Multiple amino acid substitutions in lanosterol 14 α -demethylase contribute to azole resistance in *Candida albicans*

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Lanosterol 14 α -demethylase (14DM) is the target of the azole antifungals, and alteration of the 14DM sequence leading to a decreased affinity of the enzyme for azoles is one of several potential mechanisms for resistance to these drugs in *Candida albicans*. In order to identify such alterations the authors investigated a collection of 19 *C. albicans* clinical isolates demonstrating either frank resistance (MICs ≥ 32 $\mu\text{g ml}^{-1}$) or dose-dependent resistance (MICs 8–16 $\mu\text{g ml}^{-1}$) to fluconazole. In cell-free extracts from four isolates, including the Darlington strain ATCC 64124, sensitivity of sterol biosynthesis to inhibition by fluconazole was greatly reduced, suggesting that alterations in the activity or affinity of the 14DM could contribute to resistance. Cloning and sequencing of the 14DM gene from these isolates revealed 12 different alterations (two to four per isolate) leading to changes in the deduced amino acid sequence. Five of these mutations have not previously been reported. To demonstrate that these alterations could affect fungal susceptibility to azoles, the 14DM genes from one sensitive and three resistant *C. albicans* strains were tagged at the carboxyl terminus with a c-myc epitope and expressed in *Saccharomyces cerevisiae* under control of the endogenous promoter. Transformants receiving 14DM genes from resistant strains had fluconazole MICs up to 32-fold higher than those of transformants receiving 14DM from a sensitive strain, although Western blot analysis indicated that the level of expressed 14DM was similar in all transformants. Amino acid substitutions in the 14DM gene from the Darlington strain also conferred a strong cross-resistance to ketoconazole. In conclusion, multiple genetic alterations in *C. albicans* 14DM, including several not previously reported, can affect the affinity of the enzyme for azoles and contribute to resistance of clinical isolates.

Keywords: azoles, fluconazole resistance, *Candida albicans*, lanosterol 14 α -demethylase

INTRODUCTION

Candida albicans, an asexual, diploid and dimorphic fungus, is the most common opportunistic fungal pathogen of humans (Rinaldi, 1993). Immunocompromised patients are particularly susceptible to infection by *C. albicans* and more than 80% of AIDS patients suffer from oropharyngeal candidiasis during the course

of their illness. The most commonly used antimycotic to prevent or combat mucosal and systemic candidiasis is fluconazole, because of its favourable bioavailability and safety profile (Feczko, 1992). The primary target of the azole antimycotics is lanosterol 14 α -demethylase (14DM), a key enzyme in the ergosterol biosynthesis pathway (Hitchcock, 1993; Vanden Bossche & Koymans, 1998). 14DM is a P450 haem thiolate protein which catalyses, in the presence of NADPH reductase, the oxidative removal of the 14 α -methyl group (C-32) from lanosterol (Aoyama *et al.*, 1989). 14DM is inhibited by azoles in a competitive manner. The imidazole or triazole moiety of azoles replaces water in the sixth coordination position of the haem in the active site,

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Abbreviation: 14DM, lanosterol 14 α -demethylase.

while substituent groups interact directly with the protein backbone (Hitchcock, 1993; Hitchcock *et al.*, 1990; Vanden Bossche & Marichal, 1992; Vanden Bossche & Koymans, 1998; Yoshida & Aoyama, 1985).

The prolonged and repeated periods of treatment needed by AIDS patients have led to the increasing occurrence of candidiasis resistant to fluconazole therapy. Drug susceptibility testing of *C. albicans* isolated from these patients revealed in many cases that the organism had acquired resistance to fluconazole (Denning *et al.*, 1997; Law *et al.*, 1994; Rex *et al.*, 1995; Tumbarello *et al.*, 1996; White *et al.*, 1998). Several mechanisms, independent of 14DM, have been reported which could contribute at least partially to fluconazole resistance in *Candida* species, including increased ergosterol biosynthesis (Vanden Bossche *et al.*, 1992), a lesion in $\Delta 5,6$ sterol desaturase (Kelly *et al.*, 1997), and lower intracellular accumulation of the drug (Marichal *et al.*, 1995; Parkinson *et al.*, 1995; Sanglard *et al.*, 1995; Vanden Bossche *et al.*, 1992; Venkateswarlu *et al.*, 1996, 1997). This last mechanism seems to be the most common and is associated with the overexpression of two types of multidrug transporters, the ATP-binding cassette (ABC) transporters (Albertson *et al.*, 1996; Miyazaki *et al.*, 1998; Moran *et al.*, 1998; Prasad *et al.*, 1995; Sanglard *et al.*, 1995, 1996, 1997; van Veen & Konings, 1998; White, 1997a) and the major facilitators (Albertson *et al.*, 1996; Goldway *et al.*, 1995; Moran *et al.*, 1998; Sanglard *et al.*, 1995, 1996; White, 1997a). Recently, additional *C. albicans* genes able to directly or indirectly confer resistance to fluconazole when expressed in *Saccharomyces cerevisiae* have been identified (Alarco *et al.*, 1997; Sanglard *et al.*, 1997).

An obvious mechanism for resistance to azoles would be alteration of their primary target, 14DM. Based on indirect biochemical evidence, both overexpression and mutation of 14DM have been suggested to explain acquired resistance to azoles in several fungi (Ishida *et al.*, 1988; Joseph-Horne *et al.*, 1995; Lamb *et al.*, 1995; Orozco *et al.*, 1998; Vanden Bossche *et al.*, 1990, 1992, 1994; Venkateswarlu *et al.*, 1997; White, 1997a, b). In *S. cerevisiae*, overexpression of 14DM encoded by *ERG11* leads to resistance to azoles, but the amplitude of resistance is rather weak, not exceeding fivefold (Doignon *et al.*, 1993; Kalb *et al.*, 1986; Kelly *et al.*, 1993). Several studies have supported the concept that azole resistance can be due to mutation(s) in 14DM (Lamb *et al.*, 1997; Loeffler *et al.*, 1997; White, 1997b). Substitution of the highly conserved residue T315A in the *C. albicans* 14DM gene (*CaERG11*, also called *ERG16* and *CYP51A1*) conferred a fivefold resistance to azoles when this gene was expressed in *S. cerevisiae* (Lamb *et al.*, 1997). Sequencing of *CaERG11* from a number of clinical isolates revealed the existence of numerous amino acid substitutions in the gene amplified from resistant isolates, suggesting a possible link with the resistant phenotype (Loeffler *et al.*, 1997). Lower sensitivity of sterol biosynthesis to fluconazole in cell-free extracts from a fluconazole-resistant *C. albicans* isolate correlates with the amino acid substitution

R467L in *CaERG11* at both loci in the diploid genome resulting from a loss of allelic variation (White, 1997b). Very recently, Sanglard *et al.* (1998) have formally demonstrated that several mutations in *CaERG11* can confer azole resistance upon *S. cerevisiae*. Interestingly, a single point mutation does not seem to drastically affect azole sensitivity of *CaERG11* but combinations of point mutations, principally G129A with G464S, Y132H with S405F, or Y132H with G484S, have cooperative effects (Sanglard *et al.*, 1998).

The present study was performed as part of an ongoing project to develop novel approaches to overcoming fungal drug resistance. Using a collection of fluconazole-resistant clinical isolates, we first identified potentially altered 14DM by measuring sensitivity to azole of sterol synthesis in cell-free extracts. Cloning and sequencing of *CaERG11* from the four isolates from which cell-free sterol synthesis was insensitive to fluconazole revealed the presence of several amino acid substitutions in each of them. Five of the 12 identified amino acid substitutions have not been previously reported and all their combinations are novel. Finally, we show that expression of altered *CaERG11* in *S. cerevisiae* resulted in increased resistance to fluconazole and, to a variable extent, also to ketoconazole.

METHODS

Strains. Azole-resistant clinical isolates were from patients who had failed therapy with fluconazole and were obtained from clinical centres in Europe, North America and Australia. The Darlington strain, ATCC 64124, originally isolated from a patient suffering from chronic mucocutaneous candidiasis and treated with ketoconazole (Ryley *et al.*, 1984), was obtained from the American Type Culture Collection. Species were identified using the API 20C test (bioMérieux), as well as CHROMagar (Mast Diagnostica) and Albicans ID agar (bioMérieux). The *S. cerevisiae* strains INVSC2 (*MAT α his3- Δ 200 ura3-167*) and HRI (*ura3-52 trp1 leu2 his3- Δ 200*) were obtained from Invitrogen and Anneliese Karwan (Vienna), respectively.

MIC determinations. Fluconazole, itraconazole and ketoconazole were prepared by Novartis Pharmaceuticals. Drug susceptibility testing of *Candida* species was performed in a broth macrodilution assay slightly modified from the NCCLS system (Galgiani *et al.*, 1995), as previously described (Ryder *et al.*, 1998). Drugs were prepared according to the recommendations of the NCCLS method. MIC was defined as the lowest drug concentration causing 80% inhibition of fungal growth. The assay was validated with the standard drugs fluconazole, amphotericin B (Bristol-Myers Squibb) and 5-fluorocytosine (Sigma) in three reference strains of *C. albicans*: ATCC 90028, ATCC 90029 and ATCC 24433 (Galgiani *et al.*, 1995). The reference strains of *C. krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019) were used to validate the assay with itraconazole and ketoconazole (Rex *et al.*, 1996). To ensure reproducibility, strain ATCC 24433 was run in parallel with each set of assays; MICs with this strain never varied by more than one dilution.

A microdilution assay using a 96-well plate with a flat bottom and low-evaporation lid (Costar) was developed to test drug susceptibility of *S. cerevisiae*. Under the same conditions, the

micro assay gave similar results to those initially obtained with the macro assay. Briefly, to 200 μ l per well of a cell suspension at 2.5×10^3 c.f.u. ml⁻¹, prepared in the same medium as for the macro assay but supplemented with 1.8 % glucose (final concn 2 %) and 0.2 mg adenine ml⁻¹ (as well as 0.2 mg uracil ml⁻¹ for untransformed cells), 2 μ l of a serial twofold dilution of fluconazole or ketoconazole in DMSO was added. The plate was shaken for 10 min at 900 r.p.m. and incubated for 48 h at 32 °C in maximum relative humidity. Growth was monitored spectrophotometrically at 620 nm with a Digiscan microtitre plate reader (Asys Hitech) after having shaken the plates for 30 min at 900 r.p.m. MICs were defined as the minimal drug concentration reducing growth by ≥ 80 %, in comparison with solvent controls.

Sterol biosynthesis assay in whole cells or cell-free extracts.

Whole-cell ergosterol biosynthesis was measured by incorporation of [1,2-¹⁴C]acetate into nonsaponifiable lipids as described previously (Ryder, 1985; Ryder *et al.*, 1984). Sterol synthesis in cell-free extracts, prepared as in Favre & Ryder (1997b) and Ryder (1987), was assayed in a final volume of 500 μ l, consisting of 50 mM sodium/potassium phosphate pH 7.4, 0.5 mM DTT, 1 mM NAD, 1 mM NADP, 0.1 mM FAD, 3 mM glucose 6-phosphate, 5 mM ATP, 3 mM glutathione, 2.7 mM MnCl₂, 4 mM MgCl₂ and 0.5 μ Ci (1.85×10^4 Bq) (RS)-[2-¹⁴C]mevalonate, supplemented with 1.25 mg protein from cell-free extracts and the appropriate concentration of antimycotic, added from a 50-fold concentrated stock prepared in DMSO. After 2 h incubation at 30 °C in a shaking water bath, reactions were terminated by the addition of 1 ml 15 % KOH in 90 % ethanol. Nonsaponifiable lipids were extracted and analysed by TLC (Ryder, 1985; Ryder *et al.*, 1984); the ¹⁴C-radioactivity present in each fraction was measured with an Instant Imager (Packard). Reproducibility of extraction was regularly checked by adding 15 000 d.p.m. of [1 α ,2 α (n)-³H]cholesterol, as internal standard, cutting the region corresponding to 4-desmethyl sterols and measuring the ³H-radioactivity by scintillation counting (Favre & Ryder, 1996). IC₅₀ values were calculated from a sterol biosynthesis inhibition curve determined with at least four different concentrations of the antimycotics.

Cloning and sequencing of *CaERG11*. Genomic DNA from *C. albicans* was isolated according to Philippsen *et al.* (1991). PCR amplification of *CaERG11* from genomic DNA was performed with the forward primer 5'-CTCGGATCCTAT-ATATATAAGTTTCTTTTCAAGAAGATCATAACTCA-AT-3' and the reverse primer 5'-CCGAATTCATCAGAA-CACTGAATCGAAAGAAAGTTGCCGTTTTTA-3', including a *Bam*HI and an *Eco*RI site, respectively. PCR was conducted using the polymerases mixture Expand High Fidelity (Boehringer Mannheim) and an annealing temperature of 55 °C under standard conditions. For direct sequencing, the PCR amplicons were purified with the PCR product purification kit from Qiagen. For cloning, PCR products were digested with *Bam*HI and *Eco*RI and ligated into the vector pBS⁺ (Stratagene). Sequencing on both strands of two independent clones or PCR amplicons was performed with a Li-Cor automatic sequencer, model 4000, using primers labelled with the infrared dye IRD40 (MWG Biotech) and Thermosequenase (Amersham).

Expression of c-myc-tagged *CaERG11* in *S. cerevisiae*. An *Nco*I site containing the translational initiation codon was introduced at the 5' end, whereas a c-myc tag sequence and an *Xba*I site were inserted at the 3' end of *CaERG11* ORF by two PCR amplifications from the cloned genes in pBS⁺, using the forward primer 5'-AACGTGCCATGGCTATTGTTGAAA-

CTGTCAATTG-3' and sequentially the following reverse primers: (1) 5'-CTTCAGAAATAAGTTTTTGTTCAAAC-ATACAAGTTTCTCTTTTTTCC-3' and (2) 5'-GGTCTA-GATTATTAAGATCTTCTTCAGAAATAAGTTTTTGT-TTC-3'. A similar strategy was applied to introduce the same c-myc sequence at the 5' end of *CaERG11* ORF, between the translational initiation codon ATG and the triplet encoding the second amino acid. The promoter of *S. cerevisiae* *ERG11* was PCR-amplified from genomic DNA isolated from the strain HRI with the following forward and reverse primers, which contain an *Xba*I and an *Nco*I site, respectively: 5'-CCTCTAGAGACAATACAACCAACCTTCCTCC-3' and 5'-CTTGGTAGCAGCCATGGTTGTATTACTCGTTTG-3'. The identity of the amplified fragment of approximately 1 kbp including the *ERG11* promoter (accession no. U10555) was confirmed by restriction analysis and partial sequencing. The terminator of *S. cerevisiae* *CYC1* was amplified by PCR from the plasmid pYES2 (Invitrogen) with the forward and reverse primers, which contain an *Xba*I and a *Xho*I site, respectively: 5'-ATGCATCTAGAGGGCCGCATC-3' and 5'-CACCTGCTCGAGGGCCGCAAATTAAGCCTTC-GA-3'. Amplified *CaERG11* genes, digested with *Nco*I and *Xba*I, and the *CYC1* terminator, digested with *Xba*I and *Xho*I, were subcloned together into the plasmid Litmus 28 (BioLabs). Finally, the *ERG11* promoter, digested with *Xba*I and *Nco*I, and the previous insert in Litmus 28, isolated by digesting the plasmid with *Nco*I and *Xho*I, were cloned into the vector pRS416 (Stratagene). For each final construct of c-myc-tagged *CaERG11* in pRS416, the ORF was completely sequenced to make sure that no mutation had been introduced by the PCR amplifications and the various cloning steps.

The triplet ⁷⁸⁷CTG in *CaERG11* ORF (encoding Ser in *C. albicans*: Santos & Tuite, 1995) was replaced by the triplet TCT with the QuickChange site-directed mutagenesis kit (Stratagene) using the primer 5'-CTTATATGAAAGAAAT-TAAATCTAGAAGAGAACTGTGTG-3' and its reversed, complementary primer, which contain an *Xba*I site. To preserve the amino acid substitution E266D in *CaERG11* from the strain NFI 2007, the same primers as above, except for the substitution A³² → C, were used, whereas the standard primers were applied to suppress the alteration. After site-directed mutagenesis, *CaERG11* ORFs were again completely sequenced to confirm the replacement of ⁷⁸⁷CTG by TCT (identified by restriction analysis with *Xba*I) and to ensure that no other mutations had been introduced.

S. cerevisiae INVSC2 was transformed with pRS416 containing or not containing *CaERG11* using the lithium acetate method (Becker & Guarente, 1991). Two colonies from each transformation were isolated and independently analysed.

Antibodies and Western blotting. Whole-cell extracts were prepared according to Favre & Ryder (1997a). Cell lysates from *S. cerevisiae* transformants were prepared from cultures grown in conditions as similar as possible to those for MIC determinations (medium, temperature and cell density) in 250 ml Erlenmeyer flasks containing 100 ml medium for 24 h without agitation, harvested by centrifugation, washed with phosphate-buffered saline and either immediately processed or frozen at -70 °C. Protein concentration was determined with the Bradford reagent and detergent-compatible (DC) protein assay (Bio-Rad), for samples without and with detergents, respectively. Western blot analysis with the monoclonal anti-c-myc antibodies (clone 9E10, Boehringer Mannheim), 10 μ g ml⁻¹, was performed as previously described (Favre & Ryder, 1997a). Membranes were exposed to Hyperfilm ECL (Amersham).

RESULTS

Selection of *C. albicans* isolates with enzymic resistance to fluconazole

Out of the 23 *C. albicans* isolates obtained from patients who had failed azole therapy, only 19 demonstrated either dose-dependent or frank resistance to fluconazole *in vitro* (MIC range 8 to $>128\mu\text{g ml}^{-1}$), as determined by the NCCLS method (Galgiani *et al.*, 1995; Ghannoum *et al.*, 1996). In order to identify strains which might express altered 14DM genes among these 19 isolates, we measured the sensitivity to fluconazole of the incorporation of (RS)-[2- ^{14}C]mevalonate into sterols in cell-free extracts. Disruption of the cell membrane should eliminate the effect of multidrug transporters on the sensitivity of sterol synthesis to fluconazole, whereas other mechanisms of resistance, independent of cell integrity, such as overexpression or mutation of 14DM, should still be effective. In four isolates, Darlington

(ATCC 64124), NFI 2007, NFI 2013 and NFI 2021, cell-free sterol synthesis was insensitive to inhibition by fluconazole, with a greater than 50-fold increase in the IC_{50} compared to susceptible strains (see below). These four isolates were selected for in-depth susceptibility testing, biochemical characterization of sterol biosynthesis, and molecular analysis of *CaERG11*.

Biological and biochemical characterization of four selected azole-resistant isolates

Susceptibility of the four selected fluconazole-resistant strains to several azoles was measured and compared with that of a set of four control strains, known to be susceptible to these drugs (NFI 0124, ATCC 90028, ATCC 90029 and ATCC 24433). Most of the fluconazole-resistant strains showed some degree of cross-resistance to ketoconazole (especially, as expected, the Darlington strain) and itraconazole, although to various extents (Table 1).

In parallel to MIC determinations, the sensitivity of sterol synthesis to inhibition by fluconazole, ketoconazole and itraconazole was determined both in whole cells and in cell-free extracts. In whole cells, inhibition of [1,2- ^{14}C]acetate incorporation by the antimycotics correlated with increasing MICs, with a few exceptions which might be due to the different experimental conditions used for each of the two assays. In contrast, sensitivity of sterol synthesis in cell-free extracts to the various antimycotics did not correlate with MICs (Tables 1 and 2). This suggests that at least two distinct mechanisms were responsible for the observed resistance to azoles: one at the enzymic level, mainly affecting fluconazole and sometimes ketoconazole, and a second, which influenced the action of all azoles including itraconazole and was suppressed by disruption of the cell membrane, suggesting the involvement of membrane transporters.

Table 1. Susceptibility to fluconazole (FLU), ketoconazole (KETO) and itraconazole (ITRA) of the four selected azole-resistant *C. albicans* isolates in comparison with that of four sensitive strains

Strain	MIC ($\mu\text{g ml}^{-1}$)		
	FLU	KETO	ITRA
Darlington	>128	32	>4
NFI 2007	32	1	0.25
NFI 2013	>128	0.5	>4
NFI 2021	>128	0.25	1
Normal*	0.25–0.5	0.06–0.12	0.02–0.12

* Range of values obtained with the sensitive strains NFI 0124, ATCC 90028, ATCC 90029 and ATCC 24433.

Table 2. Sensitivity of sterol synthesis to inhibition by azoles determined in whole cells and cell-free extracts from the four selected azole-resistant *C. albicans* isolates, in comparison with that of four sensitive strains

Strain	IC_{50} (μM)*					
	Fluconazole		Ketoconazole		Itraconazole	
	Cells	Cell-free	Cells	Cell-free	Cells	Cell-free
Darlington	>300	2.6	2.8	0.067	0.34	0.006
NFI 2007	>100	0.8	0.1	0.003	0.31	0.001
NFI 2013	>300	0.5	3.3	0.010	0.53	0.004
NFI 2021	>1000	0.9	0.2	0.029	0.14	0.010
Normal†	3	0.007	0.03	0.002	0.03	0.002

* The standard deviation, calculated from ≥ 2 independent experiments performed in duplicate, did not exceed 50% of the mean values.

† Mean of values obtained with the four sensitive strains NFI 0124, ATCC 90028, ATCC 90029 and ATCC 24433. The standard deviation did not exceed 50% of the mean value.

Table 3. Analysis by TLC of the distribution of incorporated [1,2-¹⁴C]acetate into squalene and sterols in whole cells of the four selected azole-resistant *C. albicans* isolates, in comparison with that of four sensitive strains

Strain	Incorporation of radioactivity (% of total)							
	4-Desmethyl sterol		4-Methyl sterol		Lanosterol		Squalene	
	Mean	SD*	Mean	SD*	Mean	SD*	Mean	SD*
Darlington	49.7	± 9.2	4.8	± 0.8	26.4	± 6.9	17.0	± 4.7
NFI 2007	42.6	± 9.8	6.2	± 1.4	26.8	± 4.9	14.7	± 5.0
NFI 2013	30.9	± 4.7	5.0	± 0.6	56.3	± 5.0	7.8	± 3.0
NFI 2021	88.4	± 3.5	2.8	± 0.6	5.3	± 1.8	3.5	± 1.8
Normal†	81.5	± 4.3	4.1	± 0.9	5.6	± 1.5	8.2	± 4.7

* $n \geq 4$.

† Mean of the values obtained with the four sensitive strains NFI 0124 and ATCC 90028, ATCC 90029 and ATCC 24433.

Analysis of the distribution of the radioactivity incorporated from the precursor [1,2-¹⁴C]acetate into squalene and sterols by TLC revealed that in strains Darlington, NFI 2007 and NFI 2013, a larger proportion of radioactivity was associated with squalene and especially lanosterol in comparison with that from sensitive strains or the fluconazole-resistant strain NFI 2021 (Table 3). Similar results were obtained from the analysis of sterol synthesis in cell-free extracts with (RS)-[2-¹⁴C]mevalonate as the sterol precursor (data not shown). These results suggest that fluconazole resistance in strains Darlington, NFI 2007 and NFI 2013 is associated with a defective 14DM activity, implying that alterations of *CaERG11* affect both the enzymic activity and the affinity for azoles of the protein. This dual effect is in fact expected since azoles bind to the active site of 14DM (Hitchcock *et al.*, 1990; Hitchcock, 1993; Vanden Bossche & Koymans, 1998; Vanden Bossche & Marichal, 1992; Yoshida & Aoyama, 1985). In contrast, strain NFI 2021, while resistant to fluconazole, incorporated [1,2-¹⁴C]acetate in a manner similar to fluconazole-sensitive strains, both qualitatively and quantitatively (Table 3).

Amino acid substitutions in *CaERG11* from the four selected azole-resistant strains

In order to identify the molecular basis for the azole insensitivity of 14DM from the four selected azole-resistant isolates, we proceeded to clone and completely sequence their *CaERG11* genes. As a control, we also sequenced *CaERG11* cloned from the sensitive strain NFI 0124. All of the sequences contained at least one cryptic nucleotide variation (not affecting the protein sequence; data not shown) when compared to the published sequence of *CaERG11* (Lai & Kirsch, 1989). In addition, all *CaERG11* genes from the azole-resistant strains contained two to four additional variations leading to amino acid substitutions in the protein sequence (Table 4). No variation leading to amino acid

substitutions was found in NFI 0124 *CaERG11*. Since PCR amplification could lead to the introduction of random mutations and cloning of PCR products can fortuitously select the mutated sequences, *CaERG11* from all five strains was PCR-amplified a second time from genomic DNA and the sequences directly determined without prior cloning. Exactly the same variations as shown in Table 4 were again found, indicating that they were not artificially introduced during the PCR amplification.

Expression of *CaERG11* with amino acid substitutions in *S. cerevisiae* confers resistance to fluconazole

To formally demonstrate that the identified amino acid substitutions in *CaERG11* from fluconazole-resistant strains could confer resistance to the antimycotic in an intact yeast cell, *CaERG11* genes from fluconazole-resistant and -sensitive clinical isolates were expressed in *S. cerevisiae*. It has been shown that *C. albicans* 14DM is active when expressed in *S. cerevisiae* (Sanglard *et al.*, 1998; Shyadehi *et al.*, 1996), and 14DM from *C. albicans* and *S. cerevisiae* exhibit similar affinities for several azoles (Hitchcock *et al.*, 1989, 1990; Vanden Bossche & Marichal, 1992; Yoshida & Aoyama, 1985). Therefore, normal expression of *CaERG11* from a fluconazole-sensitive isolate in an *erg11*⁺ *S. cerevisiae* strain should not dramatically affect the susceptibility of the cells to fluconazole. To avoid overexpression of *CaERG11*, which could have led to a resistant phenotype (Doignon *et al.*, 1993; Kalb *et al.*, 1986) and affected membrane biogenesis (Vergeres *et al.*, 1993; Zimmer *et al.*, 1997), we put the expression of the gene under the control of the *ERG11* promoter (−1036 to −3; see Methods). To monitor the expression of *C. albicans* 14DM in *S. cerevisiae* we introduced a c-myc tag (30 nucleotides long, encoding the decapeptide EQKLI-SEEDL) into its sequence. The *CaERG11* ORFs from NFI 0124 and Darlington were initially tagged either at the 5' end or at the 3' end. These tagged genes were then

Table 4. Nucleotide variations leading to amino acid substitutions identified in *CaERG11* cloned from the four selected azole-resistant isolates in comparison with the sequence from sensitive strains SC5314 (Lai & Kirsch, 1989) and NFI 0124

Strain	Amino acid substit.	DNA sequence*		Protein sequence	
		Fluconazole-sensitive	Fluconazole-resistant	Fluconazole-sensitive	Fluconazole-resistant
Darlington	1	²¹⁴ TTC	CTC	Phe ⁷²	Leu
	2	³⁹⁴ TAT	CAT	Tyr ¹³²	His
	3	¹³⁴⁸ GGG	GAG	Gly ⁴⁵⁰	Glu
NFI 2007	1	³⁷⁵ TTC	TTA	Phe ¹²⁶	Leu
	2	⁷⁹⁶ GAA	GAC	Glu ²⁶⁶	Asp
	3	¹²¹³ TCT	TTT	Ser ⁴⁰⁵	Phe
	4	¹³⁰⁹ GTT	ATT	Val ⁴³⁷	Ile
NFI 2013	1	³⁴⁶ GAT	GAA	Asp ¹¹⁶	Glu
	2	³⁹⁴ TAT	CAT	Tyr ¹³²	His
	3	¹³⁴⁵ TTT	CTT	Phe ⁴⁴⁹	Leu
NFI 2021	1	⁴²⁷ AAA	GAA	Lys ¹⁴³	Glu
	2	⁶⁸⁵ ACC	GCC	Thr ²²⁹	Ala

* Nucleotides are numbered from the translation start codon ¹ATG of *CaERG11* ORF (Lai & Kirsch, 1989).

Table 5. Increased resistance to fluconazole and ketoconazole in *S. cerevisiae* strain INVSC2 transformed with *CaERG11* genes cloned from azole-resistant strains

Transformants expressing <i>CaERG11</i> from	Position of c-myc tag	Expression of residue 263 as	MIC ($\mu\text{g ml}^{-1}$)*	
			Fluconazole	Ketoconazole
Untransformed	—	—	64	2
Vector alone	—	—	64	2
NFI 0124	N	Leu	128	4
NFI 0124	C	Leu	256	8
NFI 0124	C	Ser	128	4
Darlington	N	Leu	1024	32
Darlington	C	Leu	4096	64
Darlington	C	Ser	4096	64
NFI 2007	C	Ser	4096	16
NFI 2021	C	Ser	4096	16

* Values obtained in two or three out of three independent determinations. Variations did not exceed a twofold dilution step.

cloned into the centromere vector pRS416 in between the *ERG11* promoter and the terminator of *S. cerevisiae* *CYC1*.

S. cerevisiae INVSC2 was transformed with the vector containing *CaERG11*, or with vector alone, and then tested for susceptibility to fluconazole and ketoconazole (this strain was primarily resistant to itraconazole) (Table 5). Expression of *CaERG11* from the Darlington strain conferred an increased resistance to fluconazole in comparison with that from NFI 0124. However, the position of the c-myc tag influenced the susceptibility of the transformants to both fluconazole and ketoconazole whatever the source of *CaERG11* (Table 5). Western

blotting with anti-c-myc tag antibody revealed that this variation in susceptibility correlated with the apparent level of expression of 14DM. Proteins tagged at the carboxyl (C) terminus were more abundant than those tagged at the amino (N) terminus (Fig. 1; compare lanes 2–7 with lanes 8 and 9). The c-myc tag at the N-terminus might interfere with the targeting of 14DM to the membrane of the endoplasmic reticulum, which depends on a transmembrane domain and also probably on clusters of positively charged residues, both situated toward the N-terminus of P450 proteins (Boscott & Grant, 1994; Chen *et al.*, 1988; Sanglard *et al.*, 1993). *CaERG11* from NFI 2007 and NFI 2021 were therefore exclusively tagged at the C-terminus. A cryptic alter-

Fig. 1. Western blot analysis of the expression of c-myc-tagged *CaERG11* from whole homogenates (30 μ g protein) of *S. cerevisiae* INVSC2 transformed with the vector or plasmid containing *CaERG11* cloned from several *C. albicans* strains with anti-c-myc tag antibodies (see Methods for details). The strain from which *CaERG11* was derived, the position [amino (N) or carboxyl (C)] of the c-myc tag, and the designated amino acid residue at position 263 in the expressed protein are indicated above the Western blot. Molecular mass markers (kDa) are shown on the left. The band corresponding to c-myc-tagged *C. albicans* 14DM is arrowed. The results are representative of two independent experiments.

ation of NFI 1013 *CaERG11* introduced an *Xba*I site at position 409 of the ORF (data not shown), precluding its cloning into the expression vector, which was based on a strategy involving two digestions with *Xba*I. Since codon CUG is decoded as Ser in *C. albicans* instead of Leu (Santos & Tuite, 1995), expression of *CaERG11* in *S. cerevisiae* introduces the substitution S263L. To ensure that this would not influence the sensitivity of 14DM to inhibitors, the triplet ⁷⁸⁷CTG was systematically replaced by TCT (a Ser codon) in *CaERG11* tagged at the 3' end. Results obtained with these last plasmids are presented in Table 5. The substitution S263L in *CaERG11* gene from the sensitive strain NFI 0124 resulted in a slight decrease (one step dilution) in fluconazole and ketoconazole resistance. Transformants expressing the *CaERG11* genes from fluconazole-resistant strains were 32-fold more resistant to fluconazole than transformants expressing *CaERG11* from the sensitive strain (Table 5). The identity of residue 263, Ser or Leu, did not affect MICs to fluconazole of transformants expressing the *CaERG11* genes from resistant strains (data not shown). The amino acid substitutions present in the Darlington *CaERG11* gene simultaneously conferred a potent cross-resistance to ketoconazole (16-fold more than the control), in the other cases cross-resistance to ketoconazole was weak (4-fold) (Table 5). Transformants expressing NFI 2007 *CaERG11* without the amino acid substitution E266D were indistinguishable from those expressing *CaERG11* containing all four amino acid substitutions identified in this gene (not shown).

Western blot analysis of extracts from transformants with anti-c-myc antibodies showed that 14DM from fluconazole-sensitive or -resistant *C. albicans* strains were equally abundant, ruling out that increased ex-

pression or stability of altered proteins could account for increased MICs (Fig. 1).

DISCUSSION

In this study we have demonstrated for three *C. albicans* clinical isolates that lower susceptibility to fluconazole could be at least partly due to the presence of amino acid substitutions in *CaERG11*, since expression of the altered genes in *S. cerevisiae* led to a resistant phenotype when compared to transformants expressing *CaERG11* from a sensitive strain. In all three cases altered sensitivity of sterol synthesis to fluconazole in cell-free lysates was a good screening tool to identify altered *CaERG11*, which can confer fluconazole resistance upon a yeast cell. Therefore, it is most probable that the amino acid substitutions identified in NFI 2013 *CaERG11*, and that detected by White (1997b), R467K, are in the same way responsible for the observed biochemical insensitivity to fluconazole, and contribute to the overall fluconazole resistance of these strains. However, it remains to be investigated whether under- or overexpression of NADPH P450 reductase or cytochrome *b₅* reductase, which alters the susceptibility of *S. cerevisiae* to azoles (Sutter & Loper, 1989; Truan *et al.*, 1994), has a similar effect on the affinity of 14DM for azoles in *C. albicans* cell extracts, and whether this mode of resistance occurs in *Candida* isolates. It cannot therefore be concluded without reserve that lower sensitivity of sterol synthesis to azoles in cell-free extracts is invariably associated with mutation(s) of *CaERG11*.

Sixteen different mutations leading to amino acid substitutions have previously been identified in *CaERG11* from fluconazole-resistant isolates (Loeffler *et al.*, 1997; Sanglard *et al.*, 1998; White, 1997b). We report here five new, previously undescribed substitutions, to give a total of 21 which are now known. This multiplicity of mutations in *CaERG11* potentially leading to fluconazole resistance, and their widespread distribution along the primary structure, might be surprising at first glance. However, a molecular model for *C. albicans* 14DM has been derived from the crystallographic structure of bacterial (*Pseudomonas putida*) soluble P450 camphor hydroxylase (P450cam) (Boscott & Grant, 1994; Poulos *et al.*, 1987) (see also Loeffler *et al.*, 1997; Sanglard *et al.*, 1998). The active site of P450 proteins is buried in the core of the protein and is constituted by a complex three-dimensional arrangement of various secondary structures involving several parts of the linear protein sequence. It is thus not surprising that several amino acid substitutions along the primary structure of 14DM can affect the affinity of azoles, which act as competitive inhibitors by binding to the active site.

Fourteen of these 21 known amino acid substitutions involve residues which are conserved in all the yeast 14DM genes which have been sequenced so far. Six of these are located in a segment spanning residues 126–147, three are clustered at positions 448–450, and

<i>C. a.</i>	⁶⁸ PYEF <u>LE</u>	¹²⁴ PV <u>LGT</u> GV <u>IH</u> D	¹⁴¹ E <u>Q</u> E <u>K</u> F <u>A</u> R <u>G</u>	²²⁶ K <u>G</u> F <u>A</u> P <u>I</u>	⁴⁰² VL <u>V</u> F <u>P</u> G	⁴⁴⁷ <u>Y</u> E <u>L</u> E <u>K</u> VSKGVSSPYLPF <u>S</u> <u>G</u> <u>G</u> <u>K</u> HRC
<i>C. g.</i>	PYEFFE	PV <u>F</u> G <u>K</u> GV <u>I</u> YD	E <u>Q</u> <u>K</u> K <u>F</u> V <u>K</u> G	K <u>G</u> F <u>T</u> P <u>I</u>	VL <u>V</u> S <u>P</u> G	<u>Y</u> <u>G</u> <u>F</u> <u>G</u> <u>A</u> ISKGVSSPYLPF <u>G</u> <u>G</u> <u>G</u> <u>R</u> HRC
<i>S. c.</i>	PYEFFE	PV <u>F</u> G <u>K</u> GV <u>I</u> YD	E <u>Q</u> <u>K</u> K <u>F</u> V <u>K</u> G	K <u>G</u> F <u>T</u> P <u>I</u>	VL <u>V</u> S <u>P</u> G	<u>Y</u> <u>G</u> <u>F</u> <u>G</u> <u>A</u> ISKGVSSPYLPF <u>G</u> <u>G</u> <u>G</u> <u>R</u> HRC
<i>C. t.</i>	PYEFFE	PV <u>F</u> G <u>K</u> GV <u>I</u> YD	E <u>Q</u> <u>K</u> K <u>F</u> A <u>K</u> F	K <u>G</u> F <u>T</u> P <u>I</u>	VL <u>V</u> S <u>A</u> G	<u>Y</u> <u>G</u> <u>F</u> <u>G</u> <u>K</u> ISKGVSSPYLPF <u>G</u> <u>G</u> <u>G</u> <u>R</u> HRC
<i>C. k.</i>	PYEFFE	PV <u>F</u> G <u>K</u> GV <u>I</u> YD	E <u>Q</u> <u>K</u> K <u>F</u> A <u>K</u> V	K <u>G</u> F <u>T</u> P <u>L</u>	VL <u>I</u> S <u>P</u> G	<u>Y</u> <u>G</u> <u>F</u> <u>G</u> <u>K</u> ISKGVASPYLPF <u>G</u> <u>G</u> <u>G</u> <u>R</u> HRC
<i>P. i.</i>	PYQFFF	PV <u>F</u> GSDV <u>V</u> YD	E <u>Q</u> <u>K</u> K <u>F</u> A <u>K</u> Y	L <u>G</u> F <u>S</u> P <u>I</u>	LL <u>S</u> S <u>P</u> G	<u>Y</u> <u>G</u> <u>Y</u> <u>G</u> <u>A</u> VSKGTRSPYLPF <u>G</u> <u>A</u> <u>G</u> <u>R</u> HRC
<i>U. n.</i>	PYKFYK	PV <u>F</u> GRDV <u>V</u> FD	E <u>Q</u> <u>K</u> K <u>F</u> M <u>K</u> T	M <u>G</u> F <u>T</u> P <u>I</u>	LI <u>S</u> S <u>P</u> G	<u>Y</u> <u>G</u> <u>Y</u> <u>G</u> <u>L</u> TSTGASSPYLPF <u>G</u> <u>A</u> <u>G</u> <u>R</u> HRC
<i>S. p.</i>	PYVFFR	PV <u>F</u> G <u>K</u> DV <u>V</u> YD	E <u>H</u> <u>K</u> K <u>F</u> I <u>K</u> S	Q <u>G</u> F <u>S</u> P <u>V</u>	LL <u>A</u> A <u>P</u> G	<u>Y</u> <u>G</u> <u>Y</u> <u>G</u> <u>L</u> VTKGAASPYLPF <u>G</u> <u>A</u> <u>G</u> <u>R</u> HRC
<i>U. m.</i>	PYKFFE	PV <u>F</u> G <u>K</u> EV <u>V</u> YD	E <u>Q</u> <u>K</u> K <u>F</u> V <u>K</u> V	S <u>G</u> F <u>T</u> P <u>I</u>	IM <u>A</u> A <u>P</u> G	<u>F</u> <u>G</u> <u>F</u> <u>G</u> <u>M</u> ISTGANSPLYLPF <u>G</u> <u>A</u> <u>G</u> <u>R</u> HRC
	** *;	***** *::*	*::*** *	***::	:: : *	::* * : * ***** *****
Rat	PIEFLE	PV <u>F</u> G <u>K</u> GV <u>A</u> YD	E <u>Q</u> <u>K</u> K <u>I</u> L <u>K</u> S	G <u>G</u> F <u>S</u> H <u>A</u>	VC <u>V</u> S <u>P</u> T	-----PASGEKFAYVPF <u>G</u> <u>A</u> <u>G</u> <u>R</u> HRC
Hum.	PIEFLE	PV <u>F</u> G <u>K</u> GV <u>A</u> YD	E <u>Q</u> <u>K</u> K <u>M</u> L <u>K</u> S	G <u>G</u> F <u>S</u> H <u>A</u>	VC <u>V</u> S <u>P</u> T	-----PASGEKFAYVPF <u>G</u> <u>A</u> <u>G</u> <u>R</u> HRC
	* *	***** * : *	*::*** : *	**:	: :	: * *::*** *****

Fig. 2. Comparison of fungal and mammalian 14DM protein sequences surrounding amino acid substitutions identified in *CaERG11* from fluconazole-resistant strains (in bold and underlined), and involving conserved residues in most fungal 14DM sequences (in bold). Alignment was performed with the CLUSTAL W (1.74) program (Thompson *et al.*, 1994). Asterisks show conserved residues, colons indicate conserved replacements, and hyphens correspond to gaps. The accession numbers are, from top to bottom: *C. albicans*, X13296; *C. glabrata*, S75389; *S. cerevisiae*, M18109; *C. tropicalis*, M23673; *C. krusei*, S75391; *Penicillium italicum*, Z49750; *Uncinula necator*, U83840; *Schizosaccharomyces pombe*, Q09736; *Ustilago maydis*, Z48164; rat, D55681; human, D55653.

two are in the proximity of the thiolate (5th) ligand of the haem (Cys⁴⁷⁰) (Fig. 2). Amino acid substitutions from position 126 to 147 are close to or part of the entrance port for the substrate leading to the active site. For instance, Lys¹⁴³, substituted by Glu in *CaERG11* from isolate NFI 2021, is thought to be important for membrane binding and the correct entrance of the substrate (Boscott & Grant, 1994; Loeffler *et al.*, 1997). Amino acid substitutions between residues 405 to 450 are located in the 'helix-poor' domain connecting helices K to L, which are involved in the substrate-binding site in P450cam (Boscott & Grant, 1994; Poulos *et al.*, 1987). Surprisingly, despite the selectivity of fluconazole for fungal versus mammalian 14DM, 10 out of the 14 residues substituted in *CaERG11* from azole-resistant isolates are conserved in both fungal and mammalian 14DMs (Fig. 2).

The occurrence of two or more amino acid substitutions appears to be common in *CaERG11* from fluconazole-resistant strains (Loeffler *et al.* 1997; Sanglard *et al.* 1998; our work). It is unclear as yet whether all identified mutations are important for conferring reduced affinity for azoles. For instance, substitution E266D found in *CaERG11* from NFI 2007 and four fluconazole-resistant isolates by Loeffler *et al.* (1997) seems to be irrelevant in *CaERG11* from NFI 2007 for conferring resistance to either fluconazole or ketoconazole. Sanglard *et al.* (1998) found by introducing some amino acid substitutions in *CaERG11* from a sensitive strain that double point mutations usually have a synergistic effect over single amino acid substitution, explaining the apparent natural occurrence of more than one mutation in azole-resistant *CaERG11*. *S. cerevisiae* transformants expressing *CaERG11* from strains Darlington, NFI 2007 or NFI 2021 were up to 32-fold more resistant to fluconazole than those expressing *CaERG11* from a fluconazole-sensitive strain. Moreover, at the high concentration of 4096 µg ml⁻¹ (13 mM), fluconazole might exert non-

specific toxicity so that the real extent of resistance induced by mutations in *CaERG11* might well be higher than 32-fold.

Mutations in *CaERG11* from strains Darlington, NFI 2007 and NFI 2013 negatively affected not only the affinity for azoles but also the enzymic activity (Table 2), as expected from the competitive inhibition of 14DM by azoles. Therefore, the apparent lack of effect on 14DM activity of the new amino acid substitutions identified in *CaERG11* from NFI 2021, K143E and T229A is intriguing, although a similar phenomenon has been suggested to occur in azole-resistant *Ustilago maydis* strains (Joseph-Horne *et al.*, 1995). It is conceivable that these mutations somehow affect the binding of fluconazole without influencing enzyme activity, since there was no indication of any abnormal accumulation of ergosterol pathway intermediates in NFI 2021 (Table 3). An alternative explanation is that reduced substrate binding of 14DM is compensated by an up-regulation of 14DM and possibly other enzymes in the pathway. In support of the latter explanation, evidence has been found for overexpression of both 14DM and squalene epoxidase in this strain (authors' unpublished data), which would be compatible with the normal profile seen in Table 3. The lack of marked cross-resistance to itraconazole and ketoconazole in NFI 2021, together with the similar extent of reduction in sensitivity to azoles in the cell-free and whole-cell ergosterol biosynthesis assays (Table 2), suggests that resistance in this strain may be accounted for solely by these changes in the ergosterol pathway.

Amino acid substitutions in *CaERG11* probably do not constitute the sole mechanism of resistance to anti-mycotics present in any of the four strains examined. In contrast to NFI 2021 discussed above, strains Darlington, NFI 2007 and NFI 2013 show cross-resistance to other azoles and a more marked reduction of sensitivity of ergosterol biosynthesis in whole cells than in cell-free

assays (Table 2). For example, NFI 2013 is strongly resistant to itraconazole but its sterol synthesis in cell-free extracts is very sensitive to the same azole. Therefore, these three strains probably also express one or more transporters which reduce the intracellular concentration of the antimycotics. In support of this, reduced cellular accumulation of fluconazole was observed in strains Darlington, NFI 2007 and NFI 2013, but not in NFI 2021 (authors' unpublished data). A similar diversity of resistance mechanisms expressed in a single isolate has previously been observed (Lopez-Ribot *et al.*, 1998; Sanglard *et al.*, 1998; White, 1997a).

In conclusion, azole resistance in *C. albicans* is a complex phenomenon which can involve several different mechanisms. In addition to overexpression of the target enzyme or drug transporters, which have been widely reported, mutations in *CaERG11* can significantly contribute to a clinically occurring resistant phenotype induced by long-term treatment of patients with azoles. Our data suggest that amino acid substitutions in 14DM may contribute to resistance in about 20% of fluconazole-resistant *C. albicans* strains. The diversity of the mutations able to influence the affinity of 14DM for azoles precludes any simple analysis to detect them. Moreover, selection of more than one resistance mechanism by prolonged treatment with azoles also seems to be very common (Lopez-Ribot *et al.*, 1998; Sanglard *et al.*, 1998; White, 1997a), excluding a simple therapeutic approach to combat azole resistance. Combination of antimycotics with different modes of action, for example azole and allylamine (Barchiesi *et al.*, 1998; Ryder & Favre, 1997), is one potential approach to this problem.

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