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Complementation of a *Saccharomyces cerevisiae* ERG11/CYP51 (Sterol 14α-Demethylase) Doxycycline-Regulated Mutant and Screening of the Azole Sensitivity of *Aspergillus fumigatus* Isoenzymes CYP51A and CYP51B[▽]

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***Aspergillus fumigatus* sterol 14α-demethylase isoenzymes CYP51A and CYP51B were heterologously expressed in a *Saccharomyces cerevisiae* mutant (YUG37-*erg11*), wherein native ERG11/CYP51 expression is controlled using a doxycycline-regulatable promoter. When cultured in the presence of doxycycline, recombinant YUG37-p*cyp51A* and YUG37-p*cyp51B* yeasts were able to synthesize ergosterol and grow; a control strain harboring reverse-oriented *cyp51A* could not. YUG37-p*cyp51A* and YUG37-p*cyp51B* constructs showed identical sensitivity to itraconazole, posaconazole, clotrimazole, and voriconazole. Conversely, YUG37-p*cyp51A* withstood 16-fold-higher concentrations of fluconazole than YUG37-p*cyp51B* (8 and 0.5 μg ml^{-1}, respectively).**

Azoles are used for treatment of *Aspergillus* infections (11, 13) and also in prophylactic drug regimens for immunocompromised patients (8). The emergence (4, 14, 30, 31, 32) and potential for spread (2) of azole-resistant *Aspergillus* (hereafter focusing on *Aspergillus fumigatus*) have highlighted the need to develop diagnostic tools (6, 9) and novel antifungal agents (15). These requirements demand better understanding of the mechanisms that mediate azole resistance in *Aspergillus*.

Cytochrome P450 (CYP) was first investigated in *A. fumigatus* in 1990 (1); genome sequencing has revealed approximately 70 genes from this superfamily (29) that have not been fully annotated (manually verified) as for the 111 members of the *Aspergillus nidulans* cytochrome P450 complement (CYPome) (16). Given their importance in other pathogenic fungi (e.g., *Candida albicans* [18, 19, 21, 36]), the significance of mutations in *A. fumigatus* sterol 14α-demethylase, the CYP51 protein target of azoles, has attracted particular attention. Since the discovery that *A. fumigatus* possesses two genes (*cyp51A* and *cyp51B*) encoding sterol 14α-demethylase-like enzymes (26), it has been reasoned that the relative importance of each for ergosterol biosynthesis and/or resistance phenotypes observed in the clinic might differ. To date, the most prevalent mechanism of azole resistance in *A. fumigatus* appears to be the modification of CYP51A (5, 22, 25, 27, 28). Missense mutations in *cyp51A* are associated with cross-resistance, elevated MICs to azoles, and increased CYP51A expression (25, 27).

Research has demonstrated the essentiality of the *erg11* gene family (*cyp51A* and *cyp51B*) in *A. fumigatus* despite neither member being essential individually (15). It has also been postulated that CYP51A might provide the major 14α-demethylase activity required for growth in *A. fumigatus* and that CYP51B may serve a redundant or alternative function under certain growth conditions (28). However, despite the research interest surrounding *A. fumigatus*, it has not yet been shown that *cyp51A* and *cyp51B* both encode functional sterol 14α-demethylase. We investigated the use of a doxycycline-regulated *Saccharomyces cerevisiae erg11*/*cyp51* (sterol 14α-demethylase) mutant to heterologously express *A. fumigatus* CYP51A and CYP51B in order to demonstrate complementation for ergosterol biosynthesis. The azole sensitivity of yeast transformants expressing *A. fumigatus* CYP51A and CYP51B was then screened.

Plasmid and strain construction. Genes encoding *A. fumigatus* isoenzymes CYP51A and CYP51B (EXPASY accession no. Q4WNT5 and Q96W81) were synthesized without introns as previously described (34). The following gene-specific forward (F) and reverse (R) primers for *cyp51A* and *cyp51B* were used to amplify both genes for direct T/A ligation into the *S. cerevisiae* yeast expression vector pYES2.1 TOPO (Invitrogen): *cyp51A*F (5′-ATGGTCCCGATGCTGTG-3′), *cyp51A*R (5′-CTATTTGGAAGTGTTCTTGG-3′), *cyp51B*F (5′-ATGG GTCTGATCGCCTT-3′), and *cyp51B*R (5′-CTACGCTTTAG TCGC-3′). DNA polymerase with proofreading capacity (High Fidelity Expand; Roche) was used for all PCRs. The *S. cerevisiae* host (YUG37-*erg11*), wherein native *erg11*/*cyp51* expression is controlled using a doxycycline-regulatable promoter (10, 33), was first transformed with pYES2.1 vector containing a reverse-oriented *cyp51A* gene insertion to create the control strain (YUG37-pCTRL). Experimental yeast transformants harboring *cyp51A* and *cyp51B* plasmid DNA (hereafter YUG37-p*cyp51A* and YUG37-p*cyp51B*) and YUG37-pCTRL were all selected and maintained using glucose-based yeast minimal ($_{glc}$YM^{-dox}) medium (Difco) containing 1.34% yeast nitrogen base without amino acids, 2% glucose, leucine and

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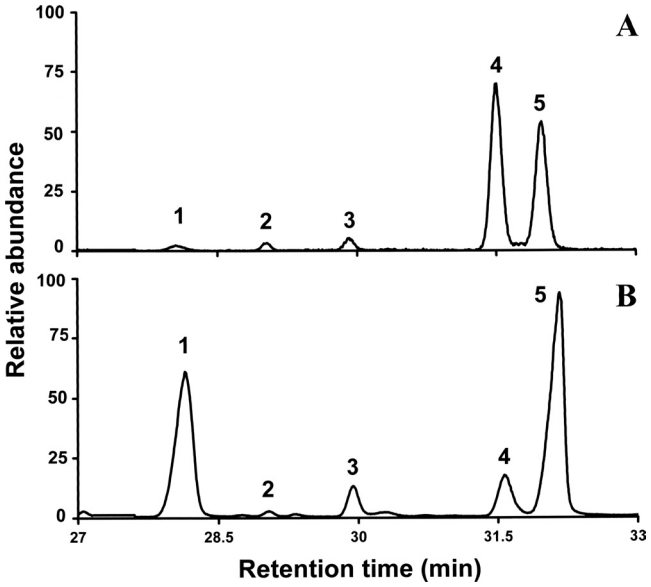

FIG. 1. Example GC-MS chromatograms for the YUG37-pCTRL construct (A) and the complementing YUG37-p*cyp51A* construct (B) cultured using $_{gal/raf}YM^{+dox}$ induction medium. 1, ergosterol; 2, 14α-methyl fecosterol; 3, 4,14α-dimethyl cholesta 8,24-dienol; 4, 14α-methyl ergosta 8,24(28) dien-3β-6α-diol; 5, lanosterol and/or obtusifoliol.

tryptophan (both 100 mg liter^{-1}), and 2% agarose (as required) (wt/vol).

Heterologous expression. For complementation experiments (Fig. 1), medium to induce plasmid expression ($_{gal/raf}YM^{+dox}$) was prepared as above except for the replacement of glucose with galactose and raffinose (2%) and the addition of 5 μg ml^{-1} doxycycline (Sigma-Aldrich). Single colonies from YUG37-p*cyp51A*, YUG37-p*cyp51B*, and YUG37-pCTRL transformation plates (all constructs in triplicate) were used to inoculate 15-ml volumes of $_{gal/raf}YM^{+dox}$; the resulting cultures were incubated for 72 h (30°C, 180 rpm) prior to checks for cell growth and subsequent sterol analyses. Sterol analysis of the YUG37-pCTRL construct cultured using $_{gal/raf}YM$ without doxycycline ($_{gal/raf}YM^{-dox}$) was also undertaken.

Sterol analysis. The sterol composition of YUG37-p*cyp51A*, YUG37-p*cyp51B*, and YUG37-pCTRL constructs cultured using $_{gal/raf}YM$ medium (Table 1) was determined by gas chromatography mass spectrometry (GC-MS) as previously described (23). Trimethylsilyl (TMS)-derivatized sterols were identified with reference to retention times and fragmentation spectra for known standards. GC-MS data files were analyzed using Agilent software (MSD enhanced ChemStation, Agilent Technologies Inc.) for derivation of integrated peak areas.

Azole sensitivity assays. The sensitivity of YUG37-p*cyp51A* and YUG37-p*cyp51B* constructs to selected azoles was assayed using standard CLSI M27-A2 broth dilution methodology, except for the use of $_{gal/raf}YM^{+dox}$ induction medium, initial inoculums equivalent to 2.5×10^3 cells ml^{-1}, and final azole concentrations of fluconazole (0.031 to 16 μg ml^{-1}), clotrimazole, itraconazole, and posaconazole (0.004 to 2.0 μg ml^{-1}), and voriconazole (0.0005 to 0.25). Owing to its inability to grow in $_{gal/raf}YM^{+dox}$ medium, azole MIC values for the YUG37-

TABLE 1. Heterologous expression of *A. fumigatus* isoenzymes CYP51A and CYP51B in an *S. cerevisiae* ERG11/CYP51 (sterol 14α-demethylase) mutant

Construct	Medium	Mean % of sterol (SD) in indicated construct[a]						MIC (μg ml^{-1})[b]				
		Ergosterol	Other 14α-demethylated sterols[c]	14α-Methyl fecosterol	4,14α-Dimethyl cholesta 8,24-dienol	14α-Methyl ergosta 8,24 (28) dien-3β-6α-diol	Lanosterol/ obtusifoliol[d]	Fluconazole	Clotrimazole	Voriconazole	Posaconazole	Itraconazole
YUG37-p*cyp51A*	$_{gal/raf}YM^{+dox}$	40.8 (2.0)		2.0 (1.3)	2.6 (1.7)	20.5 (3.1)	34.1 (4.4)	8	0.016	0.004	0.063	0.125
YUG37-p*cyp51B*	$_{gal/raf}YM^{+dox}$	39.5 (3.1)		1.7 (0.9)	2.9 (1.3)	10.5 (5.6)	45.4 (3.8)	0.5	0.016	0.004	0.063	0.125
YUG37-pCTRL	$_{gal/raf}YM^{+dox}$	4.0 (2.2)		1.9 (2.2)	3.3 (1.1)	51.1 (3.3)	40.6 (4.8)	—	—	—	—	—
YUG37-pCTRL[e]	$_{gal/raf}YM^{-dox}$	80 (3.3)	15.5 (1.1)				4.0 (1.3)	0.25	0.016	0.004	0.063	0.031

[a] Mean percentage ± SD of sterol composition of experimental constructs.
[b] MICs recorded in azole sensitivity assays. —, insufficient growth for MIC testing.
[c] Sum of all 14α-demethylated sterols (except ergosterol).
[d] 14α-Methylated sterols with identical molecular weights and GC-MS retention times.
[e] YUG37-pCTRL strain cultured in the absence of doxycycline.

pCTRL construct were determined using $_{gal/raf}YM^{-dox}$. Microtiter plates were incubated at 30°C, and MIC values (Table 1) were scored after 72 h. Azole MICs were determined as the minimum drug concentration yielding at least 80% inhibition of growth compared with growth in control wells.

The YUG37-p*cyp51A* and YUG37-p*cyp51B* constructs were both culturable using $_{gal/raf}YM^{+dox}$. The ergosterol content of each (Table 1) indicates that *A. fumigatus* CYP51A and CYP51B both complemented *S. cerevisiae* sterol 14α-demethylase function with comparable efficiency. YUG37-pCTRL cultures did not grow in $_{gal/raf}YM^{+dox}$ medium, as evidenced by GC-MS chromatograms (Fig. 1A). Briefly, 14α-methylated sterols comprised >95% of the total sterol fraction in YUG37-pCTRL as a result of downregulation of the endogenous *S. cerevisiae cyp51*. That the fungistatic sterol 14α-methyl ergosta 8,24(28)-dien-3β-6α-diol (17, 35) comprised >50% of $_{gal/raf}YM^{+dox}$-cultured YUG37-pCTRL (Table 1) is consistent with the failure of the reverse-oriented *cyp51A* gene to complement and accounts for its inability to grow. The sterol (specifically high ergosterol) content of the YUG37-pCTRL construct cultured in the absence of doxycycline is typical of wild-type *S. cerevisiae*.

MIC values from azole sensitivity assays with YUG37-p*cyp51A* and YUG37-p*cyp51B* (Table 1) agree with literature regarding the efficacy of azoles for general treatment of *A. fumigatus* infections. Specifically, the potency of voriconazole and posaconazole (in this study, MIC values of 0.004 and 0.063 μg ml^{-1}, respectively) is well documented (8, 11, 13, 24). That both YUG37-p*cyp51A* and YUG37-p*cyp51B* withstood comparatively higher concentrations of fluconazole (Table 1) is in agreement with the intrinsic resistance of *A. fumigatus* to this azole (12). It is noteworthy that YUG37-p*cyp51A* cultures withstood 16-fold-higher concentrations of fluconazole than YUG37-p*cyp51B* (MIC values of 8 and 0.5 μg ml^{-1}, respectively). This is consistent with the results of Mellado et al. (28) and indicates that the expression and properties of CYP51A may be central to fluconazole resistance in *A. fumigatus*. The MIC values for the YUG37-pCTRL construct cultured using $_{gal/raf}YM^{-dox}$ (Table 1) demonstrate the susceptibility of the endogenous yeast CYP51 to all azoles; they also indicate the potential importance of *A. fumigatus* CYP51A and CYP51B for resistance to both fluconazole (12) and itraconazole (4, 7).

It is possible that, besides variation in the structural properties of *A. fumigatus* CYP51A and CYP51B, differences in gene expression could contribute to the altered fluconazole susceptibility of the YUG37-p*cyp51A* and YUG37-p*cyp51B* constructs. However, the consistency and value of this yeast expression system for evaluating mutations in CYP51 from the fungal wheat pathogen *Mycosphaerella graminicola* has already been demonstrated (3). It is also significant that previous experimental work with *Candida albicans* CYP51 has indicated that expression levels in transformants differing by more than 1,000-fold do not alter azole MICs more than 5-fold (20). Hence, differences in the expression of CYP51A and CYP51B are unlikely to be responsible for azole MIC values observed in the present study.

Results from this study unequivocally demonstrate that *A. fumigatus cyp51A* and *cyp51B* both encode functional sterol 14α-demethylase. Given the complicating presence of both *cyp51A* and *cyp51B* in *A. fumigatus* and (owing to the efficiency of *A. fumigatus* DNA repair mechanisms) the challenge of creating stable gene knockout strains, use of the nonpathogenic *S. cerevisiae* sterol 14α-demethylase mutant to complement and assay the individual azole sensitivity of CYP51A and CYP51B constitutes a model system through which the screening of novel azole antifungals might be undertaken in the future.

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