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A Clinical Isolate of *Candida albicans* with Mutations in *ERG11* (Encoding Sterol 14α-Demethylase) and *ERG5* (Encoding C22 Desaturase) Is Cross Resistant to Azoles and Amphotericin B▽

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A clinical isolate of *Candida albicans* was identified as an *erg5* (encoding sterol C22 desaturase) mutant in which ergosterol was not detectable and ergosta 5,7-dienol comprised >80% of the total sterol fraction. The mutant isolate (CA108) was resistant to fluconazole, voriconazole, itraconazole, ketoconazole, and clotrimazole (MIC values, 64, 8, 2, 1, and 2 μg ml^{-1}, respectively); azole resistance could not be fully explained by the activity of multidrug resistance pumps. When susceptibility tests were performed in the presence of a multidrug efflux inhibitor (tacrolimus; FK506), CA108 remained resistant to azole concentrations higher than suggested clinical breakpoints for *C. albicans* (efflux-inhibited MIC values, 16 and 4 μg ml^{-1} for fluconazole and voriconazole, respectively). Gene sequencing revealed that CA108 was an *erg11 erg5* double mutant harboring a single amino acid substitution (A114S) in sterol 14α-demethylase (Erg11p) and sequence repetition (10 duplicated amino acids), which nullified C22 desaturase (Erg5p) function. Owing to a lack of ergosterol, CA108 was also resistant to amphotericin B (MIC, 2 μg ml^{-1}). This constitutes the first report of a *C. albicans erg5* mutant isolated from the clinic.

Several mechanisms can contribute to azole resistance in pathogenic fungi, such as *Candida albicans* (4, 11, 14, 32, 33). There has been an increase in research surrounding the potential importance of drug efflux transporters (26, 30) and changes in sterol 14α-demethylase (*ERG11* [*CYP51*]), the target of azole antifungals (10, 12, 13, 14, 25). Biofilm formation (2) and the possibility for azole sequestration mechanisms (15) have also attracted attention. Following the identification of defective sterol $\Delta^{5,6}$-desaturase (encoded by *ERG3*) as a mechanism of azole resistance in *Saccharomyces cerevisiae* some 20 years ago (31), this mechanism has also been reported in clinical *C. albicans* isolates (3, 8, 9, 19, 21). There remains sustained interest in the regulation of the *ERG* genes and proteins that mediate fungal sterol (specifically ergosterol) biosynthesis (Fig. 1) in azole-resistant isolates. Many antifungal compounds that are currently available to clinicians target either ergosterol (e.g., polyene antifungals) or the enzymes central to its synthesis (e.g., azole inhibitors of sterol 14α-demethylase), and hence, the potential for the emergence of cross-resistant strains exists.

Data suggest that azole inhibitors of sterol 14α-demethylase (here called Erg11p) also bind to C22 desaturase (here called Erg5p) (6); however, the possibility that the latter could constitute a target for new antifungal compounds (6, 7) remains understudied. Here, we present information on the phenotypic and genotypic characteristics of a novel azole-resistant strain (CA108) that was initially identified as an *erg5* mutant completely lacking ergosterol, using gas chromatography and mass spectrometry (GC/MS) (24). Given the importance of ergosterol for maintaining fungal cell membrane integrity (1), we were keen to investigate how CA108 was able to persist as a clinical pathogen. CA108 was discovered to be cross resistant to azoles and amphotericin B and to harbor mutations in both *ERG11* (*CYP51*) and *ERG5* (*CYP61*).

MATERIALS AND METHODS

Strains and media. The isolates of *C. albicans* included in the present study were identified from a collection of clinical specimens collected as part of a European Union (EU) FP6 project, the European Resistance Fungal Network (EURESFUN). CA108 was isolated from the oral cavity (tongue) of a patient suffering from recurrent chronic oral candidosis. Three sterol wild-type (WT) isolates (here called CA6, CA14, and CA177) in which ergosterol comprised >80% of the total sterol fraction were selected from the EURESFUN collection for comparative experiments. CA6, CA14, and CA177 were isolated from a blood culture and tracheal and throat swabs, respectively. All were maintained at 37°C on yeast extract-peptone-dextrose (YEPD) agar containing (wt/vol) 2% glucose, 2% Bacto peptone, 1% yeast extract, and 2% agar (Formedium). RPMI 1640 medium (Sigma) buffered with 0.165 M MOPS (morpholinepropanesulfonic acid) (pH 7) was used to culture isolates for sterol analyses, antifungal susceptibility testing, and morphological examinations.

Sterol analysis. Single colonies were used to inoculate 15-ml volumes of RPMI 1640 to achieve overnight cultures (18 h; 37°C; 180 rpm). Cells were harvested by centrifugation and washed with sterile H_2O prior to sterol extraction using standard methodology (24). In addition to the analysis of untreated RPMI-grown cells, the sterol compositions of all isolates, following treatment with final concentrations of fluconazole and voriconazole equivalent to half the minimum concentration required to inhibit growth, were determined using GC/MS. Briefly, cell pellets were resuspended in 2.5 ml methanol, 1.5 ml potassium hydroxide (60% [wt/vol]), and 1 ml methanol-dissolved pyrogallol (0.5% [wt/vol]) and heated at 90°C for 2 h. Nonsaponifiable sterols were extracted into glass vials with three sequential 2-ml volumes of hexane. Extracts were evaporated to dryness using a centrifugal evaporator (Heto Maxi Dry Plus) and derivatized

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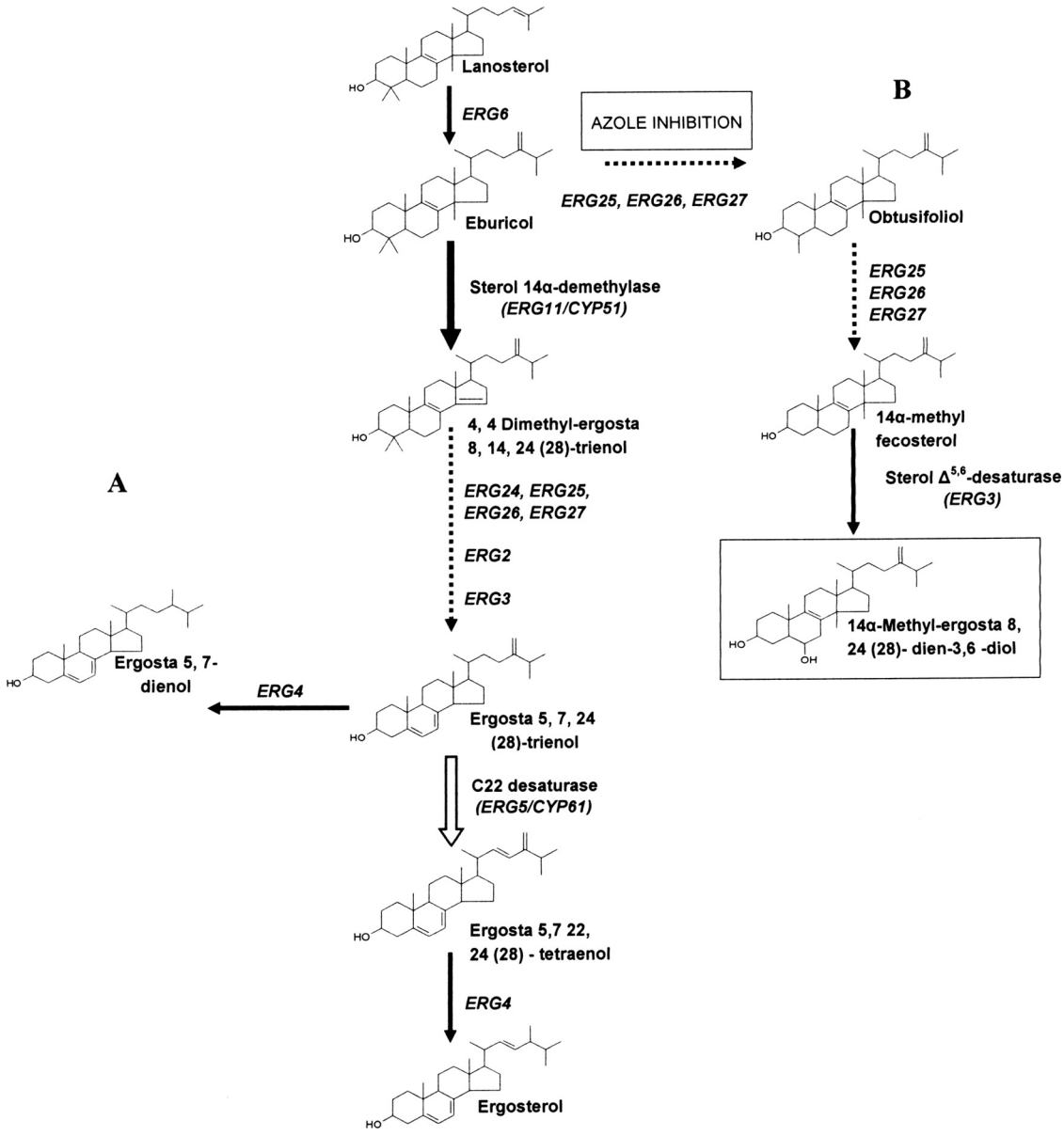

FIG. 1. Schematic representation of the ergosterol biosynthetic pathway in *C. albicans*. (A) Accumulation of ergosta 5,7-dienol with perturbation of C22 desaturase (encoded by *ERG5* [*CYP61*]) function but without azole inhibition of 14α-demethylase (encoded by *ERG11* [*CYP51*]). (B) Sterol intermediates that accumulate with classical azole inhibition of Erg11p; here, the end product is fungistatic 14α-methyl ergosta 8,24(28)-dien-3β,6α-diol. Open arrow, C22 desaturase (encoded by *ERG5*) step; broken arrows, multiple enzymatic steps; solid arrows, single enzymatic step.

{BSTFA [*N*,*O*-bis(trimethylsilyl) trifluoroacetamide]-TCMS [trimethylchlorosilane] [90:10] plus 50 μl anhydrous pyridine; 70°C} for 2 h. Tetramethylsilane (TMS)-derivatized sterols were analyzed and identified using GC/MS with reference to retention times and fragmentation spectra for known standards. The GC/MS data files were analyzed using Agilent software (MSD Enhanced ChemStation; Agilent Technologies Inc.) to determine sterol profiles for all isolates and for derivation of integrated peak areas.

Antifungal susceptibility testing. The susceptibilities of CA6, CA14, CA177, and CA108 to azole antifungals (fluconazole, voriconazole, itraconazole, ketoconazole, and clotrimazole) were determined using the standardized CLSI M27-A2 broth dilution method (20). The interpretive breakpoints for susceptibility to azoles were those employed by Sanguinetti et al. (27) and were as follows: (i) fluconazole, ≤8 μg ml^{-1}, sensitive; ≥64 μg ml^{-1}, resistant; (ii) voriconazole, ≤1 μg ml^{-1}, sensitive; ≥4 μg ml^{-1}, resistant; (iii) itraconazole and ketoconazole, ≤0.125 μg ml^{-1}, sensitive; ≥1 μg ml^{-1}, resistant; (iv) clotrimazole, ≥0.5 μg ml^{-1}, resistant. Assays for susceptibility to fluconazole and voriconazole were also performed in the presence of the multidrug efflux inhibitor (16, 27, 29) FK506 (Sigma). For these experiments, yeast cells were inoculated in microtiter plate wells containing doubling dilutions of the antifungal agent with a fixed concentration (10 μM) of FK506. Visual readings were made following 48 h of incubation at 37°C. MICs were determined as the minimum azole concentration yielding at least 80% inhibition of growth compared with the growth of control wells (16). The susceptibilities of isolates to amphotericin B were assayed as described previously (9). The MIC for amphotericin B was defined as the lowest drug concentration at which growth was completely inhibited (23).

PCR. Full-length *ERG5* and *ERG11* genes were amplified from genomic DNA (single-colony extraction: 0.2% SDS; 90°C; 10 min) using gene-specific forward (F) and reverse (R) primers: *ERG5*F, 5′-ATGAATTCAACAGAGGTC-3′; *ERG5*R, 5′-CTATAAACTCTTTAATGG3′; *ERG11*F, 5′-ATGGATATCGTA

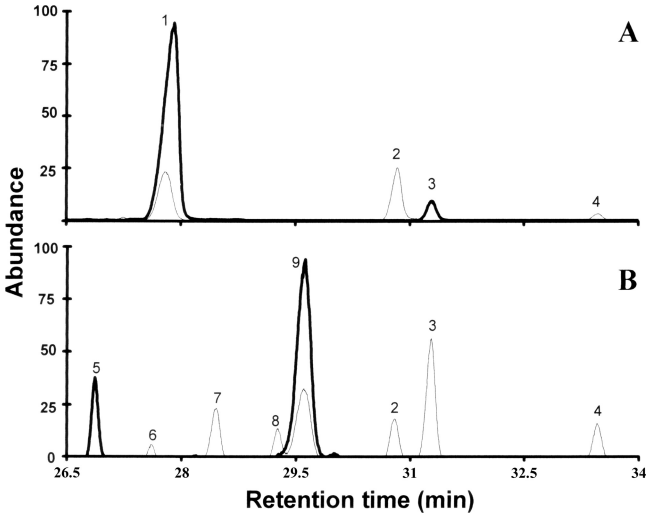

FIG. 2. Typical GC/MS chromatograms for sterol-WT (**A**) and *erg11 erg5* double mutant (CA108) (B) Isolates without treatment (bold traces) or following azole treatment (thin traces). 1, ergosterol; 2, 14α-methyl ergosta 8,24(28)-dien-3β,6α-diol; 3, lanosterol and/or obtusifoliol; 4, eburicol; 5, zymosterol; 6, 4,4-dimethylcholesta-8,14,24-trienol; 7, 14α-methylfecosterol; 8, 4,14α-dimethyl-cholesta-8,24-dienol; 9, ergosta 5,7-dienol.

CTAGAA-3′; *ERG11*R, 5′-TCATTGTTCAACATATTC-3′. DNA reads (Eurofins MWG Operon, United Kingdom) were screened for the presence of ambiguous nucleotide signals, translated into amino acid sequences (BioEdit), and aligned (Clustal X) to *C. albicans ERG5* and *ERG11* reference proteins (Uniprot accession no. O94016 and P10613, respectively).

Cell morphology. The morphologies of isolates were examined during growth on YEPD agar and 6 or 24 h following transfer of single colonies to liquid RPMI (37°C; 180 rpm). Light microscopy was used to identify yeast-like, psuedohyphal, and filamentous growth of the strains.

RESULTS

Sterol profiles of selected isolates. Untreated RPMI-grown CA6, CA14, and CA177 all exhibited WT sterol profiles (Fig. 2A) in which ergosterol comprised >85% of the total sterol fraction. No sterol intermediates indicative of perturbations in sterol 14α-demethylase (Erg11p) or C22 desaturase (Erg5p) functionality (Fig. 1A) were detected in any of these isolates. Conversely, CA108 exhibited a classical *erg5* mutant sterol profile (Fig. 2B) in which ergosta 5,7-dienol comprised >80% of the sterol fraction; C22-desaturated sterols, including ergosterol, were completely undetectable (Table 1). 14α-Methylated sterols, including the fungistatic compound 14α-methyl ergosta 8,24(28)-dien-3β-6α-diol (here called 14α-methyl-3,6-diol) accumulated in CA14 following all azole treatments (Table 1); this is indicative of classic azole inhibition of sterol 14α-demethylase activity in *C. albicans* (Fig. 1A). 14α-Methyl-3,6-diol was also seen following voriconazole, ketoconazole, itraconazole, and clotrimazole, but not fluconazole, treatment of CA108 (Table 1). The relatively high abundance of ergosta 5,7-dienol (>55% of the total sterol fraction) in fluconazole-treated CA108 is indicative of the fluconazole resistance observed (Table 2). It is noteworthy that the ergosta 5,7-dienol content of CA108 following all other (except clotrimazole) azole treatments decreased to <30%.

Antifungal susceptibilities of selected isolates. Despite displaying WT sterol profiles, CA6 and CA177 were found to be resistant to all azole antifungals (Table 2). In contrast, MIC values indicated that CA14 was sensitive to all azoles. CA108 was azole resistant, withstanding concentrations of 64, 8, 2, 1, and 2 μg ml^{-1} of fluconazole, voriconazole, itraconazole, ketoconazole, and clotrimazole, respectively (Table 2A). In the presence of the multidrug efflux inhibitor FK506, CA6, CA14, and CA177 were susceptible to fluconazole and voriconazole. Conversely, CA108 remained resistant to fluconazole and voriconazole despite inhibition of azole efflux mechanisms (efflux-inhibited MIC values, 16 and 4 μg ml^{-1}, respectively). All sterol-WT isolates were sensitive to amphotericin B (MIC values, <0.25 μg ml^{-1} for all); CA108 was not (MIC, 2 μg ml^{-1}).

***ERG11* and *ERG5* sequencing.** No amino acid substitutions were identified in the *ERG11* or *ERG5* protein translations for sterol-WT isolates (CA6, CA14, and CA177). Conversely, CA108 was found to harbor a single amino acid substitution (A114S) in Erg11p and, at position 116, a sequence insertion (duplication of the 10 amino acids ordinarily present between residues 106 and 115) in Erg5p (Fig. 3). The DNA sequence data indicated that the mutations detected were homozygous.

TABLE 1. Sterol composition of *C. albicans* sterol-WT (CA14) and *erg11 erg5* double-mutant (CA108) isolates following azole treatment

Sterol	Content (%)[a]											
	Sterol-WT							*erg11 erg5* double mutant				
	CA14	CA14 F	CA14 V	CA14 C	CA14 I	CA14 K	CA108	CA108 F	CA108 V	CA108 C	CA108I	CA108 K
Zymosterol							16.0	1.4				
4,4-Dimethylcholesta-8,14,24-trienol									3.4			
Ergosterol	86.8	45.5	47.3	30.0	33.0	36.4						
14α-Methylfecosterol								9.4	12.7			
4,14α-Dimethylcholesta-8,24-dienol								5.5	7.2			
Ergosta 5,7-dienol							82.4	57.1	27.4	42.6	25.0	24.4
Episterol							1.6					
14α-Methylergosta-8,24(28)-dien-3β,6α-diol		25.4	28.6	35.0	38.0	37.2			10.4	32.8	50.0	45.1
Lanosterol/obtusifoliol[b]	13.2	25.0	24.1	35.0	29.0	26.4		25.7	29.5	16.4	16.7	20.3
Eburicol		4.1						0.9	9.4	8.2	8.3	10.2

[a] F, fluconazole; V, voriconazole; C, clotrimazole; I, itraconazole; K, ketoconazole.
[b] 14α-Methylated sterols with identical molecular weights and retention times.

TABLE 2. MIC data for sterol-WT isolates (CA6, CA14, and CA177) and the *erg11 erg5* double mutant (CA108) determined using the broth microdilution method[a]

Isolate	MIC (μg ml^{-1})[b]							
	Standard azole assay					Presence of FK506[c]		Amphotericin B assay[d]
	FLU	VORI	ITRA	KETO	CLOT	FLU	VORI	
CA6	**256**	**16**	**16**	**16**	**1**	1.0	<0.0625	0.25
CA14	1.0	0.0625	0.0625	0.0625	0.0625	1.0	<0.0625	0.25
CA177	**256**	**16**	**16**	**16**	**1**	0.5	<0.0625	0.25
CA108	**64**	**8**	**2**	**1**	**2**	**16**	**4**	2

[a] NCCLS document M27-A2 (20).
[b] FLU, fluconazole; VORI, voriconazole; ITRA, itraconazole; KETO, ketoconazole; CLOT, clotrimazole. Values above the suggested clinical breakpoints (27) are in boldface.
[c] 10 μM (27).
[d] Reference 9.

Morphological observations. There were no obvious differences between the cell morphologies of the sterol-WT isolates and CA108 following culture at 37°C (Fig. 4). All exhibited yeast-like growth at stationary phase on YEPD agar (Fig. 4A). Following transfer to RPMI, pseudohyphae were observed in all cultures after 6 h (Fig. 4B), with extensive hyphal growth seen after 24 h (Fig. 4C).

DISCUSSION

This constitutes the first report of a clinical *C. albicans* isolate harboring mutations in both *ERG11* (encoding sterol 14α-demethylase) and *ERG5* (encoding C22 desaturase). It demonstrates, for the first time, the potential for mutations in *ERG5* to confer a selective advantage against amphotericin B treatment on a clinical isolate.

That CA108 was an *erg5* mutant could be deduced from the accumulation of ergosta 5,7-dienol and the lack of ergosterol in the GC/MS sterol profile for the isolate (Fig. 2B, bold trace); this is indicative of loss of C22 desaturase activity (Fig. 1). Complementation experiments in which *ERG5* alleles from CA108 are introduced into an *ERG5* deletion strain are now required to verify that CA108 Erg5p is entirely nonfunctional. Nonetheless, the absence of detectable ergosterol can account for the ability of CA108 to withstand concentrations of amphotericin B (up to 2 μg ml^{-1}) 8-fold higher than those tolerated by sterol-WT (>80% membrane ergosterol) comparator isolates (Table 2). However, given that the primary target of azole antifungals is sterol 14α-demethylase (Erg11p), not C22 desaturase (Erg5p), the sterol data alone provide few clues to the mechanism of azole resistance in CA108.

Because the fungistatic sterol 14α-methyl-3,6-diol (1) was not observed to accumulate in CA108 following treatment with fluconazole (Table 1), it was initially reasoned that the mutant Erg5p might function as an azole-binding cytochrome P450 that sequesters free azole away from the target Erg11p (6, 7), thus allowing the latter protein to retain activity. However, the possibility that CA108 Erg11p could harbor amino acid substitutions that might also contribute to azole resistance (10, 12, 25, 33) was never discounted. Importantly, detectable levels of 14α-methyl-3,6-diol accumulated in CA108 following treatment with voriconazole, ketoconazole, itraconazole, and clotrimazole (Table 1). This appears to be consistent with confirmation of the amino acid substitution A114S in CA108 Erg11p. A114S occurs near the substrate access channel in Erg11p (5)

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          100         110         120
....|....|  ....|....|  ....|....|
IMTVYLGPKG  HEFVFNAKLS  DVSAEDAYKH
IMTVYLGPKG  HEFVFNAKLS  DVSSEDAYKH   A
**********  **********  ***:******

          110         120         130
....|....|  ....|....|  ....|....|
VIASSRDLAR  KILSS-----  -----PKYVK
VIASSRDLAR  KILSSRDLAR  KILSSPKYVK   B
**********  *****       *****
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FIG. 3. Protein sequences for wild-type *C. albicans* (top) aligned with those for CA108, the *erg11 erg5* double mutant (bottom). (A) Sterol 14α-demethylase (encoded by *ERG11*) with the A114S substitution in boldface. (B) C22 desaturase (encoded by *ERG5*) with sequence duplication (of residues 106 to 115) in boldface.

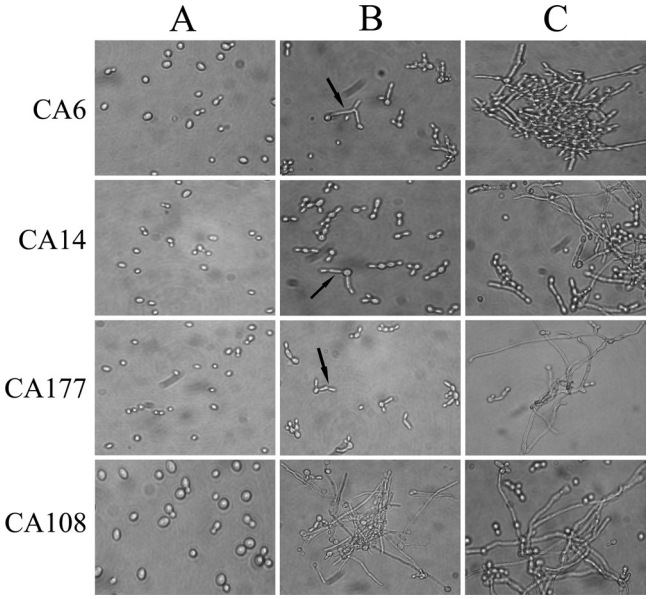

FIG. 4. Cell morphology of sterol-WT isolates (CA6, CA14, and CA177) and the *erg11 erg5* double mutant (CA108) cultured at 37°C. (A) Yeast-like growth during stationary phase on YEPD agar. (B) Pseudohyphal morphology (arrows) in RPMI medium (6 h; 180 rpm). (C) True hyphal formation in RPMI (24 h; 180 rpm).

and has been associated with fluconazole resistance in *C. albicans* (34). However, the importance of A114S for azole resistance has not been confirmed biochemically and thus requires further investigation.

Activities of drug efflux transporters provide the likely explanation for high-level azole resistance observed in sterol-WT isolates CA6 and CA177 (Table 2). Owing to their ergosterol contents, both were susceptible to amphotericin B (Table 2). However, while dual-drug therapies (e.g., fluconazole and amphotericin B [22]) constitute one option available to clinicians who encounter problematic *C. albicans* strains (e.g., CA6 and CA177), the results for CA108 highlight potential drawbacks of combination treatments. It is not known if the patient received antifungal therapy immediately before isolation of CA108; however, the patient is understood to have been treated with several antifungal drugs, including polyenes, azoles, and caspofungin. It is possible that fluconazole treatment might have selected for the A114S Erg11p mutation in CA108 with amphotericin B later (or simultaneously) exerting selection pressure toward perturbation or loss of Erg5p activity.

Traditionally, research has focused on the importance of sterol 14α-demethylase activity and membrane ergosterol for maintaining the integrity of fungal cell membranes. Nonetheless, viable *erg3* mutants lacking functional sterol $\Delta^{5,6}$-desaturase (encoded by *ERG3*), and thus ergosterol, have been isolated from the clinic (8, 9, 21). Such *erg3* mutants are able to grow with ergosta 7,22-dienol (or 14α-methylfecosterol following azole inhibition of Erg11p) as the major membrane sterol component. Due to deleterious mutations in *ERG3*, these strains do not accumulate fungistatic 14α-methyl-3,6-diol associated with the mode of azole action in *C. albicans* (Fig. 1B). Like CA108 (in which ergosta 5,7-dienol replaces ergosterol), *erg3* mutants are also understood to show cross-resistance to fluconazole and amphotericin B.

The switch between yeast-like and hyphal growth is understood to be a virulence factor in *C. albicans* (28). It is noteworthy that morphological examination revealed no impairment in the ability of CA108 to form hyphae (Fig. 4). *C. albicans erg3* mutants are reportedly impaired in the ability to form filaments (3, 19), possibly because they lack the ergosterol-rich rafts associated with the leading edge of developing hyphae (17); this does not appear to be true of *erg5* mutants. It has been postulated (3) that changes in sterol composition may adversely affect membrane-localized components of the signal transduction pathways involved in regulation of cell morphology (18). Studies of the membrane properties of CA108 now constitute one avenue for future research. Sterol modifications in *erg3* mutants influence the membrane properties and viability of fungal cells before and after fluconazole treatment (1). Further investigations of how sterol-lipid interactions might contribute to resistance in CA108 are now required.

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REFERENCES

1. **Abe, F., K. Usui, and T. Hiraki.** 2009. Fluconazole modulates membrane rigidity, heterogeneity, and water penetration into the plasma membrane in *Saccharomyces cerevisiae*. Biochemistry **48:**8494–8504.
2. **Baillie, G. S., and L. J. Douglas.** 2000. Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. J. Antimicrob. Chemother. **46:**397–403.
3. **Chau, A. S., M. Gurnani, R. Hawkinson, M. Laverdiere, A. Cacciapuoti, and P. M. McNicholas.** 2005. Inactivation of sterol Delta(5,6)-desaturase attenuates virulence in *Candida albicans*. Antimicrob. Agents Chemother. **49:** 3646–3651.
4. **Franz, R., S. L. Kelly, D. C. Lamb, D. E. Kelly, M. Ruhnke, and J. Morschhauser.** 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob. Agents Chemother. **42:**3065–3072.
5. **Jiang, W.-S., S.-S. Tan, G.-Y. Jiang, and Q.-X. Chen.** 2006. Synergistic effects of terbinafine combined with fluconazole or itraconazole on stable fluconazole-resistant *Candida albicans* induced by fluconazole in vitro. Chin. J. Immunol. **26:**360–364.
6. **Kelly, S. L., D. C. Lamb, B. C. Baldwin, A. J. Corran, and D. E. Kelly.** 1997. Characterization of *Saccharomyces cerevisiae* CYP61, sterol Delta(22)-desaturase, and inhibition by azole antifungal agents. J. Biol. Chem. **272:**9986–9988.
7. **Kelly, S. L., D. C. Lamb, and D. E. Kelly.** 1997. Sterol 22-desaturase, cytochrome P45061, possesses activity in xenobiotic metabolism. FEBS Lett. **412:**233–235.
8. **Kelly, S. L., D. C. Lamb, D. E. Kelly, J. Loeffler, and H. Einsele.** 1996. Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. Lancet **348:**1523–1524.
9. **Kelly, S. L., D. C. Lamb, D. E. Kelly, N. J. Manning, J. Loeffler, H. Hebart, U. Schumacher, and H. Einsele.** 1997. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol Delta(5,6)-desaturation. FEBS Lett. **400:**80–82.
10. **Kelly, S. L., D. C. Lamb, J. Loeffler, H. Einsele, and D. E. Kelly.** 1999. The G464S amino acid substitution in *Candida albicans* sterol 14 alpha-demethylase causes fluconazole resistance in the clinic through reduced affinity. Biochem. Biophys. Res. Commun. **262:**174–179.
11. **Lamb, D., D. Kelly, and S. Kelly.** 1999. Molecular aspects of azole antifungal action and resistance. Drug Resist. Update **2:**390–402.
12. **Lamb, D. C., D. E. Kelly, W. H. Schunck, A. Z. Shyadehi, M. Akhtar, D. J. Lowe, B. C. Baldwin, and S. L. Kelly.** 1997. The mutation T315A in *Candida albicans* sterol 14 alpha-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. J. Biol. Chem. **272:**5682–5688.
13. **Lamb, D. C., D. E. Kelly, M. R. Waterman, M. Stromstedt, D. Rozman, and S. L. Kelly.** 1999. Characteristics of the heterologously expressed human lanosterol 14 alpha-demethylase (other names: P45014DM, CYP51, P45051) and inhibition of the purified human and *Candida albicans* CYP51 with azole antifungal agents. Yeast **15:**755–763.
14. **Loeffler, J., S. L. Kelly, H. Hebart, U. Schumacher, C. Lass-Floerl, and H. Einsele.** 1997. Molecular analysis of cyp51 from fluconazole-resistant *Candida albicans* strains. FEMS Microbiol. Lett. **151:**263–268.
15. **Maebashi, K., M. Kudoh, Y. Nishiyama, K. Makimura, K. Uchida, T. Mori, and H. Yamaguchi.** 2002. A novel mechanism of fluconazole resistance associated with fluconazole sequestration in *Candida albicans* isolates from a myelofibrosis patient. Microbiol. Immunol. **46:**317–326.
16. **Maesaki, S., P. Marichal, M. A. Hossain, D. Sanglard, H. V. Bossche, and S. Kohno.** 1998. Synergistic effects of tacrolimus and azole antifungal agents against azole-resistant *Candida albicans* strains. J. Antimicrob. Chemother. **42:**747–753.
17. **Martin, S. W., and J. B. Konopka.** 2004. Lipid raft polarization contributes to hyphal growth in *Candida albicans*. Eukaryot. Cell **3:**675–684.
18. **Miwa, T., Y. Takagi, M. Shinozaki, C. W. Yun, W. A. Schell, J. R. Perfect, H. Kumagai, and H. Tamaki.** 2004. Gpr1, a putative G-protein-coupled receptor, regulates morphogenesis and hypha formation in the pathogenic fungus *Candida albicans*. Eukaryot. Cell **3:**919–931.
19. **Miyazaki, T., Y. Miyazaki, K. Izumikawa, H. Kakeya, S. Miyakoshi, J. E. Bennett, and S. Kohno.** 2006. Fluconazole treatment is effective against a *Candida albicans* erg3/erg3 mutant in vivo despite in vitro resistance. Antimicrob. Agents Chemother. **50:**580–586.
20. **National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2. National Committee for Clinical Laboratory Standards, Wayne, PA.
21. **Nolte, F. S., T. Parkinson, D. J. Falconer, S. Dix, J. Williams, C. Gilmore, R. Geller, and J. R. Wingard.** 1997. Isolation and characterization of fluconazole- and amphotericin B-resistant *Candida albicans* from blood of two patients with leukaemia. Antimicrob. Agents Chemother. **41:**196–199.
22. **Pappas, P. G., P. Chetchotisakd, R. A. Larsen, W. Manosuthi, M. I. Morris, T. Anekthananon, S. Sungkanuparph, K. Supparatpinyo, T. L. Nolen, L. O. Zimmer, A. S. Kendrick, P. Johnson, J. D. Sobel, and S. G. Filler.** 2009. A

phase II randomized trial of amphotericin B alone or combined with fluconazole in the treatment of HIV-associated cryptococcal meningitis. Clin. Infect. Dis. **48:**1775–1783.

23. **Park, B. J., B. A. Arthington-Skaggs, R. A. Hajjeh, N. Iqbal, M. A. Ciblak, W. Lee-Yang, M. D. Hairston, M. Phelan, B. D. Plikaytis, A. N. Sofair, L. H. Harrison, S. K. Fridkin, and D. W. Warnock.** 2006. Evaluation of amphotericin B interpretive breakpoints for *Candida* bloodstream isolates by correlation with therapeutic outcome. Antimicrob. Agents Chemother. **50:**1287–1292.
24. **Parker, J. E., M. Merkamm, N. J. Manning, D. Pompon, S. L. Kelly, and D. E. Kelly.** 2008. Differential azole antifungal efficacies contrasted using a *Saccharomyces cerevisiae* strain humanized for sterol 14 alpha-demethylase at the homologous locus. Antimicrob. Agents Chemother. **52:**3597–3603.
25. **Sanglard, D., F. Ischer, L. Koymans, and J. Bille.** 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14 alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob. Agents Chemother. **42:**241–253.
26. **Sanglard, D., K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille.** 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. **39:**2378–2386.
27. **Sanguinetti, M., B. Posteraro, B. Fiori, S. Ranno, R. Torelli, and G. Fadda.** 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. Antimicrob. Agents Chemother. **49:**668–679.
28. **Saville, S. P., A. L. Lazzell, C. Monteagudo, and J. L. Lopez-Ribot.** 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryot. Cell **2:**1053–1060.
29. **Sun, S., Y. Li, Q. Guo, C. Shi, J. Yu, and L. Ma.** 2008. *In vitro* interactions between tacrolimus and azoles against *Candida albicans* determined by different methods. Antimicrob. Agents Chemother. **52:**409–417.
30. **Venkateswarlu, K., D. W. Denning, N. J. Manning, and S. L. Kelly.** 1995. Resistance to fluconazole in *Candida albicans* from AIDS patients correlated with reduced intracellular accumulation of drug. FEMS Microbiol. Lett. **131:**337–341.
31. **Watson, P. F., M. E. Rose, S. W. Ellis, H. England, and S. L. Kelly.** 1989. Defective sterol C5-6 desaturation and azole resistance—a new hypothesis for the mode of action of azole antifungals. Biochem. Biophys. Res. Commun. **164:**1170–1175.
32. **White, T. C.** 1997. Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob. Agents Chemother. **41:**1482–1487.
33. **White, T. C.** 1997. The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14 alpha-demethylase in *Candida albicans*. Antimicrob. Agents Chemother. **41:**1488–1494.
34. **Xu, Y. H., L. M. Chen, and C. Y. Li.** 2008. Susceptibility of clinical isolates of *Candida* species to fluconazole and detection of *Candida albicans ERG11* mutations. J. Antimicrob. Chemother. **61:**798–804.