

## Drug resistance genes and trailing growth in *Candida albicans* isolates

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**Objectives:** To investigate possible molecular mechanisms of azole resistance among fluconazole-susceptible bloodstream isolates of *Candida albicans* that displayed the trailing growth phenomenon, and to compare these isolates with bloodstream and mucosal isolates that showed reduced susceptibilities to fluconazole.

**Methods:** Twelve *C. albicans* isolates—seven trailing and five susceptible dose dependent (SDD) or resistant (R)—were screened for *ERG11* mutations by DNA sequencing and quantification of *ERG11*, *CDR1* and *MDR1* expression by RT-PCR using the LightCycler high-speed PCR system.

**Results:** SDD and R isolates possessed more homozygous *ERG11* mutations than did the trailing isolates. Two of these, V404I and V509M, have not been described previously and were found exclusively in fluconazole SDD and R isolates. Quantification of *ERG11* expression revealed that both trailing and SDD and R isolates were capable of *ERG11* up-regulation in response to fluconazole, although the SDD and R isolates showed maximal up-regulation at higher fluconazole concentrations. Quantification of *CDR1* and *MDR1* revealed that all isolates, regardless of *in vitro* fluconazole response, were capable of *CDR1* and *MDR1* up-regulation following fluconazole exposure. Furthermore, the SDD and R isolates expressed higher constitutive levels of *CDR1* and *MDR1* or *CDR1*, respectively, in the absence of drug compared with trailing isolates.

**Conclusions:** Trailing isolates, although susceptible to fluconazole, express the same molecular mechanisms as SDD and R isolates following fluconazole exposure but regulate them differently.

Keywords: *C. albicans*, azole drug resistance, molecular mechanisms

### Introduction

The term ‘trailing’ has been used to describe the reduced but persistent growth that some isolates of *Candida albicans* and *Candida tropicalis* exhibit at drug concentrations above the MIC in broth dilution tests with azole antifungal agents, such as fluconazole and itraconazole. This trailing growth can be so great as to make an isolate that appears susceptible after 24 h of incubation appear resistant at 48 h.<sup>1</sup> However, two independent studies using murine models of invasive candidiasis and trailing isolates of *C. albicans* showed that the 24 h MIC result was consistent with the response to treatment *in vivo*.<sup>2,3</sup> These data imply that isolates demonstrating trailing growth are susceptible rather than resistant. This concept has been supported by a clinical demonstration showing that oropharyngeal candidiasis due to such isolates responded to a low dose of fluconazole used to treat typical susceptible isolates.<sup>4</sup>

It has been suggested recently that trailing may be due, at least in part, to the ability of *C. albicans* isolates to up-regulate, in response to

drug exposure, the transcription of genes encoding the azole drug target, lanosterol demethylase (*ERG11*), the terbinafine target, squalene epoxidase (*ERG1*) or the azole and terbinafine efflux transporters (*CDR1*, *CDR2* and *MDR1*).<sup>5</sup> These same mechanisms, as well as point mutations in *ERG11*, have been implicated in the development of azole drug resistance in *C. albicans*.<sup>6</sup> However, potential differences in the expression of these mechanisms—between susceptible isolates that display trailing growth and non-trailing isolates with reduced azole susceptibilities—have not been investigated.

The purpose of the present study was to examine possible molecular mechanisms of azole resistance among fluconazole-susceptible bloodstream isolates of *C. albicans* that displayed the trailing growth phenomenon, and to compare these isolates with isolates that showed reduced susceptibilities to fluconazole. We used DNA sequencing to reveal any sequence variation in the azole drug target, *ERG11*, and reverse transcription coupled with LightCycler real-time PCR to quantify expression of *ERG11* and the azole antifungal drug efflux genes, *CDR1* and *MDR1*.

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## Materials and methods

### Isolates

Twelve isolates of *C. albicans* (nine bloodstream and three mucosal) with varying *in vitro* fluconazole susceptibilities were selected. Bloodstream isolates were from a collection derived from active population-based surveillance for candidaemia conducted during 1998–2000.<sup>1</sup> Mucosal isolates were from HIV-infected persons with oropharyngeal or vaginal candidiasis.<sup>7–9</sup> Isolates were stored at  $-70^{\circ}\text{C}$  as 30% glycerol stocks in sterile water. Prior to testing, isolates were subcultured on Sabouraud dextrose agar (SAB) plates (BBL, Cockeysville, MD, USA) at  $35^{\circ}\text{C}$ .

### Broth microdilution susceptibility testing method

MICs of fluconazole, itraconazole and voriconazole were determined by the NCCLS M27-A broth dilution method.<sup>10</sup> Standard powders of fluconazole and voriconazole were received as gifts from Pfizer Pharmaceuticals Group (Groton, CT, USA), and itraconazole drug powder was purchased from Research Diagnostics, Inc. (Flanders, NJ, USA). The final concentrations of the antifungal agents were in the range fluconazole 0.125–64 mg/L and itraconazole and voriconazole 0.015–8 mg/L. The MIC endpoints were read visually following 24 and 48 h of incubation and were defined as the lowest concentration that produced a prominent reduction in growth ( $\sim 50\%$ ) compared with that of the drug-free growth control. Interpretations of MICs were assigned according to the NCCLS criteria.<sup>10</sup> Trailing growth was defined as a susceptible MIC after 24 h incubation and a resistant MIC after 48 h incubation.

### Sterol quantification method

Total cellular ergosterol was quantified as described previously.<sup>1,3</sup> The sterol quantification method MIC of fluconazole, itraconazole and voriconazole was defined as the concentration of drug that caused an 80% reduction in the total cellular ergosterol content compared with that in the drug-free control. MICs that fell between two drug concentrations (i.e. less than 80% reduction at one concentration but more than 80% reduction at the next-higher concentration) were mathematically extrapolated, based on the amount of reduction by the drug concentration that gave results closest to an 80% reduction endpoint.

### PCR amplification and sequencing

Genomic DNA from *C. albicans* isolates grown overnight in SAB broth was extracted using a QIAGEN Genomic-tip 20/G and DNA buffer set (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions, and was used as a template for amplification of *ERG11*. PCR was carried out with high-fidelity *Pwo* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA) and sequence-specific oligonucleotide primers (Table 1). PCR cycling conditions were one cycle at  $95^{\circ}\text{C}$  for 5 min, followed by 25 cycles at  $95^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 45 s, followed by one cycle at  $72^{\circ}\text{C}$  for 7 min. For direct sequencing, the PCR products were purified with the QIAquick PCR purification kit (Qiagen). Sequencing of the *ERG11* open reading frame (ORF) was separated into five parts, each containing  $\sim 400$  nt and each amplified with a unique primer pair. DNA sequencing was performed using the same primers, purified PCR products as the template and the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). After purification of cycle sequencing products using Centri-Sep spin columns (Princeton Separations, Adelphia, NJ, USA), sequence analysis was performed on an ABI 310 Genetic Analyzer (Applied Biosystems). Sequence data were assembled and compared with that of a previously reported *ERG11* sequence from a standard, wild-type, fluconazole-susceptible *C. albicans* strain<sup>11</sup> (acces-

sion number X13296) by using GCG sequence analysis software (NCD Software Co., Beaverton, OR, USA).

### Relative quantification of gene expression by LightCycler RT-PCR

**RNA extraction.** For each isolate, an overnight culture grown in 10 mL of SAB broth (Difco) was diluted 1:100 in fresh SAB broth and grown to mid-logarithmic phase ( $\text{OD}_{600}=1-1.2$ ). For detecting transcript levels in the presence or absence of fluconazole, overnight cultures of *C. albicans* isolates demonstrating fluconazole-SDD, -resistant, and trailing growth phenotypes were grown in SAB broth and diluted 1:100 in fresh SAB broth and in SAB broth containing fluconazole 8, 16, 32 or 64 mg/L. Cultures were incubated with shaking at  $35^{\circ}\text{C}$  until cells reached the mid-logarithmic phase of growth. Cells were harvested by centrifugation and washed once with sterile distilled water. Cell lysis was performed by resuspending cells in sterile distilled water plus Lysis Binding solution (Bio 101 Systems, Vista, CA, USA), transferring them to Lysis Matrix C tubes (Qbiogene, Carlsbad, CA, USA) and vortexing twice for 45 s at speed level 6 in the FastPrep high speed vortexer (Qbiogene). Following the directions of the manufacturer of the RNeasy-4PCR kit (Ambion, Austin, TX, USA), total RNA was extracted from *C. albicans* cells. To remove genomic DNA contamination, RNA samples were treated with two units of DNase I (Roche) per 100  $\mu\text{L}$  of RNA at  $37^{\circ}\text{C}$  for 1 h.

**Synthesis of cDNA.** Reverse transcription was performed in a total volume of 40  $\mu\text{L}$  with a 1st Strand cDNA Synthesis Kit for RT-PCR (Roche) using 2  $\mu\text{L}$  of RNA, AMV reverse transcriptase and random primer p(dN)<sub>6</sub>, as recommended by the manufacturer. The cDNA was purified using the QIAquick PCR purification kit (Qiagen) and quantified by spectrophotometric measurement of  $A_{260}$  and  $A_{280}$  and standard calculations.

**Quantitative real-time PCR with LightCycler.** Primers and hybridization probes were designed using LightCycler Probe Design software (Roche) and are listed in Table 1. In order to optimize the real-time PCR conditions and verify the specificity of the designed primer pairs, the optimal  $\text{MgCl}_2$  concentration and annealing temperature were determined using FastStart DNA Master SYBR Green I (Roche) in the LightCycler (Roche) and melting curve analysis. Quantification of gene expression by LightCycler is based on a standard curve for each target gene and is included in each LightCycler real-time (RT)-PCR experiment. A template for the LightCycler standard curves was generated via conventional PCR using 1–5 ng of genomic DNA from the fluconazole-susceptible *C. albicans* isolate ATCC 32354, 0.2  $\mu\text{M}$  of each PCR primer (sequence shown in Table 1), and 1.5 mM  $\text{MgCl}_2$  and 0.2  $\mu\text{M}$  of each dNTP. Cycling conditions were one cycle at  $95^{\circ}\text{C}$  for 5 min, followed by 30 cycles of  $95^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 30 s, followed by one cycle at  $72^{\circ}\text{C}$  for 7 min. The samples were held at  $4^{\circ}\text{C}$  in the thermal-cycler until retrieved.

For LightCycler relative quantification of *ERG11*, *CDR1*, *MDR1* and *ACT1* from *C. albicans* isolates with varying fluconazole susceptibility phenotypes, each specific standard curve was prepared by purification and 10-fold serial dilutions of a known concentration of amplicons (generated as described above) of each gene. Real-time LightCycler PCR reactions were performed using the FastStart DNA Master Hybridization Probe PCR mix (Roche). The reaction mixture consisted of 2.4  $\mu\text{L}$  of 25 mM  $\text{MgCl}_2$ , 0.4  $\mu\text{M}$  of each PCR primer, 0.2  $\mu\text{M}$  of each hybridization probe, 2  $\mu\text{L}$  of LightCycler DNA FastStart Hybridization mix (Roche) and PCR-grade water up to a final volume of 18  $\mu\text{L}$ . For all samples, a master mix was prepared and 18  $\mu\text{L}$  was transferred into each glass capillary. Two microlitres of cDNA from the reverse transcription step (test samples) or PCR amplicons from each dilution (standard curve) were then pipetted into all but one of the LightCycler capillaries. PCR-grade water was added to the latter in place of cDNA template to serve as a

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**Table 1.** Primers and probes used in this study

Primer/probe			GenBank accession no.	Sequence (5'–3')
For sequencing <i>ERG11</i> gene	<i>ERG11</i> ORF	forward primer	X13296	GAAAGGGAATTCAATCG
		reverse primer		TGTTAATCCAACCTAAGTAAC
	segment 1	forward primer		ATTCTTTCCATATTACTTGTCTTC
		reverse primer		AGCAGAAACATCAGATAATTTAG
	segment 2	forward primer		TATGACGGTTTATTAGGTCC
		reverse primer		AATATAGTTGAGCAAATGAACG
	segment 3	forward primer		GCTTCAAGATCTTTATTTGGTG
		reverse primer		TCACCTAAATGTAACAAGAACC
	segment 4	forward primer		CTTATGGGTGGTCAACATAC
		reverse primer		AGTATCCCATCTAGTTGGATC
segment 5	forward primer		GGTTATGCTCATACTAGTGAAAG	
	reverse primer		AACAATCAGAACACTGAATCG	
For LightCycler real-time PCR	<i>ERG11</i>	forward primer	X13296	TGGAGACGTGATGCTG
		reverse primer		AGTATGTTGACCACCCATAA
		donor probe <sup>a</sup>		AATCTCTGCTACTTATATGAAAGAAATTAACCTGAG
		acceptor probe <sup>b</sup>		GAGAACGTGGTGATATTGATCCAAAT
	<i>CDR1</i>	forward primer	X77589	AAGAGAACCATTACCAGG
		reverse primer		AGGAATCGACGGATCAC
		donor probe		CAAGACCAGCATCTCCATATACTGTAT
		acceptor probe		ATTCTTTATGCAAGTGAGGTATGGTG
	<i>MDR1</i>	forward primer	X53823	GGAGTTTAGGTGCTGT
		reverse primer		CGGTGATGGCTCTCAA
		donor probe		GCCAGTTGGAGATGGACT
		acceptor probe		TTGGTTCATGTGTATCATTCTTG
<i>ACT1</i>	forward primer	X16377	CCAGCTTTCTACGTTTCC	
	reverse primer		CTGTAACCACGTTTCAGAC	
	donor probe		CGGTATTGTTTTGGATTCTGGTG	
	acceptor probe		TGGTGTACTCACGTTGTTCC	

<sup>a</sup>Donor probes labelled at the 3' end with fluorescein.

<sup>b</sup>Acceptor probes labelled at the 5' end with LightCycler Red 640.

negative control. After 10 min of denaturation at 95°C, 40–50 PCR cycles were performed. Cycling conditions for *ERG11* and *CDR1* were 95°C for 10 s, 62°C for 15 s and 72°C for 10 s. *MDR1* was amplified with an annealing temperature of 54°C because it has a lower primer melting temperature. The concentration of each gene (*ERG11*, *CDR1*, *MDR1* and *ACT1*) was calculated by reference to the respective standard curve with the aid of the LightCycler software. Quantification of these genes was determined for each *C. albicans* isolate in three separate LightCycler PCR reactions using the same RNA preparation. Relative gene expression was calculated as a ratio of target gene (*ERG11*, *CDR1* and *MDR1*) concentration to housekeeping gene (*ACT1*) concentration, and values reported represent the mean gene expression from three experiments  $\pm$  the standard error of the mean (S.E.M.).

## Results

### Antifungal susceptibility testing

Table 2 summarizes the *in vitro* susceptibilities of the 12 isolates of *C. albicans* to fluconazole, as determined by the broth microdilution and sterol quantification methods. All fluconazole-SDD and -resistant isolates were classed as SDD and resistant by both methods. Seven bloodstream isolates (isolates 1–7) that showed trailing growth by the broth microdilution method were classed as susceptible to flu-

conazole when re-tested by the sterol quantification method. All five isolates that were classed as SDD or resistant to fluconazole also showed decreased susceptibilities to itraconazole and voriconazole (data not shown).

### Analysis of *ERG11* point mutations

The *ERG11* ORF of all 12 *C. albicans* isolates was sequenced and compared with the previously reported standard sequence<sup>11</sup> (Table 3). Trailing isolates 1 and 7, obtained from two different patients from different hospitals, had the same *ERG11* sequence. No sequence variations leading to amino acid substitution were found in isolates 4 and 6, both of which showed trailing growth in the presence of azoles. *ERG11* from the other 10 isolates contained one or more nucleotide changes that led to amino acid substitutions in the protein sequence. A total of nine different nucleotide changes in the *ERG11* alleles investigated yielded amino acid substitutions. Five of these, K143R, V404I, F449V, R467K and V509M, were found exclusively in non-trailing isolates with reduced fluconazole susceptibilities (isolates 8–12), and all were due to homozygous nucleotide substitutions.

Four homozygous nucleotide changes leading to amino acid substitutions E266D, V404I, V437I and V509M were found in the two

**Table 2.** *In vitro* susceptibilities of *C. albicans* isolates and *ERG11* expression in the absence of fluconazole

Isolate no.	Source	Fluconazole MIC (mg/L)			SQM <sup>a</sup>	Interpretation	<i>ERG11</i> expression <sup>b</sup>	Mean <i>ERG11</i> expression
		M27-A						
		24 h	48 h					
1	blood	0.125	>64	<4	trailer <sup>c</sup>	17 ± 1.2	15.4 ± 2.2 <sup>f</sup>	
2	blood	0.25	>64	<4	trailer	20 ± 2.5		
3	blood	0.25	>64	<4	trailer	20 ± 2.1		
4	blood	0.25	>64	<4	trailer	26 ± 0.6		
5	blood	1	>64	<4	trailer	17 ± 3.4		
6	blood	1	>64	<4	trailer	6 ± 1.0		
7	blood	4	>64	<4	trailer	17 ± 3.4		
8	mucosal	16	16	19	SDD <sup>d</sup>	21 ± 2.3	9.7 ± 2.6 <sup>g</sup>	
9	blood	16	32	22	SDD	7 ± 1.9		
10	blood	32	64	55	R <sup>e</sup>	5 ± 0.03		
11	mucosal	>64	>64	>64	R	5 ± 2.2		
12	mucosal	>64	>64	>64	R	14 ± 1.7		

<sup>a</sup>SQM, sterol quantification method. Values represent the fluconazole concentration that inhibits total ergosterol concentration by ≥80% relative to drug-free control.

<sup>b</sup>*ERG11* expression normalized to that of *ACT1* expression for each isolate. Values represent the mean ± S.E.M. of three independent LightCycler reactions.

<sup>c</sup>trailer, susceptible after 24 h incubation (MIC ≤ 8 mg/L) and resistant after 48 h incubation (MIC ≥ 64 mg/L).

<sup>d</sup>SDD, susceptible dose dependent (MIC 16–32 mg/L).

<sup>e</sup>R, resistant (MIC ≥ 64 mg/L).

<sup>f</sup>Value represents estimated mean *ERG11* expression ± S.E.M. for trailing isolates.

<sup>g</sup>Value represents estimated mean *ERG11* expression ± S.E.M. for SDD and R isolates.

bloodstream isolates that showed reduced fluconazole susceptibilities (isolates 9 and 10). Two of these, V404I and V509M, have not been reported previously, and were not found in the fluconazole-resistant mucosal isolates tested in this study.

#### *Expression of ERG11, CDR1 and MDR1 in C. albicans bloodstream isolates*

To investigate if distinct *ERG11* expression patterns correlated with fluconazole resistance and/or azole trailing, *ERG11* expression was quantified in all 12 *C. albicans* isolates by a LightCycler relative quantification method (Table 2). This method has been shown previously to correlate with traditional northern blotting methods for relative quantification of gene expression in *C. albicans*.<sup>12</sup> Although the data in Table 2 represent the results of three independent LightCycler experiments using the same RNA preparation, we have observed that different RNA preparations from the same isolate show a consistent trend in gene expression (data not shown). In the absence of fluconazole, *ERG11* was expressed in all isolates, regardless of fluconazole susceptibility. However, *ERG11* expression levels showed no correlation with susceptibility. In fact, the mean *ERG11* expression was higher in the seven trailing isolates (15.4 ± 2.2) than in the five isolates with reduced fluconazole susceptibilities (9.7 ± 2.6), as determined by Restricted Maximum Likelihood analysis for repeated measures with an unstructured correlation (*P* value = 0.127) (Table 2).

To evaluate the effect of fluconazole exposure on *ERG11* expression among fluconazole-trailing, -SDD and -resistant isolates, four representative bloodstream isolates (isolates 1, 3, 9 and 10) were incubated with fluconazole 0, 8, 16, 32 and 64 mg/L, and *ERG11*

expression was measured by LightCycler relative quantification (Figure 1a). It is important to note that the fluconazole concentration added to the cells cultured in SAB broth does not correlate directly with the microbroth dilution fluconazole MIC of the isolates that were determined in RPMI 1640 medium. In SAB broth, adequate growth of all isolates in all drug concentrations was achieved. Under these conditions, both the trailing and less susceptible isolates were capable of up-regulating *ERG11* in response to fluconazole exposure. However, the concentrations of fluconazole that elicited peak *ERG11* expression differed. Specifically, the fluconazole-SDD and -resistant isolates (isolates 9 and 10) demonstrated peak levels of *ERG11* expression at higher drug concentrations than the trailing isolates (isolates 1 and 3) (Figure 1a). In addition, the correlation between *ERG11* expression and the concentration of fluconazole exposure was greater for the fluconazole-SDD and -resistant isolates (0.97) than for the trailing isolates (0.56) as determined by Pearson's *r* correlation coefficient.

*CDR1* and *MDR1* expression were measured by LightCycler relative quantification in the same four selected bloodstream isolates cultivated in the presence and absence of fluconazole, as described above. *CDR2*-specific primers and probes were not included in this study. The results (Figure 1b,c) indicated distinct responses to fluconazole exposure by the trailing and less susceptible isolates. The fluconazole-SDD and -resistant isolates expressed higher constitutive levels of *CDR1* in the absence of fluconazole compared with trailing isolates. However, even though the trailing isolates expressed less overall *CDR1* relative to the SDD and resistant isolates, they were capable of *CDR1* up-regulation in response to fluconazole

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**Table 3.** Nucleotide and amino acid substitutions in *ERG11* genes from *C. albicans* clinical isolates

Amino acid substitution <sup>a</sup>	Reference nucleotide <sup>b</sup>	Isolate no.											
		1	2	3	4	5	6	7	8	9	10	11	12
F 105 F	T	T/C	C	T/C	C				T/C				T/C
<b>D 116 E</b>	T	T/A	A	T/A					T/A				T/A
K 119 K	A	A/G	G	A/G					A/G				A/G
<b>K 128 T</b>	A	A/C							A/C				A/C
S 137 S	C	C/T	T	C/T	T	T	C/T		C/T				C/T
<b>K 143 R</b>	A												<b>G</b>
H 183 H	T			T/C		C	C			C			
L 220 L	C	C/T	T	T	T	T		C/T		T			T
<b>E 266 D</b>	A		C	A/C						C			
L 320 L	G				A								
V 332 V	T			T/C		C	C			C	C		
L 340 L	A	A/G	G	A/G				A/G					G
K 342 K	A			A/G							G		
S 361 S	A						G			G			
L 370 L	C	C/T	T	T	T	T	T	C/T	T	T			T
F 380 F	T				C	C							
Y 401 Y	T				C	C	C		C	C			
<b>V 404 I</b>	G									<b>A</b>			
P 419 P	T					C				C			
D 428 D	T						C		C				
<b>V 437 I</b>	G					<b>A</b>				<b>A</b>			
G 448 G	G		G/T		A								
<b>F 449 V</b>	T								<b>G</b>				
V 452 V	T				C								
V 456 V	T				A								
<b>R 467 K</b>	G												<b>A</b>
L 480 L	A	A/G	G	G	G	G	G	A/G	G				G
N 490 N	T	T/C	C	C	C		C	T/C	C				C
<b>V 509 M</b>	G										<b>A</b>		

<sup>a</sup>Bold type indicates a mutation that created an amino acid substitution.

<sup>b</sup>Reference *ERG11* sequence (GenBank accession no. X13296).

<sup>c</sup>Relative to reference *ERG11* sequence.

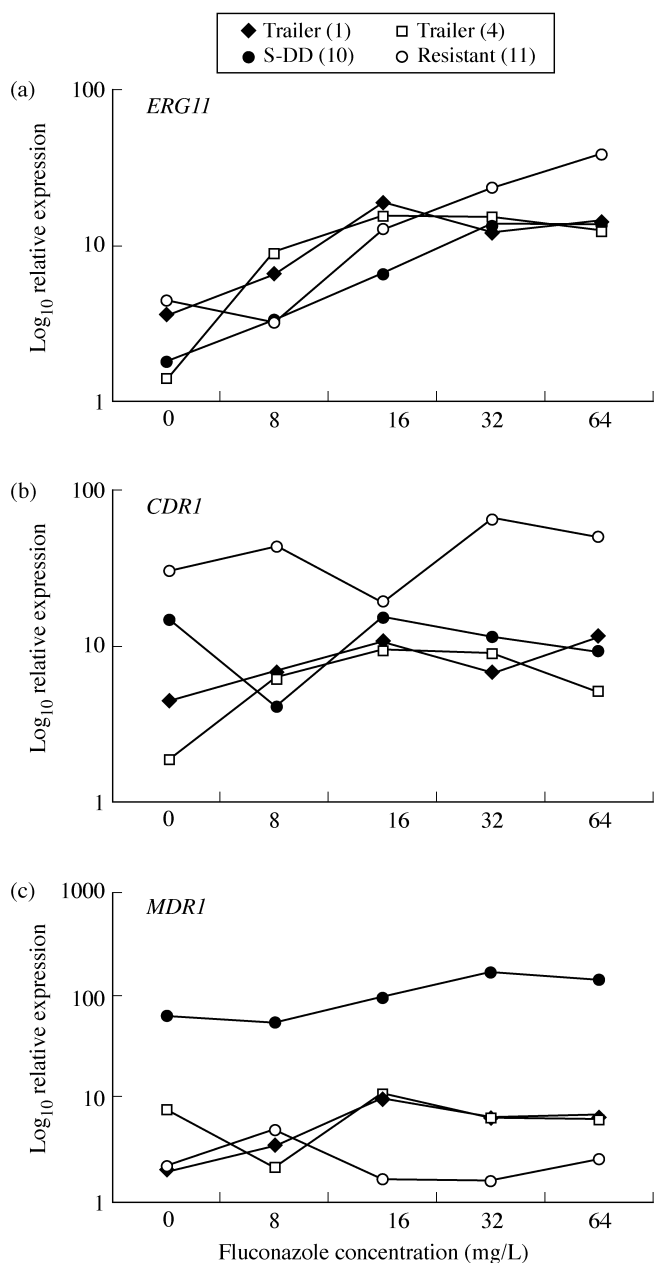
exposure, and maintained expression at levels greater than that of their respective drug-free controls at the highest fluconazole concentrations tested (64 mg/L). In the case of *MDR1*, the fluconazole-SDD isolate expressed high levels of *MDR1* in the absence of fluconazole and further up-regulated *MDR1* expression following exposure to the drug. Likewise, the trailing isolates up-regulated *MDR1* in response to fluconazole, but expressed lower overall levels compared with the fluconazole-SDD isolate. Although the resistant isolate showed an increase in *MDR1* expression at fluconazole 8 mg/L, it did not maintain over-expression at higher drug concentrations, implying that *MDR1* does not contribute to the fluconazole-resistant phenotype of this isolate.

## Discussion

The development of a reliable and reproducible broth microdilution reference procedure for the *in vitro* susceptibility testing of azole antifungals against *Candida* spp. has enabled MICs to be correlated with clinical outcomes and has permitted interpretive breakpoints to

be proposed.<sup>10</sup> However, some problems remain to be resolved. Among these is the proper interpretation of the persistent trailing growth that some isolates of *C. albicans* exhibit over an extended range of drug concentrations. In previous work, we found that 18% of *C. albicans* bloodstream isolates, collected during a population-based active surveillance for candidaemia, displayed trailing growth in broth microdilution susceptibility tests with fluconazole.<sup>1</sup> *In vitro* and *in vivo* studies, from our laboratory and others, have indicated that trailing isolates should be classed as susceptible rather than resistant.<sup>2-4</sup> To date, however, little is known about the possible genetic alterations linked to azole trailing in *C. albicans* and the role that trailing growth may have in the development of azole antifungal drug resistance.

No fewer than 35 different nucleotide substitutions leading to amino acid substitutions have been identified in *ERG11* alleles from clinical isolates of *C. albicans*.<sup>13-24</sup> Twenty of these 35 amino acid substitutions have been identified in azole-resistant isolates, including F126L, G129A, Y132H, K143E, K143R, F145L, A149V,



**Figure 1.** Relative expression (Log<sub>10</sub>) of *ERG11* (a); *CDR1* (b); and *MDR1* (c) among trailing, SDD and resistant *C. albicans* isolates in the presence of fluconazole 0, 8, 16, 32 and 64 mg/L.

T229A, S279F, K287R, G307S, S405F, G448E, G448R, F449L, V452A, G464S, G465S, R467K and I471T.<sup>13,16,17,24</sup> In this study, we found two of these mutations (K143R and R467K) in fluconazole-resistant *C. albicans* isolates derived from cases of oropharyngeal candidiasis (isolates 11 and 12). These mutations were not found in any of the nine bloodstream isolates in this study. We also found three *ERG11* amino acid substitutions that have not been described previously. Two of these, V404I and V509M, were found only in bloodstream isolates with reduced fluconazole susceptibilities. Whereas the number of mucosal isolates included in this analysis is small, these findings suggest that further study of differences between bloodstream and mucosal isolates with regard to acquisition of azole drug resistance mechanisms may be warranted.

Marichal *et al.*<sup>22</sup> have identified three hot spots within the amino acid sequence of the *ERG11* gene based on a compilation of *ERG11* mutations reported to be associated with azole resistance. These hot spots include amino acid regions 105–165, 266–287 and 405–488. Two of the newly described amino acid substitutions, V404I and V509M, fall just outside the third hot spot. A possible explanation is that nucleotide sequence data used to delineate these three hot spots were from *C. albicans* mucosal isolates rather than bloodstream isolates. Further genetic analysis of substitutions V404I and V509M is required to determine their significance to the azole drug resistance phenotype. The third newly described substitution, F449V, was found in a fluconazole-SDD vaginal isolate (isolate 8). A similar substitution, F449L, has, however, been reported elsewhere.<sup>14</sup>

A comparison of trailing isolates of *C. albicans* with non-trailing isolates with reduced fluconazole susceptibilities showed that the trailers possessed a higher number, on average, of *ERG11* nucleotide substitutions than the less susceptible isolates (10.7 versus 6.0, respectively) and that a larger proportion of these nucleotide substitutions were heterozygous (41% versus 13%, respectively). The observation that all of the *ERG11* amino acid substitutions from isolates with reduced azole susceptibility phenotypes were homozygous supports the conclusion of a previous report that a mutation occurring on one allele may not appear as a phenotype unless it becomes dominant.<sup>15</sup> In diploid cells, mutations usually occur randomly on each allele and result in heterozygosity. It is generally considered that meiosis, mating, frequent mitotic recombination and gene conversion are included in the formation of a homozygote for an altered gene. Mitotic recombination or gene conversion is a more likely explanation for *ERG11* homozygosity in fluconazole-resistant *C. albicans* isolates. However, sexual generation may not be completely excluded.<sup>25,26</sup> These results may suggest that as trailing isolates accumulate homozygous nucleotide changes leading to amino acid substitutions within critical sites of the lanosterol demethylase enzyme, susceptibility of the enzyme to azole agents decreases.

Increased mRNA levels for *C. albicans* *ERG11*, *MDR1* and *CDR1* genes have been shown previously to be associated with azole resistance.<sup>7,16,17,27–30</sup> In this study, *ERG11* expression was variable among each isolate in the absence of fluconazole pressure. Although the number of fluconazole-resistant and -SDD isolates tested was limited, our data imply that, in the absence of fluconazole, constitutive *ERG11* over-expression is not correlated with azole resistance. Other reports have also shown that *ERG11* expression, in the absence of fluconazole, is variable and appears to have no correlation with resistance.<sup>16,27</sup> This finding is logical in the sense that it is metabolically costly for an organism to over-express *ERG11* in the absence of fluconazole pressure since the enzyme, lanosterol demethylase, is believed to be involved solely in ergosterol biosynthesis. Furthermore, over-expression of one sterol biosynthetic gene in a pathway that includes multiple genes would disrupt the flow of intermediates through the pathway and the overall regulation of ergosterol biosynthesis and storage.<sup>31</sup> Therefore, it is appropriate to analyse *ERG11* expression in the presence of fluconazole as well as in its absence.

When we studied whether trailing and less azole-susceptible isolates regulated *ERG11* differently in response to fluconazole exposure, the results indicated that this was the case. Trailing isolates expressed more *ERG11* in the absence of drug than did fluconazole-SDD or -resistant isolates. This may be because acquisition of mutations that give an organism a selective advantage in the presence of drug comes at a cost which could take the form of decreased fitness or decreased virulence.<sup>32</sup> In the presence of low and intermediate concentrations of fluconazole, both groups of isolates were capable of

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*ERG11* up-regulation. However, at high drug concentrations only the fluconazole-SDD and -resistant isolates maintained peak levels of *ERG11* expression. With regard to the drug efflux genes, *CDR1* and *MDR1* expression patterns differed between the fluconazole-SDD and -resistant isolates as well as between the trailing and less susceptible isolates. These results support the hypothesis that azole drug resistance mechanisms evolve independently of each other and can be present alone or in combination in any given *C. albicans* isolate.<sup>17</sup> A limitation of this study was that sequential isolates from individual cases were unavailable, and it was not therefore possible to determine if the evolution towards fluconazole resistance corresponded to increased constitutive expression of the drug efflux genes and/or *ERG11* or the ability to up-regulate gene expression following drug exposure.

Smith & Edlind<sup>5</sup> found that regulated expression of *ERG11*, *CDR1*, *CDR2* and *MDR1* contributed to azole trailing in *C. albicans* isolates. Interference with transcriptional regulation, by addition of the histone deacetylase inhibitor, trichostatin A, eliminated trailing growth and reduced *ERG11*, *CDR1* and *CDR2* up-regulation by 50%–100%. Interestingly, the addition of trichostatin A had relatively little effect on the itraconazole MIC for two fluconazole-resistant *C. albicans* isolates. Other reports have shown that fluconazole-susceptible isolates without trailing growth up-regulated *ERG11* in response to fluconazole exposure but did not up-regulate *CDR1* or *MDR1*.<sup>18,28</sup> In these experiments, investigators exposed cells to lower fluconazole concentrations (0.1–5 mg/L) compared with this work. The ability of these susceptible, non-trailing isolates to maintain increased levels of *ERG11* expression at higher fluconazole concentrations is unknown. Taken together, our data and those of others imply that trailing and resistant or SDD isolates utilize known drug resistance genes, *ERG11*, *CDR* and *MDR1*, to yield their respective susceptibility phenotypes, but possess distinct mechanisms to regulate these genes in response to drug exposure.<sup>5,18,28</sup> These recent findings emphasize the need to continue studying *C. albicans* isolates at the molecular level to understand the role that susceptible isolates with trailing growth may play in recurrent disease and/or the emergence of azole drug resistance following exposure to azole antifungals.

In conclusion, this study has shown that susceptible isolates of *C. albicans* with trailing growth possess more heterozygous *ERG11* mutations and show different patterns of *ERG11*, *CDR1* and *MDR1* expression than non-trailing isolates with reduced fluconazole susceptibilities. Furthermore, trailing isolates possess a number of nucleotide substitutions that fall within the recently defined hot spots of the *ERG11* amino acid sequence. In addition, fluconazole-SDD and -resistant bloodstream isolates of *C. albicans* possess *ERG11* amino acid substitutions that have not been described previously. Finally, similar to fluconazole-SDD and -resistant isolates of *C. albicans*, *ERG11*, *CDR1* and *MDR1* up-regulation occurred in trailing growth isolates in response to fluconazole, but the pattern of expression was distinct.

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