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Role of *Aspergillus lentulus* 14-α Sterol Demethylase (Cyp51A) in Azole Drug Susceptibility▽

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Recent studies have demonstrated that some morphologically atypical *Aspergillus fumigatus* strains are different species belonging to the section *Fumigati*. *Aspergillus lentulus*, one of these sibling species, is increasingly reported in patients under corticosteroid treatment. MICs of most antifungals in clinical use are elevated against *A. lentulus*, and it shows primary resistance to azole drugs. Two *A. lentulus* cytochrome P450 14-α sterol demethylases, encoded by *A. lentulus cyp51*A (Alc*yp51A*) and Alc*yp51B* genes, were identified. Targeted *cyp51A* gene knockout in *A. lentulus* showed that the intrinsic azole resistance of this species is *cyp51A* dependent. The Δ*cyp51A* strain was morphologically indistinguishable from the *A. lentulus* wild-type strain, retaining the ability to cause pulmonary disease in neutropenic mice. The heterologous expression of *A. lentulus cyp51A* was performed in an *A. fumigatus cyp51A*-deficient strain, confirming that Cyp51A is responsible for the differences in *A. lentulus*-azole drug interaction.

Invasive aspergillosis (IA) commonly develops in recipients of allogeneic hematopoietic stem cell transplantation (HSCT) and in patients with persistent neutropenia (22). However, some studies indicate that the population at risk for pulmonary or disseminated IA can be expanded to patients with chronic obstructive pulmonary disease (COPD) as well as to nontransplant intensive care patients (11, 17). *Aspergillus fumigatus* is the main causative agent of IA, although the number of other *Aspergillus* species able to produce fungal disease is on the rise, increasing the complexity of this infection (24, 45).

Several studies have demonstrated that some strains morphologically identified as *A. fumigatus* are different species belonging to the section *Fumigati* (5, 7, 8). *Aspergillus lentulus*, one of these sibling species of *A. fumigatus*, is increasingly reported in hematological and cystic fibrosis patients and in those under corticosteroid treatment (4, 6, 33, 42). The risk factors associated with these cryptic species still are undefined, but it is known that they show a different antifungal drug susceptibility profile than *A. fumigatus* (3). Strains of *A. fumigatus* are intrinsically susceptible to the expanded-spectrum triazole drugs, such as itraconazole, voriconazole, and posaconazole, and to the polyene drug amphotericin B (12). However, the development of secondary resistance to azole drugs in clinical strains is well documented (10, 15, 21, 40, 47). For triazole drugs, secondary resistance involving the acquisition of resistance in a susceptible strain accounts for all of the resistance described in *A. fumigatus*. On the other hand, the MICs of azole drugs for *A. lentulus* clinical samples are high, as are those of echinocandins and amphotericin B (3, 9, 41).

The azole-derived antifungal agents inhibit the ergosterol biosynthesis pathway via the inhibition of 14-α sterol demethylase (Cyp51) (18). Analyses of *A. fumigatus* azole-resistant strains at the molecular level have already identified many underlying bases for *A. fumigatus* azole resistance. Resistance due to modifications of the azole target enzyme 14-α sterol demethylase (Cyp51A) or its overexpression are the main mechanisms involved in *A. fumigatus* azole resistance (13, 16, 26, 30, 32, 34). However, the molecular mechanism of the intrinsic resistance of *A. lentulus* remains unknown. Since *A. lentulus* isolates cause invasive disease, its primary resistance to azole drugs is a matter of concern that may have both epidemiological and clinical significance (3, 25, 28).

Similarly to what happens in *A. fumigatus*, two different *cyp51*-related genes encoding 14-α sterol demethylase-like enzymes were identified in *A. lentulus* (Alc*yp51A* and Alc*yp51B*) (29). Since the cytochrome P450 14-α sterol demethylase, encoded by the *cyp51A* gene, is responsible for the azole drug affinity in *A. fumigatus*, here we describe the characterization of *A. lentulus* Cyp51A and its role in azole drug susceptibility and in virulence by the disruption of the azole target (Cyp51A). Finally, its function was confirmed by the heterologous expression of *A. lentulus cyp51A* in *A. fumigatus cyp51A* knockout strains.

MATERIALS AND METHODS

Strains, media, and growth conditions. Fungal strains used in this study were (i) *Aspergillus lentulus* strain CM-1290 (CM refers to the fungal collection of the Mycology Reference Laboratory), with a clinical origin; (ii) *A. fumigatus* strain CM-237, previously used to describe the *cyp51A* and *cyp51B* gene sequences and also used as a control strain (29); (iii) *A. fumigatus* strain *akuB*^{KU80} (14), used as the recipient strain for some experiments of fungal transformation; and (iv) *A. fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304, used as quality-control strains for EUCAST antifungal susceptibility testing (37).

Aspergillus strains were grown at 37°C in either GYEP (2% glucose, 0.3% yeast extract, 1% peptone) or Sabouraud (2% glucose, 1% mycopeptone) medium. *Escherichia coli* JM109 was grown in Luria-Bertani (LB) medium (38) supplemented with ampicillin (100 μg/ml) for the propagation of plasmids for DNA purification. For standard cloning and subcloning procedures, the vector pGEM-T Easy vector (Promega, Madrid, Spain) was used.

***Aspergillus lentulus cyp51A* and *cyp51B* gene sequences.** *A. fumigatus* primers previously used for *cyp51A* and *cyp51B* gene amplification were used to amplify each of the genes from *A. lentulus* (29). Once the full sequence for the *A. lentulus*

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TABLE 1. Oligonucleotides used throughout this work

Name and group	Orientation	Sequence (5′–3′)	Purpose
A. *lentulus cyp51A* deletion			
L-1	Sense	CACTGTTTCTCATACAGGGAAACCTCATGG	1st PCR, 5′-end vector construction; 2nd PCR, fusion vector
LH1	Sense	GTTTTCGGATCTGACGTGGTGTATGATTGTTC GTCGACGTTAACTGATATTGAAGGAGCA	Hygromycin cassette amplification
LH2	Antisense	TGCTCCTTCAATATCAGTTAACGTCGACGAAC AATCATACACCACGTCAGATCCGAAAAC	5′-End vector construction
HL3	Sense	CAGTTAACGTCGACGAATTCGATATCAAGCG ACCTCTATCATGATCTGGACAAGGGCTTT	Hygromycin cassette amplification
HL2	Antisense	AAAGCCCTTGTCCAGATCATGATAGAGGTCG CTTGATATCGAATTCGTCGACGTTAACTG	Hygromycin cassette amplification
L-2	Antisense	CCCTGAGAAGAGCGATGAATAGTCGGTATC	1st PCR, 3′-end vector construction; 2nd PCR, fusion vector
L-3	Sense	TTGTGCCTAGCAAGGAAAAAGACAAGAAA	PCR gene deletion verification
A. lentulus cyp51A expression in *A. fumigatus*			
A	Sense	TAGAATGAGTGAGCTGATTTGCCATGGATT	1st PCR, 5′-end vector construction; 2nd PCR, fusion vector
B2	Antisense	GTAGGCCGTGAGCAATAGCATCGACACCATT TCGAGGAGACACAGGGAGGGTGAGCCCGA	5′-End vector construction
C2	Sense	TCGGGCTCACCCTCCCTGTGTCTCCTCGAAAT GGTGTCGATGCTATTGCTCACGGCCTAC	Coding Cyp51A *A. lentulus* insertion
D3	Antisense	CCTTTGAAGTCCTCGATGGTTACAACAGTCTC ACTTGGATGTGTCTTTAGAACGCTTCTC	Coding Cyp51A *A. lentulus* insertion
E3	Sense	GAGAAGCGTTCTAAAGACACATCCAAGTGAG ACTGTTGTAACCATCGAGGACTTCAAAGG	*A. fumigatus* terminator insertion
F3	Antisense	TGCTCCTTCAATATCAGTTAACGTCGACGAGC GTGCCAAGGCCAAGGCTTGATTAAGTAT	*A. fumigatus* terminator insertion
G3	Sense	ATACTTAATCAAGCCTTGGCCTTGGCACGCTC GTCGACGTTAACTGATATTGAAGGAGCA	Hygromycin resistance cassette
F4	Antisense	CTGTTCAGTCAGGGCATGTCCTTGGAGACTA AGCTTGATATCGAATTCGTCGACGTTAAC	Hygromycin resistance cassette
G4	Sense	GTTAACGTCGACGAATTCGATATCAAGCTTA GTCTCCAAGGACATGCCCTGACTGAACAG	3′-End vector construction
H2	Antisense	AGAGATAGAACCTTGGAGTCTGCTTGCCTC	1st PCR, 3′-end vector construction; 2nd PCR, fusion vector
A. lentulus cyp51A expression assessment			
Len-A4	Sense	CAGACATGATTTGGAACC	cDNA amplification
Len-Cypa2	Antisense	TTAGAACGCTTCTCCCAG	cDNA amplification
A. fumigatus cyp51A expression analysis			
A4	Sense	CAGACATGATATGGAACC	cDNA amplification
Cypa2	Antisense	TTCGACCGCTTCTCCCAG	cDNA amplification
Tub5	Sense	TGACCCAGCAGATGTT	House-keeping gene used as reference for normalization
Tub6	Antisense	GTTGTTGGGAATCCACTC	House-keeping gene used as reference for normalization

cyp51A gene was obtained, specific oligonucleotides were designed for *A. lentulus* (Table 1) when needed. All of the primers used in the present work were synthesized by Sigma Genosys (Madrid, Spain) (Table 1). The PCRs were carried out in a 50-μl volume containing 10 mM $(NH_4)_2SO_4$; 10 mM KCl; 20 mM Tris-Cl (pH 8.8); 2 mM $MgSO_4$; 10 ng bovine serum albumin (BSA); 0.1% Triton X-100; 250 μM (each) dATP, dGTP, dCTP, and dTTP (Applied Biosystem, Madrid, Spain); 1 μM each primer; 2.5 U of Titanium or Advantage 2 *Taq* polymerase (Clontech, Madrid, Spain); and 50 ng of genomic DNA. Amplification was performed in a thermal cycler (Amersham Biosciences, Madrid, Spain) for 1 cycle of 5 min at 94°C, 45 s at 56°C, and 2 min at 72°C; 30 cycles of 30 s at 94°C, 45 s at 56°C, and 2 min at 72°C; and then a final extension for 10 min at 72°C. PCR products were analyzed by electrophoresis on agarose gels and visualized by transillumination after staining them with ethidium bromide.

The full coding sequences of *cyp51A* and *cyp51B* from *A. lentulus* were amplified by PCR, and both strands were sequenced by the BigDye Terminator cycle sequencing ready reaction system (Applied Biosystems, Madrid, Spain) according to the manufacturer's instructions. Sequence analysis was performed on an ABI prism 377 DNA sequencer (Applied Biosystem) using the sequencing facilities available at the Genomic Department at Instituto de Salud Carlos III, Majadahonda, Madrid, Spain.

Construction of the deletion and heterologous expression cassettes. Two different fusion cassettes were constructed for *Aspergillus* transformation by PCR overlap as previously described (49). Basically, the first step is a conventional PCR in which oligonucleotide primers are partially complementary at their 5′ ends to the adjacent fragments that are fused to create the chimera. The second PCR step consists of the PCR amplification of the fusion of the PCR fragments generated in the first step by using the complementary extremities of the primers. The final PCR product is a chimeric vector built up with the different amplified PCR fragments (49). Oligonucleotides used for the construction of both fusion cassettes are enumerated in Table 1. Primer location and fusion vector design are

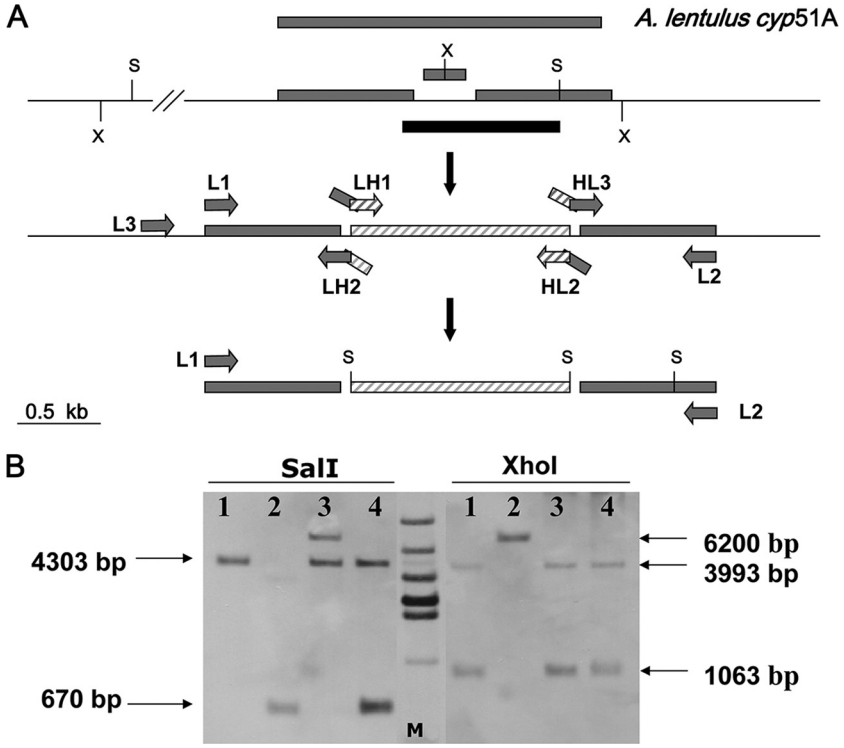

FIG. 1. Construction of the *A. lentulus cyp51A*-defective mutant strain. (A) Design of the fusion vector for *A. lentulus cyp51A* gene deletion. The location of restriction sites used for the Southern analysis are indicated before (upper section) and after (lower section) the *cyp51A* locus. The transformants were selected using the *hyg* gene (1.4 kb) flanked by SalI (S) restriction sites included in the deletion cassette (striped bars). The excised *cyp51A* coding fragment and the probe used for hybridization are indicated in gray and black bars, respectively. The location of the primers for fusion vector construction is indicated in the middle section of the diagram. (B) Southern hybridization of genomic DNA of *A. lentulus* digested with SalI and XhoI (X). Lane 1, wild-type strain; lane 2, *A. lentulus cyp51A*-deficient T38.18 strain. Lanes 3 and 4 show two ectopic transformants that maintained the wild-type *cyp51A* copy. Sizes of the expected fragment are indicated in bp on the right side. Lane M, 1-kb molecular size marker.

described in Fig. 1 and 2. The PCR conditions for the amplification of the deletion and expression cassettes were previously described (23).

***Aspergillus* transformation.** The transformation of *A. fumigatus* strains was achieved by electroporation as previously described (31), with minor modifications to adjust the differences of growth between *A. fumigatus* and *A. lentulus*. After electroporation, transformants were selected on minimal medium plates containing hygromycin B (130 μg/ml; Sigma). Mutants were named with a letter followed by two numbers (i.e., T38.18 and T52.7), indicating the transformation experiment number and the transformant number.

Deletion verification by PCR and Southern blot analysis. Gene targeting was first verified by PCR using primers selected outside the flanking regions (Table 1 and Fig. 1) used for *cyp51A* deletion fusion vector construction, in combination with primers used for resistance marker amplification. Homologous recombination at the correct locus of *A. lentulus* (Fig. 1) or *A. fumigatus* (Fig. 2) was confirmed by Southern blot analysis. Genomic DNA from hygromycin-resistant PCR-positive transformants was isolated using a rapid extraction procedure (43), digested with different restriction enzymes, and fractioned by electrophoresis through 0.8% agarose gels in Tris-acetate-EDTA (TAE) buffer. Southern analysis was performed as previously described (20). A probe for the *A. lentulus cyp51A* gene was obtained by the PCR amplification of the desired fragment, fractionation in 0.7% low-melting-point agarose gels, and further gel excision for labeling. A random-prime DNA labeling system (ECL; Amersham Pharmacia Biotech, Madrid, Spain) was used to label DNA probes according to the manufacturer's instructions.

RNA isolation and real-time reverse transcription-PCR (RT-PCR). Mycelial mats were blot dried, frozen with liquid nitrogen, and then ground to powder using a pestle and mortar. RNA was isolated from mycelial powder by using an RNeasy plant minikit (Qiagen, Madrid, Spain) by following the manufacturer's instructions. Reverse transcription was carried out in a 20-μl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 5 mM $MgCl_2$, 1 mM each deoxynucleoside triphosphate, 0.5 μg of specific primer [oligo$(dT)_{15}$ primer], 20 U rRNasin RNase inhibitor, and 15 U of avian myeloblastosis virus reverse transcriptase (AMV-RT) (reverse transcription system; Promega, Madrid, Spain) on 2.5 μg of total *A. fumigatus* RNA. The reaction conditions were 1 h at 42°C. A tube containing all of the reaction components without the AMV-RT enzyme, to check for the presence of contaminating DNA in the RNA sample, was always included as a negative control.

Two μl of cDNA products was used as target DNA for amplification using Light Cycler Fast Start (Roche). The primers Len-A4 and Len-CypA2 were used to amplify the cDNA from *A. lentulus cyp51A*, and the primers A4 and CypA2 were used for *A. fumigatus cyp51A* (Table 1).

Fold changes in expression were calculated using the $2^{-\Delta\Delta CT}$ method (39) and normalized to data for the β-tubulin housekeeping gene (GenBank accession number AY048754).

The RT-PCR products were sequenced to confirm the identity of the expressed DNA. The cDNA from *A. lentulus* also was used to check for the absence of an intron, which is present in the DNA fragment, and to delimitate its size and boundaries.

Antifungal susceptibility testing. Inoculum preparations were performed by means of counting spores in a hematocytometer chamber (1) and were adjusted to 10^5 conidia per ml. For the Etest, gradient strips of voriconazole, posaconazole, itraconazole, and fluconazole, obtained from AB Biodisk (bioMérieux, Madrid, Spain), were used. The MICs were read after 48 h of incubation at 35°C. MICs were read as the lowest concentration at which the border of the elliptical inhibition zone intersected the scale on the strip. Microcolonies inside the inhibition zone were ignored. The strips of fluconazole, voriconazole, itraconazole, and posaconazole contained concentration gradients of 0.002 to 32 mg/liter. Antifungal susceptibility testing was repeated at least twice on different days. To define susceptibility and resistance *in vitro*, the criteria used were according to the epidemiological cutoffs (designated ec-off) recently published for *A. fumigatus*. The wild-type populations were defined as isolates against which the itra-

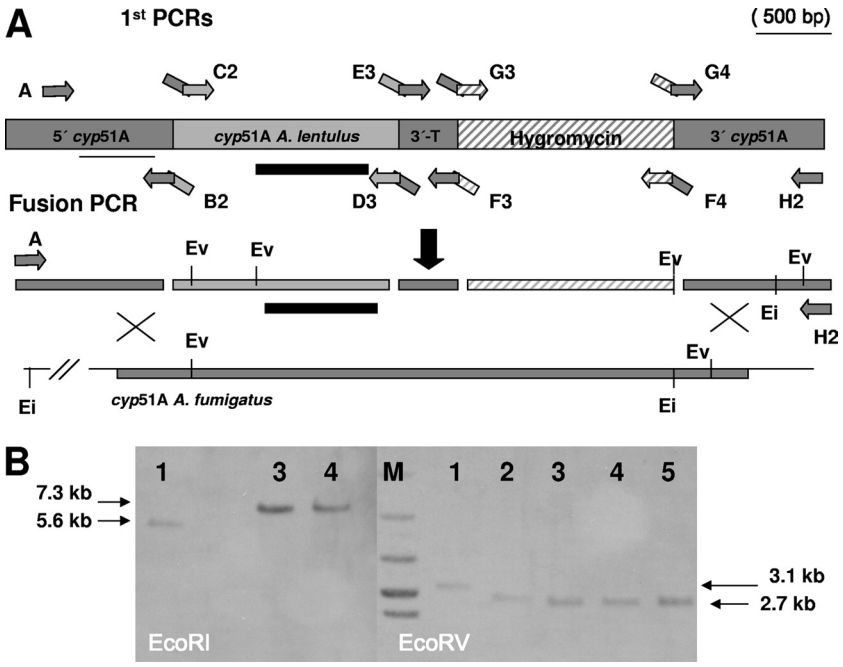

FIG. 2. Construction of the *A. fumigatus cyp51A*-defective mutant strain expressing the A. *lentulus cyp51A* gene (Al*cyp51A*). (A) Design of the fusion vector for *A. fumigatus cyp51A* gene deletion and target integration of the Al*cyp51A* gene driven by the *A. fumigatus cyp51A* promoter (5′*cyp51A*) and terminator (3′ T) and including the hygromycin cassette (*hyg*) for transformant selection. At least 1 kb of *A. fumigatus* sequence outside the *cyp51A* coding region was included on both sides of the fusion construction to facilitate the homologous recombination. The location of restriction sites used for the Southern analysis are indicated before (upper section) and after (lower section) the *cyp51A* locus. The transformants were selected using the *hyg* gene (1.4 kb) included in the deletion cassette (striped bars). The location of the primers for fusion vector construction is indicated in this section of the diagram. (B) Southern hybridization of genomic DNA with EcoRV (Ev) and EcoRI (Ei) restriction enzyme digested with *A. fumigatus* wild type (lanes 1), *A. lentulus* CM-1290 wild type (lanes 2), and three hygromycin-resistant transformants with the wild-type *cyp51A* deleted and replaced by *A. lentulus cyp51A* copy T51.7 (lanes 3), T51.8 (lanes 4), and T51.9 (lane 5). Sizes of the expected fragment are indicated in bp on the right side. Lane M, 1-kb molecular size marker. The *A. lentulus cyp51A* probe used for hybridization is indicated by a black bar.

conazole and voriconazole MICs were ≤1 μg/ml and against which the posaconazole MIC was ≤0.25 μg/ml (36).

Itraconazole, voriconazole, and posaconazole (0.015 to 8 μg/ml) also were tested by microdilution by following EUCAST procedures (37). Since amphotericin B testing is not validated by Etest testing, amphotericin B (Sigma-Aldrich Química) was tested only by microdilution (range, 0.03 to 16 μg/ml).

Experimental model of infection. The pathogenicity of the *A. lentulus* Δ*cyp51A* (T38.18) mutant strain was compared to that of its *A. lentulus* parental strain and that of *A. fumigatus* CM-237 (inoculum size of 10^5 spores/mice). Strain virulence was assessed in a neutropenic mice model (ICR; specific pathogen free; 6 weeks old; CRIFFA, Barcelona, Spain) as previously described (31, 44). Immunosuppression was induced with 175 mg/kg of body weight of cyclophosphamide (Pras-Farma, Barcelona, Spain) administered intraperitoneally (i.p.) and 112 mg/kg of cortisone 21-acetate (C-3130; Sigma) administered subcutaneously, both given 3 and 1 day before the start of the experiment. Afterwards only cyclophosphamide (175 mg/kg) was used every 3 days until the completion of the experiment. After immunosuppression, mice were infected with a 30-μl drop of a fresh suspension of *A. fumigatus* or *A. lentulus* conidia. Two inoculum sizes were tested for *A. lentulus* wild-type and mutant strains (10^5 and 10^6 spores/mice). Survival was monitored at least twice a day for a time period of 14 days, and moribund animals were sacrificed. The experiments were carried out with eight mice per group. Lungs of mice infected with conidia of different *Aspergillus* strains were analyzed to check for the recovery of the infected strain: *A. fumigatus*, the *A. lentulus* parental strain, or the *A. lentulus* Δ*cyp51A* mutant strain.

Statistical analysis. Kaplan-Meier survival analysis was used to determine differences in the pathogenicity test. Statistical analysis was done with the SPSS package (version 12.0; SPSS S.L., Madrid, Spain).

Computer analysis. The amino acid sequences of putative 14-α sterol demethylase genes *cyp51A* and *cyp51B* were deduced from nucleotide sequences and analyzed using the MegAlign software package (DNAstar, Inc., Lasergene, Madison, WI) run on a personal computer. The amino acid alignments were derived by CLUSTAL analysis (19).

Nucleotide sequence accession numbers. The full nucleotide sequences of the *cyp51A* and *cyp51B* genes from *A. lentulus* and the deduced amino acid sequences determined in this work appear in the GenBank nucleotide sequence database under accession numbers GU479991 for *Aspergillus lentulus cyp51A* and JF276895 for *Aspergillus lentulus cyp51B*. *A. fumigatus* AF338659 and AF33860 were the reference sequences used for Cyp51A and Cyp51B, respectively.

RESULTS

Identification of the *Aspergillus lentulus cyp51A* and *cyp51B* genes. PCR amplification based on *A. fumigatus* oligonucleotides allowed for the cloning and sequencing of two different *cyp51* homologue genes in *A. lentulus* (31). The two *cyp51*-related genes encoding 14-α sterol demethylases-like enzymes were named *A. lentulus cyp51A* (AlCyp51A) and *A. lentulus cyp51B* (AlCyp51B) by following *A. fumigatus* nomenclature. There was a 92% identity match between the Al*cyp51A* sequence and that of *A. fumigatus cyp51A* (Af*cyp51A*). The alignment of the two DNA sequences revealed that both have exactly the same length (1,619 bp), including the size and location of an intron of 71 bp. The second gene, *cyp51B*, has a length of 1,733 bp, including three predicted introns at the same location as Af*cyp51B* but with different lengths: 54 bp (58 bp in *A. fumigatus*), 51 bp (45 bp in *A. fumigatus*), and a third

TABLE 2. Amino acid differences between Cyp51A from *A. fumigatus* and *A. lentulus*[b]

Amino acid	*A. fumigatus*	*A. lentulus*
Pro^{3}	P3 (CCG)	P3S (TCG)
Trp^{6}	W6 (TGG)	W6L (TTG)
Met^{11}	M11 (ATG)[a]	M11T (ACG)[a]
Val^{15}	V15 (GTG)	V15M (ATG)
Ala^{18}	A18 (GCA)	A18V (GCA)
Fhe^{29}	F29 (TTT)[a]	F29Y (TAC)[a]
Ser^{49}	S49 (AGT)	S49N (AAT)
Leu^{67}	L67 (AAG)	K67R (AGG)
Asp^{161}	D161 (GAT)	D161N (AAT)
Arg^{171}	R171 (CGG)	R171Q (CAG)
Met^{172}	M172 (ATG)	M172V (GTG)
Asp^{255}	D255 (GAC)	D255G (GGA)
Cys^{270}	C270 (TGC)	C270S (AGC)
Lys^{314}	K314 (AAA)	K314Q (CAG)
Ala^{330}	A330 (GCC)	A330I (ATT)
Ser^{335}	S335 (AGT)	S335N (AAT)
Phe^{337}	P337 (CCT)[a]	P337S (TCT)[a]
His^{352}	H352 (CAT)[a]	H352Q (CAG)[a]
Ile^{354}	I354 (ATT)	I354V (GTT)
Ile^{360}	I360 (ATT)	I360L (CTT)
Ile^{367}	I367 (ATC)	I367L (CTC)
Met^{383}	M383 (ATG)	M383V (GTG)
Thr^{420}	T420 (ACT)	T420A(GCC)
Leu^{464}	L464 (CTT)	L464I (ATT)
Glu^{488}	E488 (GAA)	E488D (GAT)
Gly^{505}	G505 (GGC)	G505R (CGC)

[a] Polymorphisms between strains of *A. lentulus*.
[b] Amino acid numbers are the same for both species.

TABLE 3. Amino acid differences between Cyp51B of *A. fumigatus* and *A. lentulus*[a]

Amino acid	*A. fumigatus*	*A. lentulus*
Ile^{26}	I26 (ATA)	I26V (GTC)
Asn^{48}	N48 (AAT)	N48S (TCT)
Lys^{175}	K175 (ATG)	K175R (ATG)
Ala^{179}	A179 (GTG)	A179S (ATG)
Phe^{180}	F180 (GCA)	F180L (GCA)
Lys^{263}	K263 (TTT)	K263R (TTT)
Ala^{268}	A268 (AGT)	A268T (AGT)
Ala^{385}	A385 (AGT)	A385V (AGT)
Asn^{420}	N420 (GAT)	N420D (GAT)
Ser^{446}	S446 (CGG)	S446N (CGG)
Arg^{485}	R485 (ATG)	R485K (CGG)
Ile^{495}	I495 (GAC)	I495V (GAC)

[a] Amino acid numbers are the same for both species.

intron of 53 bp in both species. There was an overall 96% identity match between Al*cyp51B* and Af*cyp51B*.

The comparison of the deduced AlCyp51A (515 amino acids) and AlCyp51B (524 amino acids) proteins to Cyp51A and Cyp51B of *A. fumigatus* showed that the amino acid sequences of these newly described proteins had percent identity matches of 95 and 98%, respectively. Sequence alignments clearly demonstrated that these two enzymes (AlCyp51A and AlCyp51B) are the corresponding *A. fumigatus* Cyp51 homologues. Amino acid differences between both Cyp51 proteins in *A. fumigatus* and *A. lentulus* are listed in Tables 2 and 3.

Generation of the Δ*cyp51A Aspergillus lentulus* strain. To analyze the role of the azole target designated 14-α sterol demethylase A (AlCyp51A) in *A. lentulus*, the corresponding *cyp51A* gene was deleted from the *A. lentulus* wild-type strain CM-1290. For this purpose, a fusion vector containing the *cyp51A* knockout construct with the hygromycin (*hyg*) resistance marker (Fig. 1A) was generated (see Materials and Methods). A full *cyp51A*-deleted mutant was not pursued, since there is not enough *A. lentulus* sequence knowledge to allow for the excision of the full Al*cyp51A* coding sequence.

After the transformation of *A. lentulus* strain CM-1290 with the fusion vector, the integration of the Δ*cyp51A*-Hyg construct of the *cyp51A* gene in the resulting transformants was detected by PCR. To verify the results obtained by PCR, Southern blot analysis after digestion with two different restriction enzymes was performed. As shown in Fig. 1B, using SalI, the characteristic wild-type band (4.3 kb) for the Af*cyp51A* gene was absent from the Δ*cyp51A A. lentulus* mutant and was replaced by a 670-bp band because of the inclusion of an SalI site corresponding to the *Hyg* cassette (the only remaining DNA segment able to hybridize with the probe). To double check, the use of XhoI clearly detected that the two characteristic bands for the *cyp51A* gene of the wild type (3.9 and 1 kb) had disappeared from the Δ*cyp51A* mutant. Instead, there was a single band of 6.2 kb, indicative of *cyp51A* gene replacement as a consequence of the inclusion of the *hyg* resistance gene, and the disappearance of an XhoI restriction site located in the deleted section of the *cyp51A* gene (Fig. 1A and B).

Phenotype of the *A. lentulus* Δ*cyp51A* strain. The Al*cyp51A*-disrupted strain was morphologically indistinguishable from the *A. lentulus* wild-type strain CM-1290. However, antifungal susceptibility testing clearly showed that the absence of Cyp51A renders *A. lentulus* significantly more susceptible to all azole drugs, with decreased azole MICs that varied from 5- to 100-fold depending on the azole tested (Table 4 and Fig. 3). Azole MICs for the *A. lentulus* Δ*cyp51A* mutant resemble those of *A. fumigatus* Δ*cyp51A* mutants (31). Moreover, since azole MICs for *A. lentulus* strains are higher than those for *A. fumigatus*, the decrease in azole MICs was even more remarkable (Table 4). Differences in antifungal susceptibility values for itraconazole, voriconazole, and posaconazole between the *A. lentulus* wild type and the Δ*cyp51A* mutant strain (T18.38) were statistically significant ($P < 0.05$), while there were no differences in susceptibility to amphotericin B.

***Aspergillus lentulus* Al*cyp51A* gene expression in an *A. fumigatus* Δ*cyp51A* deletion strain.** To confirm the implication of AlCy51A in azole resistance, the Al*cyp51A* gene copy was expressed in an *A. fumigatus* strain, generating a *cyp51A*-deleted strain. A PCR fusion vector was constructed for this purpose, containing 1,237 bp of homology at the 5′ upstream sequence of *A. fumigatus*, followed by the full *cyp51A* gene sequence of *A. lentulus* and 270 bp of the 3′ Af*cyp51A* gene terminator, which was followed by the 1.4-kb hygromycin-selectable marker cassette and 1,000 bp of *cyp51A* sequence homologous to the 3′ downstream sequence of *A. fumigatus*. The constructed vector favors homologous recombination at the Af*cyp51A* locus, replacing its endogenous gene with the construct which includes the wild-type *A. lentulus cyp51A* gene copy expressed under the *A. fumigatus cyp51A* promoter and terminator and including the hygromycin resistance cassette for transformant selection (Fig. 2A).

After *A. fumigatus* transformation, several hygromycin-resistant mutants were analyzed by PCR. The single integration of

TABLE 4. MICs of different antifungals against *Aspergillus fumigatus* and *A. lentulus* isolates and their derived mutant strains

Isolate	Origin and/or background	Species	*cyp5A* gene copy origin	MIC (μg/ml)						
				AmB	ITC		VCZ		POS	
				EUCAST	EUCAST	Etest	EUCAST	Etest	EUCAST	Etest
CM-1290	Sputum	*A. lentulus*	*A. lentulus*	1–4	0.5–8	8–32	4	4–6	012–0.25	1
ATCC 2004305	Reference strain	*A. fumigatus*	*A. fumigatus*	0.25–0.5	0.12–0.25	2	0.25–0.5	0.25	0.03–0.12	0.19
akuB^{KU80}	BAL[a]	*A. fumigatus*	*A. fumigatus*	0.25–0.5	0.12–0.25	1.5–4	0.25	0.38–0.25	0.03–006	0.012–0.38
CM–237	Sputum	*A. fumigatus*	*A. fumigatus*	0.25–0.5	0.25	0.75–3	0.25–0.5	012–0.19	0.06–012	0.012–0.38
CM237-A–8	Mutant; CM237	*A. fumigatus*	Δ*cyp51A*	0.5	0.03	0.047	0.12	0.064	0.015	<0.02
CM1290-T29.18	Mutant; CM1290	*A. lentulus*	Δ*cyp51A*	2–4	0.03–0.06	0.032–0.064	0.25	0.064	0.015	0.064
Mutant T52.7	Mutant; *akuB*^{KU80}	*A. fumigatus*	A. lentulus	0.25	0.5–8	8–32	2	2–4	0.25	0.25–1.5
Mutant T52.8	Mutant; *akuB*^{KU80}	*A. fumigatus*	*A. lentulus*	0.25	0.25	4	1	1–2	0.06–0.12	1
Mutant T52.9	Mutant; *akuB*^{KU80}	*A. fumigatus*	*A. lentulus*	0.25	0.5	8	1	1–3	0–12	0.5–1

[a] BAL, bronchoalveolar lavage.

the fusion vector at the right locus was confirmed in three transformants (T52.7, T52.8, and T52.9) by Southern blot analysis (Fig. 2B). Also, a PCR fragment containing the whole integrated construction was sequenced in each transformant to verify the replacement of the *A. fumigatus cyp51A* sequence by *cyp51A* of *A. lentulus* and its correct nucleotide sequence.

The effect on the susceptibility to azoles in the *A. fumigatus* Δ*cyp51A* gene-replaced strains was investigated by Etest and microdilution EUCAST methods, which showed that the *A. lentulus* azole-resistant phenotype was reproduced in the Δ*cyp51A A. fumigatus* strain (Fig. 4). Accordingly, there were no changes in amphotericin B susceptibility values for any of the transformants (Table 4).

To verify that the MIC change was not due to a modification of *cyp51A* expression, we quantified the *cyp51A* mRNA levels of these mutants by real-time PCR. As is shown in Table 5, the *A. fumigatus* transformants expressed Al*cyp51A* at the same level as the homologous Af*cyp51A* in the wild-type strain (*akuB*^{KU80}) that was used as the reference for gene expression.

Virulence in an experimental model of pulmonary aspergillosis with *Aspergillus lentulus* wild-type and *cyp51A*-deleted strains. To determine whether the lack of 14-α sterol demethylase A influenced fungal pathogenicity, the *A. lentulus* Δ*cyp51A* mutant was tested in a neutropenic mouse model of invasive aspergillosis by intranasal infection. The results of the course of the infections are shown in Fig. 5. A control group remained uninfected (they were given an inhalation of saline) to monitor the influence of the immunosuppression procedure on survival. In the control groups, mortality rates were 0% in the saline group and 100% in the *A. fumigatus* group (with an inoculum size of 10^5 spores/mice). The *A. lentulus* wild-type strain was pathogenic, although there was a significant difference in the duration of the course of the infection compared to that of *A. fumigatus*. Therefore, two different inoculum sizes were tested for *A. lentulus* (10^5 and 10^6 spores/mice). Higher doses of *A. lentulus* (10^6 spores/mice) showed a survival profile similar to that of *A. fumigatus* (10^5 spores/mice). However, there were no differences, with either of the inocula sizes, between the *A. lentulus* parental and the Δ*cyp51A* mutant strains (Fig. 5). Fourteen days after infection with inocula of 10^5 or 10^6 conidia, the overall mortality rates for the *A. lentulus* mutant strain and for the parental wild-type strain CM-1290 were 10 and 20%, respectively.

DISCUSSION

The use of azoles is increasing in the management of invasive aspergillosis. Voriconazole is the recommended drug for the primary therapy of invasive aspergillosis (35). Another azole drug, posaconazole, is used prophylactically, as it has been reported to reduce the number of invasive fungal infections in neutropenic patients (48). Azole resistance in *Aspergillus* species is not yet a frequently reported event. As the number of patients exposed to azole therapy broadens, the probability of resistance is expected to increase. Unfortunately, widespread azole usage could lead to both the development of acquired resistance (especially among *A. fumigatus* strains) and a shift toward less azole-susceptible fungal species, as could be happening with *A. lentulus.*

A. lentulus prevalence is unknown, although since its identification in 2005 it is being reported more frequently in some countries (4, 7, 28, 33, 42, 45). Although this could be due to improved identification and therefore does not reflect increasing incidence, this fact is of relevance, since *A. lentulus* has been proven to be responsible for invasive aspergillosis (6). Currently, *A. lentulus* infection in susceptible patients is being described but is mostly associated with *A. fumigatus* infections (4, 33, 42). The main importance of this species is that *A. lentulus* could be considered intrinsically azole resistant if we follow the proposed breakpoints for azole resistance in *A. fumigatus* (46). Since *A. fumigatus* Cyp51A and Cyp51B were described in 2001 (29), the search for the resistance mechanism operating in *A. fumigatus* isolates has revealed at least four different mechanisms, and all of them are related to *cyp51A* alterations (13, 16, 26, 30, 32, 34). To date, *A. fumigatus* azole resistance has always been defined as secondary or acquired resistance. However, all *A. lentulus* strains have a similar antifungal susceptibility pattern (3, 28), which would indicate primary resistance operating in *A. lentulus.* It is well known that the modification of some Cyp51A amino acid residues is the most important azole resistance mechanism in *A. fumigatus* (15, 26, 28, 30, 31, 34). However, no changes have been identified in *cyp51B* in relation to azole susceptibility, suggesting that this enzyme has a different function or acts in alternative growth conditions. Backing this up, the alignment between *A. fumigatus* and *A. lentulus* Cyp51A proteins showed that there were 26 different amino acids, while both Cyp51B proteins were more conserved, with only 12 different residues.

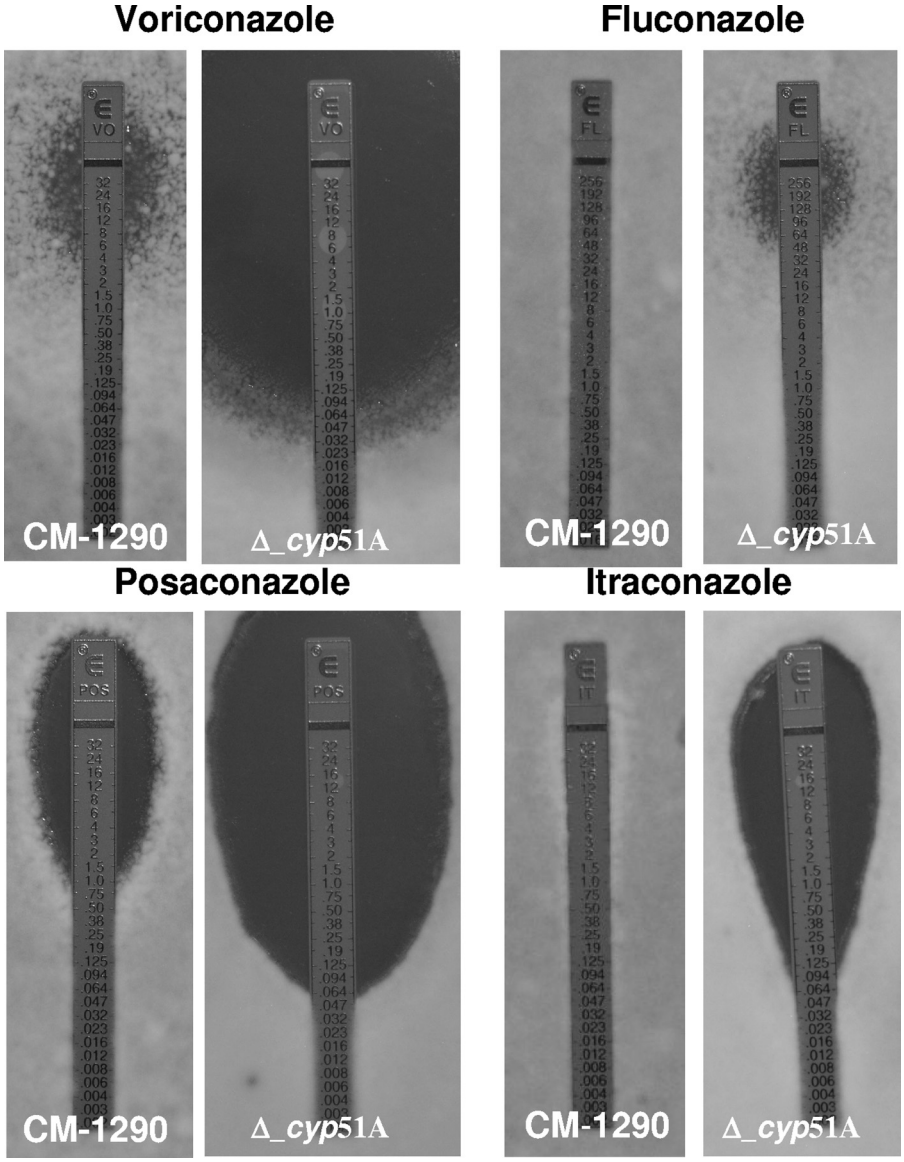

FIG. 3. Etest susceptibility testing of itraconazole (ITC), voriconazole (VCZ), fluconazole (FCZ), and posaconazole (POS) for the *A. lentulus* wild-type strain (CM-1290) and an *A. lentulus cyp51A*-deficient strain (T38.18).

A. lentulus is macro- and microscopically indistinguishable from *A. fumigatus*, although some differences in growth rate and sporulation (6, 8) could account for differences in azole drug interaction or drug uptake. In this study, we generated a *cyp51A* knockout strain of *A. lentulus*. The lack of morphological defects compared to the *A. lentulus* wild type, together with its unchanged pathogenicity, indicates that Cyp51A is not essential for *A. lentulus* viability. However, the susceptibility testing of the mutant strain showed significantly decreased MICs for all azoles, with the *A. lentulus* Δ*cyp51A* mutants being severalfold more susceptible to all azole drugs than the parental wild type. This increased susceptibility was observed even with fluconazole (Fig. 3), an azole drug to which all *Aspergillus* spp. are intrinsically resistant. The azole susceptibility profile resembled that of *A. fumigatus* Δ*cyp51A* mutants in susceptible and resistant strains (31), suggesting that the intrinsic azole resistance shown by *A. lentulus* is dependent on Cyp51A function. In addition, the replacement of the *A. fumigatus cyp51A* gene with the *A. lentulus cyp51A* gene restored the susceptibility phenotype of the *A. lentulus* wild-type strain, transforming an *A. fumigatus* (*cyp51A*-deleted) azole-hypersusceptible strain into an azole-resistant one. *A. fumigatus* strains expressing *A. lentulus cyp51A* were significantly less susceptible to azole drugs than when they were expressing their constitutive *cyp51A* gene. Some discrepancies were observed between Etest and EUCAST testing, especially with itraconazole, where the Etest showed generally higher values. Etest was used to show the results, because differences in susceptibility values are clearer with this methodology, showing clear endpoints, as well as providing clear visual reading which can be easily photographed. In any case, since we are using modified strains with the same background strain (isogenic strains), the differences

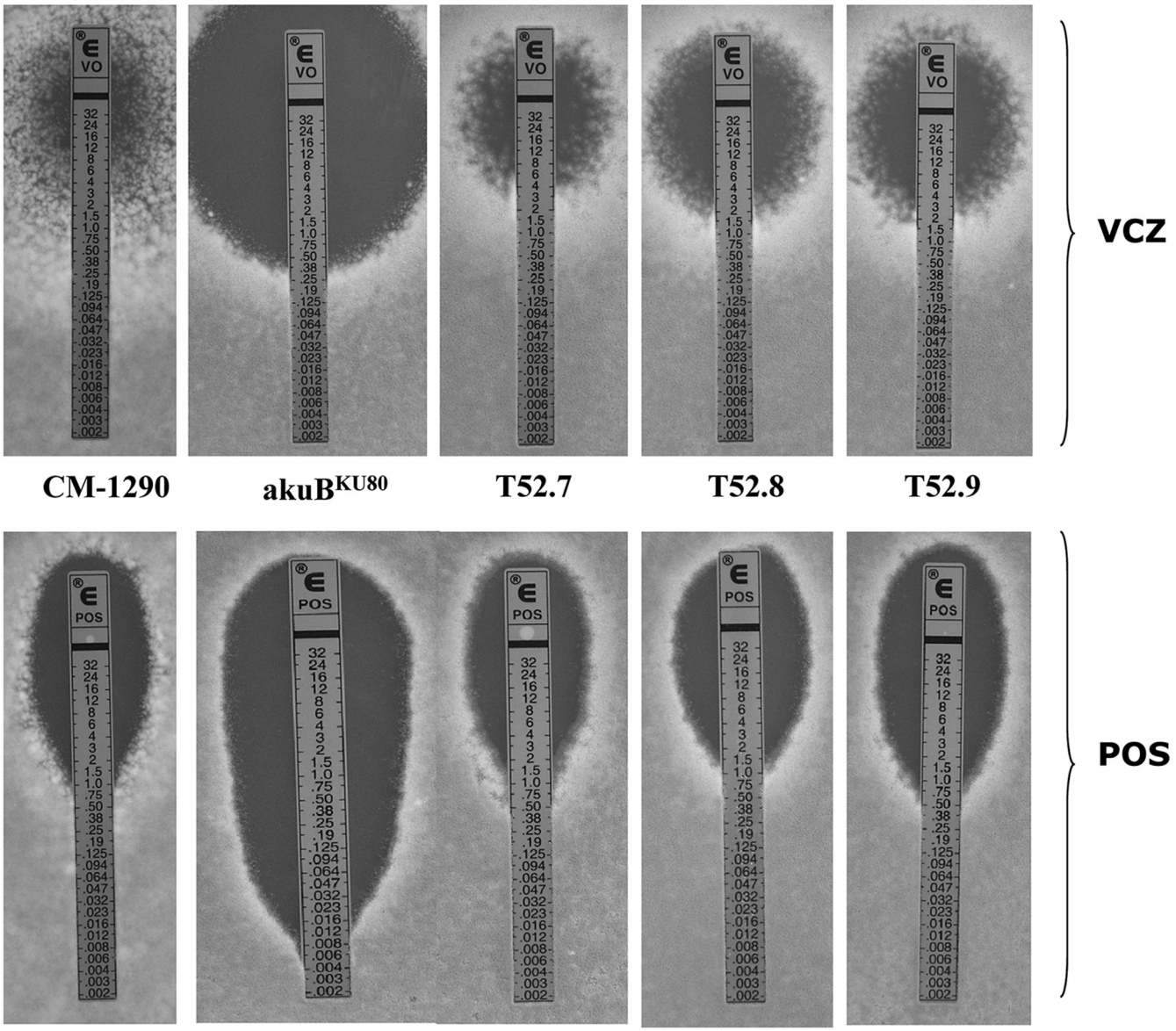

FIG. 4. Etest susceptibility testing of voriconazole (VCZ) and posaconazole (POS) for the *A. lentulus* wild-type strain (CM-1290), the *A. fumigatus* parental strain (*akuB*^{KU80}), and three mutant strains (T51.7, T51.8, and T51.9).

in susceptibility should be considered significant because we can exclude strain variability.

However, the azole MICs for *A. lentulus* are slightly higher for almost every wild-type strain tested. Therefore, it seems that an additional factor, other than protein-azole interaction, contributes to the decreased azole susceptibility shown by *A. lentulus*. Two major circumstances could be responsible for this: (i) differences in the intracellular azole concentrations achieved within *A. lentulus* because of differences in azole drug uptake or an increased expression of ABC transporters (among other efflux pumps), or (ii) due to the design of the experiment, only the coding region of the *A. lentulus cyp51A* was expressed in *A. fumigatus*. Therefore, differences in the *A. lentulus cyp51A* promoter could allow for transcription factor interaction, leading to increased *cyp51A* expression. Alternatively, differences in the *A. lentulus* 3′-untranslated region could allow for higher *cyp51A* mRNA stability, increasing the possibilities of resistance expression. Both of these possibilities have been shown to be

TABLE 5. *A. lentulus cyp51A* mRNA transcription levels with respect to *A. fumigatus* wild-type reference strain *akuB*^{KU80}

Mutant strain	*cyp51A* mRNA transcription level[a]		
	Geometric mean	SD	VC (%)
akuB^{KU80} T52.7	1.2	0.10	6.9
akuB^{KU80} T52.8	1.1	1.61	22.1
akuB^{KU80} T52.9	1.1	0.65	15.2

[a] Geometric means are from at least two different RNAs and six cDNAs. SD, standard deviation; VC, variation coefficient.

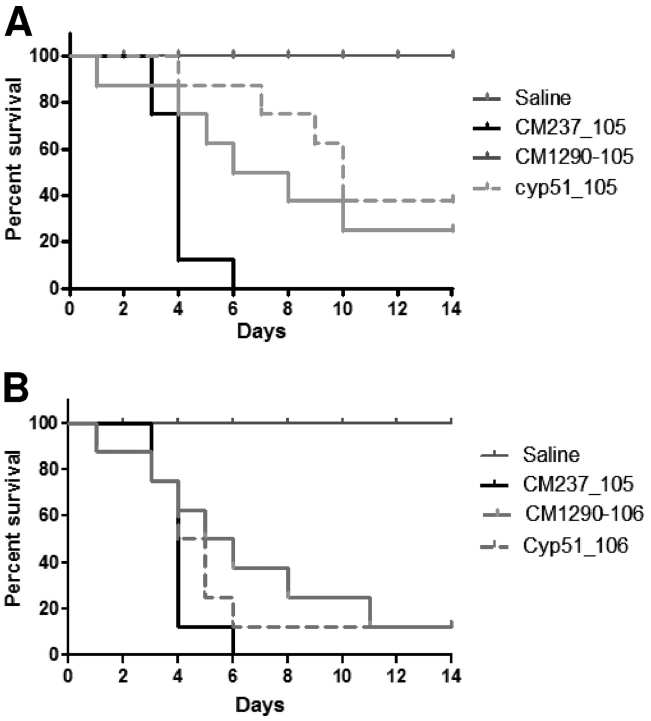

FIG. 5. Comparative analysis of parental *A. lentulus* wild-type and *cyp51A*-deficient strains in a neutropenic murine model of pulmonary aspergillosis. Survival of CD-1 immunocompromised mice infected with the *A. fumigatus* wild-type CM-237 strain (10^5 spores/mice) and the *A. lentulus* wild-type (CM-1290) and *cyp51A*-deficient (Cyp51) strains. Two inoculum sizes, 10^5 (A) and 10^6 (B) spores/mouse, were used for both *A. lentulus* strains. A control group with mice immunocompromised and inoculated only with saline also was included. Survival was monitored for a time period of 14 days, and moribund animals were sacrificed.

potential resistance determinants on their own or as contributing factors (32, 27).

The results obtained in this study proved that the *A. lentulus* wild-type strain was pathogenic in immunocompromised mice, although there was a significant difference in the duration of the course of the infection compared to that for *A. fumigatus*. This indicates a reduced virulence capability for *A. lentulus* and might explain why both species are found mainly in mixed infections (4, 33, 42). Given the differences in antifungal susceptibility between both species, this could represent a threat for patients under long-term azole treatment.

As the sequences of AfCyp51A and AlCyp51A are nearly identical (95%), it is difficult to base the different azole susceptibility profiles on single-amino-acid differences, especially since none of the different residues are located in important conserved regions of the protein (2, 50). However, the study of molecular dynamics applied to three-dimensional protein models of the molecular docking of the *A. lentulus cyp51A* and Af*cyp51A*, in combination with voriconazole, has provided information about some critical differences found in the putative closed form adopted by the proteins upon voriconazole binding. These results suggest that some major differences in the protein's BC loop differently affect voriconazole lock up and in turn correlate with *A. lentulus* differences in voriconazole susceptibility (2). The results derived could be of use for rational azole drug design or azole drug improvement in general. In addition, the *A. lentulus* Δ*cyp51A* mutant could be used in the screening for the identification of inhibitory compounds that specifically target Cyp51B activity. These findings could be confirmed by testing antifungal compounds in a murine model of invasive aspergillosis.

In summary, PCR based on *A. fumigatus* oligonucleotides allowed for the amplification and sequencing of two different *cyp51* homologue genes in *A. lentulus.* The molecular mechanisms of high azole MICs for *A. lentulus* are Cyp51A dependent but are different from what has been described previously for *A. fumigatus*. The intrinsic azole resistance shown by *A. lentulus* may represent a problem if colonization by this species becomes more frequent in the susceptible host. As the role of *A. lentulus* in invasive aspergillosis remains unclear, surveillance networks of clinical isolates are needed to determine the species distribution, their implication in clinical disease, and the outcomes of patients with invasive aspergillosis.

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