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Contribution of mutations in the cytochrome P450 14α-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*

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The cytochrome P450 14α-demethylase, encoded by the *ERG11 (CYP51)* gene, is the primary target for the azole class of antifungals. Changes in the azole affinity of this enzyme caused by amino acid substitutions have been reported as a resistance mechanism. Nine *Candida albicans* strains were used in this study. The *ERG11* base sequence of seven isolates, of which only two were azole-sensitive, were determined. The *ERG11* base sequences of the other two strains have been published previously. In these seven isolates, 12 different amino acid substitutions were identified, of which six have not been described previously (A149V, D153E, E165Y, S279F, V452A and G465S). In addition, 16 silent mutations were found. Two different biochemical assays, subcellular sterol biosynthesis and CO binding to reduced microsomal fractions, were used to evaluate the sensitivity of the cytochromes for fluconazole and itraconazole. Enzyme preparations from four isolates showed reduced itraconazole susceptibility, whereas more pronounced resistance to fluconazole was observed in five isolates. A three-dimensional model of *C. albicans* Cyp51p was used to position all 29 reported substitutions, 98 in total identified in 53 sequences. These 29 substitutions were not randomly distributed over the sequence but clustered in three regions from amino acids 105 to 165, from 266 to 287 and from 405 to 488, suggesting the existence of hotspot regions. Of the mutations found in the two N-terminal regions only Y132H was demonstrated to be of importance for azole resistance. In the C-terminal region three mutations are associated with resistance, suggesting that the non-characterized substitutions found in this region should be prioritized for further analysis.

Keywords: itraconazole, fluconazole, resistance, Erg11p, modelling

INTRODUCTION

Partly as a result of the AIDS epidemic, the incidence of fungal infections has increased during the last decade. Oropharyngeal candidiasis is the most common fungal infection in AIDS patients. Only a few classes of antifungal compounds are available to treat these infections. One important class consists of inhibitors of ergosterol biosynthesis (EBI). Several enzymes in this pathway are targets for commercialized antifungals, such as squalene epoxidase, inhibited by the allylamines, Δ^{14}-reductase and $\Delta^{8,7}$-isomerase, both inhibited by the morpholines, and cytochrome P450s catalysing 14α-demethylation and Δ^{22}-desaturation, inhibited by the azoles. A combination of factors such as the fungistatic nature of the inhibition of *Candida albicans* observed for azole compounds, impaired host immune responses in infected patients, long treatment periods and use of low antifungal doses, creates favourable breeding conditions for mutation and selection of less susceptible isolates (Cartledge *et al.*, 1997).

Over the last 5 years multiple biochemical studies have been published to elucidate the underlying causes of

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azole resistance in pathogenic fungi. These studies resulted in a series of reviews dedicated to this subject (Bodey, 1997; De Muri & Hostetter, 1995; Denning *et al*., 1997; Dupont, 1995; Frosco & Barrett, 1998; Hartman & Sanglard, 1997; Johnson & Warnock, 1995; Joseph-Horn & Hollomon, 1997; Marichal & Vanden Bossche, 1995; Odds, 1998; Rex *et al*., 1995; Sanglard *et al*., 1998a; Vanden Bossche, 1997; Vanden Bossche *et al*., 1994, 1998; White *et al*., 1998). Several mechanisms have been identified that contribute to azole resistance. Probably the most common mechanism is to effect a diminution in the concentration of active compound at the target site. In the majority of cases studied recently, this was the result of overexpression of efflux pumps. So far, two types of efflux transporters have been reported in resistant isolates. The ATP-binding-cassette type (ABC transporters; e.g. *CDR1* and *CDR2*) can export a wide variety of azoles and unrelated chemicals (Kolaczkowski & Goffeau, 1997), and the so-called 'major facilitators', (e.g. *CaMDR1*, previously described as *BEN*^{r}) with a much narrower substrate spectrum (Sanglard *et al*., 1995).

The second way in which fungi achieve effective resistance to azoles is to circumvent or compensate for the toxic consequences of the changes in sterol composition. Azole-induced growth inhibition results from both the depletion of sterol molecules able to perform some sparking hormonal function (Rodriguez *et al*., 1985), e.g. ergosterol, and coincidental accumulation of membrane-disturbing 14-methylated precursors. As well as ergosterol, sufficient quantities of 14-methylfecosterol can also fulfil this sparking function. (Watson *et al*., 1989). Accumulation of 14-methylfecosterol is achieved if cells are deficient in $\Delta^{8,7}$ isomerase and or $\Delta^{5,6}$ desaturase. This effect has been described in an azole-resistant *C. albicans* isolate (Kelly *et al*., 1997) as well as in *Saccharomyces cerevisiae* (Bard *et al*., 1993).

A third general type of mechanism for azole resistance involves changes at the level of the antifungal target. The primary target for the azole class of antifungals is the cytochrome-P450-catalysed 14α-demethylation of ergosterol precursors. This enzyme is encoded by *ERG11* (also described as *ERG16*, *CYP51*). Overexpression of this enzyme, induced either by enhanced transcription or by gene or chromosomal amplification, results in a decreased sensitivity for this class of antifungals (Marichal *et al*., 1997; Doignon *et al*., 1993). Point mutations in Erg11p [suggested by biochemical data (Vanden Bossche *et al*., 1990) or by sequence analysis], such as Y132H (tyrosine 132 is replaced by a histidine; Sanglard *et al*., 1998b), T315A (threonine 315 replaced by alanine; Lamb *et al*., 1997) or R476K (arginine 476 replaced by lysine; White, 1997), have been shown to decrease the affinity of the target for an azole. Numerous publications have listed other Erg11p mutations, but unfortunately the effect of the mutation on azole sensitivity was not always tested.

In this study, we sequenced the *ERG11* gene from a selection of five azole-resistant isolates and two sensitive isolates and evaluated the effects of amino acid substitutions on subcellular sterol biosynthesis and azole sensitivity. Computer modelling and sequence analysis were used to position the mutations found in the 3D model available and to predict their possible importance for resistance.

METHODS

Strains, media and compounds. Nine strains of *Candida albicans* were used in this study. Two of them, ATCC 28516 and ATCC 44858, were extensively used in previous biochemical studies (Vanden Bossche *et al*., 1980) and were azole-sensitive. The seven other strains were azole resistant: NCPF 3363 (Smith *et al*., 1986) was isolated from a patient suffering from chronic mucocutaneous candidiasis (CMC); J913004/I was isolated in France from an AIDS patient; B59626 and B59630 were sequential isolates from an AIDS patient in Germany, strains C40 and C26 were isolated from AIDS patients with oropharyngeal candidiasis and maintained at the Institute of Microbiology, Centre Hospital Universitaire Vaudois, Switzerland (Sanglard *et al*., 1995); strain 6406/8 was a spontaneous, laboratory-derived polyene-resistant mutant of strain 6406 and was obtained from Dr D. Kerridge, University of Cambridge. The identity of the isolates as *C. albicans* was confirmed by standard procedures. Yeasts were maintained as glycerol stocks at −80 °C. The inocula for each individual experiment were prepared from these glycerol stocks to minimize the possible influences of genotypic or phenotypic instability. Itraconazole (Janssen) and fluconazole (Pfizer) were dissolved and diluted in DMSO.

MIC determination. MICs were determined spectrophotometrically by a broth microdilution method (Odds *et al*., 1995) based on the NCCLS M27A protocol (National Committee for Clinical Laboratory Standards, 1995). In brief, cells were inoculated in RPMI buffered to pH 7·0 with 1·65 M MOPS. Results were read after 48 h at 37 °C in a microplate reader (model 3550; Bio-Rad). The MIC was the lowest concentration that inhibited growth by more than 50%; this end point showed the best reproducibility and correlation with results from the NCCLS broth macrodilution method. Quality control yeasts *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were tested in parallel and were inhibited at MICs in the correct ranges for the antifungals tested (National Committee for Clinical Laboratory Standards, 1995).

Sterol synthesis by subcellular fractions. To prepare the subcellular fractions, the *C. albicans* isolates were grown for 8 h in static culture and then for 8 h in a reciprocating shaker set at 100 strokes min^{-1} in 200 ml PYG_2 medium (l^{-1}: 10 g polypeptone, 10 g yeast extract, 40 g glucose) in 500 ml Erlenmeyer flasks at 30 °C. This method yields cells in the late exponential phase. Cells were collected by centrifugation at 1500 ***g***, washed and resuspended at a density of 10^9 cells ml^{-1} in cold homogenization buffer containing 0·1 M potassium phosphate buffer, 30 nM nicotinamide, 5 mM $MgCl_2$, 5 mM reduced glutathione and 1 mM PMSF. Cells were broken in a 350 ml Bead-Beater receptacle (Biospec Products) filled with 200 g prechilled acid-washed glass beads (diam. 0·40–0·45 mm) by three cycles of 1 min agitation with intermittent cooling. To prevent heating of the samples, the outer jacket of the Bead-Beater was filled with ice-cold water. The homogenate was differentially centrifuged at 4 °C in a Sorvall SS34 rotor, first for 5 min at 1500 ***g*** and then for 20 min at 8000 ***g***. The protein content of the S_{8000} supernatant fraction was

measured according to the Bio-Rad method using bovine serum albumin as a standard (Bradford, 1976). To measure sterol biosynthesis, 900 µl of the resultant S_{8000} fraction was added to 100 µl cofactor buffer to achieve final concentrations of 1 µM NADP, 1 µM NAD, 3 µM glucose 6-phosphate, 5 µM ATP, 2 µM $MnCl_2$, 3 µM $MgCl_2$ and 0·25 µCi [^{14}C]-mevalonic acid. One microlitre of inhibitor stock solution and/or DMSO was added to the incubation tubes prior to the addition of the S_{8000} extract. Tubes were incubated for 2 h in an orbital New Brunswick shaker set at 300 r.p.m. at 30 °C. Reactions were stopped by the addition of 1 ml 15% KOH dissolved in 90% ethanol and were saponified for 1 h at 84 °C. Non-saponifiable lipids were extracted with 3 ml n-heptane. Heptane extracts were then dried with a stream of N_2 and sterols were separated by TLC as described previously (Vanden Bossche *et al*., 1992). All experiments were repeated at least three times with different cell extracts.

Spectrophotometric analysis of cytochrome P450. Microsomes were isolated from *C. albicans* as described previously (Vanden Bossche *et al*., 1986). In summary, *C. albicans* cells, grown in semianaerobic conditions, were harvested by centrifugation, then washed and resuspended in ice-cold 0·65 M mannitol. All subsequent steps were done at 4 °C. PMSF was added to a concentration of 1 mM. Then the suspension was immediately homogenized in a Bead-Beater as described above. The homogenate was differentially centrifuged for 5 min at 1500 ***g***, 20 min at 10000 ***g*** and 1 h at 100000 ***g***. The pellet thus obtained was resuspended in 0·05 M potassium phosphate buffer containing 0·01 M EDTA (pH 7·4) and the suspension was recentrifuged for 1 h at 100000 ***g*** (Vanden Bossche *et al*., 1986). The final pellet was resuspended in 0·1 M potassium phosphate buffer (pH 7·4). The P-450 content and the effects of azoles on the interaction of CO with the reduced haem iron of P-450 were measured as described previously (Vanden Bossche *et al*., 1986).

PCR amplification and sequence analysis of the *C. albicans ERG11* gene. To amplify the *ERG11* gene encoding the cytochrome P450 14α-demethylase we used 5′-TAATACGACTCACTATAGGGAAGATCATAACTCAATATGGCTATTGTTG-3′ composed of a 19 bp T7 sequence and nucleotides 131–161 from the Lai & Kirsch (1989; accession number X13296) sequence, including the first four codons of the ORF, as sense primer and 5′-ATTTAGGTGACACTATAGGAAAGTTGCCGTTTTATTAAAACATAC-3′ composed of the SP6 sequence (18 bp) and the 1724–1750 region of the published sequence surrounding the stop codon of the ORF as antisense primer. Heat-activatable AmpliTaq Gold (0·5 units; Perkin Elmer) was used with 2·5 mM $MgCl_2$. DNA from all *C. albicans* strains was prepared by the Qiagen DNA extraction method according to the procedures of the manufacturer with zymolyase (60 U; 5000 U g^{-1}; *Arthrobacter luteus*; Seikagaku Kogyo) used as the cell-wall-degrading enzyme. The PCR parameters were 10 min at 94 °C to activate the polymerase and then 30 cycles of 1 min annealing at 60 °C, 2 min elongation at 72 °C and 1 min denaturation at 92 °C. After the reaction, the 1657 bp PCR product was purified with a Qiagen PCR cleanup kit and a sample was separated on a 1% agarose gel in 0·5 × TBE with Boehringer molecular mass standard VI. The 1657 bp amplification products from the different isolates were sequenced on both strands using the PCR primers and internal primers every 300 bp as follows: (name, sequence, nucleotide numbering according to Lai & Kirsch,1989; accession number X13296, direction) Ca ERG11-01, TTAGGTCCAAAAGGTC, 432, sense; Ca ERG11-02, CATGACCTTTTGGACC, 451, antisense; Ca ERG11-03, GACCGTTCATTTGCTC, 787, sense; Ca ERG11-04, GAGCAAATGAACGGTC, 802, antisense; Ca ERG11-05, ATTCTTATGGGTGGTC, 1057, sense; Ca ERG11-06, GCAGAAGTAGAAGCAG, 1097, antisense; Ca ERG11-07, TCTCCAGGTTATGCTC, 1360, sense; Ca ERG11-08, CCCATCTAGTTGGATC, 1439, antisense). Primers were designed by visual inspection of the sequence for stretches of 16–18 nucleotides of normal composition (40–60% GC, no palindromes, no homopolymeric stretches). Primers were ordered from Eurogentec (Seraing) and were synthesized according to the β-cyanoethylphosphoramidite method. Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, used according to the instructions of the manufacturer (Perkin-Elmer), except that half of the volume of terminator mix was replaced by HalfTerm (GenPak). Sequencing reactions were run on an Applied Biosystems 377 XL DNA sequencer (Perkin-Elmer).

Sequences were assembled from the individual runs into single contig sequences by means of Sequencher software (Gene Codes Corporation). Ambiguity positions were scored by setting the threshold as low as 30% (i.e. secondary peaks at 30% of primary peak results in ambiguity call) and by inspecting all of the ambiguity calls on all available readings.

RESULTS

MIC determination

The MIC values obtained with the NCCLS compatible methodology confirmed the previously reported susceptibility of the different strains, except for isolate 6406/8. For both sensitive ATCC reference strains, MIC values of 0·13 µg ml^{-1} were found for fluconazole and 0·016 µg ml^{-1} or lower for itraconazole (Table 1). In these test conditions isolate 6406/8 was also inhibited at low concentrations of both fluconazole and itraconazole, but showed a pronounced trailing growth effect. Complete inhibition was seen in this spontaneous laboratory mutant only at a concentration of 32 µg fluconazole ml^{-1} and 2 µg itraconazole ml^{-1}. All other strains had high MIC values for fluconazole ($>$ 32 µg ml^{-1}) and itraconazole ($\geqslant$ 0·5 µg ml^{-1}) and as such fell either at the high end of the dose-dependent sensitivity range or in the resistant range, according to the NCCLS breakpoints. The difference in ratios of MIC values obtained for fluconazole versus itraconazole for the isolates studied (Table 1) suggests that resistance to fluconazole was more pronounced in the isogenic strains B59626 and B59630, and in isolates J913004/1 and C40. In the case of this last isolate it was shown that overexpression of *CaMDR1*, a major facilitator pump that takes fluconazole and not itraconazole as a substrate, contributed to this fluconazole resistance (Sanglard *et al*., 1995).

ERG11 sequence analysis

Both strands of the coding region of the *ERG11* genes of all eight strains were sequenced and compared to the Lai & Kirsch (1989) sequence. Each strain examined carried from 9–12 nucleotide changes of which at least one and up to four resulted in an amino acid substitution relative

Table 1. Amino acid polymorphisms found in sensitive and resistant isolates together with MIC values, sensitivity of the subcellular sterol biosynthesis and cytochrome P450 CO binding for fluconazole and itraconazole

Results shown are from representative experiments. Each experiment was repeated at least three times; variation was less than 10%.

Strain	MIC (μg ml^{-1})			Subcellular sterol biosynthesis					Cytochrome P450 CO displacement(IC_{50} nM)*			Amino acid point mutation (xNNNx/y)†
	Fluconazole	Itraconazole	Ratio	Fluconazole		Itraconazole		Ratio	Fluconazole	Itraconazole	Ratio	[additional silent nucleotide variations]
				IC_{50} (nM)	[%C] 1000 nM‡	IC_{50} (nM)	[%C] 100 nM‡					
ATCC 28516	0·13	⩽0·008	>16	41	5	44	2	0·9	145	38	3·9	F72F/L, E266D [10]
ATCC 44858	0·13	0·016	8	42	3	37	2	1·1	203	36	5·6	D153E, E266D [10]
B59626	>64	1	>64	87	5	33	1	2·6	270	40	6·8	D116E, K128T, A149V [8]
B59630	64	0·5	128	65	4	21	5	3·1	530	54	9·8	D116E, K128T, A149V [8]
NCPF 3363	32	2	16	220	21	92	48	2·4	221	55	2·4	Y132H, S279S/F, G465G/S [8]
J913004/1	64	1	64	172	17	22	7	7·8		Insufficient P450§		D116E, K128T, V452A, G464S [8]
6406/8	⩽0·032	⩽0·008		90	19	14	14	6·4		Insufficient P450§		E165Y [7]
C26	>128	>2		4880	78	20	24	244	3080	30	103	D116E, Y132H, S405F [8]
C40	128	1	128	378	37	34	19	11	5030	44	114	Y132Y/H, G464S, R467K [9]‖

* Results obtained with 0·1 nmol cytochrome P450 ml^{-1} in the cuvette.

† x is the amino acid found in the published Erg11p sequence, NNN is the position in the ORF and y is the amino acid found in the isolate; if both alleles differ in sequence a slash is used to indicate this heterozygosity.

‡ [%C] 1000 nM/100 nM: percentage of control at a concentration of 1000 nM/100 nM.

§ Despite several attempts and the addition of protease inhibitor, insufficient cytochrome P450 was isolated to test the sensitivity.

‖ Mutations taken from Sanglard *et al.* (1998b).

to the reference sequence as shown in Table 1. For the representation of the substitution we used the amino acid found in the reference sequence, followed by its position and the amino acid substitute found (all amino acids are designated by the conventional one-letter code). In total 12 different amino acid substitutions were identified. Six have already been reported: F72L (Ryder & Favre, 1997), D116E (Kallakuri *et al.*, 1996; Manavathu *et al.*, 1996; Ryder & Favre, 1997; Sanglard *et al.*, 1998b; Marr *et al.*, 1998), K128T (Kallakuri *et al.*, 1996; Manavathu *et al.*, 1996; Löffler *et al.*, 1997; Sanglard *et al.*, 1998b), Y132H (Ryder & Favre, 1997; Sanglard *et al.*, 1998b; Okonogi *et al.*, 1998), E266D (Kallakuri *et al.*, 1996; Löffler *et al.*, 1997; Ryder & Favre, 1997) and G464S (Manavathu *et al.*, 1996; Löffler *et al.*, 1997; Sanglard *et al.*, 1998b; Marr *et al.*, 1998), but the other six were novel (A149V, D153E, E165Y, S279F, V452A and G465S). To verify our sequencing technique, we also included strain C26, for which the ERG11 sequence has been published (Sanglard *et al.*, 1998b). All three mutations, D116E, Y132H and S405F, were confirmed as well as the other eight silent nucleotide changes, endorsing the validity of the sequencing analysis. Both sensitive reference strains, ATCC 28516 and ATCC 44858, contained an E266D variation. Strain ATCC 28516 was in addition heterozygous with respect to a phenylalanine/leucine substitution present on only one of its alleles, represented as F72F/L. Both alleles of strain ATCC 44858 carried a novel D153E mutation. Strains B59626 and B59630 were isolated from the same patient and their sequence was identical (in the 3 amino acid substitutions found as well as in 8 silent nucleotide changes) supporting the isogenicity of these strains, as was demonstrated by hybridization with the Ca3 repetitive probe (results not shown). The alanine/valine substitution (A149V) in these strains has not been reported previously. Strain NCPF 3363 was the first strain for which Vanden Bossche *et al.* (1990) suggested a mutation in *ERG11* based on biochemical evidence such as a diminished affinity for azole antifungals and a red shift in the maximum of the CO absorption spectrum from 448 to 450 nm. This strain contained the previously identified Y132H mutation on both of its *ERG11* alleles and two heterozygotic substitutions S279S/F and G465G/S. The importance of this Y132H mutation for azole susceptibility was demonstrated by Sanglard *et al.* (1998b). Strain J913004/1, isolated from an AIDS patient, contained four mutations, of which only V452A has not been reported previously. The laboratory mutant 6406/8 contained only one mutation, E165Y. This mutation was the result of a double nucleotide change in a single codon, GAA into TAC.

Erg11p activity and sensitivity determination

In recent papers, Lamb *et al.* (1997) and Sanglard *et al.* (1998) described elegant strategies to test for alterations in affinity of variant cytochrome P450 14-demethylases for azole derivatives. Both used heterologous expression in a *S. cerevisiae* host using a strong *GAL* promoter to overexpress the *Candida* Erg11p. In the Lamb *et al.*

Table 2. Reported amino acid polymorphisms found in sensitive and resistant isolates and available characterization data

Strain	Reference	Amino acid point mutation (xNNNx/y)*	MIC (μg ml^{-1})			ERG11 activity/sensitivity measurement	
			Fluconazole	Itraconazole	Ratio	Method†	Conclusion
1	White (1997)		0·25	0·03	8	A	Flu-sensitive
17	White (1997)	R467K	>64	>2	–	A	Flu 12× less active relative to strain 1
1	Marr *et al.* (1998)	D116E	1·25	0·03	42	A	Flu- and Itz-sensitive
5	Marr *et al.* (1998)	D116E	64	0·3	213	A	Flu- and Itz-sensitive
I1	Löffler *et al.* (1997)		4	0·47	9	ND	
I4	Löffler *et al.* (1997)	F105L	128	0·064	2000	ND	
I7	Löffler *et al.* (1997)	V488I	>256	>32	–	ND	
I8	Löffler *et al.* (1997)	E266D, G464S	>256	>32	–	ND	
I12	Löffler *et al.* (1997)	F105L, E266D	8	0·25	32	ND	
TU1	Löffler *et al.* (1997)		48	1	48	ND	
TU2	Löffler *et al.* (1997)		>256	0·75	>341	ND	
TU3	Löffler *et al.* (1997)	E266D, G448E	>256	4	>64	ND	
TU4	Löffler *et al.* (1997)		3	0·125	24	ND	
TU5	Löffler *et al.* (1997)		64	0·75	85	ND	
TU6	Löffler *et al.* (1997)	F105L, E266D	>256	6	>43	ND	
TU7	Löffler *et al.* (1997)		64	1	64	ND	
TU8	Löffler *et al.* (1997)		>256	1	>256	ND	
TU9	Löffler *et al.* (1997)	K287R, G464S	>256	>32	–	ND	
P1	Löffler *et al.* (1997)		>256	>32	–	ND	
P2	Löffler *et al.* (1997)	F105L, G464S	>256	>32	–	ND	
P3	Löffler *et al.* (1997)	F105L, G450E	>256	0·75	>341	ND	
P4	Löffler *et al.* (1997)	G450E, V488I	>256	>32	–	ND	
P5	Löffler *et al.* (1997)	F105L	>256	>32	–	ND	
FS10	Löffler *et al.* (1997)	K128T	0·25	0·5	0·5	ND	
FS19	Löffler *et al.* (1997)	K147R	0·1	0·25	0·4	ND	
ATCC 90028	Okonogi *et al.* (1998)		Flu^S	Itz^S	–	A	
DUMC136	Okonogi *et al.* (1998)	Y132H, F145L	Flu^R	ND	–	A	Wild-type enzyme activity but Flu-resistant
DC	Kallakuri *et al.* (1996)	D116E, K128T, K287R	Flu^S	ND	–	ND	
B312	Kallakuri *et al.* (1996)	D116E, K143R, E266D, R267H, D278E	Flu^R	ND	–	ND	
PL01A	Manavathu *et al.* (1996)	D116E, K128T, G450E, G464S	Flu^R	ND	–	ND	
C23	Sanglard *et al.* (1998b)	D116E, K128T, S405S/F	1	0·0625	16	B	S405F allele; MIC increase for Flu (4×) and for Itz (2×)
C39	Sanglard *et al.* (1998b)	S405F	32	0·125	256	B	MIC increase for Flu (4×) and for Itz (2×)
C33	Sanglard *et al.* (1998b)	D116E, E266Q, V437I	0·25	0·0312	8	B	
C34	Sanglard *et al.* (1998b)	D116E, S405F	2	0·0625	32	B	MIC increase for Flu (4×) and for Itz (2×)
C26	Sanglard *et al.* (1998b)	D116E, Y132H, S405F	>128	4	>2·0	B	MIC increase for Flu (>64×) and for Itz (8×)
C82	Sanglard *et al.* (1998b)	D116E, S405F	32	1	32	B	MIC increase for Flu (4×) and for Itz (2×)
C27	Sanglard *et al.* (1998b)		1	0·0312	32	B	
C37	Sanglard *et al.* (1998b)	G464S, R467K	8	0·0625	128	B	MIC increase for Flu (8×) and for Itz (2×)
C40	Sanglard *et al.* (1998b)	Y132Y/H, G464S, R467K	128	1	128	B	Y132H allele; MIC increase for Flu (>64×) and for Itz (8×)
C43	Sanglard *et al.* (1998b)	D116E, E266Q	0·25	0·0312	8	B	
C56	Sanglard *et al.* (1998b)	D116E, G129A, G464S	128	>2	–	B	MIC increase for Flu (32×) and no increase for Itz
ATCC 64124	Ryder & Favre (1997)	F72L, Y132H, G450E	>128	>4	–	A	Flu-resistant
NFI2007	Ryder & Favre (1997)	F126L, E266D, S405F, V437I	32	0·25	128	A	Flu-resistant
NFI2013	Ryder & Favre (1997)	D116E, Y132H, F449L	>128	>4	–	A	Flu-resistant
NFI2021	Ryder & Favre (1997)	K143E, T229A	>128	1	>128	A	Flu-resistant
–‡	Lamb *et al.* (1997)	T315A	–	–	–	B	Enzyme less active, 5× less sensitive to Flu

ND, No data available.

*x is the amino acid found in the published Erg11p sequence, NNN is the position in the ORF and y is the amino acid found in the isolate; if both alleles differ in sequence a slash is used to indicate this heterozygosity.

† A, [^{14}C]Mevalonic acid incorporation into sterols using a subcellular fraction. B, heterologous overexpression (*gal* promoter) of the cloned *ERG11* in a *S. cerevisiae* host. Sensitivity was measured as an increase of MIC relative to the parent allele.

‡ Mutation created by PCR.

(1997) system, the recombinant protein was purified and analysed with biochemical assays, whereas in the Sanglard *et al.* (1998b) method, affinity changes were directly correlated with changes in MIC values obtained with the engineered *Saccharomyces* strains. In this study we used biochemical assays with subcellular extracts prepared from the different isolates to look for the effects of different azole concentrations on either the incorporation of [2-^{14}C]mevalonic acid into sterols or on the CO-complex formation of reduced microsomal cytochrome P450 fractions. Because two strains used in the Sanglard *et al.* (1998b) paper, C26 and C40, were included in this study, the results obtained with the different strategies could be compared.

The results obtained with the subcellular sterol bio-

synthesis method are summarized in Table 1. In the case of J913004/I and 6406/8, addition of PMSF to the homogenization buffer was found to be essential for enzyme activity, whereas no improvement was observed for the other isolates tested. For consistency reasons, PMSF was added to all preparations. According to the IC_{50} values obtained for itraconazole, only strain NCPF 3363, previously reported to contain a mutated cytochrome P450 (Vanden Bossche *et al*., 1990), was less sensitive, having an IC_{50} value of 92 nM. All other isolates gave IC_{50} values in the range 14–40 nM and as such can be regarded as itraconazole-sensitive. However, differences were seen at concentrations above the IC_{50} value, as shown in Table 1. Indeed at 100 nM, a near complete inhibition was seen with the susceptible reference strains ATCC 28516 and ATCC 44858, and with isolates B59626, B59630 and J913004/I. A leaky ergosterol biosynthesis inhibition (14–24% of control) at this concentration was seen with extracts from 6406/8, C26 and C40, suggestive of a decreased affinity for itraconazole. For fluconazole, more pronounced differences were observed. IC_{50} values ranged more than 100-fold, from 41 nM for the most sensitive isolate up to 4880 nM for the C26 isolate. As well as in this C26 strain, reductions in affinity were seen for three other isolates, NCPF 3363, J913004/I and C40. Fluconazole was as active as itraconazole with regard to inhibition of subcellular sterol biosynthesis for both reference ATCC strains, whereas for all other azole-resistant strains tested, a 2·4- to 244-fold difference was observed in favour of itraconazole, suggesting that fluconazole binding is more sensitive to the amino acid variations present in these isolates. Incomplete inhibition of ergosterol biosynthesis was more pronounced with fluconazole because even at 1000 nM, isolates NCPF 3363, J913004/1, 6406/8, C26 and C40 still produced 16–78% of the amounts found in control conditions.

Prevention of CO-complex formation in the reduced microsomal cytochrome P450 preparations is another assay that can be used to test the affinity of the protein for an azole (Vanden Bossche *et al*., 1987; Yoshida, 1988). With this method, a constant content of 0·1 nmol cytochrome P450 ml^{-1} is used, enabling direct comparison of susceptibility between different isolates. Fluconazole was consistently found to be less active compared to itraconazole, even in the sensitive reference strains ATCC 28516 and ATCC 44858. Itraconazole was able to prevent CO–cytochrome complex formation in all isolates from which an active extract could be isolated. For strains J913004/I and 6406/8 we consistently failed to obtain a fraction suitable for analysis. The addition of a protease inhibitor such as PMSF, or a protease inhibitor mix (leupeptin, PMSF, 4-amidino-PMSF) did not sufficiently improve the yield of active cytochrome P450. For the other strains, as seen in the sterol biosynthesis assays, differences were far more pronounced for fluconazole: IC_{50} values ranged almost 35-fold from 140 to 5030 nM. Again, microsomes isolated from C26 and C40 showed the largest shifts, although the C40 microsomal fraction was the least sensitive in this assay. The cytochrome P450 isolated from NCPF 3363 was as sensitive as that isolated from susceptible strains.

Computer-aided sequence analysis of identified mutations

In Table 2 the published sequence variations of *C. albicans ERG11* alleles are summarized next to the available growth sensitivity data. In total 53 full sequences were obtained, resulting in 98 variations relative to the Lai & Kirsch (1989) sequence at 29 different locations, in addition to the PCR-induced T315A mutation reported by Lamb *et al*. (1997). This is a mean of 1·85 mutations per sequence. Because not all authors mention the silent nucleotide mutations found, a precise number of nucleotide changes cannot be given. For the seven strains sequenced in this study, 59 silent mutations were found (16 positions) compared to 18 amino acid changes (12 different), which suggests a three-fold higher frequency of silent mutations. To visualize the position and the frequency of the amino acids substitutions reported, a graphical representation was made (Fig. 1). At the top of the figure, the aligned sequences of the gene products of *C. albicans ERG11* and that of the *Pseudomonas putida CYP101* gene ($P450_{cam}$) and their helical secondary structures are visualized. The alignment is taken from the model of Boscott & Grant (1994). Gaps are represented as thin lines and predicted α-helices are represented by boxes, for which the starting position and length are indicated. The letter code for the helices is indicated above the boxes. The mutations are represented by bars, for which the length is proportional to the frequency of occurrence. Different filling patterns are used to categorize the mutation: mutations found both in azole-sensitive and -resistant strains are represented by open bars and are probably not important for azole affinity. These substitutions could reflect allelic or strain variation. A filled bar is used for the mutations experimentally demonstrated to be important for the affinity of an azole for cytochrome P450. Non-characterized mutations are shown represented by hatched bars. The PCR-induced mutation is represented by an arrow. From Fig. 1 it is clear that the majority of the mutations were found in three regions: region 1 from amino acids 105 to 165, region 2 from 266 to 287 and region 3 from 405 to 488. This last region also contains C_{470}, the fifth ligand of the haem. Three of the four mutations found with the highest frequency, D116E, K128T and E266D, were also found in azole-sensitive strains; only G464S has been seen exclusively in resistant strains. No spontaneous mutation was found in the I helix, which is highly conserved over the 14-demethylase cytochrome P450 family.

A molecular three-dimensional model of *C. albicans* Erg11p was proposed by Boscott & Grant (1994; coordinates are available from these authors) after homology modelling onto the bacterial crystal structure of Cyp101p. In Fig. 2 views from two different

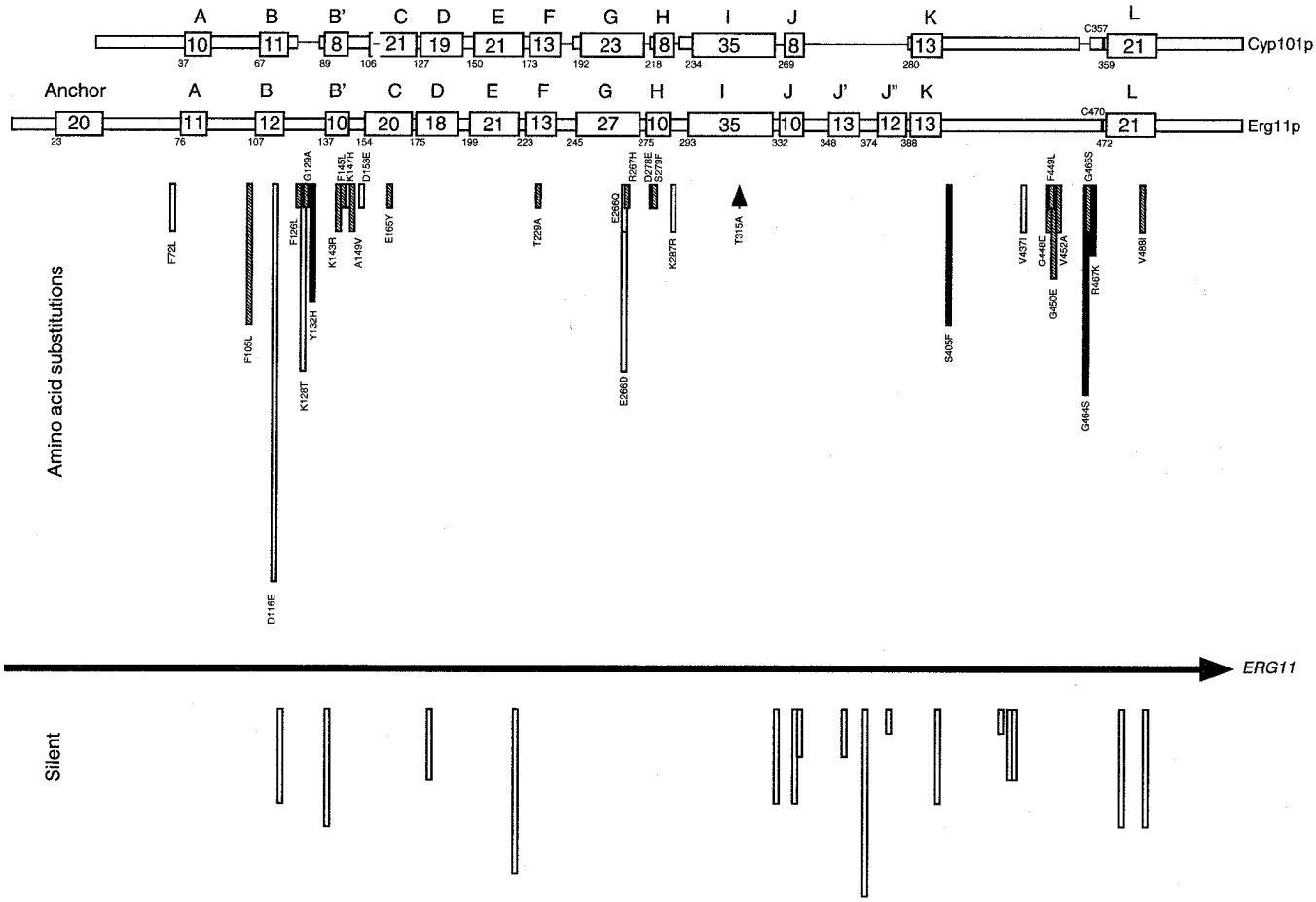

Fig. 1. Visualization of the localization and frequency of mutations in the Erg11p (Cyp51p) sequence. At the top of the figure, the aligned sequences of *C. albicans* Erg11p and *P. putida* Cyp101p and their secondary structure are visualized. Gaps are represented as thin lines and predicted α-helices are represented by boxes, for which the starting position and length are indicated. The letter code for the helices is indicated above the boxes. The amino acid mutations found are represented by bars, for which the length is proportional to the frequency of occurrence. Different filling patterns are used to categorize the mutation: mutations found both in azole-sensitive and -resistant strains are indicated by open bars; filled bars are used for the mutations for which it has been experimentally demonstrated that they were important for the affinity of an azole for the cytochrome P450; hatched bars indicate non-characterized mutations. The PCR-induced mutation is shown with an arrow. The fifth ligand of the haem, C470, is also indicated. At the bottom of the figure, the *ERG11* ORF is represented by a horizontal arrow. Silent mutations found in this study are represented by open bars. Again the length of the bars is proportional to the frequency of occurrence.

angles are given of the ribbon model. The haem is shown in green and, as an orientation aid, the central I helix is represented in orange. Because of the projection onto the plane of the paper, localization of the mutations can be misleading. To circumvent these optical artefacts, stereo views were included to allow a more precise 3D localization. The mutations discussed in this paper are placed onto this ribbon model as solid spheres with a diameter of the Van der Waals interaction range of a carbon atom. The substitutions are again categorized according to the same principle as in Fig. 1, however, a colour code is used in this model; substitutions found in both azole-sensitive and -resistant Erg11p are represented by blue spheres, substitutions experimentally demonstrated to be important for azole affinity are shown in red, non-characterized mutations are shown in black and the PCR-induced mutation is shown in green. The cytochrome P450 Erg11p catalyses the oxidative removal of the 14-methyl-group of ergosterol precursors. The prosthetic haem molecule is positioned between the proximal I helix and the distal L helix in the inner part of the enzyme as is shown in Fig. 2. This necessitates both the entry and positioning of the substrate such that its 14-methyl group is in close proximity to the activated sixth ligand of the central haem. This suggests that the substrate should position below the I helix and proximal to the haem. Because in this model no access channel is found of sufficient size to enable entry and exit of the substrate, it is hypothesized that some movement of the B′ helix and/or the coil between the F and G helices is necessary for the entry. However, because of poor sequence similarity in this

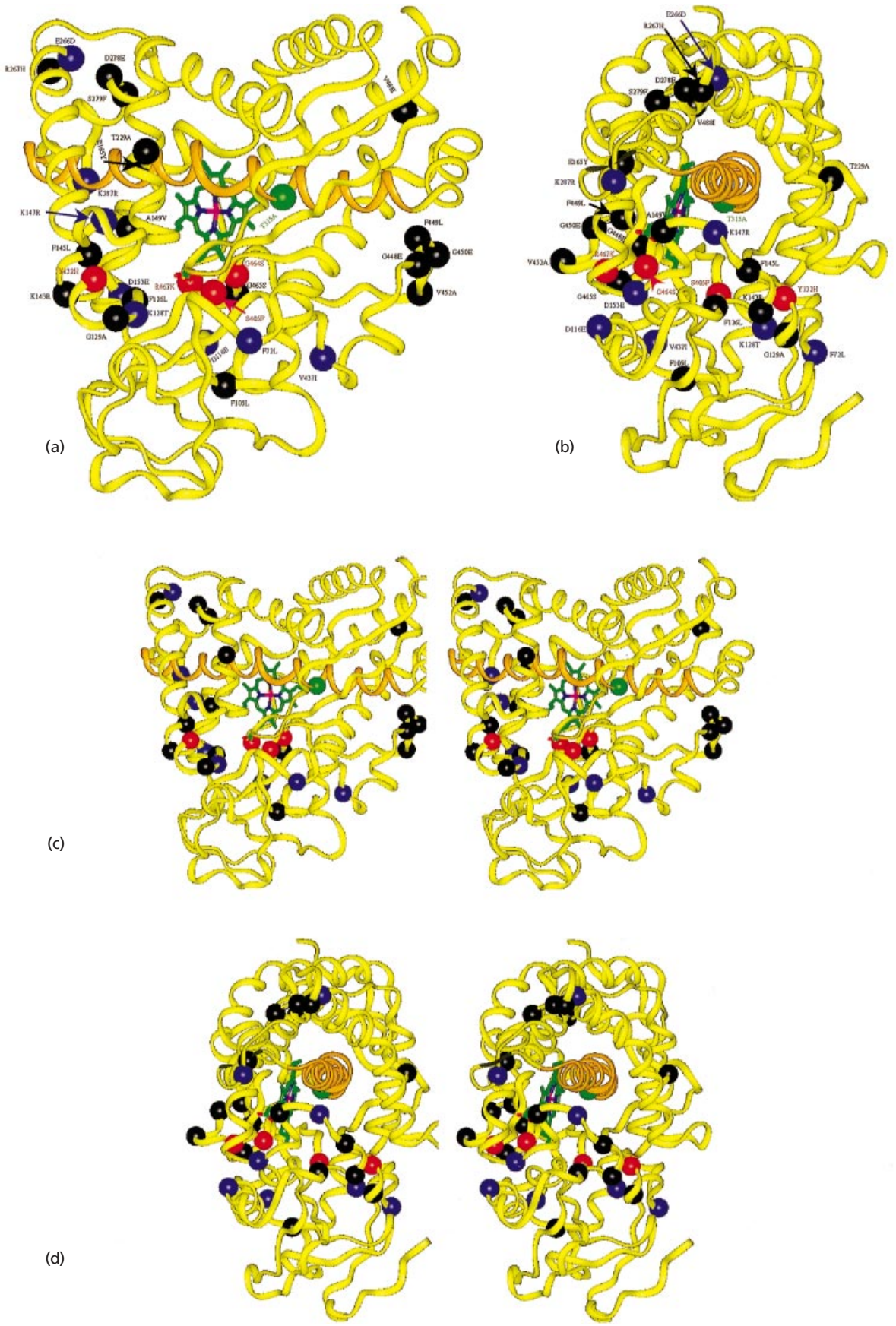

Fig. 2. For legend see facing page.

Table 3. Sequence comparison of the 19 different 14-demethylases

The sequences were aligned with CLUSTAL W. Identified amino acid substitutions found in *C. albicans* are shown. Amino acids identical to *C. albicans* are represented by a dot. Highly conserved amino acids are in bold type. '–' is indicative of a gap in the sequence alignment. The full length alignment is available on request. Amino acid numbering is taken from the *C. albicans* sequence.

Organism	GenBank/EMBL accession no.	F72L	F105L	D116E	F126L	K128T	G129A	Y132H	K143R\E	F145L	K147R	A149V	D153E	E165Y	T229A	E266D	R267H	D278E	S279F	K287R	T315A	S405F	V437I	G448E	F449L	G450E	V452A	G464S	G465S	R467K	V488I
Candida albicans	X13296	F	F	D	F	K	G	Y	K	F	K	A	D	E	T	E	R	D	S	K	T	S	V	G	F	G	V	G	G	R	V
Saccharomyces cerevisiae	M18109	.	.	A	.	.	.	.	.	.	.	.	E	.	.	K	N	.	.	.	.	.	.	.	.	.	I	.	.	.	I
Candida glabrata	S75389	.	.	A	.	.	.	.	.	.	.	.	E	.	.	.	K	.	E	.	.	.	A	.	.	.	I	.	.	.	I
Candida tropicalis	M23673	.	Y	E	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	I	.	.	.	I
Candida krusei	S75391	.	L	.	.	.	.	.	.	.	.	.	E	.	.	A	G	.	A	.	.	.	I	.	.	.	T	.	.	.	
*Candida parapsilosis**	X97681														.	.	T	.	.	.	.	.	G								
*Candida guilliermondii**	X97680																				.	F									
*Candida kefyr**	X97682																				.	.									
*Pichia anomalia**	AF19903									.	.	.	.	.	.	.	N	.	T	.	.	.	Q	.	.	.	.				
Schizosaccharomyces pombe	Z54096	.	.	E	.	.	D	.	.	.	.	G	S	.	S	–	–	I	W	R	.	A	–	.	Y	.	.	.	A	.	.
Penicillium italicum	Z49750	.	L	E	.	S	D	.	.	.	.	G	E	.	S	S	W	I	.	R	I	.	.	.	Y	.	.	.	A	.	.
Ustilago maydis	Z48164	.	.	.	.	.	E	.	.	.	.	G	E	.	.	A	A	I	A	.	.	A	Q	.	.	.	I	.	A	.	.
Uncinula necator	U72657	Y	L	E	.	R	D	F	.	.	.	.	E	.	.	–	–	M	W	.	.	.	N	.	Y	.	T	.	A	.	.
Pig	S17048	L	.	.	.	.	.	.	.	M	.	G	A	.	S	Q	S	L	Q	.	.	.	A	–	–	–	–	.	A	.	L
Rat	D55681	L	.	E	.	.	.	.	.	I	.	G	A	.	S	L	S	L	Q	.	.	.	A	–	–	–	–	.	A	.	L
Human	D55653	L	.	.	.	.	.	.	.	M	.	G	A	.	S	Q	S	L	Q	.	.	.	A	–	–	–	–	.	A	.	L
Sorghum bicolor	U74319	I	.	Q	.	P	.	F	R	.	T	.	N	.	Q	A	S	L	Q	.	.	.	E	–	–	–	–	.	.	.	L
Triticum aestivum	Y09291	I	.	Q	.	P	.	F	R	.	T	.	N	.	L	A	S	L	Q	.	.	.	E	–	–	–	–	.	.	.	L
Arabidopsis thaliana	AC002329	L	.	K	.	P	.	F	R	.	S	.	–	–	Q	R	S	L	Q	.	.	.	E	–	–	–	–	.	A	.	L

*Partial sequence available.

region between the fungal sequences and the bacterial models, no precise entry point can be identified. The antifungal azoles are believed to enter through the same access channel and inhibit cytochrome P450 by binding with their unprotonated N atom to the sixth co-ordination site of the haem and occupy the substrate-binding pocket (Vanden Bossche & Koymans, 1997). An important factor of the stability and specificity of the binding of different azoles originates from the interaction of the N1 ligand moiety of the azole with the apoprotein. Mutations in the protein could alter the affinity for an azole by interference with the entry of the azole or could change interaction points either directly or indirectly by a repositioning of the tertiary structure through changes in the positions of the structural helices as was demonstrated by the G310D mutation in *S. cerevisiae* Erg11p (Vanden Bossche & Koymans, 1997). The substitutions F126L, K128T, G129A, Y132H, K143R, F145L and K147R are close to this access channel and could interfere with the entry of the inhibitor. The G464S, G465S and R467K mutations are positioned behind the plane of the haem. The G465S and R467K mutations are probably too far away from the haem to interfere directly with the interaction of the azoles but G464S is in very close proximity. The S405F substitution is positioned just after the K helix and is close to the substrate and azole-binding pocket. Mutations E266D, R267H, D278E and S279F are located at the end of the G helix and the start of the H helix on the surface of the protein. This region could act as a pivot for the G- and F-helices to enlarge the access channel. Mutation T229A is on the F helix, but rather distant from the I helix. The V488I substitution is found at the end of the L helix. The four mutations G448E, F449L, G450E and V452A are close to the site where the I helix ends. No mutations are seen in the bottom part of the protein below the F105L mutation. This region of the protein is likely to be buried into the membrane.

Fig. 2. Ribbon presentation of the three-dimensional model of *C. albicans* Cyp51p structure from two different viewpoints (a and b). The central I helix is shown in orange, the haem is shown in green. The mutations described in this study are marked by spheres and are categorized according to the same principle as in Fig. 1; however, a colour code is used in this model; substitutions found in both azole-sensitive and -resistant strains are represented by blue spheres, substitutions experimentally demonstrated to be important for azole affinity are shown in red, non-characterized mutations are shown in black and the PCR-induced mutation is shown in green. To allow a more precise spatial localization, stereo pictures of both views are shown in (c) and (d).

It is likely that an amino acid that is important for azole interaction is conserved in the Erg11p sequences from other sensitive species. To investigate this, we first aligned all 19 known sterol 14-demethylases (13 fungal Erg11p sequences, three mammalian Cyp51p sequences and three plant Cyp51p sequences) using the CLUSTAL W multiple alignment algorithm. Table 3 lists the aligned amino acids obtained for all 29 identified mutations. The full sequence alignment is available from the corresponding author upon request. Only three amino acids were conserved for all available 14DM sequences, F126, G464 and R467. If the plant sequences were excluded, K143, K147 and E165 are conserved across the fungal and mammalian Cyp51p sequences and amino acids F145, G448, and G450 are conserved across all fungal Cyp51p sequences. Among the amino acid substitutions found in azole-sensitive strains, D116E, D153E, E266D and V437I vary even among the yeast sequences, as do the non-characterized F105L, R267H, S279F, V452A and V488I substitutions. It is therefore unlikely that these substitutions are on their own important for azole binding.

DISCUSSION

Strain 6406/8 has been described as azole-resistant but this could not be confirmed in this study. The different conditions in which sensitivity was measured could be responsible for this discrepancy. Another possibility is that long-term storage and/or distribution of the isolate to different laboratories has resulted in changes. Indeed, a similar phenomenon was demonstrated for the Darlington strain, for which isolates obtained from different locations were found to behave differently with regard to their sensitivity and small variations were seen in their restriction fragment length polymorphism (RFLP) patterns (Pearce & Howell, 1991). It is unlikely that the double nucleotide change found in a single codon was introduced at the same time. If the first mutation event was GAA to TAA, this would introduce a stop codon interrupting the translation process. After homologous recombination, this would result in a 14-demethylase-deficient strain as reported by Hitchcock *et al*. (1987). The subsequent introduction of a second mutation, TAA to TAC, would be advantageous for the isolate because it would restore the transcription of the full length of the ORF and 14-demethylase activity. The 14-demethylase deficiency reported could, however, also originate from the absence of a protease inhibitor in the enzyme preparation procedure, because we found such an addition necessary for enzyme activity. To verify the different hypotheses we would need access to the different subcultured specimens from the original 6406/8 strain.

Three different methods are available to investigate the affinity of cytochrome P450 for an azole inhibitor and each method has its strengths and limitations. The CO-binding assay is very easy to perform and allows immediate comparison between different isolates because a standard amount of cytochrome P450 is used. A drawback of this method is that preparations must be made from strains growing in high glucose and with oxygen limitation. Moreover, the preparation could contain non-14-demethylase cytochrome P450 and the long preparation time needed to obtain the washed microsomal preparation increases exposure to proteolysis. The subcellular ergosterol bioassay requires less preparation and as such is less sensitive to proteolysis. With both of these methods the enzymes are in their natural *Candida* membrane environment. This is in contrast with the third method: heterologous expression in *S. cerevisiae*. This heterologous expression method allows measurements of single allele products but because of differences in preferred codon usage between *Candida* and *Saccharomyces* could also introduce contributions from silent mutations. The use of a strong *GAL* promoter could also introduce unnaturally high concentrations of the cytochrome. Indeed Lamb *et al*. (1997) found concentrations of 2·5 nmol microsomal protein mg^{-1}, at least 25 times the basal level. This huge overexpression of membrane-bound protein forces cells to hyperproliferate their membranes, which often form multilamellar membrane structures around the nucleus (karmella) (Vergeres *et al*., 1993; Supply *et al*., 1993). It is therefore not surprising that the results obtained with the three methods do not match perfectly. The C26 Erg11p variant has a lower affinity for fluconazole according to all three methods. In contrast, the eightfold decrease in affinity for itraconazole suggested by the heterologous expression system was not confirmed by CO binding or by a decrease in IC_{50} in the subcellular assay. The incomplete enzyme inhibition at 100 nM itraconazole observed in the subcellular assay could, however, be of importance because for growth inhibition in *C. albicans* a depletion of ergosterol is needed. If a leaky inhibition is sufficient for resistance, then four out of nine isolates (NCPF 3363, 6406/8, C26 and C40) could be regarded as itraconazole-refractory and in the case of fluconazole isolate J913004/1 is also resistant. These criteria suggest that the E165Y mutation found in the laboratory mutant interferes with both itraconazole and fluconazole binding. To demonstrate this, the E165Y mutation should be introduced by mutagenesis; if one envisages heterologous expression in a *Saccharomyces* host, one should moreover prevent the unintended S263L mutation resulting from the different translation of the CUG codon in *Candida* versus *Saccharomyces*. Three other refractory alleles contain the Y132H substitution, corroborating its importance. As suggested by Sanglard *et al*. (1998b) a concomitant substitution of S405F or R467K further decreases affinity, especially for fluconazole. The smaller, hydrophilic fluconazole molecule has fewer stabilization sites in the active pocket compared to the lipophilic itraconazole molecule which may explain this difference in activity (Vanden Bossche & Koymans, 1997). Moreover, because the CO-binding assay did not reveal a diminished sensitivity for the NCPF 3363 isolate (having a homozygotic Y132H substitution), it is likely

that a concomitant mutation is necessary for maximal effect. In this isolate, the originally reported shift in CO-binding sensitivity (Smith *et al*., 1986), comparable to that found in the C26 and C40 isolates for fluconazole, could not be reproduced on later occasions; this could be due to loss of a second mutation on one or on both alleles. Unfortunately, the isolate possessing the original phenotype could not be retrieved to investigate this hypothesis.

The sequence available for *Candida guilliermondii* Erg11p also contained a phenylalanine aligning with S405 from *C. albicans*. Because different *C. guilliermondii* isolates tend to vary substantially in their fluconazole susceptibility (Odds, 1992), it would be interesting to know the MIC of fluconazole for the isolate used for sequencing and to verify whether the presence of that phenylalanine correlates with higher fluconazole MIC values. From the substitutions identified in the J913004/I isolate, D116E and K128T probably do not contribute to the decrease in fluconazole activity because these substitutions were also found in multiple azole-sensitive strains. In fact, it is likely that all substitutions found in azole-sensitive strains, and presumably also a selection of the non-characterized substitutions found only in resistant strains so far, reflect strain variation. From the substitutions found in the N-terminal and central region of the protein, only Y132H was associated with resistance. In these regions a higher proportion of the substitutions was also found in azole-sensitive strains when compared to the C-terminal region. No spontaneous substitutions have been found so far in the very conserved central I helix. A reason for this could be that substitutions in this region induce loss of function of the enzyme that would result in slower growth rates and therefore would be disadvantagous for survival. Such a mutation, G310D, has been described in *S. cerevisiae* (Ishida *et al*., 1988). The C-terminal part contains three substitutions associated with resistance, and from the remaining substitutions only one was found in azole-sensitive strains. This region therefore looks to be of greater importance for azole resistance. Further study is needed to verify to what extent V452A and G464S mutations contribute to resistance. The fact that G464S was found by several investigators in azole-resistant strains makes this a prioritized substitution for investigation. The V452A substitution, as well as the G448E, F449L and G450E mutations are situated near the end of the central I helix and the J helix, an area of the protein that could be important for the docking of cytochrome P450 reductase. Indeed it is hypothesized that this region, which is absent in the Cyp101 sequence, and as such also in the model, is creating the recognition and docking site for the reductase. It has to be stressed that the three-dimensional model used is based on a soluble isozyme of bacterial origin, with different substrate specificity. For this reason, only approximate locations of the mutations can be given. The *Mycobacterium tuberculosis CYP51*-like gene, identified by *in silico* analysis of the full genome, could become a better model because this soluble enzyme could 14-demethylate dihydrolanosterol but crystallization and structure determination has not been achieved yet (Aoyama *et al*., 1998; Bellamine *et al*., 1998). The availability of multiple sequences of Erg11p from *Candida krusei*, a species with a 14α-demethylase less susceptible to azoles (Marichal *et al*., 1995; Orozco *et al*., 1998) could provide additional comparative material to identify important regions for binding of azoles to the cytochrome P450.

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