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Two Clinical Isolates of *Candida glabrata* Exhibiting Reduced Sensitivity to Amphotericin B Both Harbor Mutations in *ERG2*

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Two novel isolates of *Candida glabrata* exhibiting reduced sensitivity to amphotericin B (MIC, 8 μg ml^{-1}) were found to be *ERG2* mutants, wherein Δ^8-sterol intermediates comprised >90% of the total cellular sterol fraction. Both harbored an alteration at Thr^{121} in ERG2; the corresponding residue (Thr^{119}) in *Saccharomyces cerevisiae* is essential for sterol Δ8-Δ7 isomerization. This constitutes the first report of *C. glabrata* harboring mutations in *ERG2* and exhibiting reduced sensitivity to amphotericin B.

Amphotericin B (AMB) is one of a limited number of antifungals that are available for the treatment of azole-resistant fungi (8). In contrast to azoles that target ergosterol biosynthesis through inhibition of sterol 14α-demethylase activity (*ERG11*) (Fig. 1), polyenes intercalate directly with membrane ergosterol (9), forming channels that leak monovalent ions (K^+, Na^+, H^+, Cl^-), causing cell lysis (2). Aside from solubility and host toxicity issues, the utility of amphotericin B is compromised by the emergence of strains with reduced sensitivity (12, 29) and by species that are intrinsically less susceptible (*Aspergillus flavus*, *Aspergillus terreus* [25], *Candida lusitaniae* [22], *Pneumocystis jirovecii* [1]).

Unlike mechanisms governing azole resistance (drug efflux [5], altered *ERG11* [17], and mutations in *ERG3* [13]), those that influence the sensitivity of pathogenic fungi to polyenes are poorly understood. Polyene susceptibility is related to fungal sterol composition and changes that result from *ERG* gene mutations (Fig. 1). Decreased sensitivity to polyenes is documented in clinical isolates of *Candida albicans* with alterations in *ERG3* (13, 19), *ERG11* (26), and *ERG5* (18). It has also been reported in an *ERG11* gene deletion strain of *Candida glabrata* (7) and in isolates harboring mutations in *ERG1* (30), *ERG6* (31, 32), and *ERG11* (10). We previously reported a clinical isolate of *Cryptococcus neoformans* with defective C8-isomerase activity, exhibiting reduced sensitivity to polyenes (14). Here we describe two novel clinical isolates of *C. glabrata* (CG852 and CG872) that showed reduced susceptibility to amphotericin B and harbored *ERG2* mutations.

Strains in the present study were obtained from the European Resistance Fungal Network (EURESFUN; EU FP6 project) collection, established for the investigation of antifungal resistance mechanisms (10, 18, 19). CG852 and CG872 were isolated from separate patients receiving treatment for fungal sepsis following organ transplantation and maintained with previously reported comparator strains (10) at 37°C on yeast extract peptone dextrose (YEPD). All were assayed for susceptibility to fluconazole (FLC), voriconazole (VRC), and amphotericin B (AMB) using standard broth dilution methodology (4) in the presence and absence of FK506, a putative multidrug efflux inhibitor (18) (Table 1). Gas chromatography-mass spectrometry (18, 19) was used to analyze sterol composition (Table 2 and Fig. 2) before and after the treatment of isolates with final concentrations of FLC and VRC equivalent to half the minimum required for growth inhibition (MIC × 0.5). *ERG11* and *ERG2* sequences were amplified from genomic DNA (single-colony extraction; 0.2% SDS, 90°C, 10 min) using the following gene-specific forward (F) and reverse (R) primers: *ERG11*F, 5′-ATGTCCACTGAAAACACT-3′; *ERG11*R, 5′-CTAG TACTTTTGTTCTGG-3′; *ERG2*F, 5′-ATGAAGTTCTTTATCAA T-3′; *ERG2*R, 5′-TTAGAACTTTTGGTTTTG-3′. PCR products were translated to amino acid sequences and aligned to *C. glabrata* *ERG11* and *ERG2* reference proteins (GenBank accession numbers P50859 and Q6FKL1, respectively). To verify the significance of amino acid substitutions detected in CG44, CG388, CG852, CG872, and CG1012 (Fig. 3), *ERG2* genes from additional EURESFUN isolates exhibiting a wild-type sterol composition (CG25, CG26, CG27, CG29, and CG30) were sequenced.

Azole treatment of *C. glabrata* is known to be compromised by the activity of drug efflux mechanisms (5, 27), and our data (Table 1; efflux-inhibited MIC values) support this knowledge. Similarly, the growth of all isolates in the presence of amphotericin B at ≥2 μg ml^{-1} also supports findings from other studies (6, 21, 23) which suggest that *C. glabrata* is inherently less sensitive to polyenes than other fungi. It is noteworthy that FK506 reduced the azole MICs of CG852 and CG872 far more than other strains (Table 1); in the absence of compensatory drug efflux mechanisms, their altered sterol content (Table 2; Δ^8-sterol intermediates were >90% of the total) may affect membrane permeability to azoles and/or azole transport. The accumulation of ergosta-5,8,22-trienol in CG852 and CG872 (Fig. 2B) may also account for their reduced sensitivity to amphotericin B; wild-type comparator strains comprising >80% ergosterol, the primary target of polyenes, were 4-fold more sensitive (Table 1).

No alterations in ERG11 protein sequences were detected in any of the study isolates; this is consistent with sterol data (Fig. 2).

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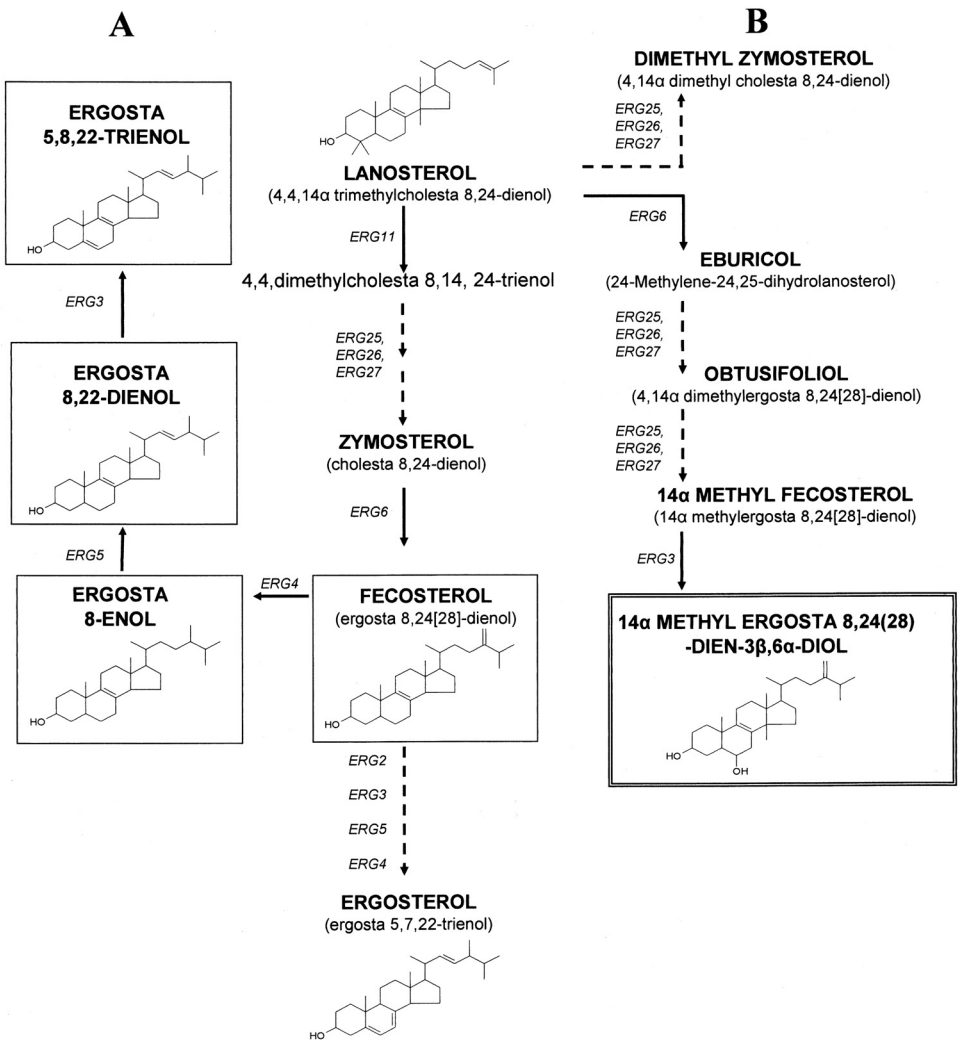

FIG 1 Schematic representation of the ergosterol biosynthetic pathway in *C. glabrata*. (A) Sterol intermediates (boxed with a single line) that accumulate due to perturbations in C8-isomerase (*ERG2* protein) activity. (B) Sterol intermediates that accumulate following azole inhibition of sterol 14α-demethylase (ERG11 protein). The fungistatic sterol 14α-methylergosta-8,24(28)-dien-3β,6α-diol is highlighted (boxed with a double line). Broken arrows, multiple enzymatic steps; unbroken arrows, single enzymatic step. *ERG3*, *ERG4*, *ERG5*, *ERG6*, *ERG25*, *ERG26*, and *ERG27* encode C5-desaturase, C24-reductase, C22-desaturase, C24-methyl transferase, C4-methyloxidase, C4-decarboxylase, and C3-ketoreductase, respectively.

Briefly, the accumulation of 14α-methylated sterols following azole treatment with FLC or VRC (Table 2) indicates classical azole inhibition of sterol 14α-demethylase activity (Fig. 1). Conversely, several amino acid changes (Fig. 3) were identified in *ERG2* protein translations and were as follows: (i) I207V, all isolates; (ii) L60F, present only in CG852 and CG29; (iii) T121V, CG852 only; and (iv) T121I, CG872 only. That replacement of Thr^{121} with valine or isoleucine (CG852 and CG872, respectively) impaired ERG2 function (Table 2; trace amounts of ergosterol) is consistent with a prior investigation of the equivalent threonine residues in human emopamil binding protein (Thr^{126}), *Zea mays* 8,7SI (Thr^{124}), and *Saccharomyces cerevisiae* ERG2 (Thr^{119}); all are required for sterol Δ8-Δ7 isomerization (20, 24). It has been postulated that this threonine residue might form a hydrogen bond with the 3-hydroxy group of the sterol substrate, locating it in the active site of the isomerase protein (24).

TABLE 1 MIC data determined for fluconazole and voriconazole (with or without 10 μM FK506) and amphotericin B[a]

Isolate[b]	MIC (μg ml^{-1})				
	CG44	CG388	CG1012	CG852	CG872
FLC	64	64	64	128	64
FLC + FK506	32	32	32	8	4
VRC	2	2	2	2	1
VRC + FK506	0.5	0.5	0.5	0.125	0.0625
AMB	2	2	2	8	8

[a] FK506 is a putative multidrug efflux inhibitor.
[b] Additional isolates (CG25, CG26, CG27, CG29, and CG30) selected for *ERG2* sequencing exhibited the same azole and polyene sensitivity as CG44, CG388, and CG1012.

Given that ERG2 is not the target of azoles or polyenes, the factors that resulted in the selection of *ERG2* mutations in CG852 and CG872 are of interest. Polyene-resistant *Candida* can be selected using amphotericin B (3), and polyene-resistant strains of *Ustilago maydis* possessing defective ERG2 have also been re-

TABLE 2 Sterol (%) composition of untreated, fluconazole-treated, or voriconazole-treated isolates of *C. glabrata*

	% of each sterol in the total sterol composition of each isolate[a]														
	Untreated					FLC-treated					VRC-treated				
Sterol	44	388	1012	852	872	44	388	1012	852	872	44	388	1012	852	872
Ergosta-5,8,22-trienol				**59.7**	**51.8**				8.4	14.3				7.3	17.3
Zymosterol	3.2	3.1	5.0												
Ergosta-8,22-dienol				4.4	4.5					1.9					
Ergosterol	**75.5**	**82.7**	**77.6**	4.1	4.2	**50.0**	**63.8**	40.0			43.5	**60.1**	37.9		
Ergosta-7,22-dienol	1.5	1.1	1.7	1.1	1.7				1.6						
Fecosterol	2.6	2.6	1.7	11.8	13.9				4.0					7.2	
4,4 dimethyl cholesta-8,24-dienol									3.4	1.4				6.6	
Ergosta-8-enol	0.5	0.6	0.4	17.6	22.4										
Ergosta-5,7-dienol	4.3	3.0	3.4												
Episterol	2.2	1.4	2.3												
Ergosta-7-enol	0.5		0.7												
14α-methyl-3,6-diol[b]							6.4	10.0	29.7	**60.4**	11.4	7.4	15.6	31.6	**51.5**
Lanosterol/obtusifoliol[c]	3.6	2.5	3.3			**50.0**	29.8	**50.0**	**52.2**	21.2	**45.2**	32.5	**46.5**	**47.3**	31.2
Unknown	1.7	0.6	0.9	1.3	1.5				0.7	0.8					
Dimethyl zymosterol	4.3	2.4	2.9												

[a] The percentage of the most abundant sterol in each isolate is shown in bold. All cultures were treated with final azole concentrations equivalent to 0.5 times the MIC. Additional isolates, CG25, CG26, CG27, CG29, and CG30, all exhibited wild-type sterol composition (>80% ergosterol).
[b] Fungistatic 14α-methylergosta-8,24(28)-dien-3β,6α-diol.
[c] 14α-methylated sterols with identical molecular weight (MW) and retention time.

ported (11). There is some evidence that clinical prophylactic use of polyenes may select for resistant fungi (15); thus, it is possible that such pressure resulted in the selection of mutations occurring in the *ERG2* genes of CG852 and CG872. Interestingly, yeast ERG2 binds several clinically relevant drugs (e.g., haloperidol, opipramol, and pentazocine), and novel compounds developed for other receptor systems also interact (16). Although specific information regarding the treatment history of the patients from whom CG852

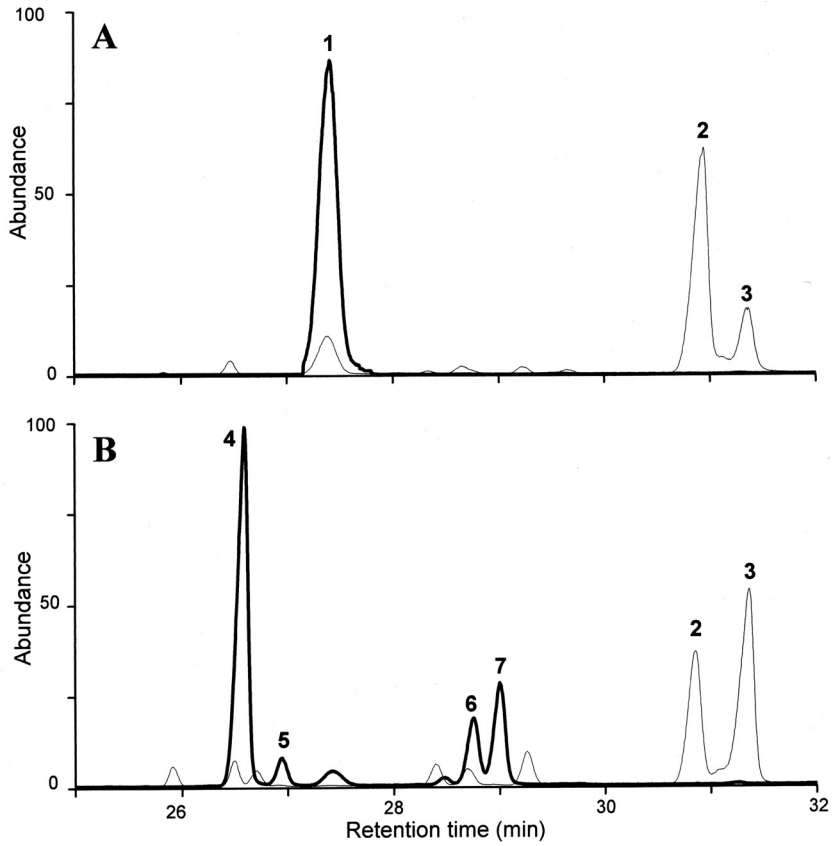

FIG 2 Typical sterol chromatograms for wild-type (WT) sterol (A) and *ERG2* mutant (B) isolates following growth on YEPD medium (bold traces) and after treatment with an FLC concentration equivalent to 0.8 times the MIC (thin traces). Sterol intermediates are as follows: 1, ergosterol (ergosta-5,7,22-trienol); 2, 14α-methylergosta-8,24(28)-dien-3β,6α-diol; 3, lanosterol; 4, ergosta-5,8,22-trienol; 5, ergosta-8,22-dienol; 6, fecosterol (ergosta-8,24[28]-dienol); 7, ergosta-8-enol.

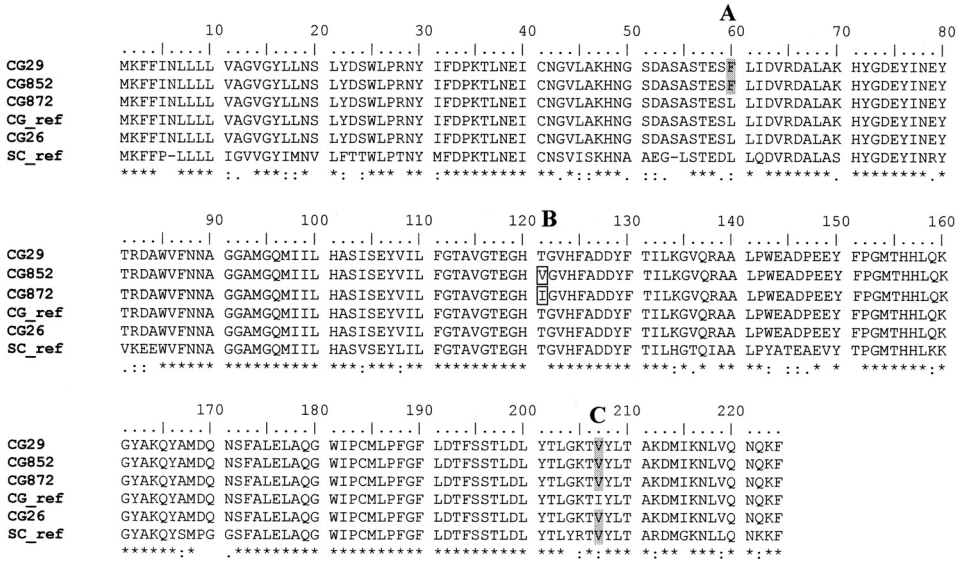

FIG 3 Sequence alignment of *Candida glabrata* (CG) and *Saccharomyces cerevisiae* (SC) C8-isomerase (*ERG2*) proteins; CG_ref and SC_ref denote reference sequences deposited in the ExPASy protein database (accession numbers Q6FKL1 and P32352, respectively). Positions of amino acid substitutions identified in experimental CG isolates are highlighted (A, B, and C). The Clustal consensus sequence indicates absolutely conserved residues (*), conserved strong (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW) groups (:), and conserved weaker (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY) groups (.) (http://www.clustal.org/).

and CG872 were isolated is limited, both were organ transplant recipients receiving immunosuppressive drugs. A novel immunosuppressant (SR 31747) has been shown to inhibit ERG2 activity in *S. cerevisiae* (28), and it is tempting to speculate that *ERG2* mutations may be selected for by unexpected or hitherto unforeseen ligands.

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