

Drug Susceptibilities of Yeast Cells Are Affected by Membrane Lipid Composition

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In the present study we have exploited isogenic *erg* mutants of *Saccharomyces cerevisiae* to examine the contribution of an altered lipid environment on drug susceptibilities of yeast cells. It is observed that *erg* mutants, which possess high levels of membrane fluidity, were hypersensitive to the drugs tested, i.e., cycloheximide (CYH), *o*-phenanthroline, sulfomethuron methyl, 4-nitroquinoline oxide, and methotrexate. Most of the *erg* mutants except mutant *erg4* were, however, resistant to fluconazole (FLC). By using the fluorophore rhodamine-6G and radiolabeled FLC to monitor the passive diffusion, it was observed that *erg* mutant cells elicited enhanced diffusion. The addition of a membrane fluidizer, benzyl alcohol (BA), to *S. cerevisiae* wild-type cells led to enhanced membrane fluidity. However, a 10 to 12% increase in BA-induced membrane fluidity did not alter the drug susceptibilities of the *S. cerevisiae* wild-type cells. The enhanced diffusion observed in *erg* mutants did not seem to be solely responsible for the observed hypersensitivity of *erg* mutants. In order to ascertain the functioning of drug extrusion pumps encoding the genes *CDR1* (ATP-binding cassette family) and *CaMDR1* (MFS family) of *Candida albicans* in a different lipid environment, they were independently expressed in an *S. cerevisiae erg* mutant background. While the fold change in drug resistance mediated by *CaMDR1* remained the same or increased in *erg* mutants, susceptibility to FLC and CYH mediated by *CDR1* was increased (decrease in fold resistance). Our results demonstrate that between the two drug extrusion pumps, Cdr1p appeared to be more adversely affected by the fluctuations in the membrane lipid environment (particularly to ergosterol). By using 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino-hexanoyl] sphingosyl phosphocholine (a fluorescent analogue of sphingomyelin), a close interaction between membrane ergosterol and sphingomyelin which appears to be disrupted in *erg* mutants is demonstrated. Taken together it appears that multidrug resistance in yeast is closely linked to the status of membrane lipids, wherein the overall drug susceptibility phenotype of a cell appears to be an interplay among drug diffusion, extrusion pumps, and the membrane lipid environment.

The incidence of the acquisition of resistance to azoles by *Candida albicans* cells has increased considerably in recent years, which has posed serious problems in successful chemotherapy of infections caused by such cells. Although the molecular basis of azole resistance in *C. albicans* is not very clear, accumulated evidence suggests that multidrug resistance (MDR) is a multifactorial phenomenon, with some of the most common mechanisms being the failure of drug accumulation mediated by drug extrusion pumps such as *CDR1*, *CDR2* (ATP-binding cassette [ABC] family), and *CaMDR1* (MFS family), alterations in Erg11p, and upregulation of *ERG11*. A combination of different resistance mechanisms has been reported to be responsible for fluconazole (FLC) resistance in clinical isolates of *C. albicans* (31, 41, 42, 44). Recent evidence also suggests that the generation of a resistant strain from a highly susceptible strain is the result of multiple mechanisms, each of which probably contributes partially to the resistant phenotype (23, 52). Of note, other mechanisms like the role of $\Delta^{5,6}$ -desaturase (*ERG3*), Δ^{22} -desaturase (*ERG5*), chromosomal alterations, modification of drugs, and membrane lipid

composition which have been found to affect azole susceptibilities also merit consideration (21, 22, 27, 35, 37, 39).

On the basis of the results of several studies, a close interaction between membrane lipids and drug extrusion pump proteins has been realized (6, 7, 11, 45). On the one hand, it has been observed that the MDR ABC protein of mammalian cells (P glycoprotein [P-gp]) and MDR proteins of yeasts (Pdr5p and Yor1p in *Saccharomyces cerevisiae* and Cdr1p and Cdr2p in *C. albicans*) can translocate phospholipids between the two monolayers of the plasma membrane, while on the other hand P-gp has been shown to participate in sterol homeostasis in mammalian cells (5, 34). Additionally, these drug extrusion pumps are found to be particularly sensitive to the nature and the physical state of the surrounding lipids (11, 19, 47).

That lipid could also play an important role in azole susceptibilities is becoming apparent from a host of recent studies. It has been shown that some of the azole-resistant *C. albicans* isolates exhibit altered membrane phospholipid and sterol compositions (15, 23, 30). Such lipid changes are observed both in clinical and in in vitro-adapted azole-resistant isolates of *C. albicans* (16, 17, 23, 30). We as well as others have observed that Cdr1p and Pdr5p are sensitive to fluctuations in the lipid environment, where functions mediated by these drug extrusion pumps are selectively affected (19, 47). Taken together, it appears that the associated changes in membrane lipid com-

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position (phospholipid and ergosterol), its order (fluidity), and asymmetry could be important determinants in the drug susceptibilities of yeast cells.

In order to study the contribution of membrane lipids in drug susceptibilities, in the present study we have exploited isogenic *erg* mutants of *S. cerevisiae* that, owing to blocks in different steps of ergosterol biosynthesis, possess increased levels of fluid membranes. We have also expressed two drug extrusion pumps, i.e., Cdr1p and CaMdr1p, in *erg* mutants to compare their functioning in an altered lipid environment (13, 43). We observed that *erg* mutants which possess higher levels of membrane fluidity were hypersensitive to the drugs tested. *erg* mutant cells elicit enhanced diffusion of drugs; however, that does not seem to solely affect drug susceptibilities. Furthermore, our results suggest that between the two pump proteins, Cdr1p, when it is expressed in *erg* mutants of *S. cerevisiae*, interacts more intimately with membrane lipids, particularly with ergosterol and sphingolipid, and thus is more severely affected by lipid perturbations.

MATERIALS AND METHODS

Materials. Medium chemicals were obtained from Difco (Detroit, Mich.) and HiMedia (Mumbai, India). Labeled 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino-hexanoyl] sphingosyl phosphocholine (NBD-sphingomyelin [NBD-SM]) was purchased from Molecular Probes (Eugene, Oreg.), lipid *N*-rhodamine-dioleoyl phosphatidylethanolamine (*N*-Rh-DOPE) was purchased from Avanti Polar Lipids Inc. (Albaster, Ala), and 1-dipalmitoyl-phosphatidylcholine (1-DPPC) was obtained from Sigma Chemical Co. (St. Louis, Mo.). The drugs cycloheximide (CYH), *o*-phenanthroline (PHE), 4-nitroquinoline oxide (4-NQO), and methotrexate (MTX) were from Sigma Chemical Co. FLC and sulfomethuron methyl (SMM) were provided as gifts by Pfizer (Sandwich, United Kingdom) and Dupont (Wilmington, Del.), respectively.

Strains used in this study. Wild-type (WT) *S. cerevisiae* strain ABC287 (*Mata* *ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4Δ::HIS3 prb1Δ1.6R can1*) and isogenic *erg* mutants designated *erg2* (strain ABC271; *Mata* *ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4Δ::HIS3 prb1Δ1.6R can1 erg2Δ::LEU2*), *erg3* (strain ABC261; *Mata* *ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4Δ::HIS3 prb1Δ1.6R can1 erg3Δ::LEU2*), *erg4* (strain ABC283; *Mata* *ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4Δ::HIS3 prb1Δ1.6R can1 erg4Δ::LEU2*), and *erg6* (strain ABC265; *Mata* *ura3-52 leu2Δ1 lys2-801 his3Δ200 pep4Δ::HIS3 prb1Δ1.6R can1 erg6Δ::LEU2*) were used in this study and were kind gifts from Anand Bachhawat (IMTech, Chandigarh, India) (19). All the *S. cerevisiae* strains were transformed with plasmid pNC39 (carrying *CaMDR1*) and plasmid pS12 (carrying *CDR1*), which were obtained from a *C. albicans* genomic library, as described earlier (13, 43). Of note, both genes had a common vector background of pYEUra3. All *erg* mutants and WT strains of *S. cerevisiae* were maintained at 30°C in yeast nutrient broth (YNB) medium containing 2% glucose and the respective auxotrophic supplements (47).

Ergosterol extraction and estimation. Sterols were extracted by the alcoholic KOH method as described by Arthington-Skaggs et al. (2, 3), in which cells grown overnight were harvested, washed in sterile water, and boiled for 1 h in 25% alcoholic KOH. The boiled cells were cooled and extracted with petroleum ether. In order to compare the efficiency of sterol extraction, yeast cells were also mechanically broken in an MSK homogenizer (Braun, Kronberg, Germany) prior to the sterol extraction. The homogenate was collected, and the sterols were extracted as described previously (2). An aliquot of sterol extract obtained by each protocol was diluted fivefold in petroleum ether and scanned spectrophotometrically between 200 and 320 nm (300 BIO UV-VIS spectrophotometer; Varian, Victoria, Australia). Both ergosterol and 24(28)-dehydroergosterol (DHE) absorb at 281.5 nm, whereas only DHE absorbs at 230 nm. The ergosterol content was calculated as a percentage of the wet weight of the cell by the following equations (2): percent ergosterol + percent DHE = $[(A_{281.5}/290) \times F]/\text{pellet weight}$, percent DHE = $[(A_{230}/518) \times F]/\text{pellet weight}$, and percent ergosterol = [percent ergosterol + percent DHE] - percent DHE, where *F* is the factor for dilution in petroleum ether, and 290 and 518 are the *E* values (in percent per centimeter) determined for crystalline ergosterol and DHE, respectively (2).

Supplementation of yeast cells and *erg* mutants with ergosterol. For ergosterol supplementation experiments, cells were grown overnight in the presence of 10

μg of ergosterol ml⁻¹, which had been dissolved in petroleum ether and added directly into the medium during inoculation (1). Sterols from supplemented cells were then extracted and analyzed by recording the wavelength scan between 200 and 320 nm as described above.

Fluorescence polarization studies. The steady-state fluorescence polarization measurements on yeast cells were carried out essentially as described earlier (19). Measurements were carried out on whole cells by using a fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). Fluorescence polarization was measured at excitation and emission wavelengths of 360 and 450 nm, respectively. The measured fluorescence intensities were corrected for background fluorescence and the light scattering from the unlabeled sample (19).

Drug uptake assay. Passive diffusion of rhodamine-6G (R6G) was determined essentially by a protocol described previously (23, 51). Approximately 10⁷ cells from an overnight culture were inoculated in 250 ml of YPD and grown for 5 to 6 h at 30°C. The cells were pelleted and washed three times with phosphate-buffered saline buffer without glucose (buffer A). The cells were subsequently resuspended as a 2% cell suspension in deenergization buffer (5 mM dinitrophenol and 5 mM 2-deoxy-D-glucose in buffer A) and incubated for 2 h at 30°C. The cells were then washed; resuspended in buffer A, to which R6G was added to a final concentration of 10 μM; and incubated. An aliquot of 1 ml was taken at various times and centrifuged at 9,000 × g for 2 min. The absorbance of the supernatant was measured at 527 nm (23). The passive diffusion of FLC was checked by using radiolabeled [³H]FLC (24, 47). The deenergized cells were incubated with 100 nM [³H]FLC (0.7 TBq mmol⁻¹); and aliquots were removed at various times, rapidly filtered, and washed three times with ice-cold buffer A containing an equimolar concentration of cold drug on a Millipore manifold filtration assembly with 0.45-μm-pore-size cellulose nitrate filter disks (Millipore, Bedford, Mass.). The radioactivity that accumulated in filtered cells and that adhered to filter disks was measured in a liquid scintillation counter with a scintillation liquid (Tri-Carb 2900TR; Packard). The radioactivity that adhered to the filter disk, which was not significant, was subtracted from the experimental values.

Drug susceptibility testing with *S. cerevisiae* strains. The susceptibilities of the yeast isolates (grown in YNB medium containing 2% glucose and the respective auxotrophic supplements) to FLC, CYH, PHE, SMM, 4-NQO, and MTX were determined by three different methods. The following stock solutions of the various drugs were made (the solvent used is given in parentheses): FLC, 1 mg ml⁻¹ (water); CYH, 0.1 mg ml⁻¹ (water); PHE, 2 mg ml⁻¹ (ethanol); 4-NQO, 0.01 mg ml⁻¹ (dimethyl sulfoxide); SMM, 1 mg ml⁻¹ (methanol); and MTX, 5 mg ml⁻¹ (10 mM Tris-Cl). The resistance to MTX was checked in the presence of 200 μg of sulfanilamide ml⁻¹ to deplete the intracellular dihydrofolate reductase pool, as previously described by Fling et al. (12). The solvents used to solubilize the different drugs were also tested, and there was no inhibition of growth due to the solvents used.

(i) **Filter disk assay.** Yeast cells (10⁵ cells ml⁻¹) were mixed with molten agar (approximately 40°C) and poured in a petri plate. After agar solidification, the filter disks were placed and the drugs, in a volume of 5 to 10 μl, were spotted onto the disks at the indicated amounts: FLC, 100 μg; CYH, 0.5 μg; PHE, 20 μg; 4-NQO, 2 μg; SMM, 5 μg; and MTX, 50 μg. The plates were incubated at 30°C, and zones of inhibition were scored after 2 to 3 days (29, 38, 47).

(ii) **Microtiter assay.** 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 (OD₆₀₀) of 0.1. The cells were then diluted 100-fold in YNB medium containing 2% glucose and the respective auxotrophic supplements. The diluted cell suspensions were added to the wells of round-bottomed 96-well microtiter plates (100 μl/well) containing equal volumes of medium (100 μl/well) and different concentrations of drugs (23, 50). A drug-free control was also included. The plates were incubated at 30°C for 48 h. The MIC test end point was evaluated both by eye and by reading the OD₆₂₀ in a microplate reader and is defined as the lowest drug concentration that gave >80% inhibition of growth compared with the growth of the drug-free controls (the MIC at which 80% of isolates are inhibited [MIC₈₀]).

(iii) **Spot assay.** The yeast cells were grown overnight on YNB medium containing 2% glucose and the respective auxotrophic supplements. The cells were then suspended in normal saline to an OD₆₀₀ of 0.1 (*A*₆₀₀). Five microliters of fivefold serial dilutions of each yeast culture was spotted onto YNB plates in the absence (control) and presence of the following drugs: 4-NQO (0.02 μg ml⁻¹), CYH (0.01 μg ml⁻¹), PHE (2 μg ml⁻¹), SMM (1 μg ml⁻¹), FLC (8 μg ml⁻¹), and MTX (1 μg ml⁻¹). Growth differences were recorded following incubation of the plates for 48 h at 30°C. Growth was not affected by the presence of the solvents used for the drugs (8, 19, 53).

Labeling of cells with NBD-SM. (i) **Preparation of donor lipid vesicles.** Donor vesicles containing fluorescent phospholipids were prepared by mixing the de-

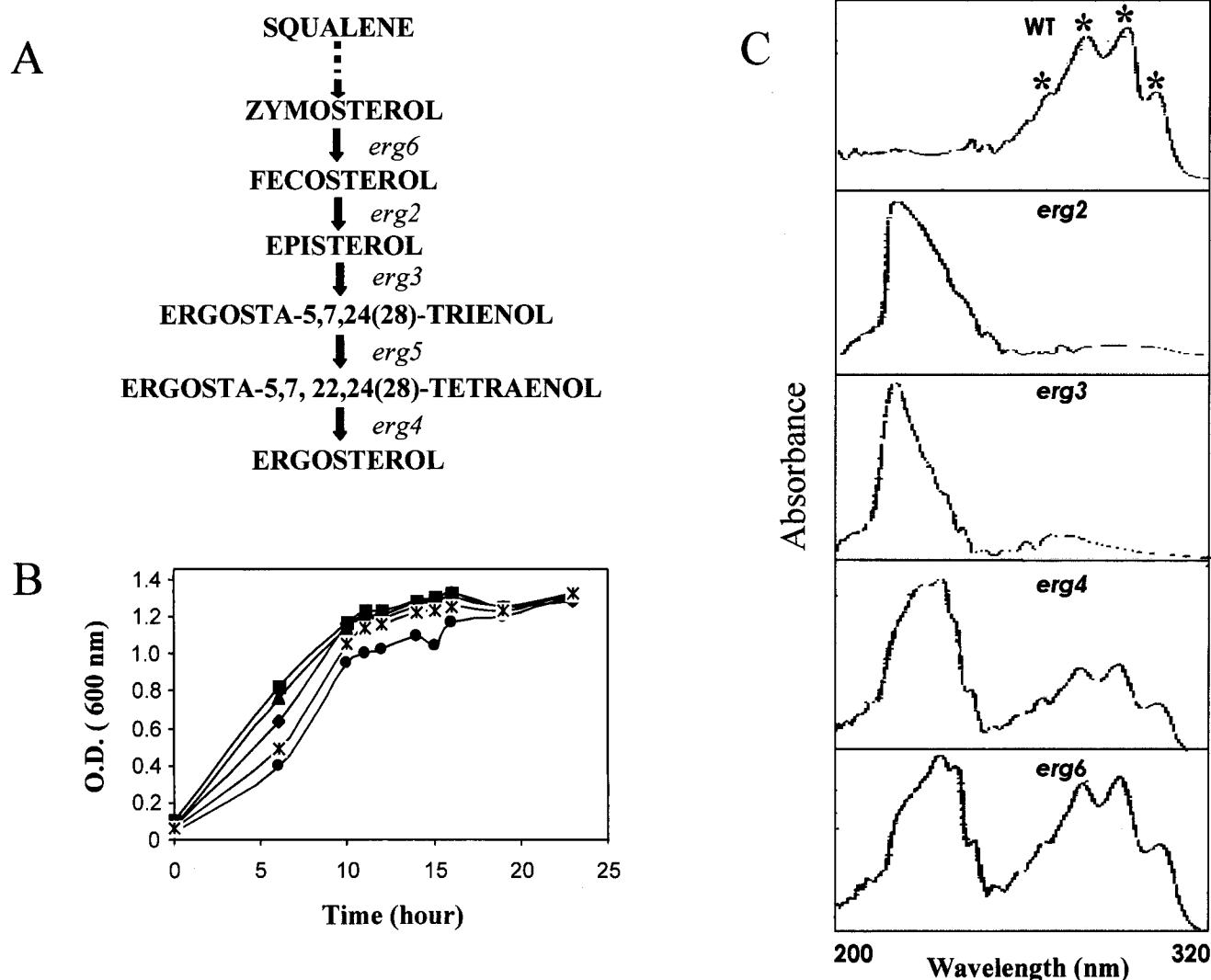


FIG. 1. (A) Schematic representation of the late stages of the ergosterol biosynthetic pathway. *erg6*, *erg2*, *erg3*, *erg5*, and *erg4* encode *S*-adenosyl methionine methyltransferase, C-8 sterol isomerase, sterol C-5 desaturase, C-22 sterol desaturase, and sterol-24(28) reductase, respectively. (B) Growth curves of WT (◆), *erg2* (■), *erg3* (▲), *erg4* (●), and *erg6* (*) strains. The specific growth rates are 1.76 h for WT, 2.01 h for *erg2*, 1.78 h for *erg3*, 1.52 h for *erg4*, and 1.71 h for *erg6*. (C) UV absorption spectra of sterols extracted from the *S. cerevisiae* WT strain and its *erg* mutants. Their absorption spectra were recorded as described in Materials and Methods.

sired proportions of the various phospholipids as described by us and others (20, 48). The typical proportion used was 40 mol% NBD-SM, 2 mol% *N*-Rh-DOPE, and 58 mol% L-DPPC. After all of these phospholipids were mixed, chloroform was evaporated under a stream of N_2 to make a thin film and kept overnight under vacuum desiccation to remove moisture and traces of solvent. Desiccated phospholipid film was then hydrated by adding 50 mM Tris buffer (pH 7.4; buffer A) with continuous vortexing. Hydrated membrane film was then sonicated for 30 min under an N_2 atmosphere with a water bath sonicator to produce donor vesicles.

(ii) **Labeling of *C. albicans* cells and *erg* mutants with NBD-SM.** Labeling of cells with fluorescent lipids was carried out essentially as described earlier (20, 48). Briefly, cells grown to the mid-log phase were harvested by centrifugation at 3,000 rpm for 5 min, washed two times with buffer B, and resuspended (5×10^9 cells ml^{-1}) in the same buffer. Donor vesicles (final concentration, 40 μM) were then added to 900 μl of the cell suspension, and every 5 min the fluorescence of the cell suspension was measured spectrofluorometrically (excitation wavelength, 475 nm; emission wavelength, 525 nm; eclipse spectrofluorometer; Varian) to check the labeling of the cells by NBD-SM. After 90 min, when labeling of the cells attained saturation, the labeled cells were washed twice with ice-cold buffer

B and resuspended in 900 μl of the same buffer (5×10^9 cells ml^{-1}). Then, fatty acid-free bovine serum albumin (BSA; final concentration, 2% [wt/vol]) was added to the cell suspension to allow the back exchange of NBD-SM from the outer monolayer of the plasma membrane to BSA. At different time intervals, aliquots of the cell suspension were taken out and centrifuged, and the fluorescence in both the supernatant and the pellet was measured. Fluorescence intensities were corrected for background fluorescence in both the supernatant and the pellet. The BSA-extracted NBD-SM fraction was calculated as follows: percent NBD-SM extracted = (fluorescence of supernatant/total fluorescence from supernatant and pellet) \times 100.

RESULTS

***S. cerevisiae erg* mutant strains lack ergosterol.** Mutants with mutations in the ergosterol biosynthetic pathway do not produce ergosterol and utilize sterol intermediates to compensate for the loss of ergosterol (19, 28, 47) (Fig. 1A). To confirm the

absence of any detectable levels of ergosterol in *erg* mutant strains, we compared the absorption spectra of sterols obtained from an *S. cerevisiae* WT strain and its isogenic *erg* mutants (1–3, 40). The method of Arthington-Skaggs et al. (2, 3) takes advantage of the unique four-peak spectral absorption pattern produced by extracted sterols between 240 and 320 nm; this pattern is indicative of the ergosterol and DHE (a late sterol pathway intermediate) contents. Both ergosterol and DHE absorb at 281.5 nm, whereas only DHE shows an intense spectral absorption band at 230 nm. Therefore, the amount of ergosterol can be determined by calculating the total ergosterol plus DHE content (by determination of the amount of absorption at 281.5 nm) and then subtracting that amount from the total amount of absorption (at 230 nm) due to DHE only (2, 3). As evident from Fig. 1C, WT cells gave four characteristic peaks for ergosterol (no absorption peak at 230 nm) which were absent for both *erg2* and *erg3* mutants. Mutant *erg6* and *erg4* cells, however, gave four characteristic peaks as well as a strong absorption peak for DHE at 230 nm. Quantitation of ergosterol from the absorption spectra (see Materials and Methods) confirmed that the WT strain had an ergosterol content of $1.3\% \pm 0.07\%$ (expressed as a percentage of the wet weight of the cells), whereas none of four *erg* mutants contained ergosterol (data not shown).

***erg* mutants of *S. cerevisiae* are susceptible to drugs.** In order to ascertain the physical state of the membrane, we used steady-state fluorescence polarization to examine the membrane order (fluidity) of *erg2*, *erg3*, *erg4*, and *erg6* mutants and the WT strain. A specific plasma membrane fluorescent probe, DPH, was used for such measurements, as described earlier (1, 19). All the *erg* mutant strains showed decreases in fluorescence polarization, thus implying decreased membrane order or enhanced fluidity of the membranes of these mutants compared to that for the WT strain (Fig. 2A). However, we did not observe any significant change in fluidity of the membrane of the *erg4* strain, which is probably because it mediates the final step of ergosterol biosynthetic pathway in yeast. In the plasma membrane of the *erg4* mutant, ergosterol is completely replaced with ergosta-5,7,22,24(28)-tetraen-3 β -ol, which is structurally highly related to ergosterol and thus is able to completely compensate for it in the membrane (53).

Whether the enhanced membrane fluidity of *erg* mutants in any way affects the drug susceptibilities of the mutant cells was checked in the following experiments. Since there is no recommended protocol for testing of the drug susceptibilities of *S. cerevisiae* cells, we simultaneously used three independent methods (filter disk, spot, and microtiter plate assays) to examine the drug susceptibilities of the *erg* mutants (8, 36, 38). As evident from the results of the filter disk assay (Fig. 3), all *erg* mutants were highly sensitive to most of the drugs tested, which was evident from the sizes of the zones of inhibition. The drug sensitivity was further confirmed by the microtiter assay, in which MIC₈₀s of the drugs for the cells of the *erg* mutant strains were lower than those for the WT cells (Fig. 4B). The drug sensitivities tested by spot assays also revealed that the mutant strains were generally sensitive to the drugs tested (Fig. 4A). Thus, the results from three independent drug susceptibility tests led us to confirm the results of the resistance profiling of the *erg* mutants. Notably, all *erg* mutant strains except strain *erg4* were highly resistant to FLC; strain *erg4* was even

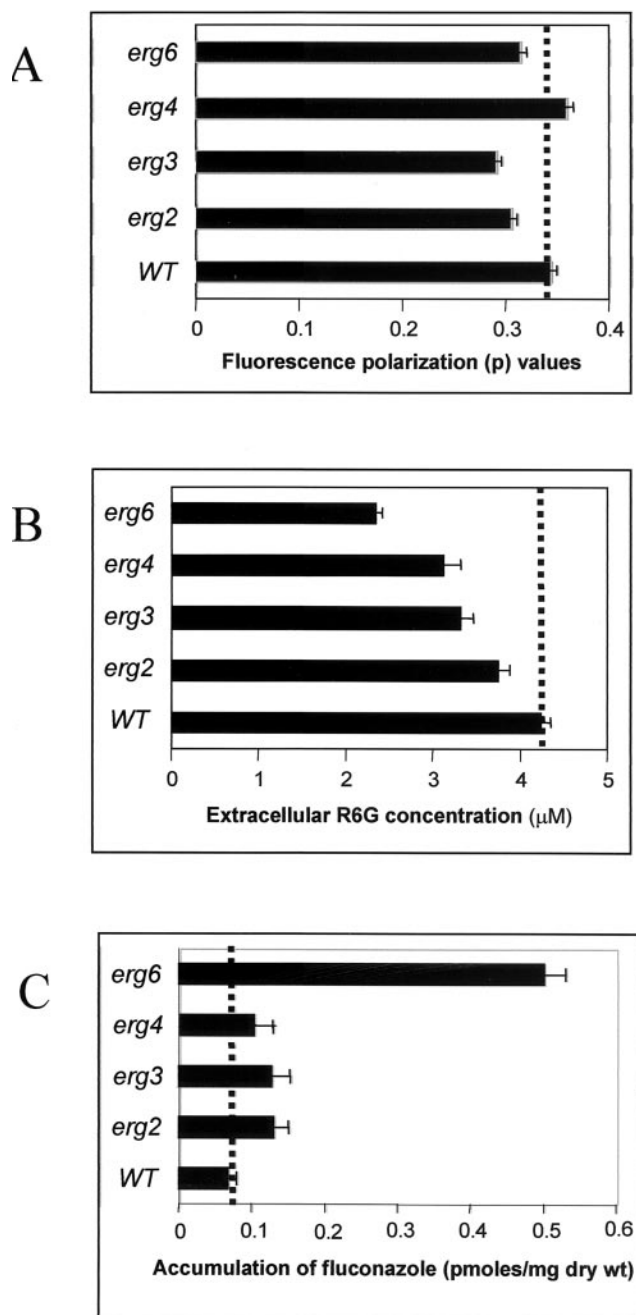


FIG. 2. (A) Steady-state fluorescence polarization measurements (p value) in an *S. cerevisiae* WT strain and its *erg* mutants. Measurements were carried out with intact cells by using DPH as the fluorescent probe at excitation and emission wavelengths of 360 and 450 nm, respectively, as described in Materials and Methods. The values are means \pm standard deviations (indicated by the bars) of three independent experiments. (B) Extracellular R6G concentration measured in *S. cerevisiae* WT and its *erg* mutants at 60-min interval. Deenergized yeast cells were incubated with R6G for 1 h, after which the cells were harvested and the extracellular concentration of R6G in the supernatant was determined spectrophotometrically by measuring the absorbance at 527 nm. The values are means \pm standard deviations (indicated by the bars) of three independent experiments. (C) Accumulation of [³H]FLC in deenergized *S. cerevisiae* WT and its *erg* mutants at 60-min intervals. The values are means \pm standard deviations (indicated by the bars) of three independent experiments.

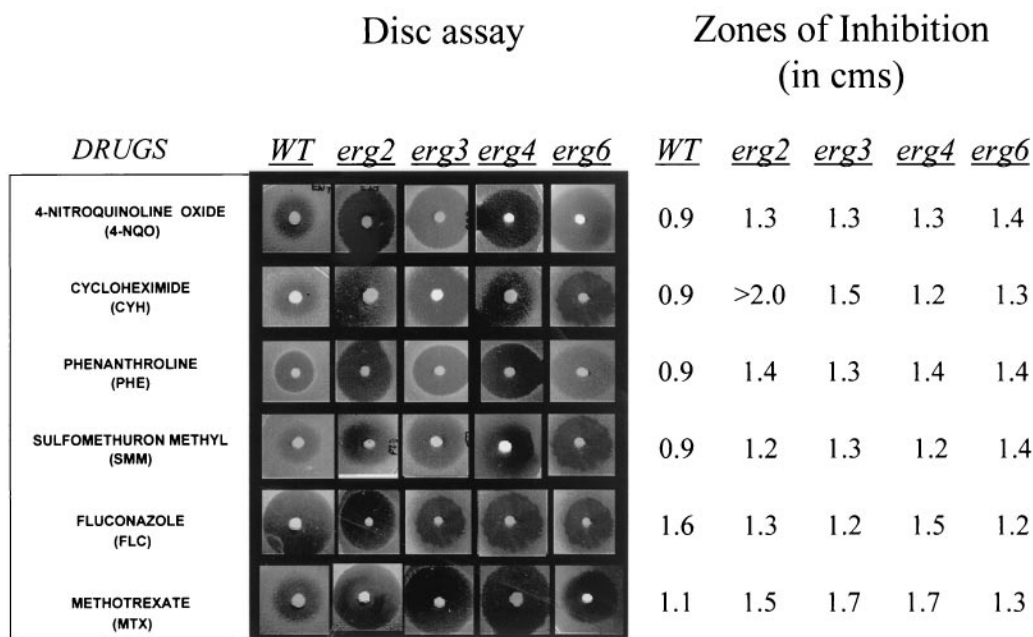


FIG. 3. Drug resistance profiles of *S. cerevisiae* WT and *erg* mutants determined by filter disk assay as described in Materials and Methods. The numbers on the right represent zones of inhibition measured from the zones shown on the left.

more susceptible than the WT (MIC_{80} , $1 \mu\text{g ml}^{-1}$ for *erg4* versus $4 \mu\text{g ml}^{-1}$ for the WT). By the disk assay, however, no major differences in the FLC susceptibilities of *erg4* and other mutant cells were evident. This points out that sometimes the MIC and the result of the filter disk assay may not match (10).

Since there was no significant difference between the growth of the *erg* mutants and the WT cells (Fig. 1B), differences in growth could not have contributed to the observed variations in drug susceptibilities. The observed resistance of *erg* mutants of *S. cerevisiae* to FLC needs special mention. While our ob-

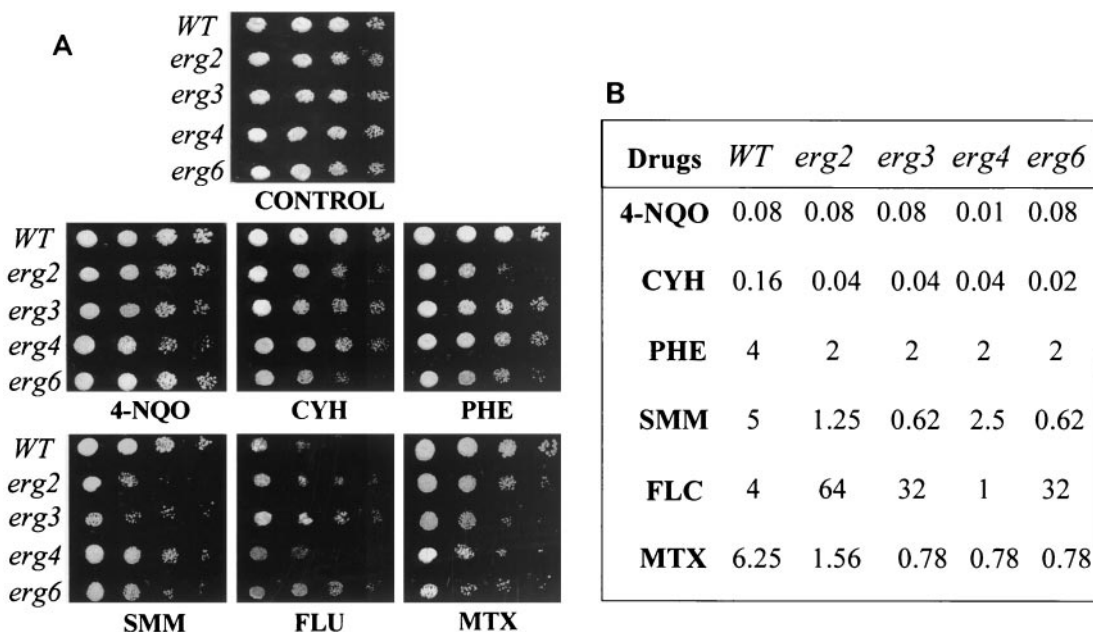


FIG. 4. Drug resistance profiles of *S. cerevisiae* WT and *erg* mutants determined by the spot assay (A) and the microtiter assay (B). (A) For the spot assay, the yeast cells were grown overnight on YNB plates at 30°C. The cells were then suspended in normal saline to an OD_{600} of 0.01 (A_{600}). Five microliters of fivefold serial dilutions of each yeast culture was spotted onto YNB plates in the absence (control) and presence of the following drugs: 4-NQO ($0.02 \mu\text{g ml}^{-1}$), CYH ($0.01 \mu\text{g ml}^{-1}$), PHE ($2 \mu\text{g ml}^{-1}$), SMM ($1 \mu\text{g ml}^{-1}$), FLC ($8 \mu\text{g ml}^{-1}$), and MTX ($1 \mu\text{g ml}^{-1}$). Growth differences were recorded following incubation of the plates for 48 h at 30°C. Growth was not affected by the presence of the solvents used for the drugs (B) The microtiter assay (MIC_{80}) was done as described in Materials and Methods.

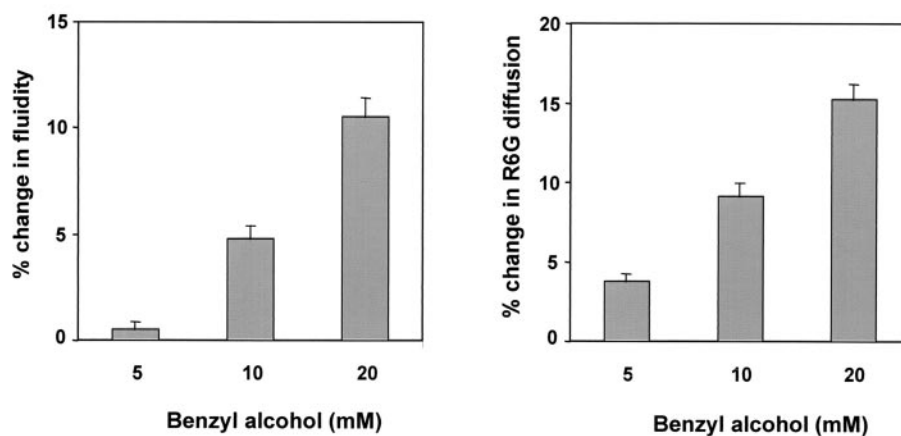


FIG. 5. Measurement of membrane fluidity and passive diffusion of *S. cerevisiae* WT cells induced by BA. Fluidity was determined by measuring the steady-state fluorescence polarization with DPH as the fluorescent probe as described in the legend for Fig. 2A. Diffusion was determined by measuring the extracellular R6G concentration as described in the legend for Fig. 2B. The values are means \pm standard deviations (indicated by the bars) of three independent experiments.

ervation is limited to the *erg* mutants of *S. cerevisiae* studied, evidence suggests that mutations in *ERG* genes could also affect azole sensitivity in *C. albicans* cells. The accumulation of ergosta-7,22-dienol-3 β -ol in an azole-resistant clinical isolate of *C. albicans* has been attributed to the absence of *ERG3* ($\Delta^{5,6}$ -desaturase) (21, 33, 35, 44). Additionally, the susceptibility patterns of *erg* mutants of *Candida* could be different. In the present study we observed that the *erg6* mutant of *S. cerevisiae* is resistant to FLC, while Jensen-Pergakes et al. (18) showed that a *C. albicans* strain with the same mutation has nearly identical susceptibilities to FLC and other azoles.

Membrane lipid phase affects passive diffusion of drugs. In order to test the possible influence of enhanced membrane fluidity on the rate of drug import via passive diffusion, we first excluded the contributions of efflux pumps, which could interfere with diffusion measurements. This was achieved by energy depletion of cells, wherein *S. cerevisiae* cells were aerated for 1 h in the presence of 5 mM 2-deoxy-D-glucose and 5 mM 2,4-dinitrophenol, which was sufficient to deplete most of the intracellular ATP (data not shown) (51). The yeast cells were then incubated in the presence of the fluorescent substrate R6G as described in Materials and Methods. The drug diffusion in energy-depleted cells was determined by measuring absorbance changes in the R6G contents in the supernatant withdrawn at different times. It is evident from Fig. 2B that after 1 h of incubation in the presence of R6G, the supernatants of all the mutant strains showed low extracellular R6G concentrations, implying increased entry (passive diffusion) of the fluorophore into the cells. The enhanced accumulation of [3 H]FLC (Fig. 2C) in energy-depleted cells (diffusion) also confirmed that *erg* mutants elicit high levels of diffusion. Thus, the drug sensitivities of the *erg* mutants could be due at least in part to an enhanced passive diffusion of the drugs, which is influenced by the membrane lipid phase.

In vitro-induced changes in membrane fluidity do not affect drug resistance. In order to further explore if the physical state of membrane lipids can indeed influence the drug resistance of yeast cells, we induced changes in the membrane fluidity by a known membrane fluidizer, benzyl alcohol (BA) (46). The

addition of 5 to 20 mM BA led to a gradual increase in membrane fluidity which was maximally enhanced to 10 to 12% in cells exposed to 20 mM BA (Fig. 5). The enhanced fluidity also led to an increase in the diffusion of R6G by 15%. However, the BA-induced changes in fluidity and passive diffusion did not alter the sensitivities to the drugs tested (data not shown). It is thus probable that enhanced membrane fluidity and the diffusion therefrom may not be the only cause of the hypersensitivity observed for the *erg* mutants.

Membrane environment selectively affects drug extrusion pumps of *Candida* when expressed in *S. cerevisiae*. In view of the intricate relationship between the membrane lipid phase and the membrane proteins, we examined the functioning of two MDR pumps of *C. albicans*, Cdr1p and CaMdr1p, in an altered membrane environment. Both the WT and *erg* mutants of *S. cerevisiae* were transformed with the *CDR1* and *CaMDR1* genes cloned on an identical vector, as described in Materials and Methods. The levels of expression of *CDR1* and *CaMDR1* were unaffected in *erg* mutants compared to the levels of expression in the WT strain (data not shown). The drug resistance conferred by the levels of expression of these proteins in an altered membrane environment was examined by a microtiter plate method (Fig. 6). Since *erg* mutant strains displayed different levels of hypersensitivity to the drugs compared to the sensitivity of the WT strain (Fig. 3 and 4), we calculated the fold change (Fig. 6) in resistance of the yeast transformants from that of their respective nontransformant strains. The Cdr1p-mediated drug resistance appeared to be adversely affected by the lipid environment. For example, the ability of Cdr1p to mediate resistance to CYH (which is a preferred substrate for this pump) was severely hampered (two- to four-fold decrease), especially in the *erg2*, *erg3*, and *erg6* mutant backgrounds. The fold resistance to FLC by Cdr1p was also decreased in the *erg* mutant strains, while the levels of resistance to 4-NQO, MTX, SMM, and PHE remained unaffected (Fig. 6). Interestingly, CaMdr1p appeared to be insensitive to membrane alterations since the fold increase in resistance that it mediated remained largely unchanged or increased between the WT and its *erg* mutant strains (Fig. 6). The resistance to

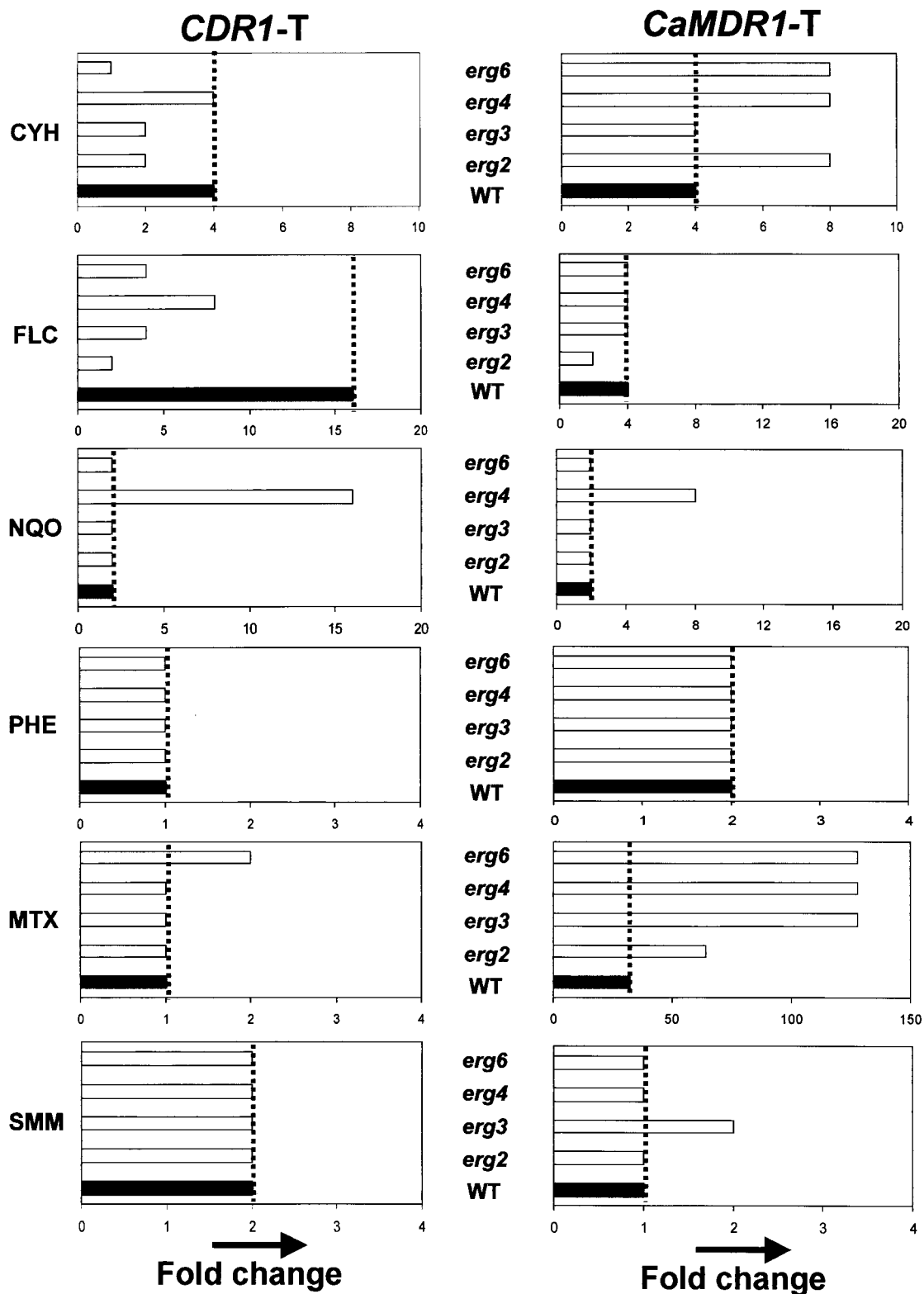


FIG. 6. Graphical representations showing the fold increase in drug resistance of WT and *erg* mutant strains of *S. cerevisiae* upon transformation with Cdr1p- and CaMdr1p-encoding plasmids (see Materials and Methods). The fold increase in resistance to each drug was calculated as the difference in resistance between the nontransformant and transformant pair for each cell type. MTX is the substrate for CaMdr1p (24), and the fold change in resistance is much higher for *CaMDR1*-transformed *erg* mutant cells than for *CDR1*-transformed *erg* mutant cells. Note the change in the x axis for fold MTX resistance.

FLC mediated by Cdr1p needs special mention. It is evident from Fig. 3 and 4 that *erg* mutants are intrinsically resistant to FLC. The WT strain, which was sensitive to FLC, exhibited a 16-fold higher level of resistance when *CDR1* was expressed in this background. On the other hand, expression of *CaMDR1* in the WT yeast strain resulted in only a fourfold enhanced level of resistance. Interestingly, while the fold resistance to FLC is more or less maintained in the case of *CaMDR1* expression, *CDR1*-transformed *erg* mutant cells could not sustain the level of resistance elicited in WT cells.

Cdr1p is more sensitive to lipid environment. It became clear from the results presented above that, compared to CaMdr1p, which is an MFS transporter, ABC drug transporter Cdr1p is more sensitive to lipid perturbations. In order to explore such an interaction between membrane ergosterol and sphingomyelin, we performed the experiment described below.

We labeled WT and *erg* mutant cells of *S. cerevisiae* with NBD-SM. The important advantage of this lipid analogue is that it can readily be inserted into biological membranes by spontaneous lipid transfer from exogenous carriers (20, 48). At 90 min postlabeling, when the level of NBD-SM incorporation was maximal, the cells were washed and the associated NBD-SM of the labeled cells was back extracted by using 2% fatty acid-free BSA. The labeling protocol and back exchange of labeled NBD-SM were described in Materials and Methods and in an earlier publication (48). Figure 7B depicts the exchangeability of NBD-SM from different labeled strains of *S. cerevisiae*. It is clear that the efficiency of extraction of NBD-SM by BSA from *erg* mutants is greater than that from the WT (Fig. 7B). It probably suggests that ergosterol depletion in *erg* mutant cells results in disruption of the interaction between ergosterol and sphingomyelin, which leads to the enhanced exchange of NBD-SM. In order to examine the lipid interaction more closely, the growth media of *erg* mutants was supplemented with ergosterol. That supplemented ergosterol is incorporated into the membrane was confirmed by spectral analysis. Figure 7A shows the appearance of characteristic peaks of ergosterol in supplemented cells. Interestingly, the exchange of NBD-SM was considerably reduced (restored to the level for WT cells) when *erg* mutant cells were grown in ergosterol-supplemented media (Fig. 7B). This shows that the ergosterol content affects the exchangeability of sphingomyelin and thus implies a close interaction between the two membrane lipid components.

DISCUSSION

In this study we have presented evidence highlighting the close interaction between membrane lipids and the drug sensitivities of yeast cells. For this we used *erg2*, *erg3*, *erg4*, and *erg6* mutants of *S. cerevisiae* which are blocked in late ergosterol biosynthesis (Fig. 1A). Due to defective ergosterol biosynthesis and, as a result, the accumulation of various intermediates, these mutants possessed high levels of membrane fluidity. These mutants were in general sensitive to several of the drugs tested; however, they were resistant to FLC (the only azole tested). The hypersensitivities of the yeast *erg* mutants could be attributed to membrane permeability changes which may involve changes in passive diffusion across the membrane or in the active transport of these drugs. In support of the former,

Van Den Hazel et al. (51) have shown that the lack of *PDR16* and *PDR17* (which encode homologues of Sec14p) *S. cerevisiae* cells results in altered phospholipid and sterol compositions and renders cells hypersensitive to many drugs due to their increased passive diffusion. In support of the latter possibility, the ABC transporter Pdr5p has been shown to function less efficiently in cells from which *erg6* is deleted. A recent report by Emter et al. (9), however, suggests that *ERG6* of *S. cerevisiae* increases the rate of passive drug diffusion without affecting Pdr5p-mediated drug export. In this study we used two substrates, i.e., the fluorophore R6G and radiolabeled FLC, and observed that *erg* mutants elicited higher levels of passive diffusion. This suggests that the enhanced diffusion of drugs could contribute to the hypersensitivities of the *erg* mutants. However, in another set of experiments, when we increased the membrane fluidity by using a membrane fluidizer, BA, we observed that a 10 to 12% increment in fluidity did not affect the susceptibilities of the *S. cerevisiae* cells to the drugs tested. Taken together, it appears that the change in membrane fluidity and the increased diffusion therefrom alone may not be sufficient to result in the observed higher susceptibilities of the *erg* mutants. Since these mutants have altered sterol compositions, it is likely that sterol interactions with other membrane lipids and transporters could be more relevant to the higher drug susceptibilities observed.

We observed that when the MFS transporter CaMdr1p and the ABC transporter Cdr1p of *C. albicans* are expressed in *erg* mutants of *S. cerevisiae*, the latter appears to be more responsive to the fluctuations in the membrane lipid environment. We show that when Cdr1p is expressed in *erg* mutants it could not restore the level of resistance to CYH and FLC compared to the level of resistance obtained when it was expressed in WT cells. Interestingly, two independent observations suggest that drug extrusion pump sensitivity to the lipid environment was not a general phenomenon: first, the Cdr1p-mediated levels of resistance to drugs like NQO, SMM, PHE, and MTX remained unchanged when Cdr1p was expressed in *erg* mutants, while the sensitivities to CYH and FLC changed drastically; second, the CaMdr1p-mediated levels of resistance to similar drugs remained unaffected and were rather improved in some instances when CaMdr1p was expressed in an *erg* background. Taken together, our results suggest that the Cdr1p ABC drug extrusion pump is selectively more sensitive to fluctuations in sterol composition.

Human P-gp, a homologue of Cdr1p, is also shown to be sensitive to fluctuations in the membrane lipid environment, where it intimately interacts with membrane sphingomyelin and cholesterol (32). In this study, we also observed a close interaction between membrane sterol (ergosterol in this case) and sphingolipids. We were able to exchange larger amounts of NBD-SM from *erg* mutants which could be reversed if the mutants were supplemented with ergosterol. A very recent (49) report shows that regulation of sphingolipid metabolism is sterol dependent in *S. cerevisiae*. This again signifies the mutual importance of the two membrane lipids in yeast (4).

Interestingly, Cdr1p-mediated phospholipid translocase activity was found to be independent of ergosterol depletion (47). This shows how functional domains of a protein respond differently to fluctuations in the lipid environment. Such selective sensitivity of an efflux pump protein (Cdr1p) to fluctuations in

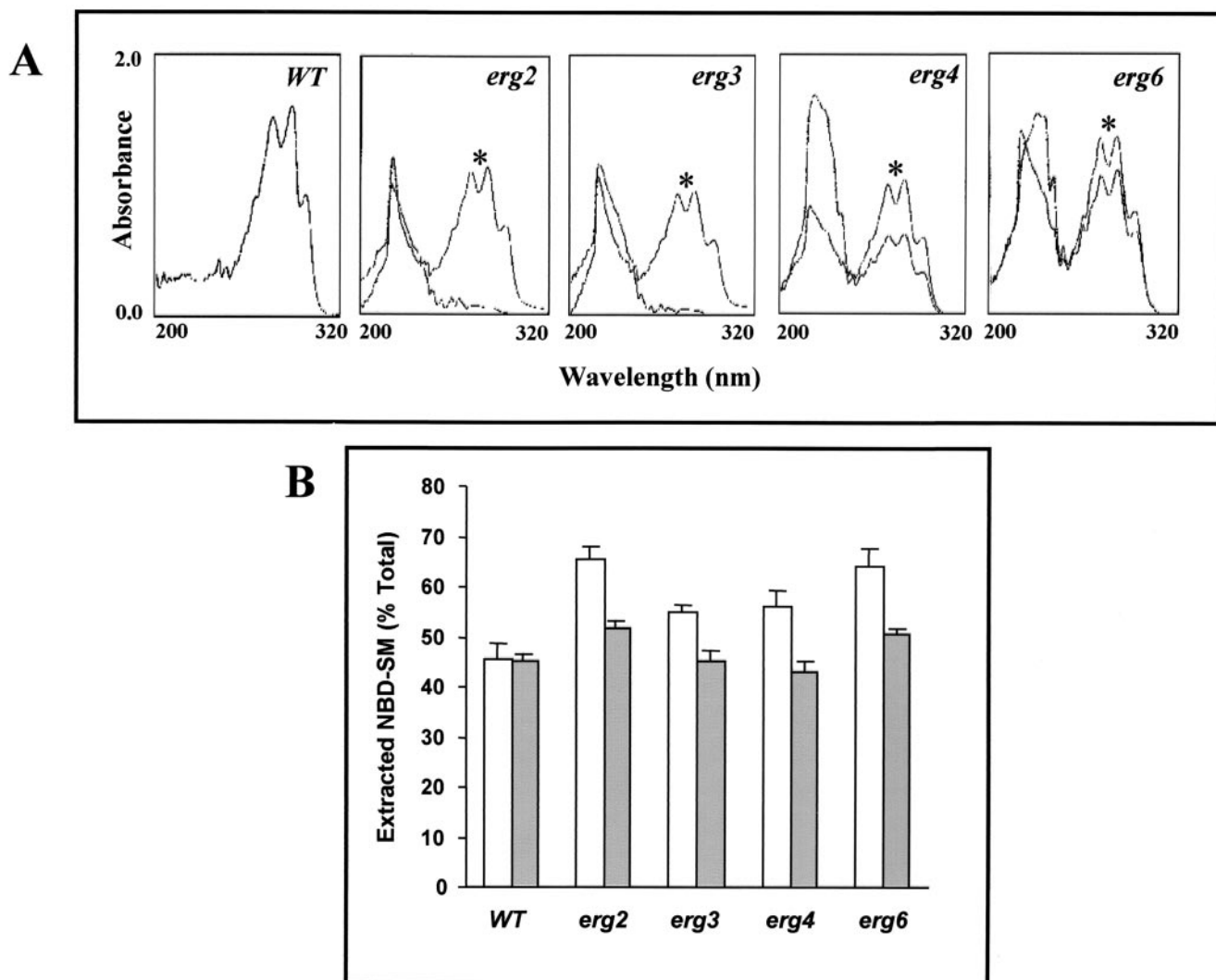


FIG. 7. (A) UV absorption spectra of sterols extracted from the *S. cerevisiae* WT strain and its *erg* mutants grown in the absence and in the presence (*) of ergosterol. The cells were supplemented with $10 \mu\text{g}$ of ergosterol ml^{-1} and grown for 20 h at 30°C before lipid extraction. The supplemented cells were harvested and washed thoroughly before extraction of sterols as described in Materials and Methods. The UV spectra of WT cells were the same as those of the unsupplemented cells; hence, only one trace is shown in the first panel. (B) Postlabeling transbilayer exchange of NBD-SM in the *S. cerevisiae* WT and its *erg* mutants. Cells were grown in the absence (open bar) and presence (filled bar) of ergosterol-supplemented media as described in Materials and Methods. NBD-SM labeled cells were washed twice with buffer A and were then incubated with 2% BSA to back exchange the NBD-SM from the labeled cells, as described in Materials and Methods. The percentage of total extracted NBD-SM was calculated (according to the formula given in Materials and Methods). The graph presents data for the 90-min time point during which the maximum back-exchanged fluorescence in the supernatant was observed. The values are the means \pm standard deviations (indicated by the bars) of three independent experiments.

the membrane lipid environment (loss of resistance to certain drugs) and selectivity within the efflux proteins (Cdr1p is more sensitive to lipid fluctuation than CaMdr1p) do point to very specific interactions between efflux pump proteins and membrane lipids.

Recently, Kontoyiannis (25) has provided genetic evidence that Pdr5p-mediated resistance to FLC is altered in the background of mutations that affect ergosterol homeostasis. It is suggested that the ABC protein functions in the maintenance of ergosterol homeostasis by extrusion or transport of sterol intermediates. In view of the observed close interaction between ergosterol and sphingomyelins in the yeast mem-

brane, such homeostasis is expected to be perturbed in *erg* mutants and could affect the functioning of drug extrusion pump proteins (14). That mitochondria and oxidative phosphorylation are physiological partners of at least Erg3p is another aspect which merits attention (26). In view of the compelling evidence from various sources in which differences in the membrane ultrastructures and the sterol and phospholipid compositions between drug-resistant and -sensitive cell lines (for a review, see reference 11) and yeast cells (30) are observed, it is important to assess the roles of such changes in the overall scenario of multidrug resistance.

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