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MECHANISMS OF RESISTANCE

Molecular Confirmation of the Linkage between the *Rhizopus oryzae CYP51A* Gene Coding Region and Its Intrinsic Voriconazole and Fluconazole Resistance

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ABSTRACT *Rhizopus oryzae* is the most prevalent causative agent of mucormycosis, an increasingly reported opportunistic fungal infection. These Mucorales are intrinsically resistant to *Candida*- and *Aspergillus*-active antifungal azole drugs, such as fluconazole (FLC) and voriconazole, respectively. Despite its importance, the molecular mechanisms of its intrinsic azole resistance have not been elucidated yet. The aim of this work was to establish if the *Rhizopus oryzae CYP51* genes are uniquely responsible for intrinsic voriconazole and fluconazole resistance in these fungal pathogens. Two *CYP51* genes were identified in the *R. oryzae* genome. We classified them as *CYP51A* and *CYP51B* based on their sequence similarity with other known fungal *CYP51* genes. Later, we obtained a chimeric *Aspergillus fumigatus* strain harboring a functional *R. oryzae CYP51A* gene expressed under the regulation of the wild-type *A. fumigatus CYP51A* promoter and terminator. The mutant was selected after transformation by using a novel procedure taking advantage of the FLC hypersusceptibility of the *A. fumigatus CYP51A* deletion mutant used as the recipient strain. The azole susceptibility patterns of the *A. fumigatus* transformants harboring *R. oryzae CYP51A* mimicked exactly the azole susceptibility patterns of this mucormycete. The data presented in this work demonstrate that the *R. oryzae CYP51A* coding sequence is uniquely responsible for the *R. oryzae* azole susceptibility patterns.

KEYWORDS azole, *CYP51*, intrinsic resistance, molecular mechanism, resistance, *Rhizopus*, fluconazole, Mucorales, voriconazole

Rhizopus oryzae is the most prevalent causative agent of mucormycosis (1–4), an increasingly reported opportunistic fungal infection (5, 6). Mucormycosis treatment is a challenge since the etiological agents are resistant to almost all available antifungals. Therapy guidelines include surgical debridement followed by treatment using amphotericin B (AMB; liposomal/lipid complex) together with the reversal of predisposing conditions. In addition, posaconazole (PSC) can be used as a rescue and/or deescalation treatment, as it is one of the few azole-based antifungal agents active against these fungi. More recently, isavuconazole was included in the antifungal armamentarium for the treatment of invasive mucormycosis (especially for patients intolerant to AMB) (7–9). Notably, Mucorales are intrinsically resistant to *Candida*- and *Aspergillus*-active antifungal azole drugs, such as fluconazole (FLC) and voriconazole (VRC), respectively (7–9).

Azole agents inhibit ergosterol synthesis by interacting with the 14-α sterol demethylases, encoded in molds by *CYP51* genes (8, 10). Reported azole resistance mechanisms in filamentous fungi include overexpression of *CYP51A* and/or point mutations in the *CYP51A* gene and overexpression of efflux pumps (8, 10). The aim of this work

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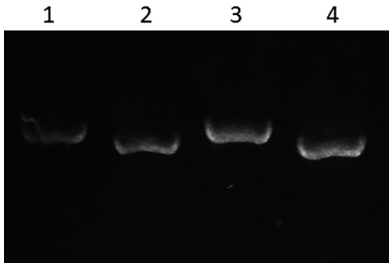

FIG 1 Agarose gel (1.5% agarose) electrophoresis at 40 V for 3 h showing the detection of *R. oryzae CYP51* transcripts using genomic DNA (lanes 1 and 3) and cDNA (lanes 2 and 4) as the templates. Lanes 1 and 2, amplification of Ro*CYP51A* (RO3G_16595) 1,626-nt and 1,524-nt bands, respectively; lanes 3 and 4, the resolved PCR bands of Ro*CYP51B* (RO3G_11790) at 1,755 nt and 1,533 nt, respectively.

was to establish if the nucleotide sequence (the open reading frame [ORF] of the gene) of a *Rhizopus oryzae CYP51* (Ro*CYP51*) gene is uniquely responsible for intrinsic VRC and FLC resistance in this mucormycete. To reach this goal, we obtained a chimeric *Aspergillus fumigatus* strain harboring a functional Ro*CYP51* gene expressed under the regulation of the wild-type *A. fumigatus CYP51A* (Af*CYP51A*) promoter and terminator. The mutant was selected after transformation by using a novel procedure taking advantage of the FLC hypersusceptibility of the *A. fumigatus CYP51A* deletion mutant used as the recipient strain.

(This study was partially presented at the 18th International Congress on Infectious Diseases of the International Society for Infectious Diseases, which was held in Buenos Aires, Argentina, in March 2018.)

RESULTS

***Rhizopus oryzae* harbors two Cyp51p paralogs that are not easily classified as *CYP51A* and *CYP51B*.** Two *CYP51* paralogous genes have been found in the *R. oryzae* genome (11), where they are classified as hypothetical ORFs and named RO3G_16595 and RO3G_11790 (11). Reverse transcription-PCR (RT-PCR) amplification demonstrated that both *R. oryzae* Cyp51ps are expressed during growth in liquid culture (Fig. 1). The first of these ORFs harbors 1,626 nucleotides (nt), while the second one harbors 1,755 nt, and the two ORFs are interrupted by 2 introns (of 50 nt and 52 nt) and 4 introns (of 56 nt, 58 nt, 58 nt, and 50 nt), respectively. These genes encode two proteins of 508 amino acids (aa) and 511 aa, respectively, and both were identified to be putative 14-α sterol demethylases by comparison with known fungal CYP51ps using the protein BLAST tool. Employing this tool, the strongest percent identity for the RO3G_16595 hypothetical protein was with the Cyp51ps of *Rhizopus microsporus* var. *microsporus*, *Parasitella parasitica*, and *Mucor ambiguus* (ranging from 78 to 74% identity). On the other hand, the RO3G_11790 ORF showed a high percent identity with the different Cyp51ps of *Rhizopus microsporus* var. *microsporus*, *Mucor circinelloides*, and *Mucor ambiguus* (78 to 75%). Considering that different filamentous Ascomycetes species harbor two different related Cyp51ps, synteny analysis was performed to unambiguously identify these genes as *CYP51A* or *CYP51B* when the sequences were compared with *Aspergillus* genome sequences. However, employing different synteny software, *R. oryzae CYP51* genes could not be classified as *CYP51A* or *CYP51B* since the genes do not colocalize within the same chromosomes as they do in *Aspergillus* spp. When *R. oryzae* Cyp51p sequences were analyzed by the neighbor-joining unweighted pair group method using arithmetic averages (UPGMA) and compared with the sequences of other Cyp51p/Erg11p paralogs, five clusters were observed. Two of the clusters included the already known filamentous ascomycete Cyp51Ap and Cyp51Bp. The third and fourth clusters included the Ascomycetes and Basidiomycetes yeast Erg11ps, respectively. All the published Mucorales Cyp51ps clustered together with the two Cyp51ps studied in this work (RO3G_16595 and RO3G_11790) (11) (Fig. 2). In this last cluster, two subclusters in which RO3G_16595 was separated from RO3G_11790 were observed.

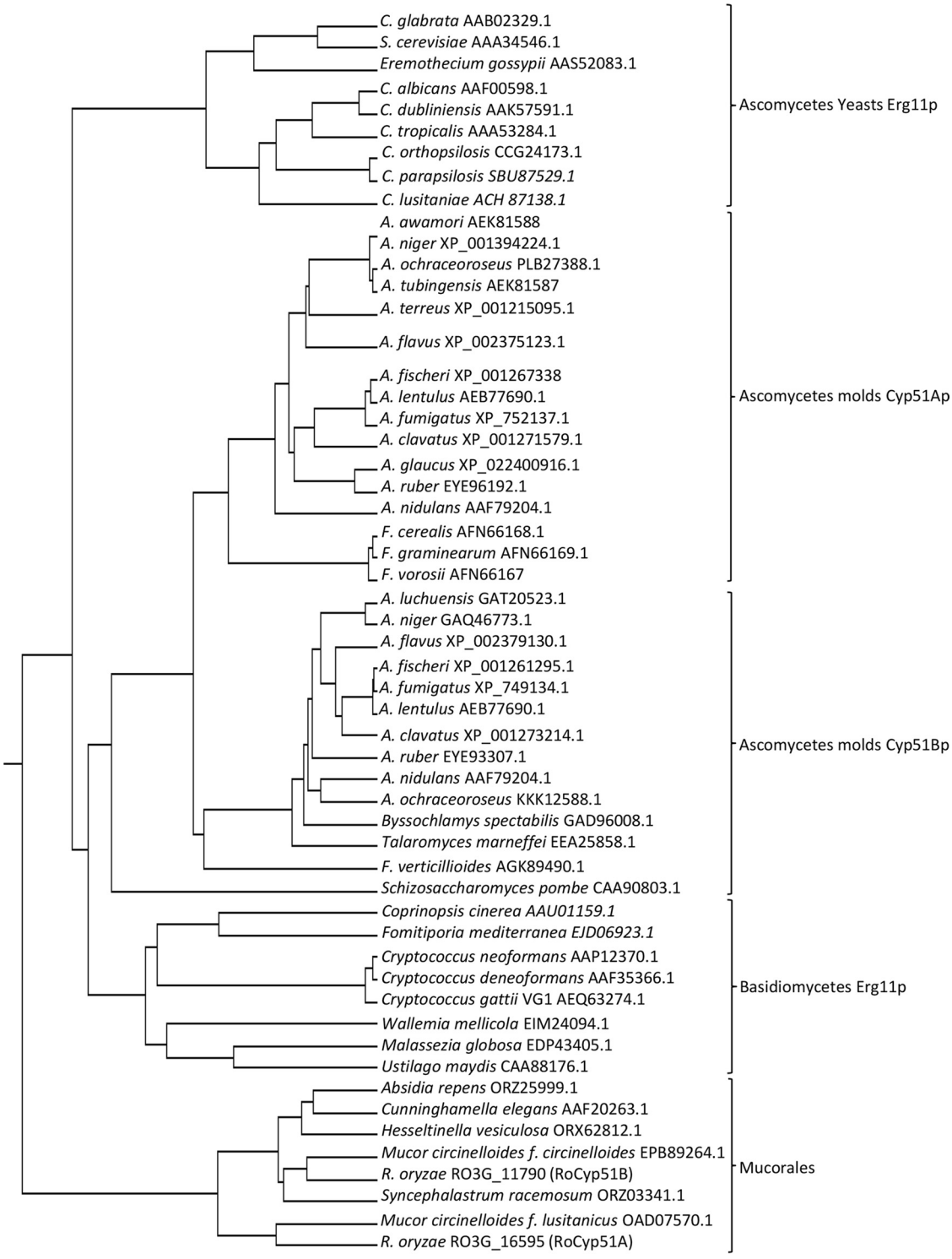

FIG 2 Neighbor-joining phylogenetic tree of 55 different fungal 14-α sterol demethylases, including enzymes from 9 Ascomycetes yeast species, 20 Ascomycetes mold species, 9 Basidiomycetes species, and 7 Mucorales species. GenBank accession numbers appear after the species names. Some fungal genus names were abbreviated as follows to esthetically improve the figure: *A.*, *Aspergillus*; *S.*, *Saccharomyces*; *C.*, *Candida*; *F.*, *Fusarium*; *R.*, *Rhizopus*.

Since no sharp differentiation could be obtained, we decided to name the RO3G_11790 gene Ro*CYP51B*, based on its low but significant amino acid sequence similarity with *A. fumigatus CYP51B* (46% identity) obtained using the BLASTP tool. On the other hand, there were few non-Mucorales 14-α sterol demethylases

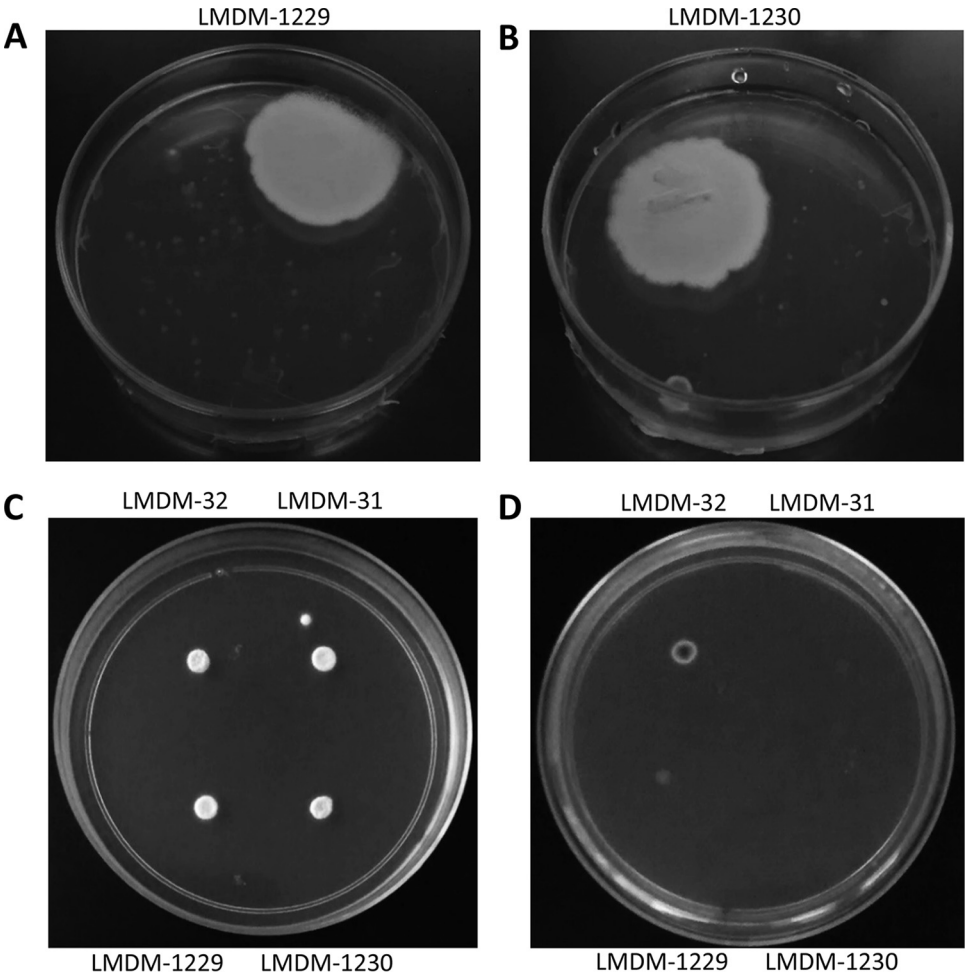

FIG 3 (A, B) Selection plates using FLC (100 μg/ml) where LMDM-1229 (A) and LMDM-1230 (B) transformants were obtained. (C, D) Replica plates used minimal medium (C) and minimal medium with 450 μg/ml hygromycin B (D).

showing a sequence similarity of over 40% with RO3G_16595. Some examples are the basidiomycete *Malassezia pachydermatis* Erg11p (GenBank accession number XP_017991977.1) and the ascomycete *Pneumocystis jirovecii* Erg11p (GenBank accession number XP_018230821.1), which showed 44 and 45% identities with RO3G_16595, respectively. Taking into account the fact that *R. oryzae* has two *CYP51* genes and RO3G_11790 has homology with the *CYP51B* genes, we decided to arbitrarily name the RO3G_16595 gene *CYP51A* and study it as the potential gene responsible for intrinsic VRC and FLC resistance.

The *R. oryzae* fluconazole and voriconazole resistance phenotype is exclusively linked with the Ro*CYP51A* coding sequence. To establish whether the Ro*CYP51A* coding sequence is the only one responsible for the phenotype of intrinsic FLC and VRC resistance and that no other molecular factors (e.g., hyperexpression of efflux pumps or other genes) are involved, we transformed azole-hypersensitive *A. fumigatus* LMDM-32 (FLC MIC, 20 μg/ml) with the coding region of the *CYP51A* gene from *R. oryzae* ATCC 11886. Thus, the transformants harbored the mucormycete gene governed by the wild-type Af*CYP51A* promoter and terminator regions. After electroporation experiments, two *A. fumigatus* FLC-resistant transformants (named LMDM-1229 and LMDM-1230) were obtained (Fig. 3). Integration and homologous recombination were phenotypically and molecularly verified by hygromycin B (Hyg) resistance evaluation and by PCRs, respectively, as described in Materials and Methods. *A. fumigatus* strain LMDM-32 is resistant to Hyg since its *CYP51A* gene was knocked out by using a Hyg

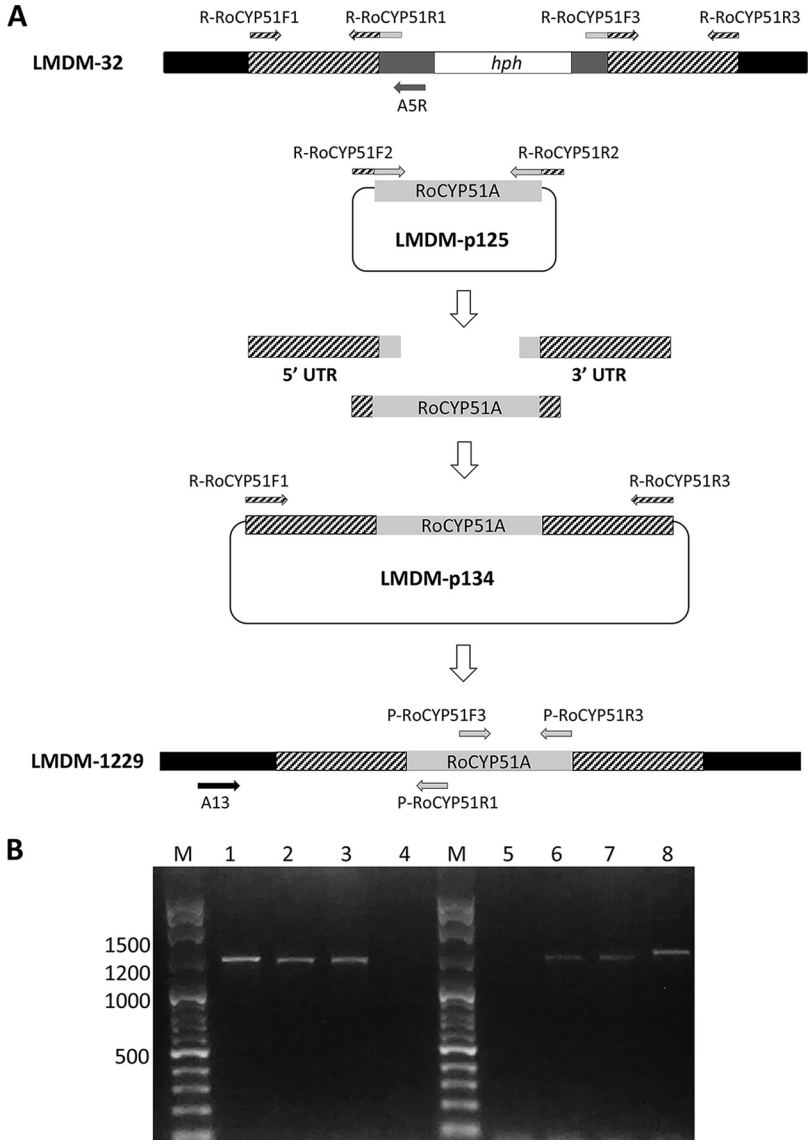

FIG 4 (A) Scheme of the LMDM-p134 vector generation process used in this work. Striped and black boxes represent the 5′ and 3′ UTRs of the Af*CYP51A* included and not included in the transformation vector, respectively. Light gray boxes denote the Ro*CYP51A* (RO3G_16595) complete coding sequence obtained from cDNA. Dark gray boxes show the C- and N-terminal fractions of Af*CYP51A* (12, 32). Thin lines in LMDM-p125 and LMDM-p134 symbolize the pGEM-T Easy vector. Arrows represent primers, and their coloring shows where they hybridized to each of the genomic or vector DNAs. (B) Integration and homologous recombination confirmation by using PCRs. Lane M, 100-nt ladder (numbers on the left are in nucleotides); lanes 1 to 4, Ro*CYP51A* (RO3G_16595) exon 3 amplifications using the P-RoCYP51F3 and P-RoCYP51R3 primers (1,374 nt); lanes 5 to 8, multiplex PCR with the A13, A5R and P-RoCYP51R1 primers to confirm homologous recombination (by the presence of the 1,286-nt band) or the lack of existence of Ro*CYP51A* (RO3G_16595) in the *A. fumigatus* genome (by the presence of the 1,461-nt band); lane 1, LMDM-p134; lanes 2 and 5, *R. oryzae* ATCC 11886; lanes 3 and 6, *A. fumigatus* LMDM-1229; lane 7, *A. fumigatus* LMDM-1230; lanes 4 and 8, *A. fumigatus* LMDM-32.

resistance cassette (12). After transformation, LMDM-p134 cassette homologous recombination would revert the Hyg resistance phenotype, given that the Hyg resistance cassette and Ro*CYP51A* (of the LMDM-p134 vector) are flanked by similar regions (5′ and 3′ untranslated [UTR] regions of Af*CYP51A*). This Hyg-susceptible phenotype was observed in two of the transformants (Fig. 3). On the other hand, Ro*CYP51A* integration into the *A. fumigatus* LMDM-1229 and LMDM-1230 mutant genomes was confirmed by the amplification of exon 3 of the mucormycete gene (as identified by a 1,374-nt PCR band obtained with the P-RoCYP51F3 and P-RoCYP51R3 primers) (Fig. 4). In addition,

TABLE 1 Antifungal susceptibility testing results for the strains used in this study

Strain	MIC or MEC value (μg/ml)[a]						
	FLC	PSC	ITC	VRC	AMB	CSF	ANF
LMDM-31	>320.00 (0)	0.25	0.12	0.12 (32)	0.50	0.06	0.03
LMDM-32	16.00 (19)	0.12	0.03	0.03 (48)	0.50	0.06	0.03
ATCC 11886	>320.00 (0)	1.00	1.00	>8.00 (0)	2.00	>8.00	>8.00
LMDM-1229	>320.00 (0)	1.00	1.00	>8.00 (0)	0.50	0.06	0.03
LMDM-1230	>320.00 (0)	1.00	1.00	>8.00 (0)	0.50	0.06	0.03

[a]MIC values were obtained on three different days by using the protocol published by CLSI (document M38-A2) (17) and are presented as geometric means. Inhibition diameters (in millimeters) for fluconazole and voriconazole are displayed in parentheses and were obtained by the agar diffusion method following the procedures described in CLSI document M51-A (37). The values are presented as the arithmetic means for three repetitions. FLC, fluconazole; PSC, posaconazole; ITC, itraconazole; VRC, voriconazole; AMB, amphotericin B; CSF, caspofungin; ANF, anidulafungin. MEC values were obtained for CSF and ANF.

these two transformants showed a unique 1,286-nt PCR band using the A13 and P-RoCYP51R1 primers, demonstrating that the LMDM-p134 vector was integrated in a homologous manner. The first primer hybridizes the Af*CYP51A* 5′ UTR in a region not included in the transformed vector (hybridization takes place upstream, where the vectors recombine), while the second oligonucleotide hybridizes the exon 1-exon 2 junction of Ro*CYP51A* (Fig. 4). Moreover, the integration of Ro*CYP51A* and its functionality were confirmed by sequencing and by evaluation of its expression. Total RNA of the *A. fumigatus* chimeras together with that of *R. oryzae* ATCC 11886 was extracted, and the Ro*CYP51A* gene was amplified by using DNA and cDNAs as the template.

The homologous recombination of the Ro*CYP51A* gene into the *A. fumigatus* genome did not modify the micro- or macromorphology of the mutants. Similarly, echinocandin drug and AMB MIC values were not modified by the incorporation of the Ro*CYP51A* gene into LMDM-32 (Table 1). On the contrary, the azole susceptibility patterns were substantially altered in the mutants. The MICs obtained for the *A. fumigatus* chimera emulated the MIC values obtained for the *R. oryzae* ATCC 11886 strain and surpassed the MICs for *A. fumigatus* LMDM-31 (the paternal strain of the recipient strain, LMDM-32). The homologous integration of Ro*CYP51A* into *A. fumigatus* increased the itraconazole (ITC) and PSC MICs 8-fold compared with the MICs for strains LMDM-31 and LMDM-32 (Table 1). Similarly, FLC MIC values increased 40-fold with respect to the MIC for the LMDM-32 strain. The highest MIC value increases were observed for VRC. The mutants showed 128- and 512-fold MIC increases compared with those for LMDM-31 and LMDM-32, respectively (Table 1). The same resistance increase was observed when FLC and VRC susceptibilities were evaluated by using paper disks. None of the strains showed inhibition diameters, with the exception of FLC and VRC against LMDM-32 (inhibition diameters, 19 mm and 48 mm, respectively) and VRC against LMDM-31 (inhibition diameter, 32 mm) (Fig. 5).

DISCUSSION

Mucormycosis is one of the most difficult to treat mycoses since its etiologic agents are naturally resistant to the first-line treatment options for aspergillosis and candidiasis (VRC and FLC, respectively) (7–9). The molecular mechanism involved in the intrinsic resistance phenotypes of Mucorales is not known. However, azole resistance mechanisms were studied to different extents in some Ascomycetes (mainly *Candida* spp. and *Aspergillus fumigatus*) and Basidiomycetes (almost exclusively *Cryptococcus* spp.). The mechanisms of azole resistance are similar regardless of the fungal species studied (10, 13–16). In addition, these mechanisms are usually similar for both intrinsic and secondary resistances. These mechanisms are mostly limited to overexpression of efflux pumps, mutations in target enzymes (*ERG11* and *CYP51A*), and/or hyperexpression of target enzymes (due to aneuploidy or production of the enzymes by alterations in

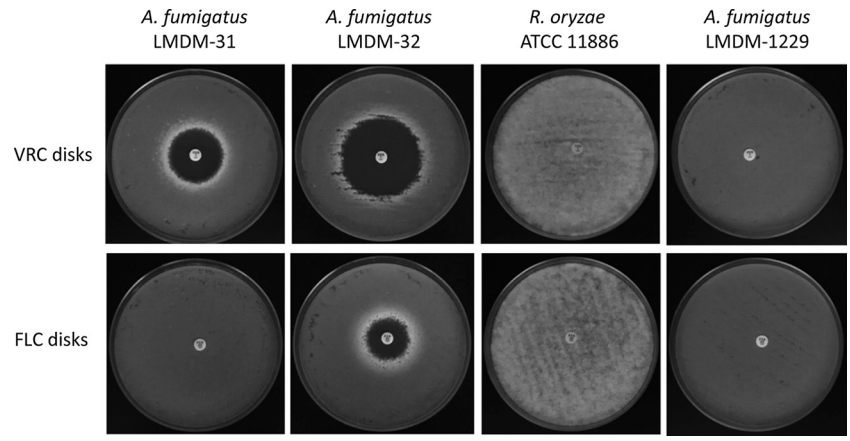

FIG 5 Disk diffusion susceptibility testing using fluconazole (FLC) and voriconazole (VRC) paper disks for LMDM-31 (parental strain akuB$_{KU80}$Δ) (31), LMDM-32 (recipient strain akuB$_{KU80}$Δ *CYP51A*Δ) (12), *R. oryzae* ATCC 11886, and *A. fumigatus* chimera LMDM-1229 (akuB$_{KU80}$Δ Ro*CYP51A*).

promoters and/or transcription factors). What does change between the different studied fungi is the prevalence of these mechanisms. Thus, in *Candida* spp. the most prevalent mechanism is hyperexpression of efflux pumps, whereas for *Aspergillus fumigatus*, the mechanism appears to be mostly related to mutations in the azole target enzymes (Cyp51Ap) (10, 13, 15–17). In *Cryptococcus* spp., the few described mechanisms include overexpression of different genes by reversible chromosomal duplications (heteroresistance), point mutations in Erg11p, and efflux pump overexpression (14, 18–22).

In this work, we used a novel transformant selection procedure which included the use of FLC as the selector agent and an FLC-hypersusceptible *A. fumigatus* mutant. The obtained results support the concept that other *CYP51* gene alleles could be studied using an *A. fumigatus CYP51A* deletion mutant as the heterologous host and FLC as the selector marker.

Using the described transformation and selection strategy, we confirm that the coding region of the *R. oryzae CYP51A* gene is uniquely responsible for its intrinsic VRC and FLC resistance phenotype. The Ro*CYP51A* homologous integration into a hypersusceptible *A. fumigatus* background produced mutants for which the MICs mimicked the azole MIC patterns observed in *R. oryzae* ATCC 11886. The implication that the Ro*CYP51A* promoter and terminator are involved in the studied phenotype was discarded since the integrated Ro*CYP51A* gene was regulated in the *A. fumigatus* chimeras by the wild-type Af*CYP51A* promoter and terminator regions (5′ and 3′ UTRs, respectively). Thus, *CYP51A* overexpression is not necessary to reproduce the observed azole resistance phenotype.

The linkage between *CYP51* genes and the relatively better activity of PSC than VRC against Mucorales species was suggested by Chau et al. by transforming *CYP51* genes into *Saccharomyces cerevisiae* (23). Also, the same authors argued that two naturally occurring amino acid substitutions in Mucorales Cyp51ps would account for their particular pattern of azole susceptibility (23). They aligned *Cunninghamella elegans*, *R. oryzae*, *Candida albicans*, and *A. fumigatus CYP51A* (*ERG11* for the yeast) sequences and hypothesized that Y132F and/or F145M substitutions (*C. albicans* Erg11p numbering) would be responsible for azole resistance in Mucorales. These substitutions were linked with secondary FLC resistance and ITC and PSC susceptibility in *C. albicans* and *Histoplasma capsulatum* isolates (24, 25). Recently, Caramalho et al. (26) resumed the Y132F natural substitution hypothesis for explaining Mucorales intrinsic VRC and FLC resistance. They generated a Ro*CYP51* homology model based on *Saccharomyces cerevisiae* Erg11p and concluded that the original suggestion of Chau et al. (23) was correct. The same Y132F substitution was also described in VRC-resistant *A. fumigatus*

(Y121F, using the *A. fumigatus* Cyp51Ap numbering). However, clinical *A. fumigatus* strains showing a similar azole susceptibility pattern, meaning that they had a high level of VRC resistance (MIC, >8 μg/ml) and 8-fold increases in PSC and ITC MIC values, harbored the Y121F substitution associated with a second amino acid substitution in the same protein (T289A) and a 46-nucleotide repetition in the *CYP51A* promoter (named TR46/Y121F/T289A) (27–29). Snelders et al. (30), using genetic recombination, demonstrated that all three modifications together are necessary to reproduce this azole resistance pattern, especially VRC MIC values of >8 μg/ml. Our group recently demonstrated that *A. fumigatus* intrinsic FLC resistance is linked to a naturally occurring T301I substitution at its Cyp51Ap (12). In addition, this substitution does not impact VRC MIC values (12). In *R. oryzae*, a threonine residue is in the equivalent position in both Cyp51ps, suggesting that intrinsic FLC and VRC resistance would be linked with other amino acid residues in this mucormycete. These data reinforce the possible implication of the involvement of the Y132F and/or F145M substitution in the observed phenotype.

The data presented in this work demonstrate that the integration of the Ro*CYP51A* coding sequence is enough to produce azole susceptibility patterns that emulate *R. oryzae* azole susceptibility patterns despite the lack of a *CYP51A* promoter alteration. Thus, molecular studies are needed to confirm or refute the hypothesis of Chau et al. (23) and Caramalho et al. (26).

MATERIALS AND METHODS

Strains. The azole-hypersusceptible *A. fumigatus* LMDM-32 strain was used in this study as the recipient strain in electroporation assays, and its DNA was the template for all the PCRs. This strain was obtained by our group in a previous work (12) by deleting the *CYP51A* gene of *A. fumigatus* LMDM-31 (parental strain akuB$_{KU80}$Δ) (31) using the plasmid pUM-102 (kindly provided by Emilia Mellado) (32). *R. oryzae* ATCC 11886 was the strain used to obtain the *CYP51* genes under study. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATTC 6258 were used as quality controls for susceptibility testing experiments. *Escherichia coli* TOP10 (Promega) was used to propagate the plasmids.

***R. oryzae CYP51* sequence analysis.** The sequences of the *R. oryzae CYP51* genes were obtained from the genomic sequences published by The Broad Institute (11) (GenBank BioProject accession number PRJNA13066) by using the BLAST tool and using the *A. fumigatus CYP51A* and *CYP51B* genes as query sequences (GenBank accession numbers AF338659 and AF338660, respectively). The primers displayed in Table 2 were designed on the basis of these hypothetical *CYP51* genes. The genes obtained from *R. oryzae* ATCC 11886 were cloned into the pGEM-T Easy vector system (Promega) and sequenced by the Sanger methodology using universal primers. Introns were identified, and the amino acid sequences were translated. Proteins were compared with known Cyp51p/Erg11p paralogs from different Ascomycetes, Basidiomycetes, and Mucorales species. A dendrogram was produced by analysis with the CLUSTAL program and was generated by the unweighted pair group method using arithmetic averages (UPGMA). The neighbor-joining method was applied to produce phylogenetic trees after an alignment step. These analysis were done using BioEdit (version 7.2.5) software (33) and Vector NTI Advance (version 11.5.4) software. Synteny analysis was performed to assess the physical localization of each of the Ro*CYP51* genes using the synteny tools of FungiBD and SynFind (http://genomevolution.org/CoGe/SynFind.pl) (34).

Nucleic acid isolation and RT-PCR. Fungal DNAs were obtained by using a phenol-chloroform-based protocol (35). Total RNA was extracted from *R. oryzae* ATCC 11886 and from the *A. fumigatus* chimeric mutants obtained (LMDM-1229 and LMDM-1230) with the RNAzol reagent (RNAzolRT; MRC Inc.), and reverse transcription was performed with the avian myeloblastosis virus reverse transcriptase enzyme (Promega, Argentina) according to the manufacturer's protocol. *R. oryzae* ATCC 11886 and *A. fumigatus* chimera cDNAs were used to amplify the complete coding sequence of the Ro*CYP51* genes and to confirm the expression of the integrated gene in *A. fumigatus*, respectively. For the first PCRs, the P-RoCYP51F1/P-RoCYP51R3 and P-RoCYP51FB/P-RoCYP51RB primer pairs were used. The P-RoCYP51F1 and P-RoCYP51R3 primers were used for the second PCRs, using LMDM-1229 and LMDM-1230 cDNAs as the template (Table 2).

Genetic constructs. The plasmid used to generate the *A. fumigatus* transformants was named LMDM-p134. It contains the gene named RO3G_16595 in the *R. oryzae* genome (11) flanked by the 5′ and the 3′ untranslated regions (UTR) of the *A. fumigatus CYP51A* gene (Af*CYP51A*) (1,000 bp each). The gene RO3G_16595 is called *R. oryzae CYP51A* (Ro*CYP51A*) here, and the reason to name it this was explained earlier. Transformation vector construction was done by using a set of four overlapping PCRs. The first two PCRs were performed to obtain the 5′ UTR and the 3′ UTR of Af*CYP51A*. They were amplified from *A. fumigatus* LMDM-32 genomic DNA using the R-RoCYP51F1 and R-RoCYP51R1 primers and the R-RoCYP51F3 and R-RoCYP51R3 primers, respectively (Table 2). The third PCR was aimed to amplify the Ro*CYP51A* coding sequence with the R-RoCYP51F2 and R-RoCYP51R2 primers and LMDM-p125 (by using the pGEM-T Easy vector with Ro*CYP51A* with no introns amplified from *R. oryzae* cDNA) as the template (Table 2). These three PCR fragments were ligated by the fourth PCR of the set by using the R-RoCYP51F1

TABLE 2 Primers used in this work

Primer	Target organism(s)	Sequence (5′-3′)[a]	Orientation	Use
R-RoCYP51F1	*A. fumigatus*	GAATATATACGTCGATCTGTGTG	Sense	LMDM-p134 construction
R-RoCYP51R1	*A. fumigatus*	GTAAGGTGGATATGATGGCCATTTCGAGGAGACACAGGGAGG	Antisense	LMDM-p134 construction
R-RoCYP51F2	*A. fumigatus*/*R. oryzae*	CCCTCCCTGTGTCTCCTCGAA**ATG**GCCATCATATCCACCTTAC	Sense	LMDM-p134 construction
R-RoCYP51R2	*A. fumigatus*/*R. oryzae*	GTCCTCGATGGTTACAACAGTC**TTA**TTGCTTTCTAGCTCTATAACG	Antisense	LMDM-p134 construction
R-RoCYP51F3	*A. fumigatus*	CGTTATAGAGCTAGAAAGCAATAAGACTGTTGTAACCATCGAGGAC	Sense	LMDM-p134 construction
R-RoCYP51R3	*A. fumigatus*	ATCCCAGCAGATACGCTGGTC	Antisense	LMDM-p134 construction
P-RoCYP51F1	*R. oryzae*	CCCCAT**ATG**GCCATCATATCCACCTTACTT	Sense	*R. oryzae CYP51A* amplification
P-RoCYP51R3	*R. oryzae*	GGGGTCGAC**TTA**AAGCTTTTGCTTTCTAGCTCTATAACGA	Antisense	*R. oryzae CYP51A* amplification, evaluation of LMDM-p134 integration
P-RoCYP51FB	*R. oryzae*	CCCCAT**ATG**GCTGTCATCTCCACTTTACTCC	Sense	*R. oryzae CYP51B* amplification
P-RoCYP51RB	*R. oryzae*	GGGGTCGAC**CTA**AAGCTTTTCTCTCCAAGTGTATTTGATG	Antisense	*R. oryzae CYP51B* amplification
P-RoCYP51F3	*R. oryzae*	CGATCGAATTTGGAAAAAACCCCATCGAATTCCTTCA	Sense	Evaluation of LMDM-p134 integration
A13	*A. fumigatus*	CTTCCCACATCCATCTTCT	Sense	Evaluation of LMDM-p134 homologous recombination
A5R	*A. fumigatus*	TCTCTGCACGCAAAGAAG	Antisense	Evaluation of LMDM-p134 homologous recombination
P-RoCYP51R1	*R. oryzae*	GGATCTTTGGGTTGAATAAACTGAGATAAAAGATGAATGAC	Antisense	Evaluation of LMDM-p134 homologous recombination

[a]Letters in bold indicate the start and stop codon of *R. oryzae* CYP51s.

and R-RoCYP51R3 primers and the PCR products of the three PCRs described earlier as the templates. The resulting construct (3.5 kb) was cloned into the pGEM-T Easy vector system (Promega), generating the plasmid LMDM-p134.

***A. fumigatus* transformation, mutant selection, and integration confirmation.** *A. fumigatus* LMDM-32 was transformed by electroporation (36) with 0.3 μg of a PCR product obtained with the R-RoCYP51F1 and R-RoCYP51R3 primers and the LMDM-p134 plasmid as the template. Transformant selection was done using FLC (100 μg/ml), taking advantage of the azole-hypersusceptible phenotype of the recipient strain (LMDM-32). An FLC resistance phenotype was expected as a consequence of the incorporation of a functional *CYP51A* gene. Genomic DNA from FLC-resistant transformants and the LMDM-32 strain were used to confirm cassette integration and its homologous recombination. Integration was confirmed by PCR using the P-RoCYP51F3 and P-RoCYP51R3 primers, with the expected PCR band of 1,374 nt (Ro*CYP51A* exon 3) being obtained. Homologous recombination in the FLC-resistant transformants into which Ro*CYP51A* was integrated in their genomes was checked by a multiplex PCR with the A13, A5R, and P-RoCYP51R1 primers (Fig. 1 and Table 2). Primer A13 was designed to hybridize to the Af*CYP51A* 5′ UTR 1,264 nt downstream of the Af*CYP51A* start codon (a region not included in the construction cloned into LMDM-p134). On the other hand, the A5R primer hybridizes 197 nt upstream of the start codon of Af*CYP51A*, a region present in the parental *A. fumigatus* strain (LMDM-32) that would be replaced by homologous integration of the LMDM-p134 construct. The last primer of the multiplex PCR, named P-RoCYP51R1, was designed to span the exon 1-exon 2 junction of Ro*CYP51A* (Fig. 1 and Table 2). Thus, if recombination of the transforming cassette was done in a homologous fashion, a 1,286-nt PCR band would be observed (with the A13/P-RoCYP51R1 primer pair). Conversely, a 1,461-nt PCR fragment would be obtained for the parental strain or for nontransformant strains. Also, a band (1,461 nt) would be observed in strains harboring ectopic integrations. In addition, Ro*CYP51A* expression in *A. fumigatus* chimeras was confirmed by PCR amplification using cDNA as the template, as described above.

PCRs. PCR amplifications were done in an Applied Biosystems thermocycler (Tecnolab-AB, Buenos Aires, Argentina) in a 25-μl final volume by using *Pfu* DNA polymerase (PBL, Buenos Aires, Argentina) according to the manufacturer's recommendations. The cycling program used included an initial 2-min step at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at the melting temperature of the primer set used, and 1 min per kilobase of the estimated PCR product size at 72°C and a final 72°C step for 10 min.

Antifungal susceptibility testing. The Clinical and Laboratory Standards Institute (CLSI) document M38-A2 broth microdilution reference method was used for the evaluation of susceptibility to itraconazole (ITC), PSC, VRC, FLC, AMB, caspofungin (CSF), and anidulafungin (ANF) (17). Antifungal drugs were purchased from Sigma-Aldrich (Argentina) or obtained from their manufacturers as standards powders. The concentration range of FLC was increased 10 times from what is published in the standardized protocol (it was modified to 640 to 1.25 μg/ml) in order to be able to differentiate FLC susceptibilities

in filamentous fungi (12). Furthermore, FLC and VRC susceptibilities were evaluated by agar diffusion using paper disks (Oxoid, Argentina) and following the guidelines in CLSI document M51-A (37). All the susceptibility testing experiments were performed in triplicate on three different days. Off-scale MIC values (values lower or higher than the maximum tested concentrations) were converted to the following concentration down or up in order to analyze the results.

Accession number(s). *R. oryzae* ATCC 11886 *CYP51A* and *CYP51B* gene sequences were deposited in GenBank under accession numbers MG872961 and MG872962, respectively.

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