templates for PCRs performed with primers A1 and A10. These oligonucleotides hybridize areas surrounding the intron of the *CYP51A* gene. Hence, fragments of 350 bp and 425 bp were observed when cDNA and DNA from both strains were used as templates, confirming the presence of *CYP51A* mRNA in both strains (Fig. 3).

DISCUSSION

It is well known that *A. fumigatus* is intrinsically resistant to fluconazole and ketoconazole and normally susceptible to the other available azole drugs (5, 8). However, clinical secondary azole resistance was described and mostly associated with several amino acid substitutions in Cyp51Ap (4, 13, 14, 18–26). In 2005, Mellado et al. reported a *CYP51A*Δ *A. fumigatus* mutant which was azole hypersusceptible, confirming the linkage between *CYP51A* and azole resistance (14). It is also clear that each particular amino acid substitution in Cyp51Ap (or the combination of them) leads to a particular azole MIC pattern and that most of the reported *CYP51A* mutant strains showed itraconazole resistance, itraconazole-posaconazole cross-resistance, isavuconazole-voriconazole cross-resistance, or pan-triazole cross-resistance (4, 13, 14, 18–26). Despite the advances in elucidating the secondary azole resistance mechanisms in *A. fumigatus*, the molecular basis of intrinsic fluconazole resistance was never studied. The first hypothesis regarding this subject was proposed by Edlind et al., who studied the *A. fumigatus* Cyp51Ap sequence (9). They suggested that the Cyp51Ap I301 residue could be implicated in fluconazole resistance since the T315A substitution in *C. albicans* Erg11p produced
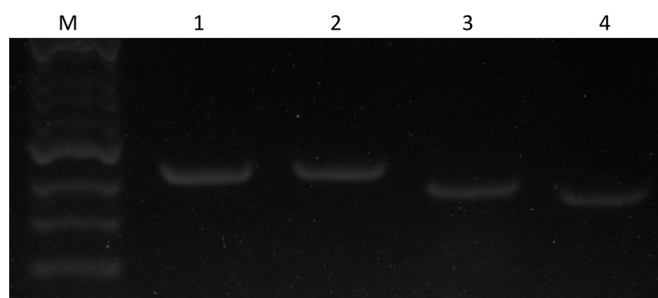
a similar phenotype. Later, Diaz-Guerra et al. gave the first laboratory clue linking fluconazole resistance with Cyp51Ap. They described that amino acid substitutions at the G54 residue of Cyp51Ap led to itraconazole resistance but 4- to 5-fold fluconazole MIC reductions, possibly due to a better interaction between fluconazole and Cyp51Ap (13).

In this work, we obtained two *A. fumigatus* mutants, one harboring a I301T substitution in Cyp51Ap and the other a *CYP51A* deletion mutant. Both mutants showed a 32-fold decrease in fluconazole MIC. To establish that fluconazole susceptibility in the engineered *A. fumigatus* mutant is due to the I301T change and not due to the loss of *CYP51A*, the expression of this gene during hyphal growth was confirmed by reverse transcription. The MICs obtained for the other tested azole drugs also confirm that the I301T substitution is necessary and sufficient to explain fluconazole MIC reduction. The LMDM-1030 strain alone showed voriconazole, posaconazole, and itraconazole MICs similar to those obtained for its parental strain, mimicking *C. albicans* susceptibility patterns (pan-azole susceptibility) (27).

The precise manner in which the I301T substitution impacts fluconazole susceptibility could be explained taking into account that in *A. fumigatus* there are two homologous *CYP51* genes (28). In both
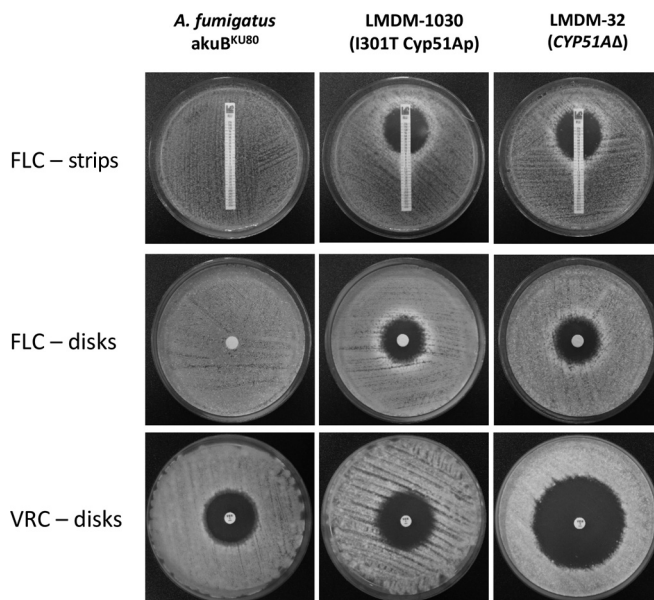

FIG 3 Agarose gel electrophoresis showing the detection of *CYP51A* transcripts. Lanes 1 and 2, PCR products using genomic DNAs; lanes 3 and 4, PCR amplification using cDNAs as the template. Lanes 1 and 3, *A. fumigatus* akuB[KU80]; lanes 2 and 4, LMDM-1030.

FIG 4 Diffusion susceptibility testing using fluconazole (FLC) disks and Liofilchem MIC test strips and voriconazole (VRC) disks for *A. fumigatus* LMDM-1030 (I301T Cyp51Ap mutant), *A. fumigatus* akuB[KU80] (parental strain), and *A. fumigatus* LMDM-32 (*CYP51A*Δ).

Cyp51p genes, four of the five residues most frequently linked with fluconazole resistance in *C. albicans* are conserved (Y132, T315, S405, G464, and R467) (10, 11). Consequently, these amino acids would not account for fluconazole resistance in *A. fumigatus*. The fifth residue (T315) is conserved only in Cyp51Bp, while in Cyp51Ap, it is naturally replaced (I301). The LMDM-1030 mutant harbors in both Cyp51p proteins a threonine, as in *C. albicans*; thus, both enzymes would be inhibited by fluconazole. The T315 residue in the *C. albicans* 14-α sterol demethylase is crucial for the correct enzyme-substrate and enzyme-drug interactions near the heme group (11, 29). Analogously, the *A. fumigatus* Cyp51Ap I301 residue is placed in the center the α-I loop (D280-Q312), which was proposed as essential for drug-enzyme interaction (30–35). Recently, Hargrove et al. reported *A. fumigatus* Cyp51Bp crystal structure complexes obtained with and without voriconazole. They confirmed that residue T315 (equivalent to I301 in Cyp51Ap) is part of the N-terminal portion of one of the substrate recognition sequence (SRS4) which showed fungus-specific features not observed in Cyp51p from other kingdoms. These data demonstrated the importance of SRS4 in the specific inhibition of sterol biosynthesis in fungi by azole drugs (31). Moreover, at the beginning of 2016, Liu et al. reported a three-dimensional (3D) structural model of *A. fumigatus* Cyp51Ap based on a crystal structure of the homologous *Saccharomyces cerevisiae* enzyme (Erg11p) (32). Itraconazole, voriconazole, and posaconazole were docked to wild-type and mutant Cyp51Ap, and their models demonstrate that the S297 residue (part of the αI helix) is adjacent to a heme group and would interact with ligands and azoles (32). When the Cyp51Ap αI helix is represented as a helical-wheel diagram, the I301 residue is the closest amino acid to S297 and would also interact with azoles (data not shown). The experimental data that we present in this work support the results obtained by Liu et al. and Edlind et al. (9, 32) and strongly implicate the Cyp51Ap I301 residue in the intrinsic resistance of *A. fumigatus* to fluconazole. Moreover, this knowledge may help to understand how the drugs interact with Cyp51Ap and in the development of new antifungals.

ACKNOWLEDGMENTS

FUNDING INFORMATION

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