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

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

Infections with *Aspergillus fumigatus*, one of the most prevalent airborne fungal pathogens causing invasive aspergillosis worldwide, result in high rates of mortality and morbidity in immunocompromised hosts (20, 21). *A. fumigatus* is intrinsically resistant to fluconazole, but other triazole drugs like itraconazole and voriconazole are active both in vitro and in vivo against this species. Also, the new triazoles, such as posaconazole and ravuconazole, have been shown to have good in vitro activities against this species (5, 7, 17, 37, 45). In many centers voriconazole has become a common choice for the primary therapy of invasive aspergillosis, as the drug has been shown to have superior efficacy compared with those of other licensed antifungal therapies, most notably, amphotericin B (19). Nevertheless, more than half of patients still fail to respond to antifungal therapy. Although this is mainly due to factors related to the host, the resistance of *Aspergillus* to triazole drugs might play a role (22, 47). Indeed, a number of *A. fumigatus* isolates with in vitro itraconazole resistance have been described (3, 8, 13, 15, 24, 31), and strains that show high MICs against the new triazole agents are being reported (29, 31, 37). We recently observed primary and breakthrough cases of invasive aspergillosis due to *A. fumigatus* isolates with a phenotype characterized by high MICs of itraconazole, voriconazole, and ravuconazole and elevated MICs of posaconazole (51). There was no apparent epidemiological link between the cases.

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

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

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

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TABLE 1. Clinical data for patients infected or colonized with azole-resistant *A. fumigatus*, genotypes, and ranges of MICs of different antifungals for the isolates^a

Sex (age)	Underlying disease	Azole exposure prior to aspergillosis diagnosis (time)	<i>A. fumigatus</i> isolate	Date of isolation (day-mo-yr)	Genotype	MIC (μg/ml)				
						AMB	ITC	VRC	RVC	PSC
F	Unknown	Unknown	M03/669 - CM2627	21-10-2003	A	0.25	>8	4–8	4–8	0.5
M (15)	X-linked CGD	ITC (6 yr)	V13/02 - CM3271	04-04-2002	B	0.25	>8	2	>8	1
			V13/03 - CM3272	05-04-2002	B	0.25	>8	2	>8	1
M (73)	No	No	V27/28 - CM3273	03-12-2003	C	0.25	>8	4	4–8	1
F (77)	AML	VRC (77 days)	V28/78 - CM3275	02-03-2004	D	0.25	>8	4	8	0.5–1
M (16)	Hyper-IgE syndrome	VRC (2 yr)	V34/75 - CM3276	19-11-2004	E	0.25	>8	4	4	0.5
			V34/76 - CM3277		E	0.25	>8	2–4	4	0.5–1
			V34/77 - CM3278		E	0.25	>8	2	4	0.5
			V34/78 - CM3279		E	0.25	>8	2	4	0.5
F (76)	Pulmonary fibrosis	No	V41/26 - CM3820	26-06-2005	F	0.25	>8	4–8	4–8	0.5–1
M (31)	CGD	ITC (>10 yr)	V45/07 - CM3819	01-11-2005	G	0.25	>8	>8	8	1
F (68)	AML	No	V48/27 - CM3936	14-02-2006	H	0.25	>8	4	8	4
F (62)	COPD	No	V49/09 - CM4023	05-04-2006	I	0.25	>8	4	8	4
M (45)	AML	ITC (>2 yr)	V49/77 - CM4050	11-05-2006	J	0.25	>8	4	4–8	1

^a IgE, immunoglobulin E; CGD, chronic granulomatous disease; AML, acute myeloid leukemia; COPD, chronic obstructive pulmonary disease; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; RVC, ravuconazole; PSC, posaconazole; F, female; M, male.

level of *cyp51A* gene expression, accounted for the resistant phenotype.

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MATERIALS AND METHODS

Fungal strains and media. The fungal strains used in the study included (i) *A. fumigatus* strain CM237 (CM refers to the fungal collection of the Mycology Reference Laboratory at the Centro Nacional de Microbiología, ISCIII) was used to describe the *cyp51A* and *cyp51B* gene sequences and was also the control strain (28); (ii) *A. fumigatus* strain *akuB*^{KU80} (10) was used as the recipient strain in some experiments of fungal transformation; and (iii) 14 clinical *A. fumigatus* strains were isolated from 10 patients (1 from Spain and 13 from the collection of the Department of Medical Microbiology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands) (Table 1). The fungi were grown at 37°C in potato dextrose agar (Oxoid, Madrid, Spain), GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone), and minimal medium (38). Conidial stocks were preserved in sterile distilled water (15).

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

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

***Aspergillus fumigatus* transformations.** Three linear DNA fragments were used throughout this work for azole-susceptible *A. fumigatus* (CM237 and *akuB*^{KU80}) strain transformations: (i) a fragment containing the coding sequence of the *cyp51A* gene with a single nucleotide change (t364a) that substitutes leucine for histidine (named the L98H fragment), (ii) a fragment that includes the *cyp51A* promoter sequence of the gene with the 34-bp tandem repeat (named the TR fragment), and (iii) a fragment containing the coding sequence of *cyp51A* gene with a t364a change that results in the L98H substitution in combination with the 34-bp TR in the *cyp51A* promoter (named the TRL98H fragment) (Fig. 1). Linear fragments were obtained by PCR amplification of *cyp51A* by using genomic DNAs from *A. fumigatus* strain CM2627 and the following primer sets: (i) primer set P450A1-P450A2, (ii) primer set A7-A5, and (iii) primer set A7-A2 (28). First, the conidia of azole-susceptible wild-type strain CM237 were used as the recipients for transformation by electroporation by separately using each of the PCR-amplified linear fragments of *cyp51A*.

Electroporation was then carried out by a protocol previously described for *Aspergillus nidulans* (41) and subsequently adapted for *A. fumigatus* (52). Transformants were selected on medium containing itraconazole (8 μg/ml or 1 μg/ml), as described previously (15). Itraconazole-resistant transformants appeared after 2 to 7 days of incubation.

Using these conditions, we were unable to recover any transformant carrying the L98H mutation at *cyp51A*. A different azole-susceptible *A. fumigatus* strain (*akuB*^{KU80}), which has an increased frequency of homologous recombination (10), was used as the recipient strain for electroporation by using the PCR-amplified linear fragments of *cyp51A* that included the TR or the L98H fragment independently. Transformants were selected on medium containing itraconazole (0.5 to 1.0 μg/ml), as described above. The transformants were labeled with “TR,” “L98H,” or “TRL98H,” followed by a number, depending on the DNA fragment used for the electroporation, and preceded by the identification number of the recipient strain (CM237 or *akuB*^{KU80}).

RNA isolation and reverse transcription-PCR. Total RNA was extracted from the *A. fumigatus* azole-susceptible strains (strains CM237 and *akuB*^{KU80}), two of the clinical isolates (isolates CM2627 and CM3275), the transformants obtained from strain CM237 (transformants CM237-TRL98H1 and CM237-TR7), and the transformants obtained from strain *akuB*^{KU80} (transformants *akuB*^{KU80}-TR2, *akuB*^{KU80}-TR3, *akuB*^{KU80}-L98H1, and *akuB*^{KU80}-L98H3). The reverse transcription reactions were performed as described before (1). First, we evaluated the *cyp51A* transcription level differences between azole-susceptible *A. fumigatus*

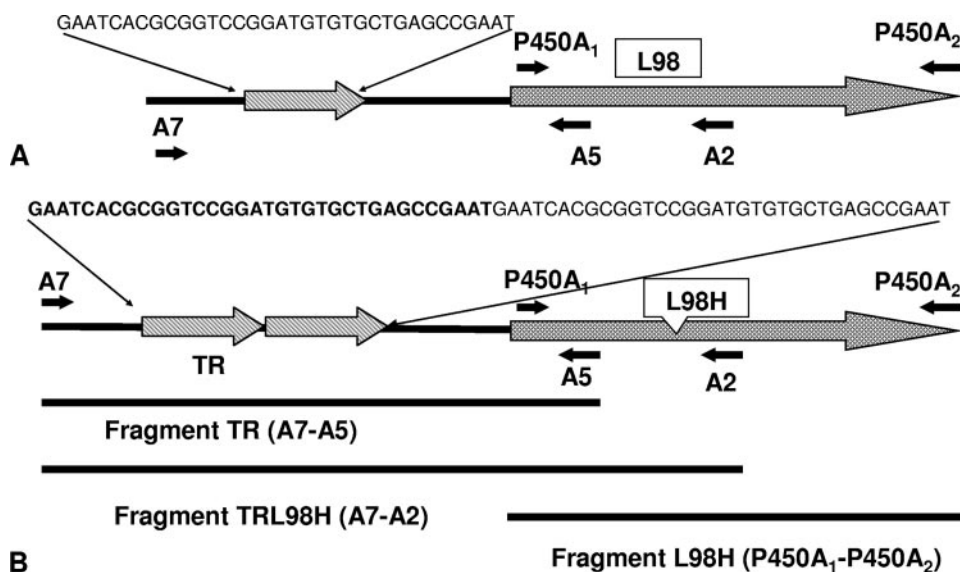


FIG. 1. Diagram of the *A. fumigatus cyp51A* gene and promoter showing the locations of the gene modifications and the primers used for PCR amplification of the different *cyp51A* gene fragments: (A) Wild-type strain CM237; (B) azole-resistant strain CM2627. The primer sets were as follows: (i) primer set A7-A2, which contains the TR and the t364a base change; (ii) primer set A7-A2, which contains only the 34-bp TR; and (iii) primer set P450A₁-P450A₂, which amplifies the full *cyp51A* gene, including only the t364a base change (L98H).

CM237 and the *akuB*^{KU80} strains used for the transformation experiments. Then, we evaluated the *cyp51A* transcription level differences between the transformants and their parental strains. For transcription level determination, a real-time PCR assay was performed as follows: the levels of expression of the target gene (*cyp51A*) were quantified for each strain by comparison with their respective control or parental strain and by use of the *A. fumigatus* β -tubulin housekeeping gene (*tub1*) as a reference for gene expression. The primer set A6-CypA2 (28) was used to amplify the cDNA from the *cyp51A* gene. The primer set Tub5 (5'-TGACCCAGCAGATGTT-3') and Tub6 (5'-GTTGTTGGGAATCCACTC-3') was used for the amplification of a fragment of the *A. fumigatus* β -tubulin gene (GenBank accession number AY048754). Real-time PCRs were set up with the FastStart DNA Master SYBR green (Roche Diagnostic). Each assay was repeated in duplicate and with RNA from at least two different RNA extractions. Each experiment included standard curves for the target gene (*cyp51A*) and for the reference gene. The PCR efficiencies of β -tubulin and *cyp51A* cDNA amplification were calculated from the slopes of the curves given by LightCycler software (Roche Diagnostic), and the efficiency values were used to validate each experiment and also for relative quantification by the method of Pfaffl (36).

Genotyping of *Aspergillus* isolates. The *A. fumigatus* isolates were genotyped by using a panel of short tandem repeats (STRs). Two sets of three STRs (M3 and M4) consisting of tri- and tetranucleotide motifs were used in separate multicolor multiplex PCRs to analyze both *A. fumigatus* azole-resistant and azole-susceptible (control) isolates. For each of the multiplex PCRs with STRs M3 and M4, the forward primers were labeled with carboxyfluorescein, hexachlorofluorescein, or tetrachlorofluorescein at the 5' end. The primer sequences were used exactly as described previously (14). Each PCR mixture contained 1 μ M all amplification primers, 3.0 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates, 1 U of FastStart Taq DNA polymerase, and 1 ng of genomic DNA in 1 \times reaction buffer. The thermoprofile was 10 min of denaturation at 95°C, followed by 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C. Before the reaction mixtures were cooled to room temperature, an additional incubation for 10 min at 72°C was performed. The fragments obtained were combined with the ET400-R size standard (GE Healthcare, Roosendaal, The Netherlands) and analyzed on a MegaBACE 500 automated DNA platform (GE Healthcare).

Data analysis. The significance of the differences in MICs was determined by Student's *t* test after logarithmic conversion of the values (unpaired, unequal variance). For statistical evaluation of the crossing point and relative expression variations, the data were analyzed by analysis of variance for significant differences. Statistical analysis was done with the SPSS package (version 14.0; SPSS S.L., Madrid, Spain). A *P* value of <0.01 was considered significant.

RESULTS

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

PCR amplification and sequence analysis of *cyp51A* and *cyp51B* genes. Sequence analysis of *cyp51A* showed the same t364a point mutation in all strains, resulting in an amino acid substitution with histidine (L98H) at the leucine 98 codon. Two isolates (both from the same patient) had two extra base changes: these were t960a and t1554a, which were responsible for amino acid changes of serine for threonine at codon 297 (S297T) and phenylalanine for isoleucine at codon 495 (F495I), respectively (Table 2). All strains also contained three different point mutations in the *cyp51B* gene that were not conserved across the 14 isolates, although all of them were silent (Table 2). The sequence alignments of the region encompassing L98 revealed that this amino acid residue is not totally conserved in all yeasts and molds. However, the fact that all azole-resistant strains carried the same mutation

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

Isolate	TR promoter	Nucleotide substitution ^a							
		<i>cyp51A</i>					<i>cyp51B</i>		
		Codon 54	Codon 98	Codon 220	Codon 297	Codon 495	Codon 35	Codon 394	Codon 464
CM237 (wild type)	(-)	GGG	CTC	ATG	TCG	TTT	TCT	CCT	ATT
<i>akuB</i> ^{KU80b}	(-)	GGG	CTC	ATG	TCG	TTT	TCT	CCT	ATT
M03/669 - CM2627	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	TCT	¹²⁸⁵ CCG	ATT
V13/02 - CM3271	(+)	GGG	³⁶⁴ CAC	ATG	⁹⁶⁰ ACG	¹⁵⁵⁴ ATT	TCT	¹²⁸⁵ CCG	¹⁴⁹⁵ ATA
V13/03 - CM3272	(+)	GGG	³⁶⁴ CAC	ATG	⁹⁶⁰ ACG	¹⁵⁵⁴ ATT	TCT	¹²⁸⁵ CCG	¹⁴⁹⁵ ATA
V27/28 - CM3273	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	TCT	¹²⁸⁵ CCG	ATT
V28/78 - CM3275	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	¹⁰⁵ TCC	¹²⁸⁵ CCG	ATT
V34/75 - CM3276	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	TCT	¹²⁸⁵ CCG	ATT
V34/76 - CM3277	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	TCT	¹²⁸⁵ CCG	ATT
V34/77 - CM3278	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	TCT	¹²⁸⁵ CCG	ATT
V34/78 - CM3279	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	TCT	¹²⁸⁵ CCG	ATT
V41/26 - CM3820	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	¹⁰⁵ TCC	¹²⁸⁵ CCG	ATT
V45/07 - CM3819	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	TCT	¹²⁸⁵ CCG	ATT
V48/27 - CM3936	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	¹⁰⁵ TCC	¹²⁸⁵ CCG	ATT
V49/09 - CM4023	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	TCT	¹²⁸⁵ CCG	ATT
V49/77 - CM4050	(+)	GGG	³⁶⁴ CAC	ATG	TCG	TTT	TCT	¹²⁸⁵ CCG	ATT
Amino acids		(G54)	(L98H)	(M220)	(S297T)	(F495I)	(S35S)	(P394P)	(I464I)

^a Nucleotides are numbered from the translation start codon ATG of *cyp51A* and *cyp51B*. The numbers indicate the position at which a base change occurs (in boldface).

^b The *akuB*^{KU80} mutant strain (10) is a genetically modified *A. fumigatus* strain with a high frequency of homologous recombination.

strongly suggested that the substitution was associated with azole drug resistance. Moreover, all strains had a duplication in tandem of a 34-bp sequence in the *cyp51A* promoter (5'-G AATCACGCGGTCCGGATGTGTGCTGAGCCGAAT3'-) located at positions -288 and -322 from the *cyp51A* start codon. A BLAST database search based on the repeated sequence unit identified no striking similarities other than similarity to the *cyp51A* promoter region of *A. fumigatus* deposited in the GenBank database (accession numbers AF338659 and AF222068).

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

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

Replacement of wild-type *cyp51A* gene with different gene modifications. Therefore, the *cyp51A* gene fragments which had been amplified from strain CM2627 by PCR and which carried the different *cyp51A* modifications were electroporated into wild-type (azole-susceptible) *A. fumigatus* strain CM237. The different PCR fragments (L98H, TR, and TRL98H) were individually electroporated into wild-type *A. fumigatus* strain CM237 (Fig. 1). When itraconazole (8 μ g/ml) was used for selection, mutants ($n = 15$) were obtained only when TRL98H

PCR fragment was used for transformation (strains CM237-TRL98H1 to CM237-TRL98H15). However, when 1 μ g/ml of itraconazole was used as the selective concentration, only one mutant (strain CM237-TR7) arose when the TR fragment obtained by PCR was used for transformation. On the other hand, after several experiments with the L98H fragment and itraconazole at concentrations of 1 μ g/ml and 8 μ g/ml, we were unable to obtain any mutant showing only the t364a base change. Therefore, a different azole-susceptible *A. fumigatus* strain (strain *akuB*^{KU80}) was used as the recipient for electroporation with the linear fragments obtained by PCR which included only one of the *cyp51A* modifications (the TR fragment or the L98H fragment). Transformants were selected on medium containing itraconazole (0.5 to 1.0 μ g/ml). By using this strain, two mutants (strains *akuB*^{KU80}-TR2 and *akuB*^{KU80}-

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

Strain	<i>cyp51A</i> mRNA transcription level ^a		VC (%) ^b
	Geometric mean	SD	
CM2627	7.70	1.45	18.8
CM3275	8.05	1.25	15.5
CM237-L98HTR1	8.10	1.36	16.8
CM237-TR7	4.23	1.02	24.0
<i>akuB</i> ^{KU80} recipient	1.48	0.10	6.9
<i>akuB</i> ^{KU80} -TR2	4.74	0.91	19.2
<i>akuB</i> ^{KU80} -TR3	8.23	1.96	23.8
<i>akuB</i> ^{KU80} -L98H1	0.65	0.15	22.9
<i>akuB</i> ^{KU80} -L98H3	1.32	0.14	17.8

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

^b VC, coefficient of variation

TABLE 4. Nucleotide and amino acids substitutions in *A. fumigatus cyp51A* and *cyp51B* genes and ranges of MICs of different antifungal drugs for *A. fumigatus* mutant strains with the TR present in the *cyp51A* promoter and/or the Cyp51A amino acid substitution at L98H

Isolate or strain ^a	<i>cyp51A</i> gene and promoter ^b				<i>cyp51B</i> gene ^b		MIC range (μg/ml) ^c				
	Codon 54	Codon 98	Codon 220	TR	Codon 35	Codon 394	AMB	ITC	VRC	RVC	PSC
CM237 (wild type)	GGG	CTC	ATG	(–)	TCT	CCT	0.25	0.25	0.5	0.5	0.12
M03/669 - CM2627	GGG	³⁶⁴ CAC	ATG	(+)	¹⁰⁵ TCT	¹²⁸⁵ CCG	0.25	8.0	4.0–8.0	4.0–8.0	0.5
VC28–78 - CM3275	GGG	³⁶⁴ CAC	ATG	(+)	¹⁰⁵ TCC	¹²⁸⁵ CCG	0.50	8.0	4.0	4.0	0.5
CM237-TRL98H1	GGG	³⁶⁴ CAC	ATG	(+)	TCT	CCT	0.5	8.0	4.0	4.0–8.0	1.0
CM237-TRL98H3	GGG	³⁶⁴ CAC	ATG	(+)	TCT	CCT	0.25	8.0	4.0	8.0	0.5–1.0
CM237-TRL98H4	GGG	³⁶⁴ CAC	ATG	(+)	TCT	CCT	0.25	8.0	4.0	4.0	0.5–1.0
CM237-TRL98H5	GGG	³⁶⁴ CAC	ATG	(+)	TCT	CCT	0.25	8.0	4.0	4.0–8.0	0.5–1.0
CM237-TR7	GGG	CTC	ATG	(+)	TCT	CCT	0.50	0.5	1.0	1.0	0.25
<i>akuB</i> ^{KU80} (recipient)	GGG	CTC	ATG	(–)	TCT	CCT	0.25	0.12–0.25	0.25–0.5	0.5	0.03–0.06
<i>akuB</i> ^{KU80} -TR2	GGG	CTC	ATG	(+)	TCT	CCT	0.50	0.5	1.0	2.0	0.12
<i>akuB</i> ^{KU80} -TR3	GGG	CTC	ATG	(+)	TCT	CCT	0.25–0.50	0.5	1.0	1.0–2.0	0.12
<i>akuB</i> ^{KU80} -L98H1	GGG	³⁶⁴ CAC	ATG	(–)	TCT	CCT	0.25–0.50	0.5	1.0–2.0	1.0–2.0	0.12
<i>akuB</i> ^{KU80} -L98H3	GGG	³⁶⁴ CAC	ATG	(–)	TCT	CCT	0.25–0.50	0.5	1.0–2.0	1.0–2.0	0.12
Amino acids	(G54)	(L98H)	(M220)		(S35S)	(P394P)					

^a Mutant nomenclature: strains CM237-TRL98H1 to CM237-TRL98H5, gene replacement of the TRL98H fragment from strain CM2627 into azole-susceptible strain CM237; strain CM237-TR7, gene replacement of the TR fragment from strain CM2627 into strain CM237; strains *akuB*^{KU80}-L98H1 and *akuB*^{KU80}-L98H3, gene replacement of the L98H fragment from strain CM2627 into the *akuB*^{KU80} azole-susceptible strain; strains *akuB*^{KU80}-TR2 and *akuB*^{KU80}-TR3, gene replacement of the TR fragment from strain CM2627 into strain *akuB*^{KU80}.

^b Nucleotides are numbered from the translation ATG start codon of *cyp51A* and *cyp51B*. The numbers indicate the position at which a base change occurs (in boldface). TR (+), presence of the 34-bp fragment duplication in tandem in the *cyp51A* promoter; TR (–), absence of the 34-bp fragment duplication in tandem in the *cyp51A* promoter.

^c AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; RVC, ravuconazole; PSC, posaconazole.

TR3) were obtained when the PCR-generated TR fragment was used for transformation and five mutants (strains *akuB*^{KU80}-L98H1 to *akuB*^{KU80}-L98H5) were obtained when the PCR-generated L98H fragment was used for transformation.

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

In order to rule out the possibility of the integration of extra copies of *cyp51A* in the transformation procedure, DNAs from every transformant were digested with a restriction enzyme, blotted, and hybridized (29) with a labeled fragment of *cyp51A* (data not shown).

The *cyp51A* gene including the promoter area and the *cyp51B* genes from all transformants were sequenced. All mutants analyzed (nine) had incorporated the mutated *cyp51A* allele corresponding to the fragment that was introduced (Table 4). With the exception of the L98H mutation and/or duplication of the repeat in tandem, none of the transformants had any other mutation in either *cyp51A* or *cyp51B* compared to the sequence of wild-type strain CM237. The *A. fumigatus* transformants' susceptibilities to triazole drugs were determined as described above. All the transformants with both *cyp51A* alterations (TR and L98H) exhibited susceptibility profiles similar to those of the clinical isolates (Table 4). However, transformant CM237-TR7 had a slight but not significant increase in azole drug MICs ($P > 0.01$) that did not correspond to an azole-resistant phenotype. Similarly, transformants obtained with the *akuB*^{KU80} strain (containing either the TR or the L98H modification independently) had a slight but not significant increase in azole drug MICs ($P > 0.01$) that did not correspond to an azole-resistant phenotype (Table 4).

The levels of *cyp51A* mRNA expression in two transfor-

ants (strains CM237-TRL98H1 and CM237-TR7) in comparison with those in *A. fumigatus* azole-susceptible recipient strain CM237 and two azole-resistant clinical strains (strains CM2627 and CM3275) were analyzed. The relative increases in the levels of *cyp51A* expression obtained in CM237-TRL98H1 were eightfold compared to the level of expression in the wild-type strain, whereas the levels of *cyp51A* transcription in CM237-TR7 were increased only fourfold compared to the level of transcription in the wild type (Table 3). Also, the levels of *cyp51A* mRNA expression in four mutants (strains *akuB*^{KU80}-TR2, *akuB*^{KU80}-TR3, *akuB*^{KU80}-L98H1, and *akuB*^{KU80}-L98H3) in comparison with that in their *A. fumigatus* recipient *akuB*^{KU80} strain were analyzed. The relative increases in the levels of *cyp51A* expression obtained in strains *akuB*^{KU80}-TR2 and *akuB*^{KU80}-TR3 were between five- and eightfold those in the *akuB*^{KU80} parental strain, whereas the levels of *cyp51A* transcription in strains *akuB*^{KU80}-L98H1 and *akuB*^{KU80}-L98H3 were similar to that in the parental *akuB*^{KU80} strain (Table 3).

DISCUSSION

In *Candida* spp., the majority of reports on azole drug resistance are related to the increased efflux of azole drugs due to the overexpression of efflux pumps of different types (4, 39, 42). In addition, a number of reports have identified polymorphisms in the *erg11* gene (which is homologous to *cyp51A*) from clinical *Candida albicans* isolates that are responsible for and/or that are associated with fluconazole resistance (16, 26, 43). On the other hand, the resistance of filamentous fungi to different demethylase inhibitors (DMIs) used for agricultural purposes has mainly been correlated with one amino acid change (Y136F) in the azole target, Cyp51 (11, 12, 53), or with its overexpression (18, 23, 44, 46). In *A. fumigatus*, azole drug resistance has been described for both laboratory mutants and

clinical strains and has mainly been attributed to alterations in the target enzyme (Cyp51A) (2, 15, 25, 28, 32). Even though analysis of the *A. fumigatus* genome has shown the existence of more than 40 ATP binding cassette transporter (ABC) homologs and more than 100 major facilitator transporter genes (five times more than the number in yeast) (49), isolates that overexpress efflux pumps as an azole resistance mechanism have rarely been described. Nevertheless, there are a number of reports of efflux pump overexpression related to azole resistance in *A. fumigatus*, although these have been *A. fumigatus* mutant strains generated in the laboratory and not clinical isolates (9, 32). Only one clinical strain (strain AF72) has been found to overexpress (by five times) the *atrF* gene (which encodes an ABC transporter efflux pump), but only when it is grown in the presence of itraconazole (46).

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

The results obtained with the mutants (strains CM237-TR7, *akuB*^{KU80}-TR2, and *akuB*^{KU80}-TR3) suggest that the duplication of the 34-bp TR by itself seems to be insufficient for reproduction of the resistant phenotype, even though there was a four- to eightfold increase in the level of *cyp51A* expression which corresponded to a slight increase in azole MICs. Previous reports have related azole resistance to the high level of *cyp51/erg11* expression in both yeast and filamentous fungi. The mechanisms that have been associated with increased expression include gene or chromosome duplication and promoter modification. The first mechanism, known in mammals as gene amplification, was described in *Candida glabrata* (27) and in an engineered laboratory strain of *Aspergillus niger* (50). The latter mechanism has been seen in an *A. nidulans* triazole-resistant strain obtained by amplification or overexpression of the *A. nidulans cyp51* gene expressed in a high-copy-number plasmid (34). An increased level of *cyp51* expression has been suggested as a possible mechanism for sterol DMI resistance in the plant pathogen *Mycosphaerella graminicola* (48). Similarly, a DMI resistance mechanism has been described for another plant pathogenic mold, *Venturia inaequalis*. The high level of *cyp51* expression was correlated with the presence of 553-bp insertion located in the gene promoter (44). Also, just recently, the analysis of 59 DMI-resistant isolates of *Bumeriella jaapii* has shown 5- to 12-fold increased levels of *cyp51* expression related to various forms of a truncated non-long terminal direct repeat, long interspersed nuclear retrotransposon element (23). In *Penicillium digitatum* a mechanism similar to the one that we describe has been reported for strains that have a 126-bp sequence tandemly repeated in the *cyp51* promoter and that is directly related to a pattern of resistance to different DMIs. Nevertheless, the number of tandem repeats proved to

be essential for the production of a high level of DMI resistance (18). Therefore, the results obtained in this study suggest that the 34-bp sequence in the promoter region of *cyp51A* might be a transcriptional enhancer and that its duplication is responsible for the increased level of expression of *cyp51A* and the higher *A. fumigatus* MICs for azole drugs. However, these results also suggest that the TR duplication by itself is not enough to confer the azole cross-resistant phenotype shown by these strains. On the other hand, the introduction of the L98H amino acid substitution at Cyp51A alone seems to be responsible for only a slight increase in the *A. fumigatus* MICs for azole drugs without influencing the *cyp51A* expression levels. In the *A. fumigatus* Cyp51A protein, the leucine at position 98 is close to the first substrate recognition site (SRS1). This residue is conserved throughout yeast and filamentous fungi but not in plant or human Cyp51 proteins. An homology model based on the X-ray crystal structure of Cyp51 from *Mycobacterium tuberculosis* was used to predict the specific Cyp51A residues within *A. fumigatus* Cyp51A in combination with azole drugs (54). However, the specific residue L98 has not been implicated in any azole drug interactions with the protein (54). The search for Cyp51A mutations at L98 in *A. fumigatus* strains with intermediate MICs of azole drugs will help to clarify whether this amino acid substitution is present in strains isolated in the clinical setting and/or in environmental isolates (these experiments are already under way in our laboratory).

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

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

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