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Research Article

CaALK8, an alkane assimilating cytochrome P450, confers multidrug resistance when expressed in a hypersensitive strain of *Candida albicans*

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Abstract

We report the isolation of a novel *C. albicans* gene designated *CaALK8*, by its ability to complement drug hypersensitivity of a *pdr5* (ABC: ATP-binding cassette drug extrusion pump) null mutant of *S. cerevisiae* (JG436). *CaALK8* in JG436 conferred resistance to drugs such as cycloheximide (CYH), fluconazole (FCZ), *O*-phenanthroline (PHE) and 4-nitroquinoline oxide (NQO). The gene was so designated because its sequence was identical to a partial sequence entry named as *ALK8* in the *Candida* database (http://alces.med.umn.edu/candida.html). *CaALK8* encodes for a putative 515 amino acid protein highly homologous to alkane-inducible cytochromes P450 (*CYP52* gene family) of *C. maltosa* and *C. tropicalis*. The ability of *CaALK8* to confer drug resistance was also established by its expression in another drug-hypersensitive strain of *S. cerevisiae* (AD 1234568), which was deleted in seven ABC efflux pumps. The homozygous disruption of *CaALK8* in a wild-type *C. albicans* strain (CAI4) did not result in altered drug susceptibilities. The overexpression of *CaALK8* in CAI4 resulted in only FCZ resistance. However, a distinct MDR phenotype was evident when *CaALK8* was overexpressed in a drug-hypersensitive *C. albicans* strain disrupted in both *CDR1* and *CDR2* (ABC drug extrusion pumps of *C. albicans*). Alk8p, similar to other Alk proteins from *C. maltosa* and *C. tropicalis*, could hydroxylate alkanes and fatty acids. In this study we demonstrate that several drugs could compete with the hydroxylation activity by directly interacting with CaAlk8p. Taken together, our results suggest that a member of the *CYP52* gene family could mediate MDR in *C. albicans*, although it does not seem to be involved in the development of azole resistance in clinical isolates. The nucleotide sequence reported in this paper has been submitted to GenBank under Accession No. Y14766.

Keywords: **multidrug resistance; alkane-inducible cytochrome P450; *Candida albicans***

Introduction

The opportunistic fungus *Candida albicans* is a major cause of infections in humans, and is predominantly rampant in immunocompromised individuals (Prasad, 1991; Odds, 1988; Prasad *et al*., 1996; Cannon *et al*., 1998; White *et al*., 1998). *C. albicans* infections are treated with antifungal agents, particularly with the triazole derivative fluconazole (FCZ). The repeated use of FCZ to treat candidiasis has led to the emergence of resistant species of *Candida*, which earlier had often been correlated with an alteration or an over-expression of the target enzyme of azoles, viz. 14 α-lanosterol demethylase (P45014DM) involved in sterol biosynthesis (Vanden Bossche *et al*., 1992; Marichal *et al*., 1999). However, the characterization of the first ATP-binding cassette (ABC) protein Cdr1p (Prasad *et al*., 1995) and the subsequent identification (Cdr2p, another ABC protein) and implication of other efflux pumps (CaMdr1p, a major facilitator) (Sanglard *et al*., 1997; Fling *et al*.,

1991), due to their overexpression in azole-resistant clinical isolates, has led to the suggestion that these transporters represent another mechanism involved in the multidrug resistance (MDR) scenario of *C. albicans* (Krishnamurthy *et al.*, 1998a; Sanglard *et al.*, 1997; Gupta *et al.*, 1998; Lopez-Ribot *et al.*, 1998; Marr *et al.*, 1998). A defect in $\Delta^{5,6}$-desaturase, an enzyme responsible for the conversion of ergosta-7,22-dienol into ergosterol, has also been shown to contribute to azole resistance (Kelly *et al.*, 1997). The lack of overexpression of known MDR genes of *C. albicans* in some azole-resistant clinical isolates suggested possibilities of as yet unknown mechanisms that could be contributing to such resistance. Evidence accumulated so far suggests that several mechanisms involved in conferring azole resistance can simultaneously exist in a single resistant isolate (White *et al.*, 1998).

The modification of drugs to their non-toxic forms mediated by cytochromes P450 represents another mechanism by which a cell could confer resistance to different drugs. The role of cytochrome P450 as the detoxifying enzymes in prokaryotes, as well as in eukaryotes, is well-established (Graham-Lorence and Peterson, 1996; Omura, 1999). Although, in yeasts, the existence has been shown of two different classes of cytochromes P450, viz. P45014DM and P450alk (alkane-inducible), neither has been linked to xenobiotic metabolism (Kappeli, 1986). P450alk genes represent a large family of genes in *Candida* which make them unique in utilizing straight-chain hydrocarbons. Eight members of P450alk genes have already been identified in *C. maltosa* and *C. tropicalis* and there is evidence that at least four such genes also exist in *C. albicans* (**http://alces.med.umn.edu/candida.html**) (Ohkuma *et al.*, 1995; Seghezzi *et al.*, 1992). *S. cerevisiae*, on the other hand, lacks this class of cytochrome P450 and thus is unable to assimilate hydrocarbons.

In this report we describe the molecular characterization of *CaALK8*, an alkane-inducible cytochrome P450 gene of *C. albicans*, and demonstrate that it could confer multidrug resistance in a hypersensitive strain of this pathogenic yeast.

Materials and methods

Strains and media

JG436 (*Mat***a**, *pdr5::Tn5, leu2, met5, ura3-52, mak71, KRB1*) and AD 1234568 (*Matα*, *pdr1-3*, *his*1, *ura*3, *pdr5*Δ, *snq2*Δ, *pdr10*Δ, *pdr11*Δ, *pdr15*Δ, *yor1*Δ, *ycf1*Δ) transformants were grown in YNB as described previously (Prasad *et al.*, 1995; Krishnamurthy *et al.*, 1998b). *C. albicans* strain CAI4 *(*Δ*ura3::imm434/*Δ*ura3::imm434*) and DSY1025 (Δ*cdr1::hisG/*Δ*cdr1::hisG/*Δ*cdr2::hisG/* Δ*cdr2:: hisG*) were grown in YEPD.

Isolation of *CaALK8*

The pYEURA3-based centromeric library used for functional complementation was a kind gift from C. Nombela, Madrid, Spain. One of the many transformants (obtained after functional complementation in *pdr5* null mutant of *S. cerevisiae*) displaying a multidrug resistance profile was analysed by restriction mapping and found to carry a 2.6 kb genomic DNA insert (pALK8Δ). DNA sequence analysis of pALK8Δ revealed a truncated ORF. To obtain a full-length clone, a *C. albicans* fosmid library was screened with the 2.6 kb fragment as a probe, yielding six positive fosmids (B.B. Magee, University of Minnesota, St. Paul, MN). A 2.0 kb *Xba*I–*Eco*RI fragment of the above fosmids was further subcloned into pBKS$^+$, generating pSLP. pSLP was digested with *Sal*I and the 1.6 kb fragment was cloned into *Sal*I digested pALK8Δ, generating pALK8. Sequencing of the *CaALK8* gene was performed on both DNA strands of the entire 3.8 kb genomic DNA fragment, with T3, T7 and internal synthesized oligonucleotides, using the automated sequencing facilities of the Indian Institute of Science, Bangalore, India. The sequences were assembled and edited using Laser gene software (DNA star). The search for homologies was carried out using NCBI services. Analysis of ORFs and restriction sites was performed using the University of Wisconsin Genetics Computer Group programs.

Drug resistance assays

The drug resistance profile of the JG436 and AD1234568 transformants carrying plasmids pYEURA3 and pALK8 was determined by a filter-disc assay, as described previously (Leppert *et al.*, 1990; Prasad *et al.*, 1995). The diameter of the zone of inhibition was scored after 48 h. Microtitre plate assays in JG436 transformants and spot assays in the DSY1025 transformants were essentially done as described previously (Talibi and Raymond, 1999).

Construction of plasmids

The homozygous disruption of *CaALK8* was carried out by using a 'URA blaster' cassette (Fonzi and Irwin, 1993). For the disruption of *CaALK8* in *C. albicans*, a 0.9 kb *Hpa*I fragment was removed from the plasmid pALK8. A blunt-ended 4.0 kb *Sal*I–*Bgl*II fragment from pMB-7 (Fonzi and Irwin, 1993), containing the *hisG–URA3–hisG* 'URA blaster' cassette, was inserted into the *Hpa*I-digested pALK8 plasmid to generate pYE/*alk8*Δ::*hisG-URA3-hisG*. A linear 6.8 kb *Xho*I–*Sma*I fragment from pYE/*alk8*Δ::*hisG–URA3–hisG* was used for *CaALK8* disruption. Cloning in the vector YPB–ADHpt (Bailey *et al*., 1996) was carried out by PCR-amplifying the *CaALK8* ORF. The PCR amplification of the 1.5 kb DNA fragment comprising the full-length ORF was carried by using the primers 5′-CGGGATCCTCCA TAAATTCAACAATC-3′ and 5′-CGGGATCCG TATCAATTAGTAATAAC-3′. PCR amplification was carried out by using the high fidelity *Pfu* DNA polymerase (Stratagene). The resulting 1.5 kb PCR fragment was digested with *Bam*HI, gel-purified and then ligated to the *Bgl*II-digested YPB–ADHpt vector, thereby generating the clone YPB–ADH–ALK8.

CaALK8-mediated lauric acid hydroxylation and competition assays

Isolation of microsomes and microsomes-bound lauric acid hydroxylation was assayed as described earlier (Venkateswarlu *et al*., 1997; Sanglard *et al*., 1984). The hydroxylation activity in the CAI4 transformant carrying the YPB–ADH–ALK8 plasmid was assessed by incubating 1.0 ml of microsomal fraction (1 mg protein) in the presence of 185 μm (^{14}C) lauric acid (100 000 dpm) and 1mM NADPH, under vigorous shaking on a gyratory shaker. For assaying the competition of hydroxylation activity, the microsomal preparations were pre-incubated for 5 min with 100-fold excess (0.0185 M) of FCZ, itraconazole (ITZ) and NQO. All other assay conditions were similar to those described earlier (Sanglard *et al*., 1984).

Results

Isolation and sequence analysis of *CaALK8*

The *pdr5* null mutant of *S. cerevisiae*, JG436, is hypersensitive to several drugs and has earlier been used successfully by us for cloning *C. albicans* MDR genes, such as *CDR1* and *CaMDR1* (Prasad *et al*., 1995; Gupta *et al*., 1998). In that study, we had identified several clones conferring multidrug resistance by functional complementation of JG436 with genomic libraries of *C. albicans* (Prasad *et al*., 1995; Gupta *et al*.,1998). One such clone, designated as NC36, displaying an MDR phenotype (data not shown), upon analysis revealed 76–82% nucleotide sequence identity with alkane-inducible cytochromes P450 of *C. maltosa* and *C. tropicalis*. Further analysis of the 2.6 kb *Xho*I–*Sma*I fragment from NC36 revealed the presence of an incomplete ORF (pALK8Δ, –272 amino acids from C-terminal) (Figure 1). Localization of *CaALK8* to chromosome R of *C. albicans* (**http://alces.med.umn.edu/candida.html**) led to the identification of six fosmids harbouring *CaALK8* sequence (17D5, 2H8, 15H3, 2D2, 19A2, 8A7). A full-length *CaALK8* gene was obtained from the above fosmid clones by hybridizing with a ^{32}P-labelled 0.5 kb *Sal*I fragment derived from the 3′-end of pALK8Δ (Figure 1). The six fosmids were found to share a 2.0 kb *Xba*I–*Eco*RI fragment, which was cloned into pBKS^{+}, generating pSLP. Nucleotide sequence analysis of the 2.0 kb *Xba*I–*Eco*RI fragment in pSLP revealed the presence of a complete ORF. The full-length clone in pYEURA3 was finally constructed by ligating the 1.6 kb *Sal*I fragment from pSLP at the *Sal*I site of pALK8Δ. The resulting plasmid, pALK8 (Figure 1), with a 1548 bp complete ORF, was also functional in conferring resistance to CYH as well as to other drugs (discussed below). Analysis of the 5′- flanking region led to the identification of a putative TATA box (TATAAAA), which is 66 bp upstream of the ATG codon.

BLAST and FASTA homology searches of protein sequence databases with the full-length CaAlk8p sequence revealed closest homology with alkane-inducible cytochromes P450 of *C. maltosa* and *C. tropicalis*. *CaALK8* codes for a protein of 515 amino acids, with a calculated molecular mass of 56.6 kDa. This protein has the structure characteristic of the P450 superfamily, comprising well-conserved heme binding domains (HR1 and HR2) and a single 22 amino acid amino-terminal hydrophobic domain, which is responsible for anchoring this protein to the endoplasmic reticulum (ER), like the Alk proteins from *C. maltosa* and *C. tropicalis* (Menzel *et al*., 1996; Schunck *et al*., 1991).

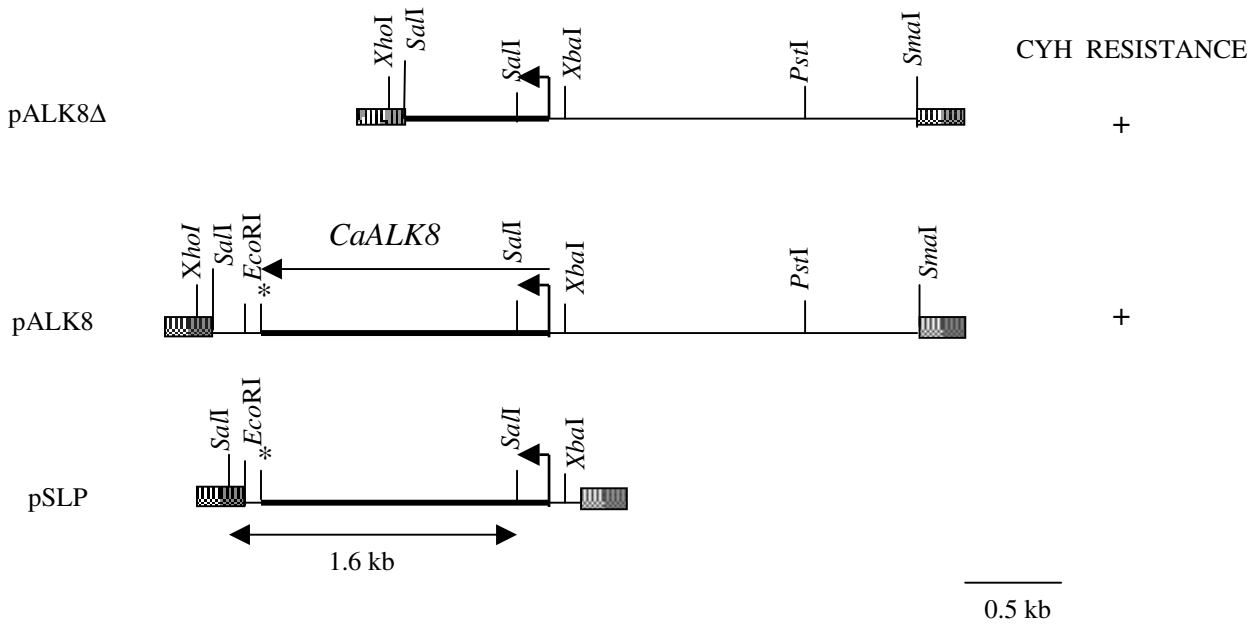
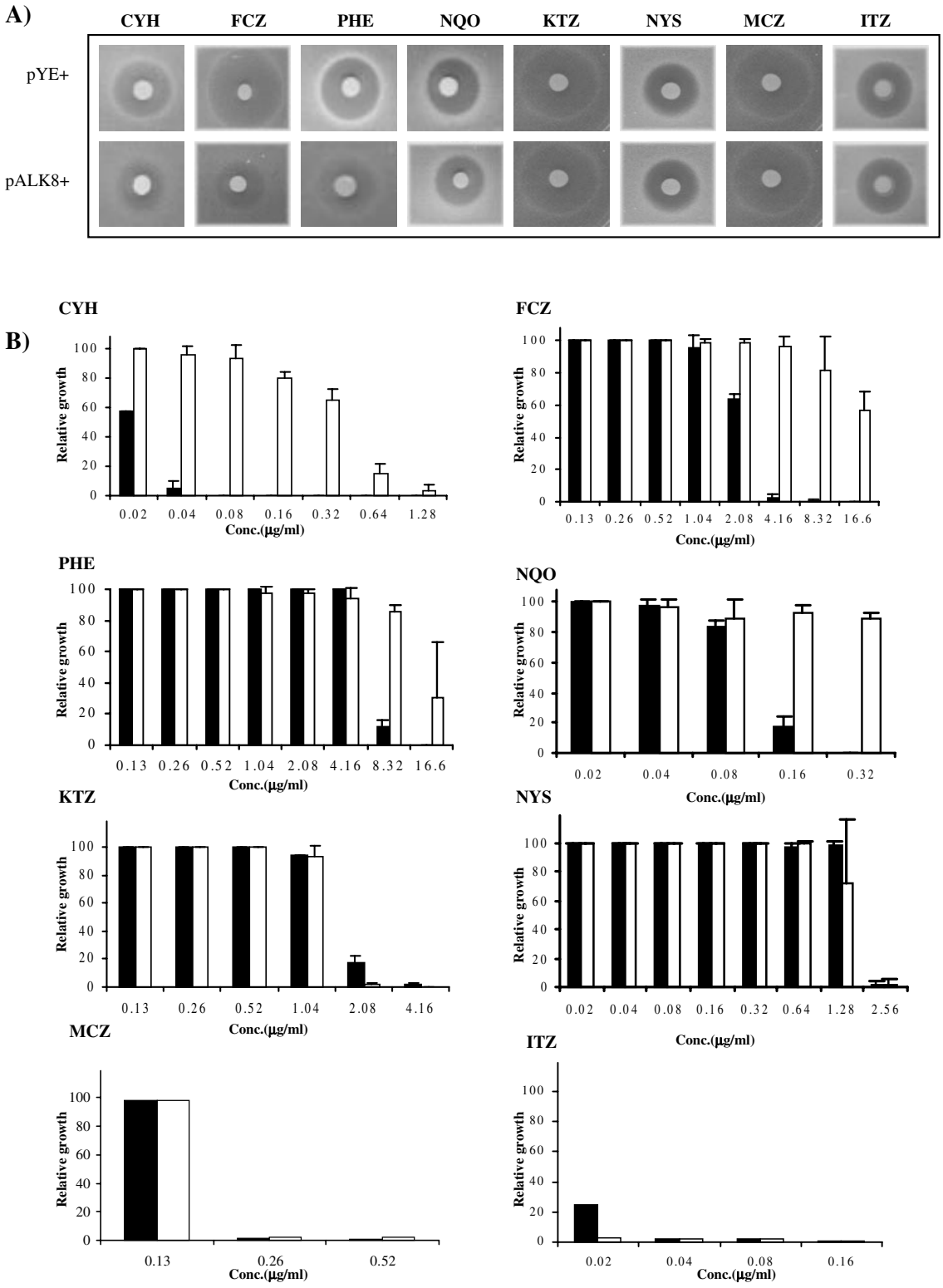


Figure 1. Restriction map of *C. albicans* genomic DNA clone conferring resistance to cycloheximide (CYH). The hatched boxes represent the vector pYEURA3 and the thin lines represent the genomic DNA insert. The ability of pALK8Δ and the clone pALK8 to confer resistance to cycloheximide is indicated (+ confers resistance). The restriction sites used to generate pALK8 are shown. The arrow on the clone maps indicate the position of the initiator codon at the beginning of the incomplete and the complete ORFs identified in clones pALK8Δ and pALK8, respectively. The ORF in pALK8Δ and pALK8 is shown in a thick bold line in all the constructs. A 2.0 kb *Xba*I–*Eco*RI fosmid fragment overlapping the complete ORF was subcloned into pBKS$^+$ generating the clone pSLP (bottom). A 1.6 kb *Sal*I fragment from pSLP overlapping the complete ORF was subcloned into pALK8Δ at the *Sal*I site to generate pALK8. The initiators (arrows) and the stop codons (asterisks) are shown

CaALK8 confers multidrug resistance when expressed in *S. cerevisiae*

The susceptibilities of the JG436 transformants carrying plasmids pYEURA3 and pALK8 to different drugs was compared by the filter disc assay, as mentioned in Materials and methods. The host JG436, which was hypersusceptible to CYH and other drugs due to the absence of functional *PDR5*, was rendered resistant to several drugs when transformed with the plasmid carrying *ALK8* gene. The host transformed only with the vector (pYEURA3) remained sensitive to the drugs tested. As shown in Figure 2A, pALK8 was able to confer resistance to CYH, FCZ, NQO and PHE, while it was ineffective against drugs such as nystatin (NYS), itraconazole (ITZ), miconazole (MCZ) and ketoconazole (KTZ). That pALK8 transformant could confer multidrug resistance was further confirmed by another drug susceptibility assay. Microtitre plate assay is routinely used to determine the MIC of a given drug (Espinel-Ingroff *et al.*, 1998). The results depicted in Figure 2B clearly show higher MIC values (resistance) for the similar spectrum of drugs as was observed with filter disc assay (Figure 2A). Thus, the results in

Figure 2. *CaALK8* confers drug resistance when expressed in *S. cerevisiae*. (A) JG436 cells transformed with plasmids pYEURA3 (pYE+) and pALK8 (pALK8+) were tested for resistance to various drugs by filter disc assay . The diameter of the zone of inhibition was scored after 48 h of growth at 30°C. The panel shows the profile of the transformants at that amount of drug at which maximum difference between the control (pYE+) and the pALK8 transformant (pALK8+) was observed. The following amounts of drugs were used: 0.5 μg CYH, 100 μg FCZ, 50 μg PHE, 5 μg NQO, 10 μg KTZ, 5 μg NYS, 100 μg MCZ and 10 μg ITZ. (B) The degree of resistance to the indicated drugs was determined by a microtitre plate assay, as described previously (Talibi and Raymond, 1999). The percentage of growth in different concentrations of drugs is expressed relative to growth in drug-free medium (100%). ■, pYE+; □, pALK8+



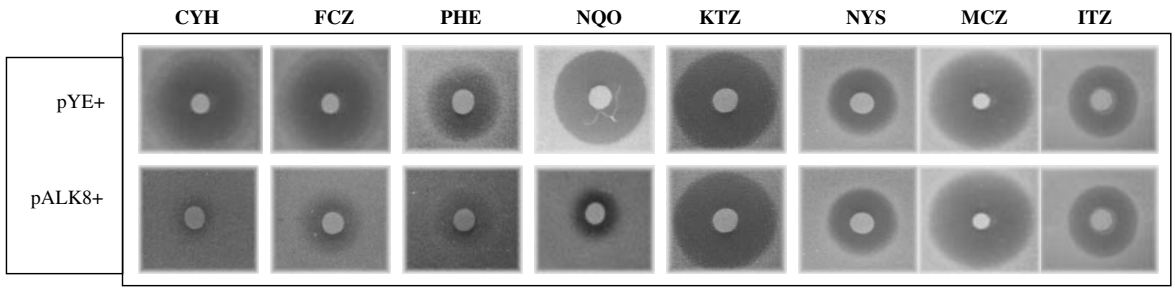


Figure 3. Drug resistance profile of AD 1234568 transformants. Drug resistance profile of AD 1234568 cells transformed with pYEURA3 (PYE+) and pALK8 (pALK8+) was determined by filter disc assays. The following amounts of drugs were used: 0.5 μg CYH, 100 μg FCZ, 50 μg PHE, 5 μg NQO, 10 μg KTZ, 5 μg NYS, 100 μg MCZ and 10 μg ITZ. The amounts reported are those at which there is maximum difference between the control (pYE+) and the transformant (pALK8+)

Figure 2A, B confirmed that *CaALK8* transformants could confer MDR in *S. cerevisiae*.

CaALK8 confers MDR independent of ABC transporters

In order to confirm the MDR phenotype of *CaALK8*, it was expressed in another drug-hypersensitive *S. cerevisiae* host (AD 1234568), which was deleted in seven ABC transporters, viz. *PDR5*, *PDR10*, *PDR11*, *PDR15* (pleiotropic drug resistance), *SNQ2* (sensitive nitroquinoline oxide resistance gene), *YCF1* (yeast cadmium factor gene) and *YOR1* (yeast oligomycin resistance gene). As shown in Figure 3, the spectrum of resistance and sensitivities elicited to various drugs by pALK8 in AD 1234568 was similar to what was observed with the pALK8 transformant in JG436 (Figure 2A). Therefore, *CaALK8*-mediated MDR phenotype was established in two different genetic backgrounds, demonstrating that the phenotype is independent of the strain where *CaALK8* is expressed. It is also apparent from these results that *CaALK8*-mediated drug resistance represents an independent mechanism that does not function in concert with at least the seven deleted plasma membrane efflux pumps.

Chromosomal deletion of *CaALK8* in *C. albicans* CAI4 did not lead to a hypersensitive phenotype

In order to investigate the functional relevance of *CaALK8* in *C. albicans*, both copies of this gene were deleted in a diploid strain CAI4, using the 'ura blaster' strategy (Fonzi and Irwin, 1993). Southern blotting was carried out to ensure the correct localization of the disruption of both the *ALK8* alleles (Figure 4). It may be noted that an extra band of 3.2 kb was seen on Southern blots when probed with the 3.8 kb full *CaALK8* fragment (Figure 4C), which could be due to the presence of other homologues of *CaALK8* in *C. albicans* (**http://alces.med.umn.edu/candida.html).**

The growth characteristics of CAI4 and CSLP4 (*CaALK8* disruptant) were checked by growing them in a medium supplemented with glucose or alkane of different chain lengths. It was observed that while there was no substantial difference between CSLP4 and CAI4 with respect to growth rates in minimal medium with glucose (Figure 5A), the growth of the disruptant strain CSLP4 on various alkanes was relatively slower as compared to its parent (Figure 5B). However, the observation that CSLP4 was able to grow in a medium supplemented with alkanes of different chain lengths indicated that *CaALK8* is not the sole gene required for alkane assimilation in *C. albicans*. Furthermore, the homozygous disruption of the *CaALK8* gene did not result in hypersensitivity to the tested drugs (Figure 5C). The masking of any altered phenotype following homozygous disruption of MDR genes is not very uncommon (see Discussion).

Overexpression of *CaALK8* leads to drug resistance

Since *CaALK8* expression was not detected in the glucose-grown CAI4 cells (data not shown), it was used for overexpression studies. In order to achieve this, the *CaALK8* gene was cloned in YPB–ADHpt vector (Bailey *et al.*, 1996). The resulting clone YPB–ADH–ALK8 was introduced into a wild-type strain of *C. albicans* (CAI4) and the transformant was used for spot assays, as shown in Figure 6A. CAI4 strain transformed with the YPB–ADH–ALK8 plasmid was resistant only to FCZ.

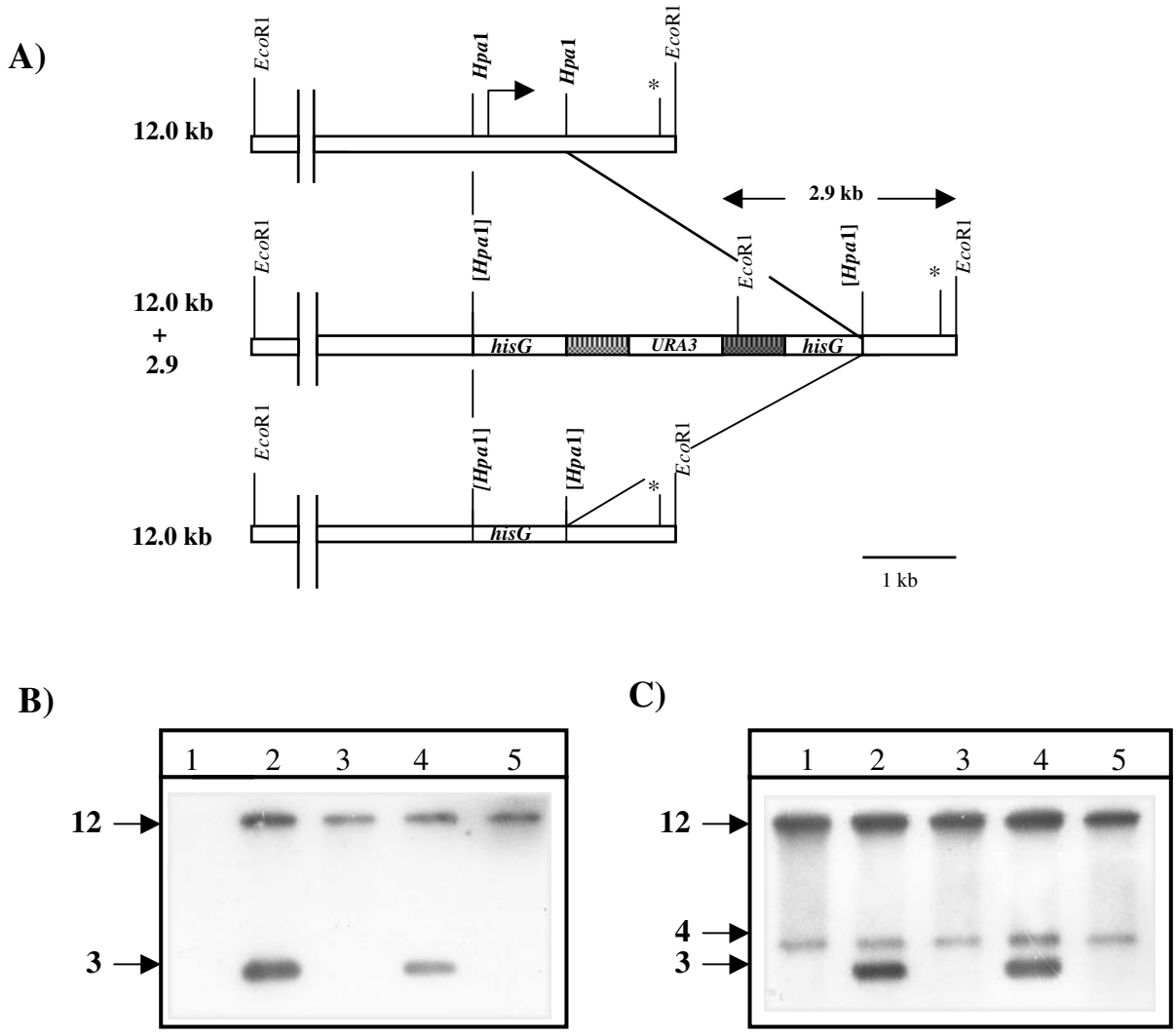
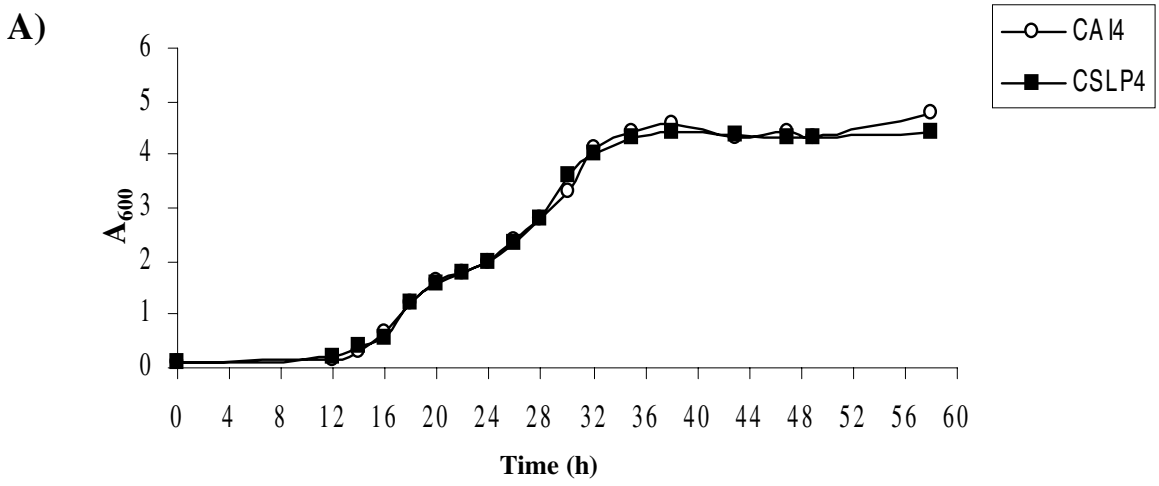
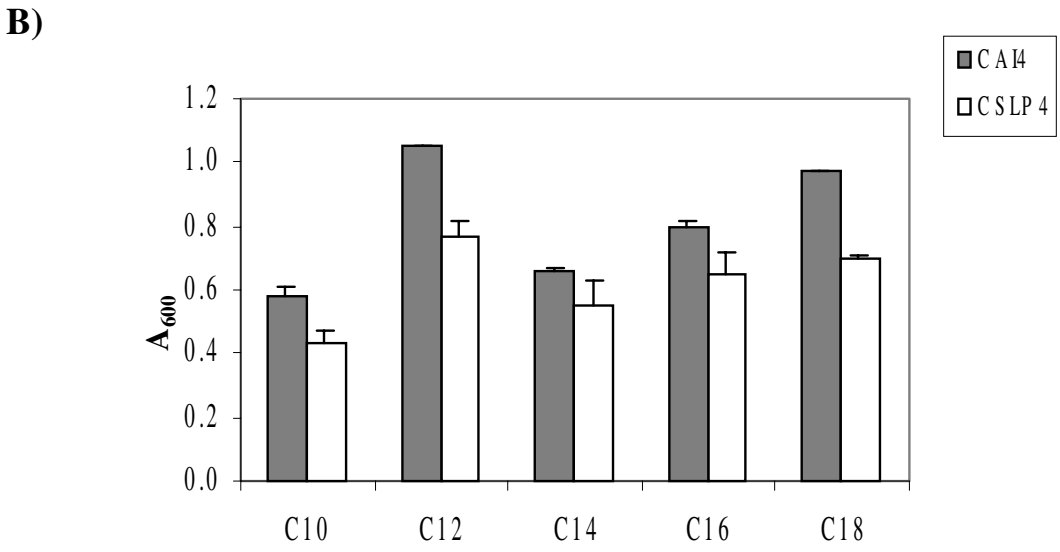
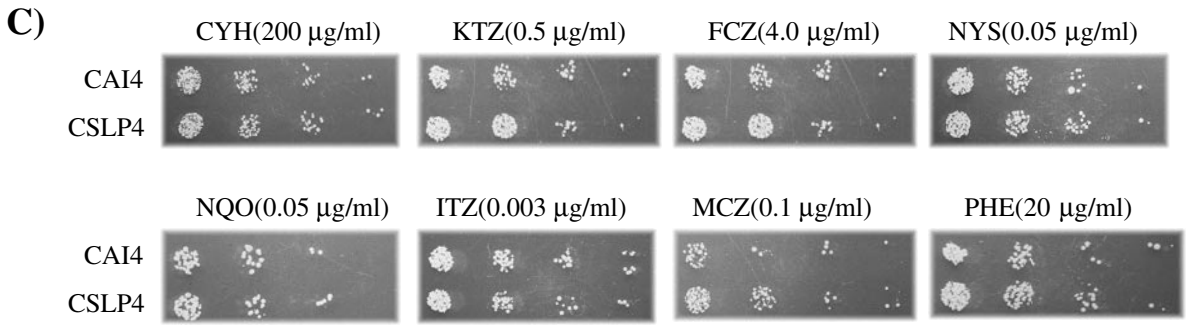


Figure 4. Chromosomal deletion of *CaALK8* in CAI4. *A*, The *CaALK8* locus is contained within a 12.0 kb *Eco*RI fragment (top). The start (arrow) and the stop (asterisk) codons of the *CaALK8* ORF are indicated. The disruption cassette (middle) was generated by replacing a 0.9 kb *Hpa*I fragment by the 4.0 kb *hisG–URA3–hisG* cassette. The disruption cassette upon digestion with *Eco*RI should generate a 12.0 kb band and a 2.9 kb band. After counterselection on 5-FOA, recombination between the two *hisG* repeats should generate only the 12.0 kb band (bottom). (B, C) Southern blot analysis was used to characterize the different steps of disruption. Genomic DNA was extracted from strains: CAI4, *ALK8/ALK8* (lane 1); CSLP1, *ALK8/alk8*Δ*::hisG-URA3-hisG* (lane 2); CSLP2, *ALK8/alk8*Δ*::hisG* (lane 3); CSLP3, *alk8*Δ*::hisG-URA3-hisG/alk8*Δ*::hisG* (lane 4); and CSLP4, *alk8*Δ*::hisG/ alk8*Δ*:hisG* (lane 5). DNA samples (1 μg) were digested in duplicate with *Eco*RI, separated by electrophoresis on agarose gels, and transferred to nylon membranes. The blots were then probed with a 0.9 kb *Bam*HI–*Bgl*II *hisG* fragment (B) or the 3.8 kb fragment comprising the entire wild-type *CaALK8* gene (C). Positions of molecular size markers (in kilobases) are indicated on the left. Membranes were exposed to a Kodak AR X-ray firm for 4 h at −80°C with two intensifying screens

In order to circumvent the masking of the *CaALK8*-mediated phenotype in CAI4 owing to the presence of other efflux pumps in *C. albicans* (Fling *et al.*, 1991), *CaALK8* was overexpressed in a hypersensitive *C. albicans* host, DSY1025, disrupted in both *CDR1* and *CDR2* genes (Sanglard *et al.*, 1997). The spot assays with the *CaALK8* overexpression in *cdr1cdr2* null background demonstrated that it







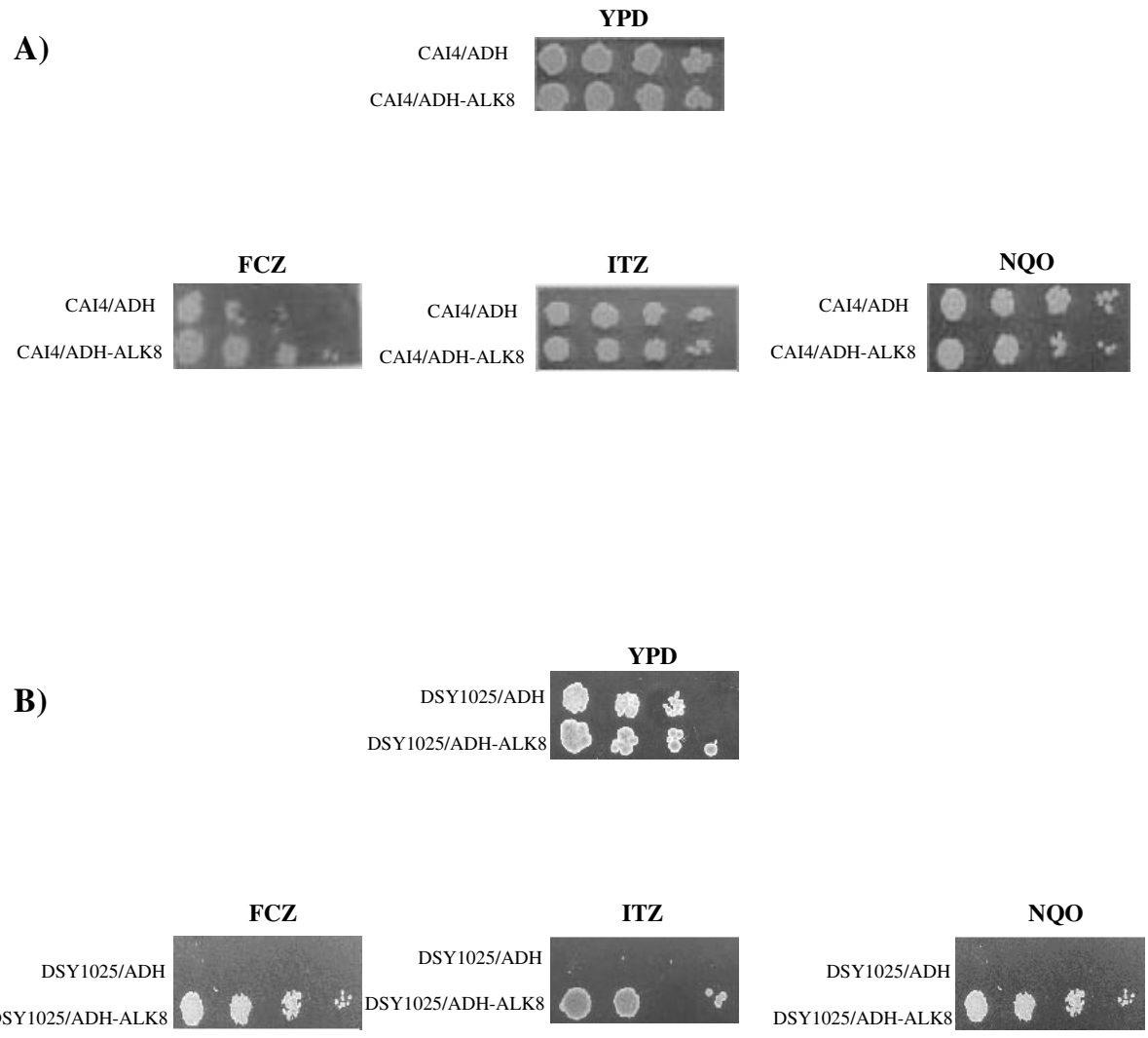


Figure 6. Drug resistance profile in *C. albicans* CAI4 and DSY1025 transformants. (A) Strains CAI4/ADH and CAI4/ADH-ALK8 were analysed by spot assay. Growth differences were monitored after 48 h at 30°C. The concentrations of drugs used were 50 μg/ml FCZ, 0.003 μg/ml ITZ and 0.05 μg/ml of NQO. (B) Spot assays of strains DSY1025/ADH and DSY1025/ADH-ALK8. Growth differences were monitored after 48 h at 30°C. The concentrations of drugs used are 4 μg/ml FCZ, 0.06 μg/ml ITZ, 0.05μg/ml NQO

Figure 5. Growth and drug resistance profile of CAI4 and CSLP4. (A) Growth of CAI4 and CSLP4, the *ALK8* disruptant, in glucose. (B) Growth of CAI4 and CSLP4 in minimal media supplemented with alkanes. The primary cultures of *C. albicans* strain CAI4 and the *CaALK8* disruptant, CSLP4, were grown in YEPD medium overnight. The cells were then harvested and washed with fresh medium without glucose. After washing, the cells were cultivated in YNB medium (without amino acids) to an initial A_{600} of 0.1 using either 2% glucose or 1% alkanes of various chain lengths as the carbon source. The alkanes used were *n*-decane (C10), *n*-dodecane (C12), *n*-tetradecane (C14), *n*-hexadecane (C16), and *n*-octadecane (C18). The A_{600} was recorded at different time intervals. The growth points at the 96 h are shown. (C) Drug susceptibility of *C. albicans* wild-type strain CAI4 and the *CaALK8* disruptant, CSLP4. Yeast strains were spotted in serial dilutions on YEPD medium containing the drug at the corresponding concentrations as described earlier (Talibi and Raymond, 1999). The plates were incubated at 30°C for 48 h

conferred distinct resistance to ITZ, FCZ and NQO, while susceptibilities to other tested drugs did not change (Figure 6B).

Drugs could compete *CaALK8*-mediated hydroxylation of lauric acid

Earlier, lauric acid has been used as a substrate to check for the microsome-bound P450alk enzyme activity in well-characterized Alk proteins of *C. maltosa* and *C. tropicalis* (Sanglard *et al.*, 1984; Sanglard and Loper, 1989; Seghezzi *et al.*, 1991). We were able to demonstrate that the microsomes prepared from *C. albicans* wild-type strain CAI4 overexpressing Alk8p could hydroxylate lauric acid. CAI4 transformed with the vector YPB–ADH only did not show any spot of hydroxylated lauric acid (data not shown). In order to check whether drugs to which Alk8p conferred resistance could affect the hydroxylation of radiolabelled (^{14}C) lauric acid, enzyme activity was assayed in the presence and absence of drugs. Figure 7 shows a prominent spot corresponding to lauric acid and another spot with a lower Rf value of 12-OH lauric acid (confirmed by mass spectrometry). Interestingly, the presence of 100-fold excess of FCZ, ITZ and NQO considerably reduced the hydroxylation activity, which was evident from the disappearance of the 12-OH lauric acid spot when competed with ITZ and NQO (Figure 7). The presence of the 12-OH lauric acid spot, albeit of a low intensity in lane 3, implies that FCZ could not compete with lauric acid as efficiently as to other drugs (Figure 7). Therefore, an interaction of drugs with CaAlk8p was evident from these experiments.

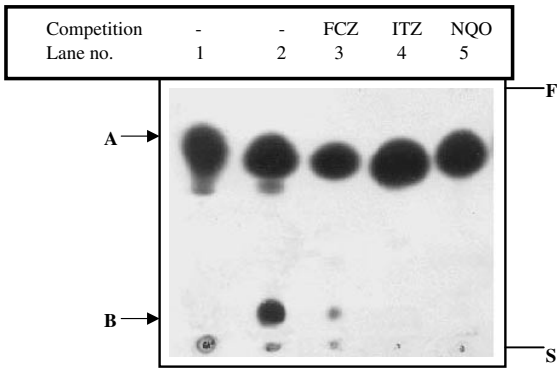


Figure 7. Drugs could compete *CaALK8* mediated hydroxylation of lauric acid. Fluorogram of the (^{14}C)-lauric acid hydroxylation product and competition of hydroxylation in the microsomes of *C. albicans* CAI4 transformant viz. CAI4/ADH-ALK8. Lane 1, control in which the reaction was stopped at the beginning by adding sulphuric acid; lane 2, reaction in the presence of (^{14}C)-lauric acid; lanes 3, 4 and 5, ^{14}C-lauric acid hydroxylation in the presence of FCZ, ITZ and NQO. Assay details followed by extraction and thin layer chromatography on a 1 mm silica gel 60 plate (Merck) were performed as described in Materials and methods. After development of the thin layer chromatography, the thin layer plate was sprayed with Enhance^{3}H (NEN™ Life Sciences product), exposed to a Kodak AR X-ray film between two intensifying screens and exposed for 24 h at −80°C. Compounds A and B co-migrated with lauric acid and 12-OH lauric acid, respectively. S, start; F, solvent front

Discussion

In this study, we report the molecular characterization of *CaALK8*, a new gene of *C. albicans*, capable of conferring MDR. *CaALK8* belongs to a family of hydrocarbon-assimilating genes (CYP52 gene family) unique to the genus *Candida*. A host of such a family of genes is well characterized in *C. tropicalis* and *C. maltosa* (Seghezzi *et al.*, 1992; Ohkuma *et al.*, 1995). *CaALK8* represents the first gene of this family in *C. albicans*, which is shown to be involved in multidrug resistance in this pathogenic yeast. The entries in genome database of *Candida* (**http://alces.med.umn.edu/candida.html**) point out that there are at least four more *ALK* genes present in *C. albicans*.

The homozygous disruption of *CaALK8* did not lead to a hypersensitive phenotype. The masking of any detectable phenotype following homozygous disruption of MDR genes is not very uncommon. For example, disruption of *CDR2* (ABC drug extrusion pump) did not show any enhanced sensitivity to drugs, which could only be seen when both *CDR1* and *CDR2* genes were disrupted (Sanglard *et al.*, 1997). *CaMDR1* (MFS) disruption in *C. albicans* (CAI4) also did not lead to hypersensitivity to the tested drugs (Morschhauser *et al.*, 1999). A recently characterized multidrug transporter gene, *FLU1*, gives a phenotype only when disrupted in a *C. albicans* background, where several multidrug efflux transporter genes, such as *CDR1*, *CDR2* and *CaMDR1*, have been deleted (Calabrese *et al.*, 2000).

Furthermore, in the present case, overexpression of *CaALK8* in a wild-type *C. albicans* strain (CAI4) resulted in resistance to FCZ only while susceptibilities to the other tested drugs remained unaltered. This could also be attributed to the presence

of other efflux pumps in CAI4, which could be masking the *CaALK8*-mediated phenotype. Nonetheless, we found that the overepression of *CaALK8* in a hypersensitive strain, DSY1025, which was disrupted in both *CDR1* and *CDR2* genes, conferred distinct resistance to ITZ, FCZ and NQO. This confirmed for the first time that an alkane-assimilating gene, *CaALK8*, could affect drug susceptibilities of *C. albicans*.

The alkane-inducible cytochrome P450s are known to catalyse the terminal hydroxylation of alkanes and fatty acids. Lauric acid has been used in *C. maltosa* and *C. tropicalis* to assay the enzyme activity of P450alks (Sanglard *et al*., 1984; Sanglard and Loper, 1989; Seghezzi *et al*., 1991). Using radiolabelled lauric acid, we have shown that, like the other *ALK* genes, CaAlk8p is capable of converting lauric acid to hydroxylauric acid. The same drugs to which it conferred resistance could compete with the CaAlk8p-mediated hydroxylation of lauric acid. The competition of hydroxylation by different drugs clearly demonstrated an interaction of drugs with CaAlk8p. It is thus possible that the incoming drugs could be modified by *CaALK8*, thereby rendering cells resistant to drugs. It must, however, be pointed out that azoles at least have been shown to be predominantly inert to metabolism (White *et al*., 1998; Hitchcock, 1993). It is also a well-established fact that the modification or degradation of drugs represents one of the important drug resistance mechanisms in a variety of bacterial and eukaryotic systems. Kelly *et al*. have shown that CYP61 (Δ^{22}-desaturase), which is involved in 22-desaturation in ergosterol biosynthesis in *S. cerevisiae*, can also metabolize xenobiotics. Thus, a CYP superfamily member has been shown to metabolize aflatoxins, dimethylnitrosamine and various cyclopenta(a)phenanthrenes (Kelly *et al*., 1997). In light of this, drug resistance mediated by *CaALK8* (a member of the *CYP52* gene family) could represent another mechanism in which chemical modification of the incoming drug could affect the drug susceptibilities of *C. albicans*. The mechanism by which *CaALK8* or the P450alk proteins would be rendering the drugs non-toxic still remains to be investigated. In view of the fact that CYP51 (P45014DM) is the prime target of azoles, it would be interesting to investigate how the CYP52 (P450alk) gene family could be involved in the overall resistance scenario of *C. albicans*.

In view of the presence of a multigene family of P450alk genes in *Candida*, it would also be worthwhile to ascertain their role in MDR scenario associated with azole-resistant clinical isolates. Fluconazole resistance in clinical isolates of *C. albicans* has been associated with combinations of several distinct mechanisms (White *et al*., 1998; Perepnikhatka *et al*., 1999). However, the lack of overexpression of efflux pumps, viz. *CDR1*, *CDR2*, *CaMDR1*, and of *ERG11* (P45014DM) in some clinical isolates, suggested that there are still some unknown mechanism(s) that may contribute to azole resistance (Perepnikhatka *et al*., 1999). It is worth mentioning that a recently characterized multidrug efflux transporter gene (MFS) from *C. albicans*, *FLU1*, conferred resistance to FCZ and other drugs; however, its expression did not vary significantly between azole susceptible and resistant clinical isolates (Calabrese *et al*., 2000). In a preliminary study, we also could not detect *CaALK8* transcript in azole-resistant clinical isolates in which the contribution of other mechanisms of resistance have been excluded (data not shown). It would mean that genes such as *CaALK8* and *FLU1* could be employed in mediating the MDR phenomenon in *C. albicans*, albeit in a selective scenario yet to be characterized. Since *CaALK8* and *FLU1* are shown to confer resistance to azoles, particularly to FCZ, it is possible that the activation of these genes takes place after upregulation of known targets and could thus represent secondary mechanisms of azole resistance. The stage at which these genes would begin to express during azole therapy in a patient remains to be examined. However, an in-depth analysis of more clinical isolates and sequential drug-adapted resistant strains is necessary for a final dissection of the role of genes like *CaALK8* and *FLU1* in affecting the drug susceptibilities of this human pathogen.

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References

Bailey DA, Feldmann PJF, Bovey M, Gow NAR, Brown AJP. 1996. The *Candida albicans HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J Bacteriol* **178**: 5353–5360.

Calabrese D, Bille J, Sanglard D. 2000. A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (*FLU1*) conferring resistance to fluconazole. *Microbiology* **146**: 2743–2754.

Cannon RD, Fischer FJ, Niimi K, Niimi M, Arisawa M. 1998. Drug pumping mechanisms in *Candida albicans*. *Jpn J Med Mycol* **39**: 73–78.

Espinel-Ingroff A, Barchiesi F, Hazen KC, Martinez-Suarez JV, Scalise G. 1998. Standardization of antifungal susceptibility testing and clinical relevance. *Med Mycol* **36**: 68–78.

Fling ME, Kopf J, Tamarkin A, *et al*. 1991. Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. *Mol Gen Genet* **227**: 318–329.

Fonzi WA, Irwin MY. 1993. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**: 717–728.

Graham-Lorence S, Peterson JA. 1996. P450s: Structural similarities and functional differences. *FASEB J* **10**: 206–214.

Gupta V, Kohli AK, Krishnamurthy S, *et al*. 1998. Identification of polymorphic mutant alleles of *CaMDR1*, a major facilitator of *Candida albicans* which confers multidrug resistance and its *in vitro* transcriptional activation. *Curr Genet* **34**: 192–199.

Hitchcock CA. 1993. Resistance of *Candida albicans* to azole antifungal agents. *Biochem Soc Trans* **21**: 1039–1047.

Kappeli O. 1986. Cytochromes P-450 of yeasts. *Microbiol Rev.* **50**: 244–258.

Kelly SL, Lamb DC, Baldwin BC, Corran AJ, Kelly DE. 1997. Characterisation of *Saccharomyces cerevisiae* CYP61, sterol Δ^{22}-desaturase, and inhibition by azole antifungal agents. *J Biol Chem* **272**: 9986–9988.

Kelly SL, Lamb DC, Kelly DE. 1997. Sterol 22-desaturase, cytochrome P45061, possesses activity in xenobiotic metabolism. *FEBS Lett* **412**: 233–235.

Krishnamurthy S, Gupta V, Prasad R, Panwar SL, Prasad R. 1998a. Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: *In vitro* transcriptional activation by heat shock, drugs and human steroid hormones. *FEMS Microbiol Lett* **160**: 191–197.

Krishnamurthy S, Gupta V, Snehlata P, Prasad R. 1998b. Characterisation of human steroid hormone transport mediated by Cdr1p, multidrug transporter of *Candida albicans*, belonging to the ATP binding cassette superfamily. *FEMS Microbiol Lett* **158**: 69–74.

Leppert G, McDevitt R, Falco SC, Van Dyk FiM, Golin J. 1990. Cloning by gene amplification of two loci conferring multiple drug resistance in *Saccharomyces*. *Genetics* **125**: 13–20.

Lopez-Ribot JL, McAtee RK, Lee LN, *et al*. 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. *Antimicrob Agents Chemother* **42**: 2932–2937.

Marichal P, Koymans L, Willemsens S, *et al*. 1999. Contribution of mutations in the cytochrome P450 14α-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. *Microbiology* **145**: 2701–2713.

Marr KA, Lyons CN, Bowden RA, White TC. 1998. Rapid, transient fluconazole resistance in *Candida albicans* is associated with increased mRNA levels of CDR. *Antimicrob Agents Chemother* **42**: 2584–2589.

Menzel R, Kargel E, Vogel F, Bottcher C, Schunck W-H. 1996. Topogenesis of a microsomal cytochrome P450 and induction of endoplasmic reticulum membrane proliferation in *Saccharomyces cerevisiae*. *Arch Biochem Biophys* **330**: 97–109.

Morschhauser J, Michel S, Staib P. 1999. Sequential gene disruption in *Candida albicans* by FLP-mediated site-specific recombination. *Mol Microbiol* **32**: 547–556.

Odds FC. (ed.). 1988. *Candida and Candidosis: A Review and Bibliography*. London: Ballière Tindall.

Ohkuma M, Muraoka S-I, Tanimoto T, *et al*. 1995. *CYP52* (cytochrome P450alk) multigene family in *Candida maltosa*: identification and characterisation of eight members. *DNA Cell Biol* **14**: 163–173.

Omura T. 1999. Forty years of cytochrome P450. *Biochem Biophys Res Commun* **266**: 690–698.

Perepnikhatka V, Fischer FJ, Niimi M, *et al*. 1999. Specific chromosome alterations in fluconazole-resistant mutants of *Candida albicans*. *J Bacteriol* **181**: 4041–4049.

Prasad R. (ed.). 1991. *Candida albicans: Cellular and Molecular Biology*. Berlin: Springer-Verlag.

Prasad R, Krishnamurthy S, Prasad R, Gupta V, Lata S. 1996. Multidrug resistance: an emerging threat. *Curr Sci* **71**: 205–213.

Prasad R, Worgifosse PD, Goffeau A, Balzi E. 1995. Molecular cloning and characterisation of a novel gene of *C. albicans*, *CDR1*, conferring multiple resistance to drugs and antifungals. *Curr Genet* **27**: 320–329.

Sanglard D, Ischer F, Monod M, Bille J. 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterisation of *CDR2*, a new multidrug ABC transporter gene. *Microbiology* **143**: 405–416.

Sanglard D, Kappeli O, Fiechter A. 1984. Metabolic conditions determining the composition and catalytic activity of cytochrome P-450 monooxygenases in *Candida tropicalis*. *J Bacteriol* **157**: 297–302.

Sanglard D, Loper JC. 1989. Characterisation of the alkane inducible cytochrome P450 (P450alk) gene from the yeast *Candida tropicalis*: identification of a new P450 gene family. *Gene* **76**: 121–136.

Schunck W-H, Vogel F, Gross B, *et al*. 1991. Comparison of two cytochromes P450 from *Candida maltosa*: primary structures, substrate specificities and effects of their expression in *Saccharomyces cerevisiae* on the proliferation of the endoplasmic reticulum. *Eur J Cell Biol* **55**: 336–345.

Seghezzi W, Meili C, Ruffiner R, *et al*. 1992. Identification and

characterisation of additional members of the cytochrome P450 multigene family *CYP52* of *Candida tropicalis*. *DNA Cell Biol* **11**: 767–780.

Seghezzi W, Sanglard D, Fiechter A. 1991. Characterisation of a second alkane-inducible cytochrome P450 encoding gene, *CYP52A2* from *Candida tropicalis*. *Gene* **106**: 51–60.

Talibi D, Raymond M. 1999. Isolation of a putative *Candida albicans* transcriptional regulator involved in pleiotropic drug resistance by functional complementation of a *pdr1 pdr3* mutation in *Saccharomyces cerevisiae*. *J Bacteriol* **181**: 231–240.

Vanden Bossche H, Marichal P, Odds FC, Le Jeune L, Coene MC. 1992. Characterization of an azole resistant *Candida glabrata* isolate. *Antimicrob Agents Chemother* **36**: 2602–2610.

Venkateswarlu K, Denning DW, Kelly SL. 1997. Inhibition and interaction of cytochrome P450 of *Candida krusei* with azole antifungal drugs. *J Med Vet Mycol* **35**: 19–25.

White TC, Marr KA, Bowden RA. 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin Microbiol Rev* **11**: 382–402.