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SUSCEPTIBILITY

Point Mutation or Overexpression of *Aspergillus fumigatus cyp51B*, Encoding Lanosterol 14α-Sterol Demethylase, Leads to Triazole Resistance

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ABSTRACT *Aspergillus fumigatus* is the most common cause of invasive fungal mold infections in immunocompromised individuals. Current antifungal treatment relies heavily on the triazole antifungals which inhibit fungal Erg11/Cyp51 activity and subsequent ergosterol biosynthesis. However, resistance, due primarily to *cyp51* mutation, is rapidly increasing. *A. fumigatus* contains two Cyp51 isoenzymes, Cyp51A and Cyp51B. Overexpression and mutation of Cyp51A is a major cause of triazole resistance in *A. fumigatus*. The role of Cyp51B in generating resistance is unclear. Here, we show that overexpression or mutation of *cyp51B* results in triazole resistance. We demonstrate that introduction of a G457S Cyp51B mutation identified in a resistant clinical isolate results in voriconazole resistance in a naive recipient strain. Our results indicate that mutations in *cyp51B* resulting in clinical resistance do exist and should be monitored.

KEYWORDS *Aspergillus fumigatus*, triazole antifungals, drug resistance, *cyp51B*, azole resistance

Aspergillus fumigatus is the most common invasive mold pathogen in humans. It can cause a wide range of diseases in humans, with high mortality rates in immunocompromised patients (1, 2). The first line of treatment for invasive *A. fumigatus* infections is triazole antifungals that inhibit Erg11/Cyp51 lanosterol demethylase activity, blocking ergosterol biosynthesis. However, triazole-resistant strains of *A. fumigatus* are increasingly encountered, leading to increased mortality (3). To date, the most common triazole resistance mechanisms in *A. fumigatus* are alterations in the *erg11A/cyp51A* gene or promoter followed by overexpression of efflux pumps and mutations in *hmg1*, encoding 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (3).

The *A. fumigatus* genome contains two *cyp51* genes, *cyp51A* and *cyp51B*, showing 60% identity (4). Neither gene is essential, but deletion of both is lethal, suggesting they act in a compensatory fashion (5, 6). Deletion of *cyp51A* alone increases triazole susceptibility, whereas deletion of *cyp51B* has only a minor effect (6, 7). Cyp51A and Cyp51B green fluorescent protein (GFP)-tagged proteins localize primarily to the endoplasmic reticulum (ER), and their level of expression is upregulated after treatment with voriconazole (VRZ) (6). Expression of Cyp51A is strongly increased after deletion of Cyp51B, and vice versa, suggesting a potential compensatory response (6).

The involvement of Cyp51A in clinical triazole resistance has been well established and includes point mutations in codons G54, L98, Y121, T289, G138, and M220 and duplications in the promoter region (TR34 and TR46) that lead to overexpression (3, 8).

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TABLE 1 MIC values of voriconazole and itraconazole in WT and mutant strains with various doxycycline concentrations

Doxycycline concn (ng/ml)	MIC (mg/liter)							
	VRZ in strain:				ITZ in strain:			
	WT	Δ*cyp51B*	B+/A_tetOFF	B−/A_tetOFF	WT	Δ*cyp51B*	B+/A_tetOFF	B−/A_tetOFF
200	0.5	0.5	0.0625	Inviable	0.5	0.5	0.125	Inviable
100	0.5	0.5	0.0625	Inviable	0.5	0.5	0.25	Inviable
50	0.5	0.5	0.0625	Inviable	0.5	0.5	0.25	Inviable
25	0.5	0.5	0.0625	Inviable	0.5	0.5	0.25	Inviable
12.5	0.5	0.5	0.125	0.0625	0.5	0.5	0.25	0.0625
6.25	0.5	0.5	0.25	0.0625	0.5	0.5	0.25	0.0625
3.13	0.5	0.5	0.5	0.125	0.5	0.5	0.5	0.125
1.56	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.25
0.78	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.25
0.39	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.25
0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

In contrast, the involvement of Cyp51B in clinical triazole resistance has not been fully revealed. *cyp51B* gene overexpression has been observed in two clinical triazole-resistant *A. fumigatus* isolates without *cyp51A* mutations, but a direct link between *cyp51B* overexpression and resistance was not made (9). Overexpression of *cyp51B* in a laboratory strain of *A. fumigatus* using a strong constitutive promoter resulted in moderately increased VRZ resistance (10). Recently, a clinical triazole-resistant *A. fumigatus* strain containing mutations in both Cyp51B (G457S) and Hmg1 (F390L) was described (11). However, the relative contribution of the ***c****yp51B* mutation toward resistance remained unclear.

Our goals, therefore, were to clearly define the contributions of *A. fumigatus cyp51B* overexpression and mutation toward triazole resistance. To achieve this, we expressed *cyp51B* under the inducible promoter *xylP* and under the *cyp51A* wild type (WT) and tandem repeat (TR)-containing promoter, in both WT and *cyp51A* null strains, and also introduced point mutations predicted to confer resistance. Our findings suggest that both *cyp51B* overexpression and mutation can lead to triazole resistance, indicating that this gene may have a more important role than previously realized in generating clinical triazole resistance. We show that the Cyp51B G457S mutation found in a clinical isolate confers voriconazole resistance when introduced into a susceptible strain, indicating a role for *cyp51B* as a generator of clinical resistance.

RESULTS

***A. fumigatus cyp51A* is more important than *cyp51B* in conferring triazole resistance.** Previous work has demonstrated that deletion of *cyp51A* alone increases triazole susceptibility, whereas deletion of *cyp51B* has only a minor effect (6, 7). We repeated, verified, and extended these findings. Deletion of *cyp51A* resulted in 4- to 16-fold increased sensitivity to the three triazoles tested, while deletion of *cyp51B* only slightly increased sensitivity (2-fold) to VRZ (Table 1). To quantify the effect of *cyp51A* expression levels on triazole susceptibility, we constructed two additional mutant strains with the tetOFF promoter regulating *cyp51A* expression in the WT (*B+/A_tetOFF*) or Δ*cyp51B* background (*B−/A_tetOFF*) (Table 1). Strain *B−/A_tetOFF* under doxycycline (Dox) at 25 ng/ml and higher concentrations was inviable, indicating, as expected, that loss of both *cyp51A* and *cyp51B* was lethal in *A. fumigatus*. In both *B+/A_tetOFF* and *B−/A_tetOFF* strains, increasing Dox concentrations resulted in growing sensitivity to VRZ and itraconazole (ITZ), consistent with a progressive downregulation of the gene. Interestingly, strain *B−/A_tetOFF* was more sensitive than the *B+/A_tetOFF* strain under the same increasing Dox concentrations, indicating that *cyp51B* is important when *cyp51A* expression is compromised.

***A. fumigatus cyp51B* mRNA expression is triazole induced, but *cyp51B* is expressed at lower levels than *cyp51A*.** Previous studies have shown that *A. fumigatus cyp51B* and *cyp51A* can be induced by triazoles (6, 9). Because gene expression may vary among

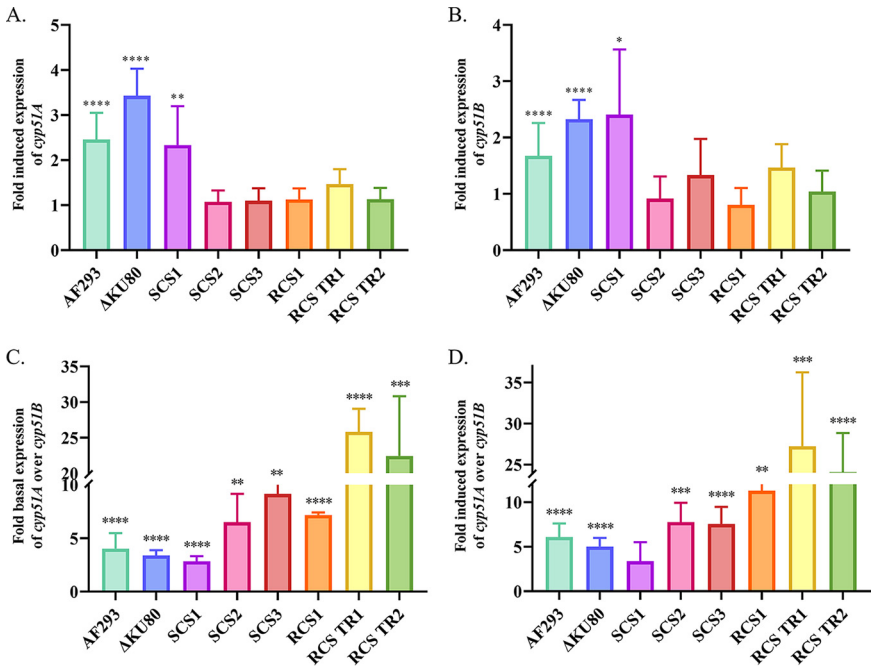

FIG 1 Expression of *cyp51A* and *cyp51B* in clinical isolates of *A. fumigatus*. Fold expression of *cyp51A* compared to that of *cyp51B* calculated as $2^{-\Delta\Delta CT}$. SCS, susceptible clinical strain; RCS, resistant clinical strain. RCS TR1 and RCS TR2 possess a Cyp51A TR34/L98H mutation, RCS1 possesses a Cyp51A F46Y/M172K/E427K mutation. (A) Fold induced expression of *cyp51A* after VRZ treatment (treated versus untreated). (B) Fold induced expression of *cyp51B* after VRZ treatment (treated versus untreated). (C) Fold basal expression of *cyp51A* compared to that of *cyp51B* in VRZ-untreated samples. (D) Fold induced expression of *cyp51A* compared to that of *cyp51B* in VRZ-treated samples. *, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P < 0.0001$.

isolates, we used quantitative PCR (qPCR) to assess *cyp51A* and *cyp51B* expression in eight clinical strains, including Δ*KU80* and Af293 laboratory strains, three triazole-susceptible strains (SCS1, SCS2, and SCS3), and three resistant strains (RCS1 [F46Y/M172K/E427K Cyp51A] and RCS-TR1 and RCS-TR2, both with TR34/L98H Cyp51A mutations) (see Tables S1 and S2 in the supplemental material). Mycelium was grown for 20 h and exposed to 0.5 MIC VRZ for 4 h, and total RNA was prepared for qPCR. The results show that VRZ induced the expression of *cyp51A* and *cyp51B* in three of the eight strains (Δ*KU80*, Af293, and SCS1) between 2- and 4-fold, indicating that induction is apparently strain specific at this time point and drug concentration (Fig. 1A and B).

Previous studies had not directly compared the basal and triazole-induced expression levels of *cyp51A* and *cyp51B*. Here, we show that *cyp51A* is expressed at higher basal and triazole-induced mRNA levels than *cyp51B* in all eight strains (Fig. 1C and D). Especially, high expression of *cyp51A* compared to that of *cyp51B* was observed in two resistant clinical strains (RCS-TR1 and RCS-TR2) resulting from a known Cyp51A TR34/L98H mutation that leads to overexpression.

Overexpression of *A. fumigatus cyp51B* leads to decreased triazole susceptibility. Based on our results showing higher expression of *cyp51A* than of *cyp51B*, we hypothesized that increased expression of *cyp51B* may lead to triazole resistance. We therefore generated strains in which the *cyp51B* promoter was replaced (i) by the strong inducible promoter *xylP* in the Δ*KU80* strain (generating strain A+/XylpB) and the Δ*cyp51A* strain (generating strain A−/XylpB), (ii) by the *cyp51Ap* promoter (*Ap*) in the Δ*KU80* strain (generating strain A+/ApB) and the Δ*cyp51A* strain (generating strain A−/ApB), and (iii) by the *cyp51A-TRp* tandem repeat promoter (*ATRp*), in the Δ*KU80* (generating strain A+/ATRpB) and Δ*cyp51A* (generating strain A−/ATRpB) strains. Strain susceptibility to the triazoles VRZ, ITZ, and posaconazole (POS) was tested by broth microdilution (Table 2) and point inoculation on yeast extract-dextrose-$MgSO_4$ (YAG) agar plates,

TABLE 2 MIC values of voriconazole, itraconazole, and posaconazole in strains overexpressing *cyp51B*

Strain	MIC (mg/liter)		
	VRZ	ITZ	POS
Δ*KU80*	1	0.5	0.25
A+/ApB	1	0.5	0.25
A+/$A_{TR}pB_{L113H}$	4	>16	1
A+/XylpB (+xylose)	4	>16	1
Δ*cyp51A*	0.25	0.06	0.016
A−/ApB	1	0.25	0.125
A−/$A_{TR}pB_{L113H}$	4	2	0.5
A−/XylpB (+xylose)	4	1	0.25
Δ*cyp51B*	0.5	0.5	0.25

which we found to be more sensitive to minor changes in triazole susceptibility (Fig. 2). Using both methods, decreased triazole susceptibility was seen in both Δ*KU80* and Δ*cyp51A* backgrounds containing *cyp51B* under *xylP* (plus xylose) and *cyp51A-TRp* (Table 2 and Fig. 2). Moderately decreased triazole susceptibility was also seen by point inoculation in the Δ*KU80* strain containing *cyp51B* under *cyp51Ap* (strains A+/ApB and A−/ApB) (Fig. 2A) and by broth microdilution in strain A−/ApB compared to that in the Δ*cyp51A* strain (Table 2).

Point mutations in *A. fumigatus cyp51B* lead to decreased triazole susceptibility. Point mutations in *cyp51A* are known to confer triazole resistance. However, whether this mechanism is also true for *cyp51B* is not clear. Therefore, we generated strains containing *cyp51B* mutations homologous to known triazole resistance mutations in Cyp51A. Mutations included (i) G69E (homologous to Cyp51A G54E), (ii) G153C (homologous to Cyp51A G138C), and (iii) G457S, identified in a clinical resistant *A. fumigatus* isolate (12) and homologous to Cyp51A G448S (see Fig. S10). Strain susceptibility to the triazoles VRZ, ITZ, and POS was tested by broth microdilution (Table 3) and point inoculation on YAG agar plates (Fig. 3). By broth microdilution, VRZ resistance was seen with Cyp51B G457S (4-fold increase in MIC versus that in the Δ*KU80* control), while moderately decreased ITZ and POS susceptibilities (2-fold increase in MICs versus that in the Δ*KU80* control) were seen with Cyp51B G153C. Moderately decreased POS susceptibility (2-fold increase in MIC versus that in the Δ*KU80* control) was also seen with Cyp51B G69E (Table 3). By point inoculation, strongly decreased VRZ susceptibility with Cyp51B G457S and decreased ITZ and POS susceptibilities with Cyp51B G69E and Cyp51B G153C were seen (Fig. 3). By point inoculation, modestly increased VRZ susceptibility was seen with Cyp51B G69E and Cyp51B G153C, and modestly increased ITZ and POS susceptibilities were seen with Cyp51B G457S. The control Cyp51B WT-*hph* strain, in which a normal version of *cyp51B* had been introduced into the Δ*KU80* strain, showed the same susceptibility pattern as the WT Δ*KU80* strains. Strain growth in the absence of drug was not compromised by the mutations, as seen in the "no drug" control plate (Fig. 3).

DISCUSSION

Fungal enzymes of the Cyp51/ERG11 family mediate lanosterol demethylation, a crucial step in the biosynthesis of the fungal-specific sterol ergosterol. They have been intensively studied, as they are the target of azole antifungals, the main drug group used to treat fungal infections (13). However, due to their extensive medical use in the last 30 years and their massive application to treat mold infections in agricultural crops, resistance mutations in *cyp51/ERG11* have emerged and are rapidly compromising treatment efficacy (8). Filamentous *Pezizomycotina* fungi such as the aspergilli are unique in that their genome contains 2 to 3 *cyp51* copies (4, 14, 15). In *A. fumigatus*, which contains two *cyp51* genes (*cyp51A* and *cyp51B*), *cyp51A* appears to be more dominant and important in generating azole resistance, while *cyp51B* has been considered more redundant

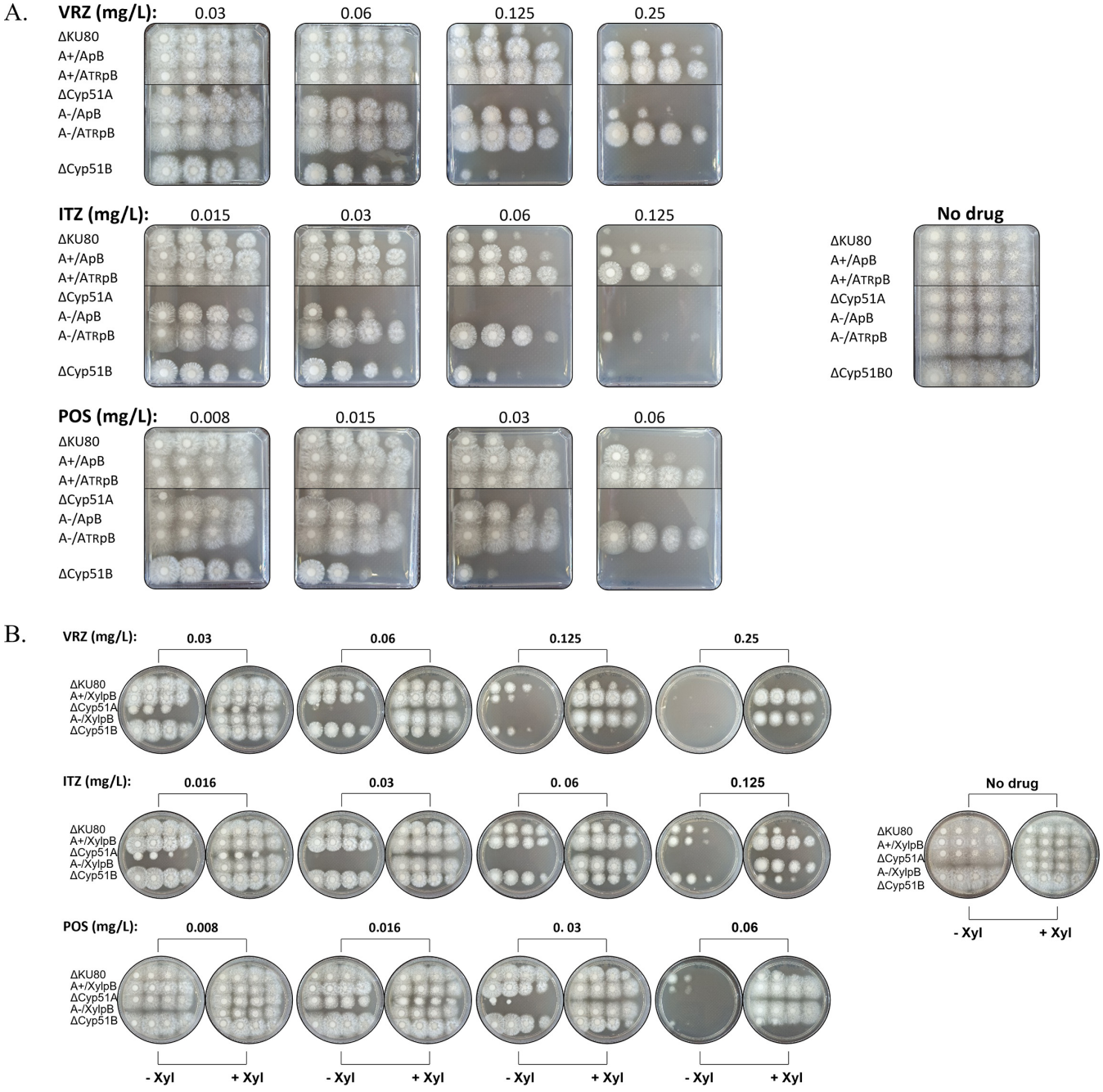

FIG 2 Triazole susceptibility of *A. fumigatus* strains overexpressing cyp51B. Droplet assay on RPMI-MOPS agar plates with increasing concentrations of VRZ, ITZ, and POS without (A and B) and with (B) xylose added to the medium. Each droplet column (from left to right) contained 10^4, 10^3, 10^2, or 10 spores per 10-μl droplet. A+/ApB, *cyp51A*-positive strain expressing *cyp51B* under *cyp51A* promoter; A−/ApB, *cyp51A*-negative strain expressing *cyp51B* under *cyp51A* promoter; A+/A_{TR}pB, *cyp51A*-positive strain expressing Cyp51B L113H under *cyp51A* TR34 promoter; A−/A_{TR}pB, *cyp51A*-negative strain expressing Cyp51B L113H under *cyp51A* TR34 promoter; A+/XylpB, *cyp51A*-positive strain expressing *cyp51B* under xylose promoter; A−/XylpB, *cyp51A*-negative strain expressing *cyp51B* under xylose promoter.

and less involved in resistance (6, 7, 9, 10). The aim of this study was to better understand the role of *cyp51B* in generating triazole resistance in *A. fumigatus*.

In overview, our findings confirm that *A. fumigatus cyp51B* is less dominant than *cyp51A* in generating triazole resistance, as we show that deletion of *cyp51A* results in greater triazole susceptibility than deletion of *cyp51B*. Interestingly, the relevance of *cyp51B* is higher when *cyp51A* expression becomes compromised (Table 1). Therefore, it is tempting to speculate that the presence of *cyp51B* in *A. fumigatus* may have facilitated the development of mutations in *cyp51A*, serving as a backup system to maintain

TABLE 3 MIC values of voriconazole, itraconazole, and posaconazole in strains with point mutations in *cyp51B*

	MIC (mg/liter)		
Strain	**VRZ**	**ITZ**	**POS**
Δ*KU80*	0.5	0.25	0.25
Cyp51B G69E	0.5	0.25–0.5	0.5
Cyp51B G153C	0.5	0.5	0.5
Cyp51B G457S	2	0.25	0.25
Cyp51B WT-*hph*	0.5	0.25	0.25

some level of resistance if Cyp51A function becomes compromised after mutation. Additionally, we provide several lines of evidence suggesting that this is primarily because *cyp51B* is driven by a weaker promoter: First, in multiple clinical strains, *cyp51B* is expressed at lower levels than *cyp51A*. Second, overexpression of *cyp51B* under the inducible *xyl* promoter or TR-*cyp51A* promoter generates substantial triazole resistance. Third, expression of *cyp51B* under the native *cyp51A* promoter in a strain lacking *cyp51A* (strain A−/ApB) (Table 2 and Fig. 2) results in greater resistance than a strain containing only *cyp51A* (strain Δ*cyp51B*) (Table 2 and Fig. 2).

If, as we have shown, overexpression of *cyp51B* can lead to triazole resistance, why has it not been found in clinical isolates? We believe that a very real possibility is that it has been overlooked. An additional explanation, although not exclusive, is that mutations in the *cyp51B* promoter that lead to overexpression may be difficult to generate. *cyp51A* overexpression occurs by tandem-repeat duplication of the two serum response element (SRE) promoter binding sites to generate four SRE sites, thereby increasing binding by the positive transcriptional regulator SrbA (16). The *cyp51B* promoter contains a single SRE site (see Fig. S11 in the supplemental material) that could be duplicated under selection, but it is unclear if two SRE sites will generate sufficiently high levels of *cyp51B* expression to generate resistance. Future research should aim to investigate this possibility in the laboratory.

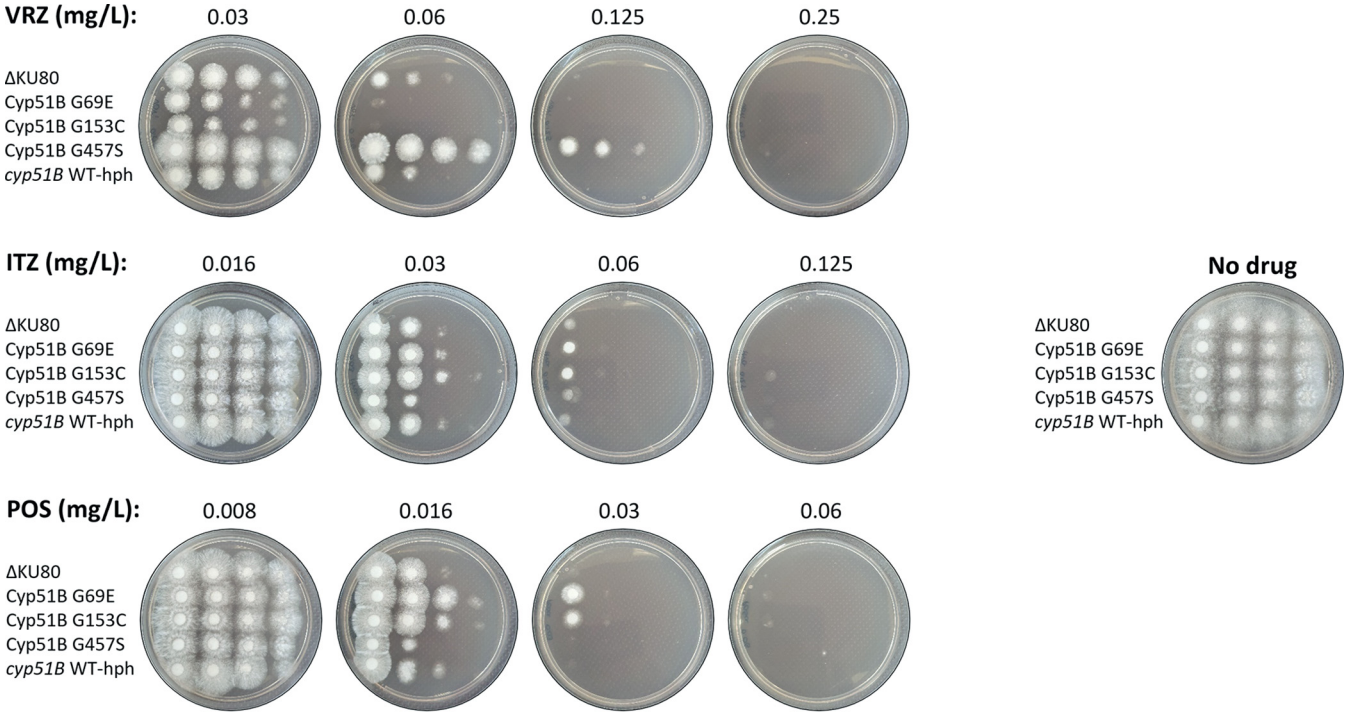

FIG 3 Triazole susceptibility of *A. fumigatus* strains with cyp51B mutations. Droplet assay on RPMI-MOPS agar plates containing increasing concentrations of VRZ, ITZ, and POS. Each droplet column (from left to right) contained 10^4, 10^3, 10^2, or 10 spores per 10-μl droplet.

To explore the possibility that point mutations in *A. fumigatus cyp51B* can confer triazole resistance, we introduced mutations G69E and G153C, homologous to the Cyp51A mutations G54E and G138C, respectively. The G54E Cyp51A mutation is situated in the substrate access channel and appears to interact directly with the triazole drug. It leads to ITZ and POS resistance but typically does not affect VRZ susceptibility (3). Interestingly, the G69E Cyp51B also resulted in up to 2-fold increase in the MICs to ITZ and POS but not to VRZ. Point inoculation analysis, which we find is more sensitive than the standard MIC test, showed increased VRZ susceptibility in the Cyp51B G69E mutant.

The G138C Cyp51A mutation is located in the substrate access channel near the heme cofactor and leads to VRZ, POS, and ITZ resistance (10, 17). The analogous Cyp51B G153C mutation resulted in an up to 2-fold increase in the MICs to ITZ and POS but not to VRZ. Taken together, our results show that *cyp51B* mutations mimic their analogous *cyp51A* mutations with respect to their triazole resistance profile, but, probably because of reduced expression of *cyp51B*, they achieve a lower level of resistance. It will be interesting to investigate if combinations of tandem repeats (TRs) in the promoter region with point mutations in the open reading frame (ORF) can confer higher levels of resistance, as is the case with the TR34/L98H and the TR46/Y121F/T289A mutations in *cyp51A*.

The most important finding described here is that a *cyp51B* point mutation occurring in a clinical resistant isolate is responsible for triazole resistance. The Cyp51B G457S mutation was originally described in the pan-azole-resistant strain CM9460, which also contains an F390L mutation in Hmg1. The glycine in position 457 in Cyp51B is at the same position as glycine 448 in Cyp51A, which when mutated to serine, leads to VRZ resistance and weak ITZ and POS resistance, apparently by altering the conformation of the heme-binding site (11, 12). Here, we show that reconstitution of the Cyp51B G457S mutation in a triazole-sensitive strain of *A. fumigatus* results in resistance to VRZ (MIC increased 4-fold to 2 μg/ml) but not to ITZ or POS. In contrast, strain CM9460 showed MICs of $>$8 μg/ml for VRZ, ITZ, and POS (11), which suggests that either its resistance is mainly driven by the mutation in *hmg1* or simultaneous presence of both mutations has a synergistic effect on triazole resistance, as has been suggested for *hmg1* and *cyp51A*. This should be confirmed by reconstitution of mutated *cyp51B* and *hmg1* alone and in combination in a triazole-susceptible strain. Taking into consideration that the *cyp51B* mutations described here have a smaller role in generating triazole resistance than parallel *cyp51A* mutations, we predict that they will be important as secondary drivers alongside stronger mutations in *cyp51A* and *hmg1*.

In summary, mutations in *cyp51B* can lead to triazole resistance in *A. fumigatus*, and we suggest this should be considered when analyzing resistant clinical isolates.

MATERIALS AND METHODS

Media and strains. Strains were grown on YAG agar plates (0.5% yeast extract, 1% dextrose, 0.01 M $MgSO_4$, trace elements solution, vitamin mix, 1.5% agar) for 48 to 72 h. MIC experiments were performed in RPMI-MOPS broth (10% 10$\times$ RPMI medium, 3.45% morpholinepropanesulfonic acid [MOPS]), and droplet assays were performed on RPMI-MOPS agar plates. Transformation of *A. fumigatus* spores was made on YPGS agar plates (2% yeast extract, 0.5% peptone, 2% D-glucose, 1 M sucrose, 1.5% agar for plates or 0.7% for top, pH 6) with 200 μg/ml hygromycin B for hygromycin B selection or on AMM sucrose agar plates (1$\times$ salts solution, 1% dextrose, 1 M sucrose, 0.012 M KPO_4, pH 6.8, 0.1% trace elements solution, 1.5% agar for plates or 0.7% for top, pH 6) with 0.1 μg/ml pyrithiamine for pyrithiamine selection.

This study included the wild-type strains Af293 and Δ*KU80*, six patient isolates (see Table S1 in the supplemental material), *cyp51A* and *cyp51B* single knockout strains constructed from the Δ*KU80* strain, *cyp51B* promoter replacement strains constructed from either Δ*KU80* or Δ*cyp51A* strains, and *cyp51B* point mutation strains constructed from the Δ*KU80* strain. A detailed description of the construction and verification of these strains is provided in the Tables S1, S3, and S4 and Fig. S1 to S9.

Antifungal susceptibility testing. MIC was determined by CLSI M38-A2 broth microdilution methodology. Briefly, stock solutions of either voriconazole (VRZ), itraconazole (ITZ), or posaconazole (POS) (Merck Sigma) were diluted in RPMI-MOPS and loaded into 96-well plates; spores were diluted to 5×10^4 spores/ml and were loaded into the wells. Plates were incubated at 37°C for 48 h, and then the lowest concentration of triazole completely inhibiting fungal growth (observed by inverted light microscope) was set as the MIC. The CLSI epidemiological cutoff values (ECVs) used were $>$1 mg/liter for itraconazole, $>$1 mg/liter for voriconazole, and $>$0.25 mg/liter for posaconazole (18). Droplet susceptibility testing was performed by inoculation of 10^4, 10^3, 10^2, or 10 spores in 10 μl distilled water on the surface of RPMI-MOPS agar plates containing different concentrations of antifungal and incubation for 48 h at 37°C.

Determination of gene expression by qPCR. Strains were grown on YAG agar plates for 72 h, and 6×10^7 spores were collected and inoculated in 150 ml YAG broth in 250-ml flasks. Flasks (6 per strain) were incubated under shaking for 20 h at 37°C, and then 0.5 MIC of VRZ (Table S2) was added to 75 ml for 4 h, and 75 ml was left untreated. Mycelium was collected, lyophilized, and crushed; RNA was extracted using the Qiagen RNeasy plant minikit. RNA concentration was assessed using a NanoDrop, and then equal amounts of RNA from each sample were converted to cDNA using the Verso cDNA synthesis kit. Equal amounts of cDNA (based on RNA amounts) from each sample were loaded into Applied Biosystems MicroAmp optical 96-well plates with Applied Biosystems Fast SYBR green master mix and primer for either β-tubulin (housekeeping control gene), *cyp51A*, or *cyp51B*. Comparative threshold cycle ($2^{-\Delta\Delta CT}$) analysis was performed. Statistical analysis was performed with one-way analysis of variance (ANOVA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.7 MB.

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We declare no conflict of interest.

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