

Specificity of drug transport mediated by *CaMDR1*: a major facilitator of *Candida albicans*

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CaMDR1 encodes a major facilitator superfamily (MFS) protein in *Candida albicans* whose expression has been linked to azole resistance and which is frequently encountered in this human pathogenic yeast. In this report we have overexpressed CaMdr1p in *Sf9* insect cells and demonstrated for the first time that it can mediate methotrexate (MTX) and fluconazole (FLC) transport. MTX appeared to be a better substrate for CaMdr1p among these two tested drugs. Due to severe toxicity of these drugs to insect cells, further characterization of CaMdr1p as a drug transporter could not be done with this system. Therefore, as an alternative, CaMdr1p and Cdr1p, which is an ABC protein (ATP binding cassette) also involved in azole resistance in *C. albicans*, were independently expressed in a common hypersensitive host JG436 of *Saccharomyces cerevisiae*. This allowed a better comparison between the functionality of the two export pumps. We observed that while both FLC and MTX are effluxed by CaMdr1p, MTX appeared to be a poor substrate for Cdr1p. JG436 cells expressing Cdr1p thus conferred resistance to other antifungal drugs but remained hypersensitive to MTX. Since MTX is preferentially transported by CaMdr1p, it can be used for studying the function of this MFS protein.

1. Introduction

The opportunistic dimorphic fungus *Candida albicans* is a major cause of life threatening fungal infections in humans, and is predominantly rampant in immunocompromized individuals. *C. albicans* infections are treated with antifungal agents, particularly with the triazole derivative fluconazole (FLC). In order to combat the effects of antifungals, *Candida* has evolved a variety of mechanisms to acquire resistance to these drugs (Cannon *et al* 1998; Marichal 1999; White *et al* 1998). The resistance to azoles in *C. albicans* primarily occurs through an alteration or an overexpression of the 14 α -lanosterol demethylase (P45014DM) involved in ergosterol biosynthesis. Recent identification of ATP binding cassette

(ABC) proteins viz. Cdr1p and Cdr2p (*Candida* drug resistance) (Prasad 1995; Sanglard *et al* 1997) and their overexpression in azole resistant clinical isolates has led to the suggestion that these transporters could represent another mechanism involved in multidrug resistance (MDR) scenario of *C. albicans* (Albertson *et al* 1996; Krishnamurthy *et al* 1998a).

Fling *et al* (1991) had earlier identified the *CaMDR1* gene in *C. albicans* which encodes a major facilitator superfamily (MFS) pump and conferred resistance to benomyl and methotrexate (MTX) (Ben Yaacov *et al* 1994; Gupta 1998). Recently, the overexpression of *CaMDR1* has also been linked to azole resistance in *C. albicans* (White *et al* 1998). On the basis of the complete yeast genome sequence, at least 28 MFS proteins have

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Abbreviations used: ABC, ATP binding cassette; BEN, benomyl; CYH, cycloheximide; FLC, fluconazole; MDR, multidrug resistance; MFS, major facilitator superfamily; MTX, methotrexate; P-gp, P-glycoprotein.

been identified in *Saccharomyces cerevisiae* (Goffeau et al 1997) and sequencing efforts of *Candida* genome reveal that this pathogen could also have similar number of MFS proteins. In spite of the existence of a large number of these proteins, only a few have been associated with drug resistance, while a vast majority as MDR determinants remains unknown. Although the molecular basis of drug resistance in *Candida* is not very clear, but accumulated evidences suggest that MDR is a multifactorial phenomenon where a combination of mechanisms could contribute to azole resistance (Bossche et al 1994; White et al 1998). Here we report the overexpression of CaMdr1p in *Sf9* insect cells for the first time, to show that it could transport MTX and FLC and that MTX is much preferred substrate of CaMdr1p over FLC.

2. Materials and methods

2.1 Materials

The chemicals used were from Sigma Chemical (USA). All media components were purchased either from Difco (USA) or HiMedia (India). FLC was kindly provided by Pfizer Ltd., Sandwich, Kent, UK. The [³H]FLC and [³H]MTX were obtained from Amersham (UK).

2.2 Yeast strains and growth media

S. cerevisiae strain JG436, which lacks a functional *PDR5* gene (pleiotropic drug resistance) and is hypersensitive to drugs (*Mat a*, *PDR5::Tn5*, *leu2*, *met5*, *ura3-52*, *mak71*, *KRBI*) was a kind gift from Dr J Golin, Catholic University of America, Washington DC, USA. JG436 was transformed with plasmid pNC39 (carrying *CaMDR1*) and plasmid pS12 (carrying *CDR1*) that were obtained from *C. albicans* genomic library as described earlier (Prasad et al 1995; Gupta et al 1998). It should be noted that both the genes had a common vector background of pYEUra3. The resulting JG436 transformants were designated as JGCaMDR1 and JGCDR1. The strains were grown on YNB w/o amino acids with the respective supplements as described earlier (Prasad et al 1995).

2.3 Cloning of CaMDR1 in Sf9 cells

CaMDR1 was cloned into pBacPAK8TM (Clontech, USA) as a 2.1 kb *EcoRV* fragment of pNC39 using standard recombinant techniques resulting in a plasmid pVGAcCaMDR1. *Sf9* cells were co-transfected with wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA and pVGAcCaMDR1 using LipofectinTM (Clontech, USA) as described earlier (Krishnamurthy et al 1998b). The resulting virus was designated as

vAcCaMDR1. Recombinant baculoviruses were identified by plaque assay and the integration of *CaMDR1* at the *polh* locus of vAcCaMDR1 was confirmed by Southern hybridisation using [³²P]-labelled *CaMDR1* specific probe. The recombinant virus was amplified to 10⁸ plaque forming units ml⁻¹ for further studies.

2.4 Drug transport in virus infected insect cells

To study the transport of FLC, 0.5 × 10⁶ *Sf9* cells were seeded in a 24-well culture plate. The cells were infected with recombinant virus, vAcCaMDR1 and cells infected with wild type virus, AcNPV were taken as control. After 48 h post infection, the cells that adhere to the culture plate were washed with PBS (pH 7.4). [³H]FLC (0.7 TBq mmol⁻¹) was then added at a final concentration of 100 nM. At different time intervals, the cells were washed with ice cold PBS and lysed with 0.5 M NaOH. For studying MTX transport [³H]MTX (0.2 TBq mmol⁻¹) was added at a final concentration of 25 μM. The total radioactivity accumulated in cells was measured in liquid scintillation counter using cocktail-T (Spectrochem, India) scintillation liquid. The protein content of the lysed cells was quantitated by a modified Bradford method (Pande and Murthy 1994).

2.5 Antifungal susceptibility testing

Susceptibility of yeast isolates to FLC, MTX, cycloheximide (CYH) and benomyl (BEN) were determined by micro dilution test in SD-URA media as described previously (Talibi and Raymond 1999). The MIC test end point was defined as the lowest drug concentration, which gave > 80% inhibition of growth compared with drug free controls.

2.6 Radio-labelled drug accumulation and energy dependence in *S. cerevisiae* cells

Accumulation of labelled drugs in yeast cells was determined essentially as described previously by Smriti et al (1999). For competition experiments, 100 folds excess of cold drugs (2.5 mM for [³H]MTX and 10 μM for [³H]FLC competition) viz BEN, CYH, FLC and MTX were added to the cell suspension (5 × 10⁸ cells ml⁻¹) 5 min before the addition of 100 nM [³H]FLC or 25 μM [³H]MTX. Similarly, inhibitors like sodium azide (10 mM) and sodium orthovanadate (100 μM) were also added 5 min before the commencement of the transport. The accumulation of drug was measured at 30 min time point. The differences in intracellular accumulation of radio-labelled drug between JGCaMDR1 cells incubated with and without competitor were calculated as per cent inhibition of efflux.

3. Results and discussion

3.1 Cloning of CaMDR1 in baculovirus expression system

Although ABC transport proteins have been extensively studied in the context of active drug efflux (Marichal 1999; White *et al* 1998), MFS proteins of *C. albicans* have not received similar attention. In the present work we have studied drug transport mediated by a MFS protein, CaMdr1p, by expressing it for the first time in a baculovirus system which has been increasingly used for high level expression of foreign genes in insect cell lines *Spodoptera frugiperda* (*Sf9*). *CaMDR1* was cloned into pBacPAK8™ (Clontech, USA) baculovirus shuttle vector as described in § 2. The resulting recombinant plasmid pVGAcCaMDR1 included full ORF of *CaMDR1* preceded by ~ 110 bp of 5'-untranslated region under the transcriptional control of very strong polyhedrin (*polh*) promoter. Homologous recombination *in vivo* between the *AcNPV* sequences in the transfer vector and the *AcNPV* genomic sequence generated a recombinant baculovirus vAcCaMDR1, which was confirmed by Southern hybridization (figure 1).

The expression of CaMdr1p in *Sf9* cells was checked using [³⁵S]methionine labelling as described earlier

(Krishnamurthy *et al* 1998b). The time course analysis of CaMdr1p synthesis in vAcCaMDR1 infected *Sf9* cells revealed that it was maximally expressed at 48 h post infection and accordingly this time point was selected for subsequent transport experiments (data not shown).

3.2 CaMdr1p mediates FLC and MTX transport in insect cells

Although *CaMDR1* has been linked to drug resistance, its mechanism of action remains poorly understood. The overexpressed CaMdr1p in baculovirus system provided an opportunity to understand its mechanism of drug transport. We selected two drugs FLC and MTX for transport studies since these drugs are known to transcriptionally activate *CaMDR1* (Albertson *et al* 1996; Gupta *et al* 1998). The choice of concentration of FLC selected for transport assay was based on a previous report (Smriti *et al* 1999). However, for MTX, a concentration dependent transport assay revealed that 25 μM concentration was optimum (data not shown). It is evident from figure 2a, b that insect cells infected with vAcCaMDR1 showed significantly reduced accumulation of FLC and MTX as compared to cells infected with wild type *AcNPV* virus. This lower accumulation of MTX suggested that most of

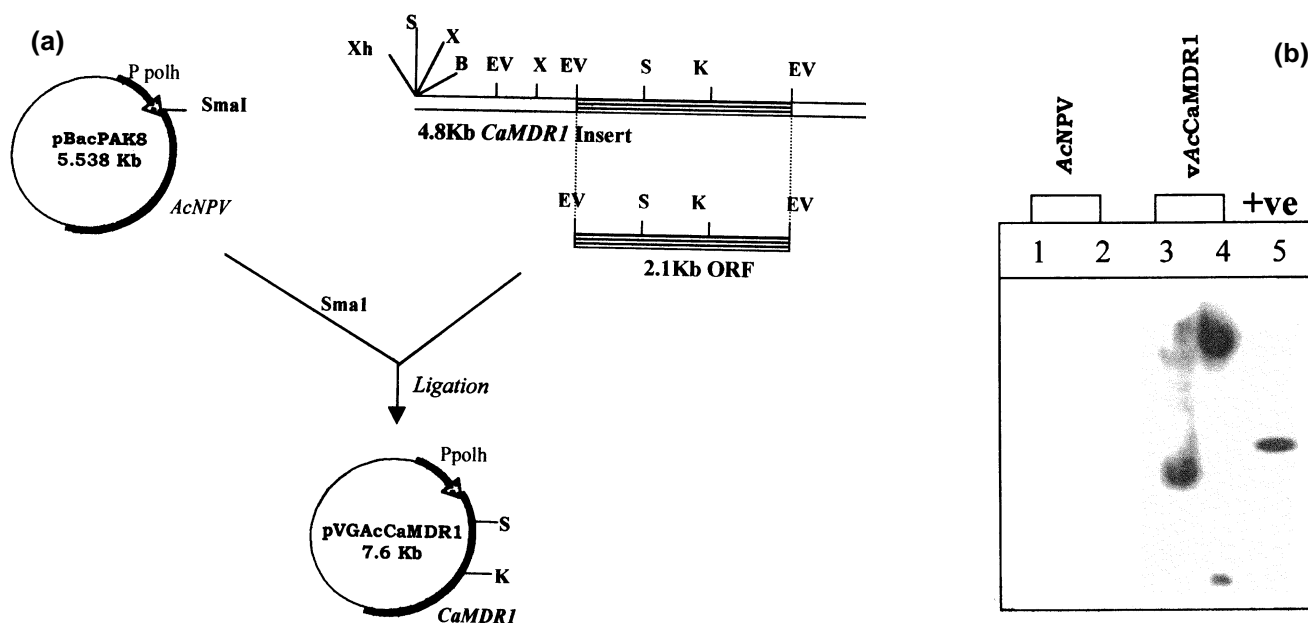


Figure 1. Cloning of *CaMDR1* in *pBacPak8* vector and its integration into *polh* locus of *AcNPV* genome. (a) Schematic diagram depicting construction of pVGAcCaMDR1. A 2.1 kb *EcoRV* fragment of pNC39 harbouring *CaMDR1*, was subcloned into pBacPak8 resulting in a plasmid pVGAcCaMDR1 as described in § 2. *Sf9* cells were co-transfected with wild type *Autographa californica* nuclear polyhedrosis virus (*AcNPV*) DNA and pVGAcCaMDR1. The resulting virus was designated as vAcCaMDR1. (b) Southern hybridization of DNA extracted from *Sf9* cells infected with the wild type virus, *AcNPV* (lanes 1 and 2) and recombinant virus vAcCaMDR1 (lanes 3 and 4). Lanes 1 and 3 contain DNA digested with *SalI* and lanes 2 and 4 contain DNA double digested with *SalI* and *KpnI*. Lane 5 contains 2.1 kb *EcoRV* fragment of *CaMDR1* containing the ORF which was also used as a probe for hybridization.

the drug was effluxed out from the insect cells expressing CaMdr1p within first 15 min. However, at later time points due to MTX induced cytotoxicity to insect cells, the level of drug accumulation decreased even in the control cells. Between the two drugs, MTX efflux was very rapid (low accumulation) suggesting it to be a preferred substrate of CaMdr1p (figure 2a, b). ABC type of drug transporters have earlier been overexpressed in baculovirus expression system, but virus infected intact insect cells could not be used to ascertain if expression led to MDR phenotype because post infected cells are prone to viral-induced lysis (Germann 1998; Krishnamurthy *et al* 1998b). As an alternative, membrane vesicles of insect cells were used to study drug transport and drug protein interactions. Hence, this is the first report where we could use intact *Sf9*

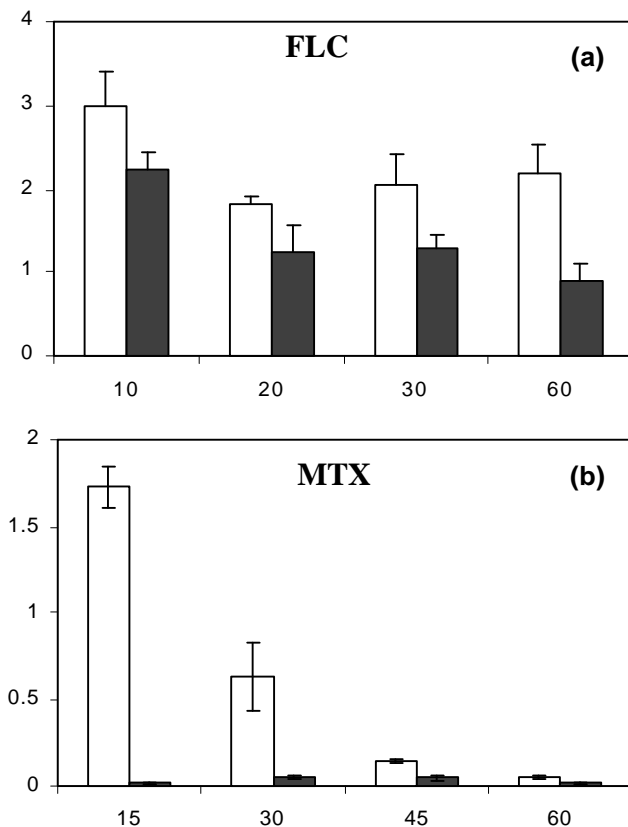


Figure 2. Drug transport mediated by CaMdr1p in *Sf9* cells. (a) FLC transport mediated by CaMdr1p in *Sf9* insect cells at 48 h post infection was done as described in § 2. Briefly (□) wild type AcNPV and (■) vAcCaMDR1 virus infected *Sf9* cells were incubated in the presence of 100 nM of [³H]FLC for various time points up to 60 min. Cells from each time point were washed with PBS and radioactivity associated with the cells was measured. (b) Time course of [³H]MTX transport. Cells were incubated in the presence of 25 μM [³H]MTX. The uptake of MTX was done as described above for FLC. The results are average of 3–4 separate experiments where variation was less than 5%.

insect cells over expressing CaMDR1 to study drug transport.

3.3 FLC and MTX binding sites in CaMdr1p do not overlap

Further studies pertaining to the kinetics and specificity of CaMdr1p expressed in insect cells could not be performed because of drug toxicity. In order to circumvent this problem, CaMdr1p was expressed in a hypersensitive *S. cerevisiae* strain. As shown in figure 3a and figure 4a, host

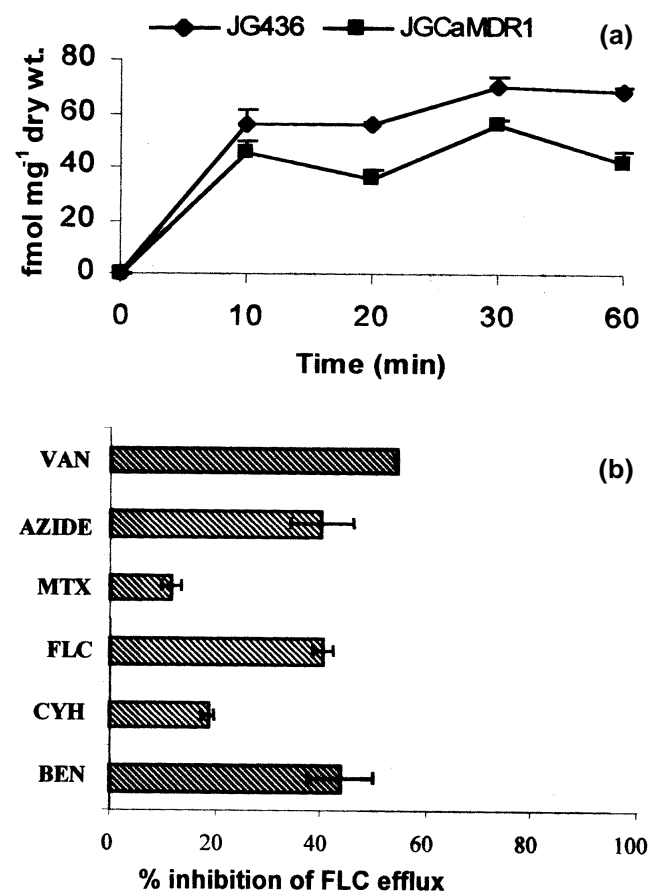


Figure 3. Kinetics of [³H]FLC accumulation in yeast JG436 transformed with CaMDR1. (a) Time course of [³H]FLC transport. Mid log phase yeast cells JG436 and JGCaMDR1 were incubated with 100 nM of [³H]FLC for 60 min as described in § 2. (b) Specificity of FLC transport mediated by CaMdr1p. Competition of [³H]FLC transport in JGCaMDR1 cells was measured in the presence of 100-fold excess of cold drugs as described in § 2. Energy inhibitors Van, sodium orthovanadate and Azide were added to the cells at a concentration of 100 μM and 10 mM respectively. The accumulation of [³H]FLC in yeast cells was measured and compared at 30 min time interval. The bar in each figure shows standard deviation and values are the means of three independent experiments.

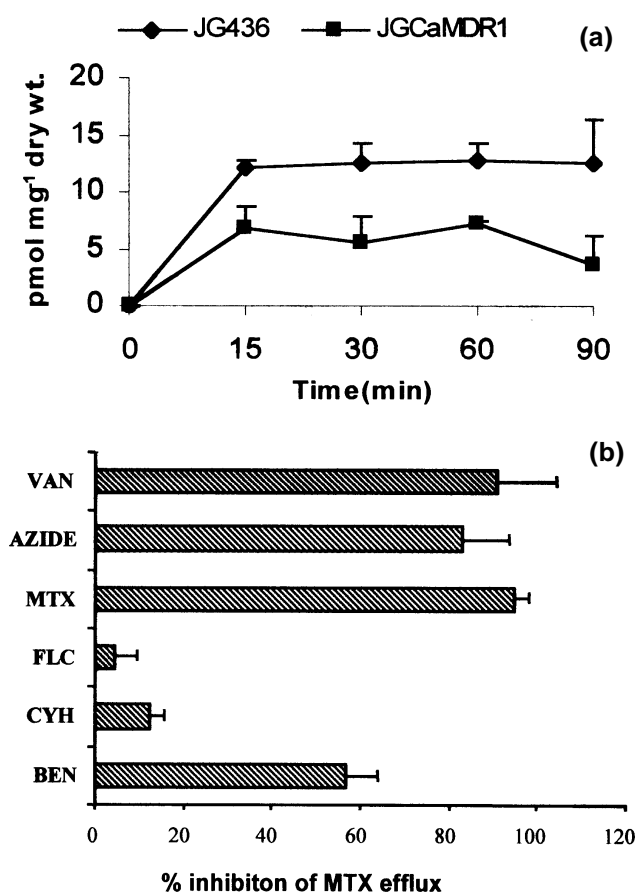


Figure 4. Kinetics of [^3H]MTX accumulation in yeast JG436 transformed with *CaMDR1*. (a) Time course of [^3H]MTX transport. Mid log phase yeast cells JG436 and JGCaMDR1 were incubated with 25 μM [^3H] of MTX for 180 min as described in § 2. (b) Specificity of MTX transport mediated by CaMdr1p. Competition of [^3H]MTX transport in JGCaMDR1 cells was measured in the presence of 100-fold excess of cold drugs as described in legend to figure 3b.

JG436 showed enhanced accumulation of [^3H]FLC and [^3H]MTX with time, while the transformant expressing *CaMDR1* showed reduced accumulation of both the drugs.

As mentioned earlier *CaMDR1* confers resistance to a variety of unrelated drugs implying that these drugs could also be transported by this transporter (Gupta *et al* 1998; Sanglard *et al* 1996). Therefore, in order to check the specificity of CaMdr1p mediated drug transport, the accumulation of MTX and FLC was followed either alone or in presence of high concentration of other drugs. In the following experiments, JGCaMDR1 cells were pre-incubated with 100-fold concentrations of MTX, FLC, CYH and BEN before assaying for drug accumulation as described in § 2. Interestingly, the excess MTX did not affect the accumulation of FLC. The accumulation of MTX also remained unaffected by high concentration of FLC (figures 3b and 4b). For all such experiments, a parallel control experiment was performed in *S. cerevisiae* host JG436, in which no marked difference in the levels of accumulation of radiolabelled drugs was observed upon incubation with excess cold drugs (data not shown). Therefore, while CaMdr1p transports both the drugs, the binding sites for MTX and FLC in CaMdr1p appeared to be distinct. The excess of BEN on the other hand could block the efflux of both MTX and FLC, which suggested that its binding to CaMdr1p overlaps with that of two drugs. Interestingly, CYH did not interfere with the efflux of either of the drugs, indicating that it had discrete binding sites. The efflux of both the drugs mediated by CaMdr1p was energy dependent as it could be inhibited by sodium orthovanadate and sodium azide (figures 3b and 4b).

In another experiment we observed that MTX which was a substrate for CaMdr1p, could not be transported by

Table 1. Susceptibility of yeast and its MDR transformants to antifungal agents.

Strains	Genotype	MIC ($\mu\text{g ml}^{-1}$)			
		FLC	MTX	CYH	BEN
JG436*	<i>Dpdr5^a</i>	2	3.12	0.019	10
JGCDR1	<i>Dpdr5 + CDR1</i>	32	3.12	0.156	10
JGCaMDR1	<i>Dpdr5 + CaMDR1</i>	8	12.0	0.156	10

MIC were determined by microtiter plate assay as described in § 2 (Talibi and Raymond 1999). For MTX, sulphanilamide was added to the media at a concentration of 200 $\mu\text{g ml}^{-1}$. Cells were grown for 48 h at 30°C to obtain single colonies which were resuspended in a 0.9% normal saline solution to give an optical density at 600 nm (OD_{600}) of 0.1. The cells were then diluted 100-folds in SD-URA media. The diluted cell suspensions (100-folds) were added to round bottomed 96-well microtiter plates (100 $\mu\text{l/well}$) in wells containing equal volumes of medium (100 $\mu\text{l/well}$) with different concentrations of drugs. End point was read at OD_{620} after 48 h incubation of plates at 30°C.

^a*PDR5* has been disrupted in this strain (see § 2).

*The relative drug sensitivity of vector transformed JG436 cells did not show any variations as compared to non transformant JG436 cells.

Cdr1p an ABC protein also involved in azole resistance in *C. albicans* when expressed in similar host background (data not shown). The selectivity of the pump was further evident from microtitre plate assay where it was observed that JGCDR1 (harbouring *CDR1*) had a higher MIC value for FLC but was hypersensitive to MTX (table 1). It is notable that MTX could strongly enhance the expression of *CaMDR1* while it had no effect on *CDR1* expression (Gupta et al 1998). That MTX is not a substrate of ABC proteins was also supported by previous observations by De Graaf et al (1996) where MTX resistance of a cell was shown to be distinct from MDR mediated by P-glycoprotein (P-gp). MTX which inhibits dihydrofolate reductase, is hydrophilic in nature and is only removed from the cells by P-gp when under certain conditions MTX enters cells via membrane. Our results show that Cdr1p, which poorly transports MTX, is a structural and functional homologue of P-gp, thus illustrating the conservation of functions down the evolutionary scale.

It is becoming increasingly evident from mammalian ABC transporters that they harbour discrete drug binding domains that are clustered for some drugs and scattered over the entire protein for some other drugs (Gottesmann and Pastan 1993; Ambudkar and Gottesman 1998). Cdr1p, an ABC transporter of *C. albicans* has also been shown to possess distinct drug binding sites at its C terminal (Krishnamurthy et al 1998b). The deletion of 79 aa from its C-terminal encompassing TM12 could lead to hypersensitivity to several drugs, while the truncated protein could still confer resistance to drugs like FLC. In this study we have shown for the first time that CaMdr1p also has different drug binding sites. Precise knowledge of the mechanism by which transporters recognize their substrates might have important clinical implications. Understanding antifungal resistance in general and the specificity of drug transport (this study) in particular is important for the improved management of *C. albicans* infection.

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