The yeast multidrug resistance pump, Pdr5p, confers reduced drug resistance in *erg* mutants of *Saccharomyces cerevisiae*

Rupinder Kaur and Anand K. Bachhawat

Author for correspondence: Anand K. Bachhawat. Tel: +91 172 690004/690908.
Fax: +91 172 690585/690632. e-mail: abachhawat@excite.com

Institute of Microbial
Technology, Sector 39-A,
Chandigarh – 160036,
India

Mutants of *Saccharomyces cerevisiae* bearing lesions in the ergosterol biosynthetic pathway exhibit a pleiotropic drug-sensitive phenotype. This has been reported to result from an increased permeability of the membranes of the mutant strains to different drugs. As disruption of the yeast multidrug resistance protein, Pdr5p, results in a similar pleiotropic drug-sensitive phenotype, the possibility that Pdr5p may be functioning with a reduced efficiency in these altered sterol backgrounds was examined. To do this, the function of Pdr5p in isogenic strains of *S. cerevisiae* that have disruptions in the late stages of the ergosterol biosynthesis pathway (*ERG6*, *ERG2*, *ERG3*, *ERG4*) was studied. A reduced ability of Pdr5p to confer resistance to different drugs in these strains was observed, which did not appear to be dependent solely on the permeability of the membrane towards the drug. A simultaneous examination was made of how the lipid composition might be altering the efficiency of Pdr5p by similar studies in strains lacking phosphatidylserine synthase (encoded by *CHO1*). The results indicated that the drug sensitivity of the *erg* strains is, to a significant extent, a result of the reduced efficiency of the Pdr5p efflux pump, and that the membrane environment plays an important role in determining the drug resistance conferred by Pdr5p.

Keywords: *Saccharomyces cerevisiae*, multidrug resistance pump, membrane environment, sterols, *erg* mutants

INTRODUCTION

Multidrug resistance (MDR), the acquisition of simultaneous resistance to a wide variety of structurally and functionally unrelated compounds, is often associated with the overexpression of one or more members of a superfamily of ATP-binding cassette transporters (Gottesman & Pastan, 1993). These proteins are comprised of 12 membrane-spanning α -helices and two ATP-binding domains (Borst & Schinkel, 1997). MDR is a ubiquitous phenomenon and the membrane proteins responsible for this phenomenon are conserved throughout the evolutionary scale. The mechanism by which these proteins are able to confer resistance to such a structurally diverse range of compounds is still not known (Gottesman *et al.*, 1996). Structure–function

analyses from different groups (Loo & Clarke, 1993; Beaudet & Gios, 1995; Hanna *et al.*, 1996; Kwan & Gros, 1998) have not identified any single domain that is responsible for the diverse substrate specificity of MDR pumps, although mutations affecting substrate specificity appear to be clustered around the transmembrane domains and the substrates have been shown to bind within the membrane bilayer (Sharom, 1997).

The yeast *Saccharomyces cerevisiae* has also been shown to contain a MDR efflux pump, Pdr5p, that belongs to the family of ABC transporters. Pdr5p has been cloned independently by several groups (Bissinger & Kuchler, 1994; Balzi *et al.*, 1994; Hirata *et al.*, 1994; Kralli *et al.*, 1995) and has been shown to confer resistance to a wide range of compounds and metal ions (Mahe *et al.*, 1996). Overproduction of this protein confers pleiotropic drug resistance, whilst disruption of the gene encoding it leads to a pleiotropic drug-sensitive phenotype. Recent work with Pdr5p, however, could not identify any single domain that affects the substrate specificity of this pump

Abbreviations: DPH, diphenylhexatriene; TMA-DPH, trimethylammonium diphenylhexatriene; MDR, multidrug resistance; P-gp, phosphoglycoprotein; wt, wild-type.

and it was suggested that the folded structure of the Pdr5p protein, and possibly the membrane composition, might play an important role in determining its substrate specificity (Egner *et al.*, 1998). Yeast *erg* mutants, which carry mutations in the ergosterol biosynthetic pathway, are resistant to antifungal antibiotics that target this pathway, but are otherwise pleiotropically drug-sensitive to a wide variety of structurally diverse compounds. This has been reported to be due to an increased permeability of their membranes to different drugs (Bard *et al.*, 1978). However, considering that disruption of the yeast Pdr5 pump also results in similar pleiotropic drug sensitivities, and the possible importance of the membrane environment in the functioning of these pumps, we decided to re-examine the drug-sensitive phenotypes of the *erg* mutants and investigate how the Pdr5 pump functions in these strains. An earlier investigation had reported that ergosterol, the major sterol of the yeast plasma membrane, inhibits the drug-binding ability of mammalian phosphoglycoproteins (P-gps) when expressed in yeast (Saeki *et al.*, 1991); however, this has not been substantiated by later workers (Kuchler & Thorner, 1992). A possible role of lipids in the modulation of the drug-binding activity of P-gps has been suggested by the observations that many characteristics of P-gps, including the pattern of ATPase stimulation or inhibition by drugs, is affected by the lipid environment (Doige *et al.*, 1993; Saeki *et al.*, 1992; Urbatsch & Senior, 1995).

Our results, described in this report, indicate that the membrane environment/composition plays a significant role in determining the functioning of MDR pumps, and that the drug sensitivity of *erg* mutants is, to a large extent, due to the decreased drug efflux mediated by Pdr5p in these strains.

METHODS

Chemicals. The chemicals used were of analytical grade. All media components were either purchased from HiMedia or Difco. Rhodamine 6G, cycloheximide, crystal violet, emetine and β -oestradiol were obtained from Sigma. Diphenylhexatriene (DPH) and trimethylammonium diphenylhexatriene (TMA-DPH) were obtained from Molecular Probes. Oligonucleotides were purchased from Ransom Hill Biosciences. Restriction/modification enzymes were obtained from New England Biolabs.

Yeast strains, media and growth conditions. The yeast strains used in this study are listed in Table 1. They were grown at 30 °C and maintained on yeast extract/peptone/dextrose medium (YPD). YPD and synthetic media (SD) were prepared as described by Rose *et al.* (1990).

Stock solutions of crystal violet, cycloheximide and emetine were prepared in water, whilst rhodamine 6G and β -oestradiol solutions were made in ethanol. Plates containing different compounds were made by adding the required volume of the stock solution of the compound to YPD medium during pouring.

Recombinant DNA methods. DNA manipulations including PCR were essentially carried out as described by Sambrook *et*

al. (1989). Yeast genomic DNA was isolated by the glass bead lysis method (Rose *et al.*, 1990). Yeast transformations were carried out by the lithium acetate method (Ito *et al.*, 1983).

Strain construction

(1) **Construction of *erg4Δ* strain.** Plasmid pCM2 containing the *ERG4* gene disrupted with *URA3* with flanking repeats (obtained from C. Marcireau, Rhone-Poulenc Rorer, Vitry-Sur-Seine, France) was cleaved with *SacI*/*HpaI* and the 6.94 kb *erg4Δ::URA3* fragment was transformed into strain ABC 709 and selected for uracil prototrophy. The disruption at the *ERG4* locus was confirmed by phenotypic analysis (drug sensitivity) and the *URA3* region was popped out by selecting on 5-fluoroorotic acid plates, rendering *erg4Δ* a uracil auxotroph. The disruptant strain thus obtained was crossed with strain ABC 710 and tetrads were dissected to obtain an *erg4Δ* ABC 283 strain in a protease-deficient background.

(2) **Construction of *erg3Δ* strain.** The fragment containing *erg3Δ::LEU2* was amplified from strain ABC 102 (*erg3Δ::LEU2*, obtained from L. Parks, North Carolina State University, Raleigh, USA) with the primers for the flanking regions of this fragment. Strain ABC 287 [constructed from a cross of ABC 229 and ABC 710, which was used as a wild-type (wt) strain] was transformed with the purified 2.4 kb PCR product and selected for leucine prototrophy. The disruptant ABC 261 was confirmed by phenotypic characterization and PCR.

(3) **Construction of *erg2Δ* strain.** The disruption of *erg2Δ::LEU2* was done by introducing a 2.2 kb *LEU2* gene at the *NdeI* site within the coding region of *ERG2* in the plasmid pFLA 2-7 (the plasmid was obtained from J. Heinisch, Heinrich Heine Universität, Dusseldorf, Germany). A 3.2 kb *StuI*-*PstI* fragment containing the *erg2Δ::LEU2* construct was used to transform strain ABC 709 to leucine prototrophy. The strain was confirmed for the disruption at the right locus by phenotypic analysis and PCR. The disruptant was mated with strain ABC 710 and tetrad dissection was done to obtain an *erg2Δ* strain (ABC 271) in a protease-deficient background.

(4) **Construction of *erg6Δ* strain.** An *ERG6* (*SED6*) plasmid was obtained from H. Pelham, MRC, Cambridge, UK, and an *ERG6* disruption was made by inserting a *LEU2* fragment into the *KpnI* site of *ERG6* that was first subcloned into the vector pGEX-2T. A 3.2 kb *ScaI*-*BamHI* fragment containing the *erg6Δ::LEU2* fragment was transformed into ABC 709 and selected for leucine prototrophy. The disruption at the *ERG6* locus was confirmed by PCR. An *erg6Δ* strain (ABC 265) in a protease-deficient background was obtained by crossing the resultant disruptant with ABC 710 and subsequently dissecting tetrads.

(5) **Construction of *pdr5Δ* strain.** A diploid was made by crossing ABC 710 with ABC 152 (*pdr5Δ::TRP1*) and a 3.2 kb *ScaI*-*BamHI* fragment of the plasmid bearing *erg6Δ::LEU2* was transformed into the diploid strain. The transformants were selected for leucine prototrophy and then plated on the sporulation medium. Tetrad dissection was done to obtain a *pdr5Δ* (ABC 288) strain in a protease-deficient background.

(6) **Construction of *cho1Δ* strain.** The *cho1Δ::LEU2* strain was constructed by the PCR-mediated direct gene disruption method (Baudin *et al.*, 1993). The primers CHDEL1 (5' CACAGAGACGAAAATGACGGGTATGCCTCAGATGAAGTTCGACTACGTCGTAAGGCCG 3') and CHDEL2 (5' GACACAATATGCCATACCCAACACCAAAGCTAGAGTGGTAATGGAATCCCAACAATTACA 3') were used

Table 1. Yeast strains used in the present study

Strain	Genotype	Source*
ABC 102 (SY12-D)	MATa <i>ura3-52 leu21 his4 erg3-12Δ::LEU</i>	L. Parks
ABC 152 (YKKA-7)	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 ade2-101 trp1 Δ63 sts1Δ::TRP1</i>	K. Kuchler
ABC 154 (YPH499)	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 ade2-101 trp1 Δ63</i>	K. Kuchler
ABC 229	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 erg2Δ::LEU2</i>	This study
ABC 261	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 erg3Δ::LEU2</i>	This study
ABC 265	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4::HIS prb1Δ1.6R can1 erg6Δ::LEU2</i>	This study
ABC 271	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4::HIS prb1Δ1.6R can1 erg2Δ::LEU2</i>	This study
ABC 283	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 erg4Δ</i>	This study
ABC 287	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4::HIS3 prb1Δ1.6R can1</i>	This study
ABC 288	MATα <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 pdr5Δ::TRP1</i>	This study
ABC 625	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 ade2-101 trp1Δ63 cho1Δ::TRP1</i>	This study
ABC 626	MATα <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 cho1Δ::LEU2</i>	This study
ABC 706 (BRS1188)	MATα <i>ura3-52 ade2-101 trp1Δ63 cho1Δ::TRP1</i>	S. Henry
ABC 709 (BJ5418)	MATa <i>ura3-52 leu2Δ1 lys2-801 his3Δ200</i>	E. Jones
ABC 710 (BJ5458)	MATα <i>ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 trp1Δ63</i>	E. Jones

*L. Parks, North Carolina State University, Raleigh, USA; K. Kuchler, University and Biocenter of Vienna, Austria; S. Henry and E. Jones, Carnegie Mellon University, Pittsburgh, USA.

for making the disruption. PCR was done from a *LEU2*-based plasmid, pSP1, and the 2.2 kb amplified product was used to transform ABC 287 strain to leucine prototrophy. The putative disruptant (leucine prototroph) (ABC 626) was checked for disruption by phenotypic analysis (choline auxotrophy) and PCR.

To construct strain *cho1Δ::TRP1* in a background that was not protease-deficient, PCR was done on strain ABC 706 (*cho1Δ::TRP1*) with CHFOR (5' TCACATGGACCCATC-TAAAGA 3') and CHREV (5' TTTGACGCCAGGCAT-GAACAA 3') primers. The amplified product (2.6 kb) having *cho1Δ::TRP1* was used to transform strain ABC 154 to tryptophan prototrophy. The disruption at the right locus was confirmed by phenotypic characterization and PCR in strain ABC 625.

Construction of plasmid. The plasmid *PDR5/YEplac195* was constructed by digesting pSTSI (obtained from K. Kuchler, University and Biocenter of Vienna, Austria) with *SphI*/*Apal*, purifying the 7.5 kb fragment and cloning in the *SmaI* site of the multicopy plasmid *YEplac195* (Gietz & Sugino, 1988).

In vivo drug sensitivity assay. Pdr5p-mediated resistance to different compounds was tested by spotting serial dilutions of

yeast culture onto plates containing drugs. The transformants were pregrown in SD broth lacking uracil to late-exponential phase and then reinoculated into fresh medium to a cell concentration of 5×10^6 cells ml⁻¹. After incubation for 6–7 h at 30 °C, the optical density was measured at 600 nm and the number of cells per ml of culture was calculated. Serial dilutions containing 10^7 , 10^6 , 10^5 and 10^4 cells ml⁻¹ were then made in sterile water and 10 µl of each serially diluted culture was spotted onto YPD plates containing either the solvent or the drug, and onto SD lacking uracil (SD – ura) selection plates to check for plasmid stability.

MIC estimations. The MIC of a drug was defined as the minimum concentration at which no growth was observed when 10 µl of the second dilution (i.e. 10^6 cells ml⁻¹) of a culture was spotted onto the plate containing the drug.

Rhodamine 6G efflux assay in whole cells. The rhodamine efflux study was essentially carried out as described by Kolaczowski *et al.* (1996) with a few modifications. The strains were pregrown in YPD broth for 12–14 h and then reinoculated into fresh media to an initial OD₆₀₀ of 0.2. After incubation for 5–6 h at 30 °C, equal numbers of cells from an exponential-phase culture were harvested, washed four times with water and resuspended (wet wt = 40 mg) in 0.5 ml

HEPES buffer (50 mM HEPES-NaOH, pH 7.0). 2-Deoxy-D-glucose and rhodamine 6G were added to a final concentration of 5 mM and 10 μ M, respectively, and incubated at 25 °C for 2 h. After incubation, rhodamine-6G-loaded cells were quickly spun down, washed twice with HEPES buffer and finally resuspended in 4 ml HEPES buffer. The final suspension was aliquoted into two parts (2 ml each), 1 mM glucose was added to one aliquot to initiate the active, Pdr5p-mediated efflux of rhodamine 6G. The cell suspension was incubated at room temperature. At time zero, i.e. immediately after the addition of glucose, a 0.5 ml sample was withdrawn from both aliquots (with and without glucose), cells were quickly spun down and the fluorescence of rhodamine extruded in the assay buffer was measured at an excitation and emission wavelength of 529 nm and 553 nm, respectively (slit width = 5) on a Perkin-Elmer LS 50 B spectrofluorimeter. After 7 and 21 min further incubation, a 0.5 ml sample was again withdrawn, cells were removed by centrifugation and the fluorescence of rhodamine 6G extruded in the supernatant was measured.

Plasma membrane fluidity measurement. The fluidity of the yeast plasma membrane was measured in the whole cells using DPH and TMA-DPH as the fluorescent probes (Obrenovitch *et al.*, 1978; Kuhry *et al.*, 1985). The solutions of the probes were prepared in DMSO. Yeast cultures were grown overnight in YPD at 30 °C and then reinoculated into YPD at an OD₆₀₀ of approximately 0.2–0.3. After incubation for 5–6 h, cells were harvested, washed twice with sterile water and then suspended in buffer 1 (100 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.4) to a density of 8 mg wet weight ml⁻¹. The cells were incubated at 20 °C for 5 min and the DPH and TMA-DPH probes were added to a final concentration of 2 μ M or 5 μ M, respectively. The same volume of the solvent (DMSO) was added to the cells as a control. Incubation was continued for 20 min at 20 °C, after which the cells were immediately washed twice with buffer 1 and then suspended in the same volume of buffer 1. Fluorescence polarization was measured on a Perkin-Elmer LS 50 B spectrofluorimeter. The excitation and emission wavelengths were 360 nm and 450 nm, respectively. The measured fluorescence intensities were corrected for background fluorescence and light scattering from the unlabelled sample, i.e. the sample treated with the solvent alone. The degree of steady-state anisotropy (r_s) was calculated as described by Haggerty *et al.* (1978).

RESULTS

Effect of Pdr5p overexpression on drug resistance in *erg* strains

Strains with disruptions in the late steps of the ergosterol biosynthetic pathway are viable and accumulate sterols other than ergosterol in the membrane (Lees *et al.*, 1995). The predominant sterols that accumulate are the ones shown in Fig. 1. These strains have also been demonstrated to display an increased sensitivity to many drugs owing to an increased permeability of the membranes (Bard *et al.*, 1978). We decided to examine if the yeast MDR protein, Pdr5p, might be affected in the membranes with these different sterol contents. We constructed disruptions in the *ERG6*, *ERG2*, *ERG3* and *ERG4* genes as described in Methods. The strains were constructed in a vacuolar-protease-deficient (*pep4 prb1*) background to minimize differences in the turnover rates of Pdr5p; the turnover of Pdr5p has been shown to

Zymosterol
↓ *ERG6*
Fecosterol
↓ *ERG2*
Episterol
↓ *ERG3*
Ergosta-5,7,24(28)-trienol
↓ *ERG5*
Ergosta-5,7,22,24(28)-tetraenol
↓ *ERG4*
Ergosterol

Fig. 1. Schematic representation of the late stages of the ergosterol biosynthesis pathway from zymosterol to ergosterol. *ERG6*, *ERG2*, *ERG3*, *ERG5* and *ERG4* encode S-adenosyl methionine methyltransferase, C-8 sterol isomerase, sterol Δ^5 desaturase, C-22 sterol desaturase and sterol-24(28) reductase, respectively.

occur in the vacuole (Egner *et al.*, 1995; Egner & Kuchler, 1996).

Pdr5p was overexpressed in different *erg* strains by introducing the *PDR5* gene on a *URA3*-based multicopy plasmid, transforming the different *erg* strains with this plasmid, and then examining the resistance conferred to different drugs. We used drug resistance conferred by Pdr5p overexpression in these strains as a means of evaluating Pdr5p function. Overexpression of Pdr5p itself did not affect the growth of the strains relative to the vector only, nor were there any differences in plasmid stability in the different strains (data not shown).

We observed that all the *erg* strains showed an increased sensitivity to cycloheximide although to different extents (Fig. 2). Control experiments without drugs, or with only the solvents in which the drugs were dissolved, were carried out in these and subsequent experiments, to eliminate any differences that might have occurred in the absence of drugs (data not shown). As additional controls, the resistance conferred in wt and *pdr5* Δ strains was also simultaneously determined and these data are included in all the figures. The *erg6* Δ and *erg4* Δ strains were the most sensitive, and the sensitivity of the *pdr5* Δ strain was also comparable to that of the *erg6* Δ and *erg4* Δ strains. The sensitivities of the strains are a reflection of the intracellular accumulation of the drug under steady-state conditions. When we overexpressed Pdr5p in these strains, we found that overexpression of the Pdr5p in these different strains did not lead to drug resistance to the same levels (Fig. 2). Pdr5p overexpression led to significantly higher levels of drug resistance in the *erg4* Δ strain as compared to the *erg6* Δ strain, and even as compared to the *erg2* Δ strain, which otherwise displayed lesser sensitivity. In the *pdr5* Δ

Fig. 2. Pdr5p-mediated cycloheximide resistance in wt, *erg4Δ*, *erg3Δ*, *erg2Δ*, *erg6Δ* and *pdr5Δ* strains of *S. cerevisiae*. Strains transformed either with the control plasmid YEplac195 (V) or with the *PDR5*-overexpressing plasmid *PDR5/YEplac195* (P) were grown to exponential phase in SD-ura at 30 °C. An equal number of cells of each strain was harvested, resuspended in sterile water to a density of 1×10^7 cells ml⁻¹ and 10 µl of undiluted cell suspension, 1:10, 1:100 and 1:1000 dilutions was spotted onto YPD plates containing different concentrations of cycloheximide. Growth was scored after 3 d incubation at 30 °C. The growth pattern of the first two suspensions (10^7 and 10^6 cells ml⁻¹) is shown in the first and second rows, respectively.

strain, however, which otherwise has no altered sterols in the membrane, the level of resistance conferred was up to the wt levels. These results indicate firstly that alteration in the sterol content of the cells led to a decreased drug resistance conferred by Pdr5p as compared to the cells having ergosterol in their membranes, and secondly that the decreased resistance was not a simple consequence of increased permeability and sensitivity of the strains to the drug.

To examine whether our observation with cycloheximide was a general phenomenon, we extended this study to other structurally unrelated drugs, crystal violet, emetine and oestradiol. The conclusions we reached with crystal violet (data not shown), were very similar to those with cycloheximide. In the case of emetine, however, Pdr5p virtually failed to confer any significant resistance in *erg6Δ* and *erg2Δ* strain backgrounds (Fig. 3), although it could mediate efflux of the drug in the other strain backgrounds. The results with oestradiol were interesting in that we were able to observe oestradiol toxicity for the first time in yeast using the *erg6Δ* strain. However, we observed that oestradiol toxicity could be effectively reversed even in an *erg6Δ* background by Pdr5p overexpression (Fig. 4), indicating that Pdr5p was functioning in this background to efflux some, but not all of the drugs.

Rhodamine 6G efflux in ergosterol-deficient strains of *S. cerevisiae*

Since the decreased drug resistance conferred by Pdr5p was indirect evidence of Pdr5p efficiency under steady-state conditions, we decided to measure Pdr5p efficiency directly by monitoring the Pdr5p-mediated efflux of rhodamine 6G in *erg* backgrounds at different time intervals.

Rhodamine 6G is a known substrate of Pdr5p and is reported to be effluxed by Pdr5p in an energy-dependent manner (Kolaczowski *et al.*, 1996). To check the functioning of Pdr5p in altered sterol backgrounds, we carried out the rhodamine efflux assay in *erg* strains along with the wt and *pdr5Δ* strains serving as the positive and negative controls. The energy-dependent Pdr5p-driven extrusion of rhodamine was initiated by the addition of 1 mM glucose and fluorescence of extruded dye in the assay buffer was measured after different time intervals. The *erg4Δ*, *erg2Δ* and *erg6Δ* strains were found to efflux rhodamine 6G at a slower rate than the wt cells while *erg3Δ* cells showed a relatively higher rate of efflux as compared to other *erg* strains (Table 2). Rhodamine efflux was seen in the wt, *erg4Δ* and *erg3Δ* strains 7 min after glucose addition; however, very little efflux was seen in *erg2Δ* and *erg6Δ* strains after 7 min. We also examined the efflux after

Fig. 3. Pdr5p-mediated emetine resistance in wt, *erg4Δ*, *erg3Δ*, *erg2Δ*, *erg6Δ* and *pdr5Δ* strains of *S. cerevisiae*. The experiment was essentially done as described in Methods and in the legend to Fig. 2. V, control plasmid; P, *PDR5*-overexpressing plasmid.

21 min. At this time point, both *erg2Δ* and *erg6Δ* cells were found to be able to efflux rhodamine in the presence of glucose. Very little release of rhodamine was seen at 21 min in the *erg2Δ* and *erg6Δ* mutants in the absence of glucose indicating that efflux was not due to mere leakage of the dye across the membranes. The cells lacking Pdr5p (*pdr5Δ* strain) showed very low levels of active efflux of the dye even 21 min after glucose addition (Table 2). This experiment demonstrated that although Pdr5p was functional in these strains, the altered sterol environment had a significant effect on its efflux capabilities.

Does Pdr5p function in *erg* strains correlate with membrane fluidity?

Alteration in sterol content of the membrane is known to primarily affect its fluidity. To determine whether the differences in Pdr5p efficiency that we observed in different strains was correlated with membrane fluidity, we measured the membrane fluidity of the strains. This was done with whole cells using both DPH and TMA-DPH as probes. The fluorescence anisotropies of these probes in membranes correlate inversely with membrane fluidity. Although we did observe an increase in membrane fluidity in *erg* mutants relative to the wt, we could not find any correlations between the membrane fluidity of these strains and the efficiency of the Pdr5p pump (Table 3).

Fig. 4. Pdr5p-mediated β -oestradiol resistance in wt, *erg4Δ*, *erg3Δ*, *erg2Δ*, *erg6Δ* and *pdr5Δ* strains of *S. cerevisiae*. The experiment was essentially done as described in Methods and in the legend to Fig. 2. V, control plasmid; P, *PDR5*-overexpressing plasmid.

Pdr5p pump efficiency in a phosphatidylserine-deficient mutant

As alteration of the sterol composition led to a significant reduction in the ability of Pdr5p to confer drug resistance, we wished to determine if alteration in the phospholipid composition of the membrane might also affect Pdr5p efficiency. We therefore constructed a *cho1Δ* strain in the same background. These cells are choline auxotrophs but are otherwise viable. *CHO1* encodes phosphatidylserine synthase and its disruption results in the membrane being completely devoid of phosphatidylserine (Hikiji *et al.*, 1988). The lack of phosphatidylserine is largely compensated for by an increase in the phosphatidylinositol and phosphatidylcholine levels (Atkinson *et al.*, 1980).

When we overexpressed Pdr5p from a multicopy plasmid in such a *cho1Δ* strain in a similar manner to that done for *erg* mutants, we observed that the plasmid bearing *PDR5* was highly unstable, making a comparison of the drug resistance profiles of Pdr5p difficult to study (data not shown). We therefore carried out the

Table 2. Pdr5p-mediated energy-dependent efflux of rhodamine 6G in wt, *erg4Δ*, *erg3Δ*, *erg2Δ*, *erg6Δ* and *pdr5Δ* strains of *S. cerevisiae*

Cells were harvested, prepared and loaded with rhodamine 6G as described in Methods. Rhodamine 6G efflux was initiated by adding 1 mM glucose. The experiment was repeated twice and the values given are of a representative experiment.

Strains	Rhodamine 6G fluorescence intensity at (min):		
	0	7	21
wt – glucose	112	143	170
wt + glucose	123	473	795
<i>erg4Δ</i> – glucose	114	133	143
<i>erg4Δ</i> + glucose	116	245	226
<i>erg3Δ</i> – glucose	119	133	147
<i>erg3Δ</i> + glucose	134	358	608
<i>erg2Δ</i> – glucose	121	151	172
<i>erg2Δ</i> + glucose	107	158	352
<i>erg6Δ</i> – glucose	120	142	154
<i>erg6Δ</i> + glucose	120	170	290
<i>pdr5Δ</i> – glucose	120	152	174
<i>pdr5Δ</i> + glucose	114	147	200

Table 3. Fluorescence anisotropies of DPH and TMA-DPH in wt, *erg4Δ*, *erg3Δ*, *erg2Δ* and *erg6Δ* strains of *S. cerevisiae*

Measurements were carried out on whole cells and fluorescence anisotropy values were calculated as described in Methods. The values represent the mean of three experiments $\pm$ SD (each experiment was done in triplicate).

Strain	DPH	TMA-DPH
wt	0.388 $\pm$ 0.056	0.561 $\pm$ 0.055
<i>erg4Δ</i>	0.346 $\pm$ 0.032	0.559 $\pm$ 0.042
<i>erg3Δ</i>	0.291 $\pm$ 0.009	0.490 $\pm$ 0.020
<i>erg2Δ</i>	0.300 $\pm$ 0.012	0.505 $\pm$ 0.030
<i>erg6Δ</i>	0.344 $\pm$ 0.020	0.513 $\pm$ 0.024

analysis in a strain that is not deficient in the vacuolar proteases *PEP4* and *PRB1*. In this background, the plasmid was stable, which allowed the comparison of Pdr5p overexpression in this strain (Fig. 5).

The *cho1Δ* strain was mildly resistant to cycloheximide as compared to the wt strain, as seen in Fig. 5. Pdr5p overexpression in the *cho1Δ* strain was, though, able to confer resistance to cycloheximide to a significant level; however, it did not reach the wt levels of resistance.

In the case of crystal violet, the *cho1Δ* strain was significantly more resistant as compared to the wt cells,

Fig. 5. Stability of *PDR5*-overexpressing plasmid in wt (ABC154) and *cho1Δ* (ABC625) strains of *S. cerevisiae*. wt and *cho1Δ* strains were transformed with either the control plasmid YEplac195 (V) or the *PDR5*-overexpressing plasmid *PDR5*/YEplac195 (P). The wt transformants and the *cho1Δ* transformants were grown to exponential phase in SD–ura broth and SD–ura broth supplemented with 1mM choline, respectively. Cells were spotted onto plates of YPD, SD–ura containing 1mM choline, and YPD containing different concentrations of cycloheximide as described in the legend to Fig. 2. The growth pattern of the first two suspensions (10^7 and 10^6 cells ml^{-1}) is shown in the first and second rows, respectively.

and the level of resistance conferred by Pdr5p was also significantly higher than that in the wt strain (Fig. 6). This drug selectivity indicates that lipid composition also plays a role in modulating the level of drug resistance conferred by the Pdr5p pump, and in a drug-specific manner.

DISCUSSION

In this report, we have investigated whether the pleiotropic drug sensitivity of *erg* mutants might in part result from an altered functioning of the yeast MDR pump, Pdr5p. These *erg* strains are used extensively in different drug-based studies (Graham *et al.*, 1993; Hemenway & Heitman, 1996) and the tacit assumption has been that these strains are hyperpermeable to different drugs. However, the studies described in this report indicate that this assumption is not entirely valid and one also has to take into account the fact that the efflux pumps have a reduced efficiency in these backgrounds. We have not examined the localization of Pdr5p in these strains and it is possible that the reduced efficiency of Pdr5p in

Fig. 6. Pdr5p-mediated crystal violet resistance in wt and *cho1Δ* (ABC 625) strains of *S. cerevisiae*. The experiment was essentially done as described in Methods and in the legend to Fig. 5. V, control plasmid; P, *PDR5*-overexpressing plasmid.

altered sterol backgrounds could be due to partial mislocalization of the protein. However, complete mislocalization had not occurred since the pumps could mediate efflux of some of the drugs, although to lesser extents. Some other important conclusions may be drawn from this study. Firstly, the drug resistance profiles were affected in a manner that was dependent on both the strain background and the drug being tested. Thus the *erg6Δ* strain could efflux some, but not all, of the drugs. This suggests that although the lipid bilayer is not the primary determinant for substrate specificity, it plays an important role in modulating this specificity in addition to modulating efficiency. Furthermore, previous studies have indicated that the ability of the MDR pump to mediate efflux of a drug depends on the permeability of the membrane to the drug (Eytan *et al.*, 1996, 1997), but we observed that the reduced ability of Pdr5p to efflux drugs in different strains is not dependent solely on the permeability of the membrane towards the drugs.

Our results also highlight the importance of evaluating the function and substrate specificity of MDR pumps over a range of drug concentrations to attain more complete resistance profiles.

The decreased efflux of drugs in the *erg6Δ* strain leading to the drug hypersensitivity phenotype is in contrast to the cation-sensitivity phenotype of this strain, where the Li^+ and Na^+ hypersensitivity was primarily due to

increased uptake rather than the decreased efflux (Welihinda *et al.*, 1994). *erg6Δ* cells also exhibit defective tryptophan uptake, presumably due to a defect in tryptophan permease (Gaber *et al.*, 1989). In addition to this, the transport kinetics of a few other permeases of yeast is also known to be affected by the lipid composition of the membrane (Keenan *et al.*, 1982; Calderbank *et al.*, 1985).

Recently, Egner *et al.* (1998) have initiated detailed studies of mutants of Pdr5p with altered function by random mutagenesis. As mutants with altered substrate specificity were observed to occur in no single domains, and a few of the mutants were also found to have defects in folding, these authors have suggested a possible role for the folded structure of Pdr5p as a major determinant of its diverse substrate specificity. Lipids have been shown to function as molecular chaperones (Bogdanov *et al.*, 1996) and it is possible that in strains with altered lipid/ergosterol content, some defect in folding of membrane proteins may occur and that this might be partly responsible for both their altered efficiency and their altered substrate specificity.

The observation that Pdr5p overexpression in a phosphatidylserine-deficient mutant in a protease-deficient background led to significant differences in plasmid stability suggests that overexpression of this protein is, for some reason, deleterious to cells in this background. Mammalian P-gps have also been shown to destabilize membranes (Arsenault *et al.*, 1988) and it may be interesting to examine whether the destabilization is affected by the composition of the membrane itself.

The present study, for the first time, reports that the reduced efficiency of the MDR pump, Pdr5p, in *S. cerevisiae erg* strains is responsible to a significant extent for their pleiotropic drug-sensitive phenotypes. This observation is of great significance in view of the fact that the majority of antifungal compounds that are being extensively used to treat fungal infections inhibit the enzymes of the ergosterol biosynthetic pathway. Thus it will be very interesting to study whether the reduced efficiency of the MDR pumps of these yeasts might allow use of other drugs in combating the fungal infection. The present study, if extended to pathogenic yeast-like *Candida* spp., may shed some light on the complex drug resistance phenomenon of this yeast, and will stimulate further dissection of the role of the membrane in the functioning of MDR pumps.

ACKNOWLEDGEMENTS

We are indebted to Dr K. Kuchler for providing us with the *PDR5* plasmid and disruptants. We also thank Drs C. Marcireau, L. Parks, J. Heinisch, H. Pelham, S. Henry and E. Jones for their generous gifts of strains and plasmids. We would like to thank Mr G. Subba Rao, Mr Bharat L. Dixit and other members of Dr C. M. Gupta's laboratory for helping us in conducting rhodamine efflux studies and the membrane fluidity studies. R. Kaur is a Senior Research Fellow of the Council of Scientific and Industrial Research, India. This

work was supported in part by a Grant-in-aid project (BT/R&D/15/40/93) from the Department of Biotechnology, Government of India.

REFERENCES

- Arsenault, A. L., Ling, V. & Kartner, N. (1988). Altered plasma membrane ultrastructure in multidrug-resistant cells. *Biochim Biophys Acta* **938**, 315–321.
- Atkinson, K. D., Jensen, B., Kolat, A. I., Storm, E. M., Henry, S. A. & Fogel, S. (1980). Yeast mutants auxotrophic for choline or ethanolamine. *J Bacteriol* **141**, 558–564.
- Balzi, E., Wang, M., Leterme, S., Van Dyck, L. & Goffeau, A. (1994). PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. *J Biol Chem* **269**, 2206–2214.
- Bard, M., Lees, N. D., Burrows, L. S. & Kleinhans, F. W. (1978). Differences in crystal violet uptake and cation-induced death among yeast sterol mutants. *J Bacteriol* **135**, 1146–1148.
- Baudin, A., Kalogeropoulos, O. O., Denouei, A., Lacroute, F. & Cullin, C. (1993). A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **21**, 3329–3330.
- Beaudet, L. & Gros, P. (1995). Functional¹ dissection of P-glycoprotein nucleotide-binding domains in chimeric and mutant proteins. *J Biol Chem* **270**, 17159–17170.
- Bissinger, P. H. & Kuchler, K. (1994). Molecular cloning and expression of the *Saccharomyces cerevisiae* STS1 gene product. *J Biol Chem* **269**, 4180–4186.
- Bogdanov, M., Sun, J., Kaback, H. R. & Dowhan, W. (1996). A phospholipid acts as a chaperone in assembly of a membrane transport protein. *J Biol Chem* **271**, 11615–11618.
- Borst, P. & Schinkel, A. H. (1997). Genetic dissection of the function of mammalian P-glycoproteins. *Trends Genet* **13**, 217–222.
- Calderbank, J., Keenan, M. H. J. & Rose, A. H. (1985). Plasma-membrane phospholipid unsaturation affects expression of the general amino-acid permease in *Saccharomyces cerevisiae* Y185. *J Gen Microbiol* **131**, 57–65.
- Doige, C. A., Yu, X. & Sharom, F. J. (1993). The effects of lipids and detergents on ATPase-active P-glycoprotein. *Biochim Biophys Acta* **1146**, 65–72.
- Egner, R. & Kuchler, K. (1996). The yeast multidrug transporter Pdr5 of the plasma membrane is ubiquitinated prior to endocytosis and degradation in the vacuole. *FEBS Lett* **378**, 177–181.
- Egner, R., Mahe, Y., Pandjaitan, R. & Kuchler, K. (1995). Endocytosis and vacuolar degradation of plasma membrane-localized Pdr5-ATP binding cassette multidrug transporter in *Saccharomyces cerevisiae*. *Mol Cell Biol* **15**, 5879–5887.
- Egner, R., Rosenthal, F. E., Kralli, A., Sanglard, D. & Kuchler, K. (1998). Genetic separation of FK 506 susceptibility and drug transport in the yeast Pdr5 ATP-binding cassette multidrug resistance transporter. *Mol Biol Cell* **9**, 523–543.
- Eytan, G. D., Regev, R., Oren, G. & Assaraf, Y. G. (1996). The role of passive transbilayer drug movement in multidrug resistance and its modulation. *J Biol Chem* **271**, 12897–12902.
- Eytan, G. D., Regev, R., Oren, G., Hurwitz, C. D. & Assaraf, Y. G. (1997). Efficiency of P-glycoprotein-mediated exclusion of rhodamine dyes from multidrug-resistant cells is determined by their passive transmembrane movement rate. *Eur J Biochem* **248**, 104–112.
- Gaber, R. F., Copple, D. M., Kennedy, B. K., Vidal, M. & Bard, M. (1989). The yeast gene *ERG6* is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. *Mol Cell Biol* **9**, 3447–3456.
- Gietz, R. D. & Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527–534.
- Gottesman, M. M. & Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62**, 385–427.
- Gottesman, M. M., Pastan, I. & Ambudkar, A. (1996). P-glycoprotein and multidrug resistance. *Curr Opin Genet Dev* **6**, 610–617.
- Graham, T. R., Scott, P. A. & Emr, S. D. (1993). Brefeldin A reversibly blocks early but not late protein transport steps in the yeast secretory pathway. *EMBO J* **12**, 869–877.
- Haggerty, D. F., Kalra, V. K., Popjak, G., Reynolds, E. E. & Chiappelli, F. (1978). Fluorescence-polarization measurements on normal and mutant human skin fibroblasts. *Arch Biochem Biophys* **189**, 51–62.
- Hanna, M., Brault, M., Kwan, T., Kast, C. & Gros, P. (1996). Mutagenesis of transmembrane domain 11 of P-glycoprotein by alanine scanning. *Biochemistry* **35**, 3625–3635.
- Hemenway, C. S. & Heitman, J. (1996). Immunosuppressant target protein FKBP12 is required for P-glycoprotein function in yeast. *J Biol Chem* **271**, 18527–18534.
- Hikiji, T., Miura, K., Kiyono, K., Shibuya, I. & Ohta, A. (1988). Disruption of the *CHO1* gene encoding phosphatidylserine synthase in *Saccharomyces cerevisiae*. *J Biochem* **104**, 894–900.
- Hirata, D., Yano, K., Miyahara, K. & Miyakawa, T. (1994). *Saccharomyces cerevisiae* YDR1, which encodes a member of the ATP-binding cassette (ABC) superfamily, is required for multidrug resistance. *Curr Genet* **26**, 285–294.
- Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**, 163–168.
- Keenan, M. H. J., Rose, A. H. & Silverman, B. W. (1982). Effect of plasma-membrane phospholipid unsaturation on solute transport into *Saccharomyces cerevisiae* NCYC 366. *J Gen Microbiol* **128**, 2547–2556.
- Kolaczowski, M., Rest, M. V., Kolaczowska, A. C., Soumillion, J. P., Konings, W. N. & Goffeau, A. (1996). Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p. *J Biol Chem* **271**, 31543–31548.
- Kralli, A., Bohen, S. P. & Yamamoto, K. R. (1995). LEM1, an ATP-binding-cassette transporter selectively modulates the biological potency of steroid hormones. *Proc Natl Acad Sci USA* **92**, 4701–4705.
- Kuchler, K. & Thorner, J. (1992). Functional expression of human MDR1 in the yeast *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **89**, 2302–2306.
- Kuhry, J. G., Duportail, G., Bronner, C. & Lautriat, G. (1985). Plasma membrane fluidity measurements on whole living cells by fluorescence anisotropy of trimethylammonium diphenylhexatriene. *Biochim Biophys Acta* **845**, 60–67.
- Kwan, T. & Gros, P. (1998). Mutational analysis of the P-glycoprotein first intracellular loop and flanking transmembrane domains. *Biochemistry* **37**, 3337–3350.
- Lees, N. D., Skaggs, B., Kirsch, D. R. & Bard, M. (1995). Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae* – a review. *Lipids* **30**, 221–226.
- Loo, T. W. & Clarke, D. M. (1993). Functional consequences of

proline mutations in the predicted transmembrane domain of P-glycoprotein. *J Biol Chem* **268**, 3143–3149.

Mahe, Y., Lemoine, Y. & Kuchler, K. (1996). The ATP binding cassette transporters Pdr5 and Snq2 of *Saccharomyces cerevisiae* can mediate transport of steroids *in vivo*. *J Biol Chem* **271**, 25167–25172.

Obrenovitch, A., Sene, C., Negre, M. T. & Monsigny, M. (1978). Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene embedded in membranes of mouse leukemic L 1210 cells during the cell cycle. *FEBS Lett* **88**, 187–191.

Rose, M. D., Winston, F. & Hieter, P. (1990). *Methods in Yeast Genetics: a Laboratory Course Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Saeki, T., Shimabuku, A. M., Azuma, Y., Shibano, Y., Komano, T. & Ueda, K. (1991). Expression of human P-glycoprotein in yeast cells – effects of membrane component sterols on the activity of P-glycoprotein. *Agric Biol Chem* **55**, 1859–1865.

Saeki, T., Shimabuku, A. M., Ueda, K. & Komano, T. (1992).

Specific drug binding by purified lipid-reconstituted P-glycoprotein: dependence on the lipid composition. *Biochim Biophys Acta* **1107**, 105–110.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sharom, F. J. (1997). The P-glycoprotein efflux pump: how does it transport drugs? *J Membr Biol* **160**, 161–175.

Urbatsch, I. L. & Senior, A. E. (1995). Effects of lipids on ATPase activity of purified chinese hamster P glycoprotein. *Arch Biochem Biophys* **316**, 135–140.

Welihinda, A. A., Beavis, A. D. & Trumbly, R. J. (1994). Mutations in *LIS1 (ERG6)* gene confer increased sodium and lithium uptake in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1193**, 107–117.

Received 22 September 1998; revised 27 November 1998; accepted 18 December 1998.