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Multiple drug resistance in *Candida albicans**

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By functional complementation of a *PDR 5* (pleiotropic drug resistance) null mutant of *S. cerevisiae*, we have recently cloned and sequenced a multidrug resistance gene *CDR 1* (*Candida* Drug Resistance). Transformation by *CDR 1* of a *PDR 5* disrupted host hypersensitive to cycloheximide and chloramphenicol resulted in resistance to these as well as other unrelated drugs. The nucleotide sequence of *CDR 1* revealed that, like *PDR 5*, it encodes a putative membrane pump belonging to the ABC superfamily. *CDR 1* encodes a protein of 169.9 kDa whose predicted structural organisation is characterised by two homologous halves, each comprising a hydrophobic region, with a set of six transmembrane stretches, preceded by a hydrophilic binding fold. We now have evidence to suggest that there are several *PDR* homologues present in *C. albicans* which display multidrug resistance and a collateral sensitivity pattern different from *PDR 5* and *CDR 1*. The functions of such genes and their products in the overall physiology of *C. albicans* is not yet established.

Candida albicans is an opportunistic human pathogen which most frequently infects the mucous of epithelial tissues of the oral and urogenital tracts, particularly in the immunocompromised host. *C. albicans* can cause deep seated as well as systemic infections [1]. A number of factors are thought to influence the pathogenicity of this fungus [1, 2], one of which is its ability to undergo a dimorphic switching from a budding yeast to a hyphal form which probably helps the pathogen to evade the host immune system [3–5]. Although both yeast and hyphal forms exist at infected sites [1, 6], the hyphal form appears better adapted to penetrate the epithelia [7], suggesting that dimorphism plays a crucial role in its pathogenicity. Genes which play an important role in regulation of dimorphism are being identified. *Saccharomyces cerevisiae*, which also displays dimorphic transition [8] is being exploited as a model to identify morphogenetically regulated genes, since it is easy to manipulate this model system for higher eukaryotes. The homologues of *S. cerevisiae CHS 1* and *CHS 2*, which code for chitin synthase, have been cloned and sequenced in *C. albicans* [9, 10]. Both *CHS 1* and *CHS 2* show different levels of transcription between yeast and the hyphal forms of *C. albicans* [10]. Recently, the regulation of the gene encoding the translation elongation factor 3 (*TEF 3*) during growth and morphogenesis in *C. albicans* has been studied [11]. The observed

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Abbreviations: ABC, ATP-binding cassette; CDR, Candida drug resistance; MDR, multidrug resistance; MFS, major facilitator's superfamily; ORF, open reading frame; PDR, pleiotropic drug resistance; TEF, translation elongation factor.

changes in *TEF 3* expression during dimorphic transition reflected the underlying physiological rather than morphogenesis-dependent changes. However, *TEF 3* represents an attractive antifungal target, since it is essential for translation in fungi and has no role in mammalian systems.

Nearly 40% of all deaths from hospital-acquired infections are due to fungal infections, and in most cases the culprit is *Candida* [12]. Recently this organism has been documented as one of the main agents in the morbidity and mortality of patients suffering from acquired immunodeficiency syndrome, AIDS [13, 14]. The situation is further aggravated due to acquired antifungal resistance and the emergence of resistant isolates in response to widespread and prolonged drug treatment, particularly with azoles. The major challenge in antifungal therapy at present is in dealing with the increase in the resistance of *Candida* to presently available antifungal agents. Therefore, an understanding of the basis of drug resistance in *C. albicans* could assist in the development of new therapeutic approaches.

DRUG RESISTANCE IN *CANDIDA ALBICANS*

Candida albicans has generally been found to be more resistant to metabolic inhibitors than *S. cerevisiae* and there are instances of acquired additional drug resistance [15, 16]. Dimorphism of *C. albicans* enables it to produce a large number of genotypic and phenotypic variants with physiological characteristics such as reduced susceptibilities to azole antifungals, even without previous exposure to these agents [17]. A variety of clinical regimens exist for systemic candidiasis [18]. Primary amongst them are treatment with amphotericin B, 5-fluorocytosine and imidazole. Other antifungal agents less toxic than amphotericin B are azoles, including ketoconazole, fluconazole and itraconazole.

The incidence of azole resistance in *C. albicans* with cross-resistance to other antifungals is on the increase. There are several mechanism by which *C. albicans* could acquire resistance to an azole. For example the cells could take up less drug or could efflux more of it, the target site(s) could be altered or the drug could be inactivated after entry by subsequent metabolism [16, 19]. Given the heterogenity of the mechanism of action shown by some azoles, the exact mechanism of resistance is not yet known which may depend on the type of azole used, the fungal strain and the nature of medium on which it is grown [16]. Recent studies with ketoconazole, itraconazole, fluconazole and some other azole derivatives have shown that the mechanism of resistance could be divided broadly into changes in cytochrome P-450-dependent 14α-sterol demethylase, changes in sterol $\Delta^{5,6}$ desaturase, or permeability resistance [16, 18–20]. Interestingly, all of the resistant organisms are also cross-resistant to all of the other azoles (for details see Chapter by P. Marichal & Vanden Bossche in this volume).

Acquired fluorocytosine resistance has also been observed in *C. albicans*, resulting from a defect in uracil phosphoribosyl transferase, the enzyme involved in the synthesis of both 5-FdUMP and 5-FUTP, and of uridylate in the pyrimidine salvage pathway [18]. Whether the multidrug resistance phenomenon observed in a variety of organisms also exists in pathogenic fungi, including *C. albicans*, was not known till recently (discussed below).

MULTIDRUG RESISTANCE IS NOT RESTRICTED TO MAMMALS

Multidrug resistance (MDR) is a well-known phenomenon in mammalian cancer cells. The molecular mechanism underlying the multidrug resistance of cancer cells exposed to chemotherapy is due to overexpression of an ATP-dependent extrusion pump (P-glycoprotein) which enhances the efflux of cytotoxic compounds [21]. The phenomena of MDR is not restricted to mammalians cells. Homologues of mammalian MDR genes have now been identified in several genera of bacteria such as *emr* and *mdl* of *E. coli* [22, 23], *Qac C* of *Staphylococcus* [24] and *Pseudomonas* [25], in protozoan parasites including *Pfmdr* of *Plasmodium falciparum* [26, 27], *Leishmania donovani ldmdr* [28–30] and *Entamoeba histolytica* [31], in the fungi *S. cerevisiae* [32, 33] and also the *MDR 50* multidrug resistance gene homologue of *Drosophila* [34].

Multidrug resistance in yeast *S. cerevisiae* was earlier described as a generalised resistance of

a number of functionally and structurally unrelated drugs and was termed as PDR (Pleiotropic Drug Resistance) [35]. Recent molecular analysis of PDR mutations in yeast have confirmed the existence of at least 20 different genes [36]. Some of the PDR-encoded gene products identified in yeast are listed in Table 1. These molecules belong to three different

Table 1
Drug resistance genes in yeast

Species	Gene	Drugs	Features
S. cerevisiae	*PDR 5/STSI/YDR1*	cyh, chl, ery, amy, sts, flu, smm, com, cer, lyn	ABC membrane protein. Duplicated. (NBD-TM)2
S. cerevisiae	*SNQ 2*	4-NQO, NMNG, flu, sts, tri, phen	ABC membrane protein. Duplicated. (NBD-TM)2
S. cerevisiae	*STE 6*	Val	ABC membrane protein. Duplicated. (TM-NBD)2
S. cerevisiae	*YCF 1*	Cd	ABC membrane protein. Duplicated. (TM-NBD)2
S. cerevisiae	*PDR 10*	–	ABC membrane protein. Duplicated. (NBD-TM)2
S. pombe	*pmd 1*	lep, cyh, val	ABC membrane protein. Duplicated. (TM-NBD)2
C. albicans	*CDR 1*	cyh, chl, mic, amy	ABC membrane protein. Duplicated. (NBD-TM)2
S. cerevisiae	*ADP 1*	–	ABC membrane protein. Half sized. (NBD-TM)
S. cerevisiae	*YKL 741*	–	ABC membrane protein. Half sized. (TM-NBD)
S. cerevisiae	*MDL 1*	–	ABC membrane protein. Half sized. (TM-NBD)
S. cerevisiae	*MDL 2*	–	ABC membrane protein. Half sized. (TM-NBD)
S. cerevisiae	*Ssh 1*	–	ABC membrane protein. Half sized. (TM-NBD)
S. cerevisiae	*Ssh 2*	–	ABC membrane protein. Half sized. (TM-NBD)
S. pombe	*HMT 1*	heavy metals (cd)	Vacuolar. Half sized. (TM-NBD)
S. cerevisiae	*ATM 1*	–	Mitochondrial ABC membrane protein. Half sized. (TM-NBD)
S. cerevisiae	*ATR 1/SNQ 1*	atr, 4-NQO	Membrane facilitator
S. cerevisiae	*YCL 069w*	–	Membrane facilitator
S. cerevisiae	*YCL 023c*	–	Membrane facilitator
S. cerevisiae	*YCL 070c*	–	Membrane facilitator
S. cerevisiae	*YKR 105c*	–	Membrane facilitator
S. cerevisiae	*YKR 106w*	–	Membrane facilitator
C. albicans	*ORF 1*	ben, met	–
C. maltosa	*CYHR*	cyh	–
S. pombe	*car 1*	aml	–

Table 1 (continued)

Species	Gene	Drugs	Features
S. cerevisiae	*PDR 1*	cyh, chl, oli, nys, ner, muc, ebr, bor, tet, smm, car, dqc, acf, amy, cer, gen, par, neo	Transcription regulators
S. cerevisiae	*PDR3*	muc, chl, cyh, oli, tet, ner	Tanscription regulators
S. cerevisiae	*yAP 1/PDR 4 SNQ 3/PAR 1*	Cd, Zn, cyh, tre, smm, NQO, phe, NMNG, nin	Tanscription regulator
S. cerevisiae	*CAD 1/YAP 2*	Cd, Zn, phe	Transcription regulator
S. pombe	*pap 1*	sts	Transcription regulator
S. cerevisiae	*PDR 7*	cyh, smm	–
S. cerevisiae	*PDR 9*	cyh, smm	Transcription regulator
S. cerevisiae	*RPD 1*	cyh	Transcription regulator
S. cerevisiae	*RPD 3*	cyh	Transcription regulator
S. cerevisiae	*YGL 022*	cyh, smm	–
S. cerevisiae	*PDR 6*	cyh, bor, hyg B	–
S. cerevisiae	*PDR 8*	oli, smm	–
S. pombe	*sts 1*	cyh, sts, caf, chl, divalent cation	–
S. cerevisiae	*cpr*	van	Soluble
S. cerevisiae	*HOM 3*	bor	Soluble
S. cerevisiae	*AMY 1*	amy	–
S. pombe	*RIM-C*	cyh	Soluble, ribosomal binding protein
S. cerevisiae	*ZRC 1*	Zn, Cd	Transporter

Drugs are abbreviated as follows: acf, acriflavin; atr, aminotriazole; amy, antimycin; aml, amiloride; ben, benomyl; bor, borrelidin; caf, caffeine; car, carbomycin; cer, cerulenin; chl, chloramphenicol; cyh, cycloheximide; com, compactin; dac, dibenzyldimethylammonium chloride; dqc, dequalinium chloride; ery, erythromycin; ebr, ethidium bromide; flu, fluphenazine; gen, gentamycin; hyg B, hygromycin B; lep, leptomycin; lym, lyncomycin; mic, miconazole; muc, mucidin; nin, 1-nitroso-2-naphtol; neo, neomycin; NMNG, *N*-methyl-*N'*-nitrosoguanidine; 4-NQO, 4-nitroquinoline *N*-oxide; ner, Neutral red; met, methotrexate; oli, oligomycin; par, paromomycin; phe, 1,10-phenanthroline; smm, sulfomethuron methyl; sts, staurosporine; tet, tetracycline; val, valinomycin; van, vanadate; tri, triaziquone; tre, trenimon; cd, cadmium; Zn, zinc. Other abbreviations are, NBD, nucleotide binding domain; TM, transmembrane region; ABC, ATP binding cassette; (NBD-TM)2, NBD precedes TM and *vice versa* and has 2 halves. (The table is compiled from [36, 37, 40–43]).

classES of proteins: membrane proteins of the ATP-binding cassette (ABC) superfamily, such as *SNQ 2*, *STE 6*, *PDR 5* and *YCF 1*, the major facilitator's superfamily (MFS) such as *ATR 1* and *SGE 1* and the transcription regulators, such as *PDR 1*, *PDR 3*, *PDR 7*, *PDR 9*, *YAP 1* and *YAP 2*. The network of genes involved in the multidrug resistance of the yeast *Saccharomyces cerevisiae* has been recently reviewed [37]. The transcription regulators *PDR 1*, *PDR 3*, *PDR 7* and *PDR 9* control expression of *PDR 5*, which encodes a protein of the ATP-binding cassette and functions as a drug extrusion pump. Several other target genes, encoding membrane proteins of the ABC type such as *SNQ 2*, *STE 6*, *PDR 10*, *PDR 11*, *YOR 1*, are also found to be controlled by *PDR 1*.

MULTIDRUG RESISTANCE IN *C. ALBICANS*

The existence of MDR/PDR homologue(s) in *C. albicans* has been recently reported. The *CDR 1* (Candida Drug Resistance) gene has been cloned by functional complementation of a null mutant of *PDR 5* of *S. cerevisiae* [38] (Fig.1). The expression of *CDR 1* in *S. cerevisiae* (as single or low copies) conferred multidrug resistance and collateral sensitivity to various drugs, while

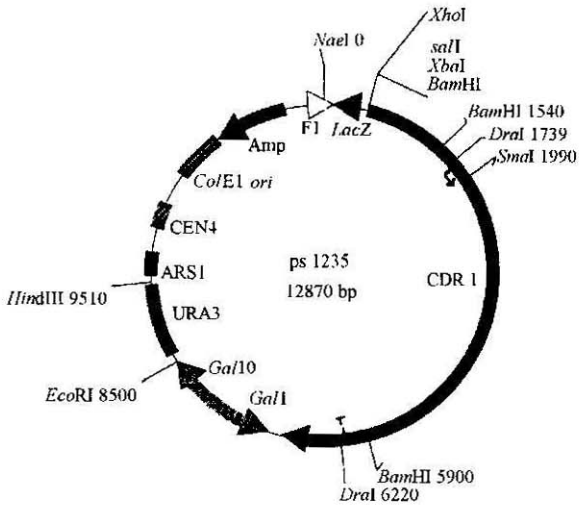

Fig. 1. Construct of subcloned 6.2 kb fragment in pYEURA3 showing the CDR 1 gene and restriction sites: (→) denotes the ORF and (T) denotes the termination site.

multiple copies of the homologous *Saccharomyces PDR 5* gene [39] were required to yield drug resistance. The level of resistance to drugs like cycloheximide was much stronger as compared to that conferred by *PDR 5*, which suggested that *CDR 1* could be intrinsically much more effective in *C. albicans*. That *C. albicans* is more resistant to metabolic inhibitors and drugs as compared to other yeasts could be the reason for *CDR 1* efficiency. The exact phenotype of *CDR 1* expression will, however, be known only when the gene is expressed/overexpressed in *C. albicans*.

The nucleotide sequence of *CDR 1* (6202 bp) and the subsequent deduced protein sequence revealed great similarity to other homologues of *S. cerevisiae*, e.g. *PDR 5* (56% identity, 73% similarity), and *SNQ 2* (42% identity, 60% similarity), etc. The similarity to these genes was not restricted to nucleotide binding domains but was conserved on the entire length of the protein. The CDR 1 protein displays the structure of a typical protein of the ABC superfamily. It belongs to the four-domain type of ABC-transporters, consisting of two homologous halves, each comprising one hydrophobic region, with a set of six predicted transmembrane spans, preceded by one hydrophilic nucleotide-binding fold (Fig. 2). The structural arrangement is identical to that of *S. cerevisiae* ABC proteins PDR 5 and SNQ 2. It mirrors the architecture of the yeast a-mating pheromone transporter STE 6, as well as the mammalian drug resistance P-glycoprotein (MDR1) and cystic fibrosis factor CFTR [38]. The significance of such domain inversion in some ABC proteins is not clear. The

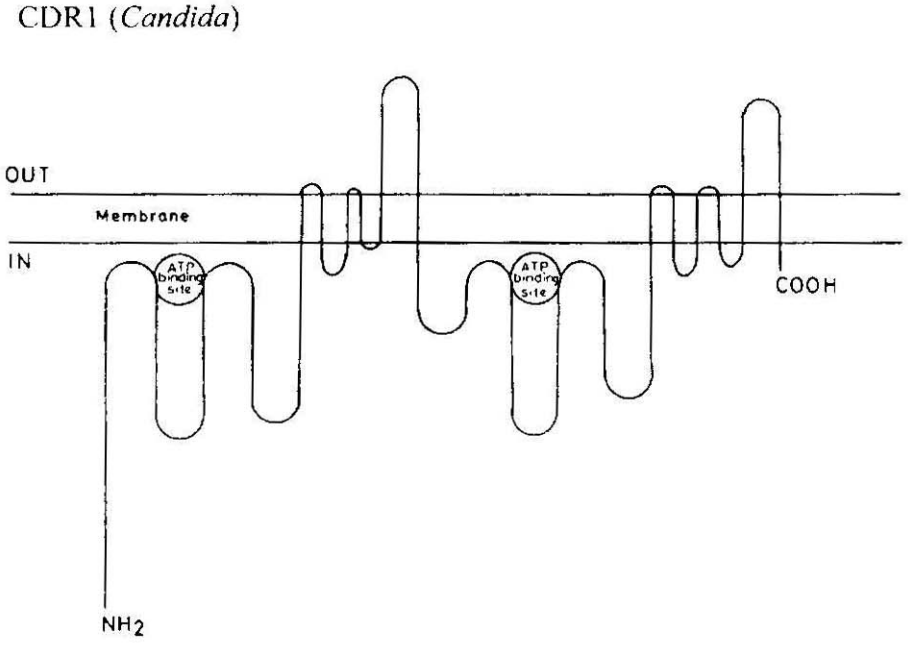

Fig. 2. Predicted structure of the CDR 1 protein, considered to be composed of two repeated halves, each comprising one hydro- philic domain, followed by a hydrophobic domain. Two hydrophilic domains are cytoplasmic (IN) and each contains one ATP-binding site. The two hydrophobic domains are considered to be spanning the membrane.

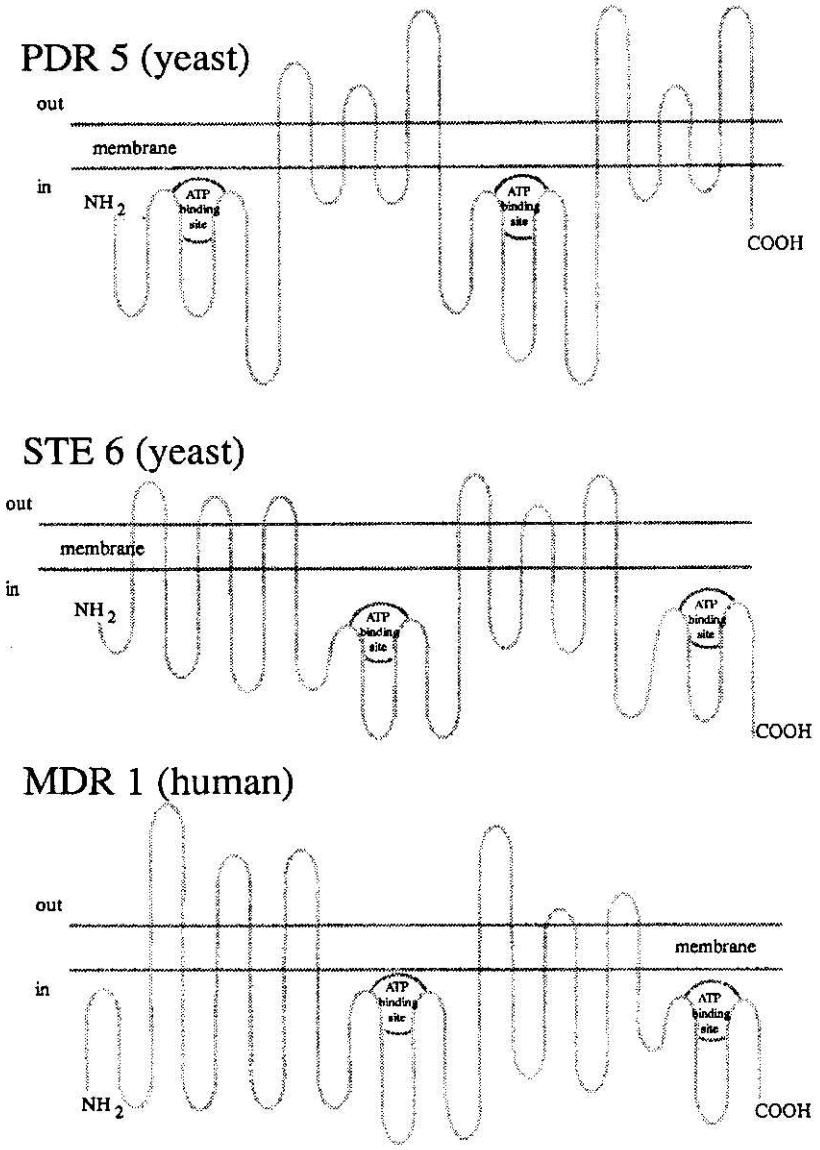

Fig. 3. Predicted structure of the PDR 5, STE 6 and MDR 1 proteins. The sequence of domain inversion between CDR 1 (Fig. 2) and STE 6 can be seen from the figure.

Table 2

Resistance of different transformants with respect to drugs and antifungals

Clone No.	CYH 5 μg	NYS 1 μg	FIL 100 μg	MIC 100 μg	PHE 20 μg	ERY 50 μg	CHL 500 μg	OLI 0.2 μg	AMY 0.01 μg
NC 34	HR	R	N	R	HS	S	N	N	R
NC 36	HR	R	N	R	HR	S	HR	R	N
NC 55	R	N	R	R	R	S	N	R	S
NE 2	HR	N	N	N	HHR	R	R	N	R
NE 4	HR	N	N	R	HHR	R	R	N	N
NE 18	HHR	R	N	S	HS	R	S	HS	N

Quantitative measurement of drug resistance was done by the filter disc assay as described [44]. Cycloheximide (cyh), nystatin (nys), filipin (fil), miconazole (mic) and phenanthroline (phe) were tested on YNB medium supplemented with methionine and leucine. The mitochondrial inhibitors *viz.*, chloramphenicol (chl), oligomycin (oli), erythromycin (ery) and antimycin (amy) were tested on YPG medium. The relative degree of drug resistance was determined by comparing the diameter of zone of inhibition to that of null mutant JG436 after two days for YNB medium and four days for YPG medium. Other abbreviations used are HHR, hyper-hyper resistant; HR, hyper resistant; R, resistant; N, no change; S, sensitive; HS, hypersensitive.

predicted structures of PDR5, STE6 and MDR1 proteins are shown in Fig. 3 for comparison.

OTHER MDR GENES IN *CANDIDA*

While screening for drug resistance of transformants which complemented the *PDR 5* null mutants, we obtained several clones which displayed multiple drug resistance. Subsequent plasmid escape tests revealed that there are several transformants which harbour more of these pleiotropic drug resistance genes. The multiple drug resistance of at least six interesting transformants is shown in Table 2. Each transformant elicited different multidrug resistance and collateral sensitivity to various mitochondrial inhibitors and antifungals. Furthermore, initial restriction and Southern analysis suggested that each transformant has a different gene (other than *CDR 1*) responsible for selective and specific drug resistance/sensitivity.

Considering the fact that *C. albicans* which is capable of infecting most human body locations and that only a limited number of anti-*Candida* drugs are available, the demonstration of multidrug resistance genes in *C. albicans* is an important development for the elucidation of permeability constraints of the pathogenic yeast which are still poorly understood. Furthermore, such genes responsible for multidrug resistance in *C. albicans* could also become potential targets for new antifungals.

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