 Cytometry 39:26–35 (2000)

Rapid Detection of Efflux Pumps and Their Relation With Drug Resistance in Yeast Cells

Cristina Prudêncio,[1,2] Filipe Sansonetty,[3] Maria João Sousa,[1] Manuela Côrte-Real,[1] and Cecília Leão[1]*

[1]Centro de Ciências do Ambiente — Departamento de Biologia, Universidade do Minho, Braga Codex, Portugal
[2]Escola Superior de Tecnologia da Saúde do Porto, Porto, Portugal
[3]Laboratório de Citometria, Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Porto, Portugal

Received 26 February 1999; Revision Received 27 July 1999; Accepted 28 September 1999

Background: Cell drug resistance can be due to the presence of active efflux pumps (AEP). Identification of yeast cells with a resistance phenotype is important either from a clinical, agricultural or biotechnological point of view. Rapid and reliable methods to detect AEP can be therefore very useful.

Methods: Some yeast cells change their staining by calcein-AM, BCECF-AM, rhodamine 123 and $DiOC_5$, when pretreated with verapamil, CCCP or ATP depletion, or when pretreated with specific antimicrobial agents. This fact may be interpreted as an indication of the presence/absence of AEP. Six yeast species were tested with a flow cytometric method (FCM) and an epifluorescence microscopic method (EFM), and ten other species were evaluated only by EFM. The minimum inhibitory concentration (MIC) of penconazol, benomyl and cycloheximide for *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, were determined by growth inhibition on solid medium and were compared to the staining changes detected by FCM.

Results: The FCM and the EFM allowed the detection of AEP in all the yeast species tested. High MIC values for a drug were related with the presence of at least one AEP indicated by the cytometric data.

Conclusions: The FCM revealed to be a robust assay whereas the EFM can be used as a preliminary test. It is possible to identify resistance/sensitivity patterns in yeast cells through cytometric detection methods of different efflux pumping systems. Cytometry 39:26–35, 2000.

Key terms: active efflux pumps; yeast drug resistance; antimicrobial agents

The involvement of active efflux pumps (AEP) in the mechanisms responsible for drug resistance has been described in different cell types like human neoplastic cells and several pathogenic microbial cells (1,2).

Currently the functional assay of these AEP in eukaryotic cells involves the use of fluorescent compounds, such as daunorubicin, doxorubicin, rhodamine derivatives and more recently calcein-AM. The first two compounds are essentially used as anticancer drugs, and due to its fluorogenic characteristics they allow an easy discrimination of resistant cell lines expressing multidrug resistance (MDR), from non-resistant cells. Furthermore, the detection of MDR proteins with fluorescent probes was improved by the use of inhibitory conditions leading to a functional block of AEP and, secondarily to an intracellular probe retention with a consequent cell fluorescence increase.

In some yeast species, genes responsible for generalized resistance to a large number of structurally and functionally unrelated drugs have been reported (1,3–6). This phenotype is known as pleiotropic drug resistance (PDR) and is related to multidrug membrane transporters that drive different compounds out of the cell. In general, the PDR proteins are classified in four distinct transporter families: ABC (ATP-binding cassette), MFS (major facilitator superfamily), RND (resistance-nodulation-cell division) and SMR (small multidrug resistance), the last two appearing to be specific of prokaryotes (5). While the proteins belonging to the ABC family seem to require ATP, the MFS proteins are H^+/substrate antiporters. Actually, the former possess a highly conserved domain (of about 200 amino acids) that binds and hydrolyzes ATP (6), and the latter depend on the proton gradient established by the H^+-ATPase (7). Although extensive work has been done in the genetic characterization of PDR proteins and on the elucidation of the mechanisms underlying PDR, very few of these transporters have been functionally characterized (1,5,7,8).

Grant sponsor: PAMAF; Grant number 6088.
*Correspondence to: Cecília Leão, Department of Biology, University of Minho, 4710-057 Braga Codex, Portugal.
E-mail: cleao@bio.uminho.pt

Recently, a strong homology between human MDR proteins, such as P-glycoprotein, and yeast members of the PDR protein superfamilies has been reported (3,8). Therefore, the yeast cell emerges as a good model for the elucidation of the mechanisms underlying drug resistance.

In agriculture, the development of PDR to fungicides by different phytopathogens is becoming a problem (9). The recent discovery of PDR 12 involved in the yeast resistance to weak organic acids used as preservatives in the food industry, brought new insights to this field in biotechnology (10). Also, a screening for PDR phenotype in yeast clinical isolates is relevant for an early elimination of the pathogen, because infection progression, in some cases, depends more on an efficient therapy than on the host due to severely reduced defenses. This is particularly important when dealing with immunocompromised patients and patients receiving broad-spectrum antibiotic therapy (11). This approach may contribute to a more rational antimicrobial susceptibility testing. Thus, functional studies directed to the determination of a resistance phenotype and modulator agents that reverse it (12–15) might have a great clinical importance prior to treatment. Due to the lack of an adequate methodology (8), these tests are not done routinely.

In this paper, we adapted the methodologies referred to above to yeast cells, for the study of AEP in other eukaryotic cells, including the use of different inhibitory conditions for PDR proteins. Furthermore, since our aim was to detect as many of these AEPs in yeast as possible, we used four fluorescent probes among those generally referred in the literature for such purposes, namely: rhodamine 123, $DiOC_5$, calcein-AM, and BCECF-AM. The first two probes have been extensively used as MDR substrates in different types of cells (11,16,17) and are also membrane-potential-sensitive probes. Therefore, they are taken up and retained by the cell, having an intracellular fluorescence dependent on the membrane potential. While rhodamine123 is supposed to be mitochondria specific, $DiOC_5$ is also dependent of membrane potential of plasma or other organelle membranes (18). On the other hand, calcein-AM and BCECF-AM are two esterase substrates that are hydrolyzed to fluorescent charged products calcein and BCECF, respectively (12–14). While the former has a pH insensitive fluorescence, the latter has a pH dependent fluorescence, which intensity increases with pH. Calcein-AM and BCECF-AM have been reported to be transported out of the cell either in the ester or hydrolyzed form (12).

The ultimate goal of this work was to develop a method for a rapid detection of AEP in yeast, which could be used as a tool to evaluate to what extent PDR phenotype is generalized in these microorganisms. In addition, we evaluated the relationship between the resistance to the antimicrobial agents, penconazol, benomyl, and cycloheximide, with the presence and/or induction of transporter proteins (pumps) detected by FCM in the yeasts, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

The yeast species studied were: *Saccharomyces cerevisiae* IGC 4072, *Zygosaccharomyces bailii* ISA 1307, *Pichia membranifaciens* IGC 2487, *Kluyveromyces marxianus* IGC 2671, *Debaryomyces hansenii* IGC 2968, *Dekkera anomala* IGC 5133, *Pichia anomala* IGC 4121, *Rhodotorula mucilaginosa* IGC 4791, *Torulaspora delbrueckii* ISA 326, *Candida utilis* IGC 2578, *Lodderomyces elongisporus* ISA 1421, *Saccharomycodes ludwigii* ISA 1083, *Issatchenkia orientalis* IGC 3806, *Kloeckera apiculata* ISA 1189, and *Candida sheatae* IGC 3504. The abbreviation ISA is used for the collection of the Instituto Superior de Agronomia and the other yeast strains were obtained from the Portuguese Yeast Culture Collection (PYCC), Universidade Nova de Lisboa, Portugal. *Schizosaccharomyces pombe* G2, was isolated at the Institut Coopératif du vin (Montpellier, France). The following *S. cerevisiae* strains (19) were kindly provided by A. Goffeau (Unité de Biochimie Physiologique, Faculté des Sciences Agronomiques, Université Catholique de Louvain, Belgique): US50-18C (*Matα, PDR1-3, ura3, his1*), SUPERYOR (Δyor1::hisG, Δsnq2::hisG, Δ*pdr5::PDR5PROM-YOR1-PDR5STOP,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG,* Δ*pdr3::hisG*) and AD12345678 (Δ*yor1::hisG,* Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG,* Δ*pdr3::hisG,* Δ*pdr15::hisG*). In the two latter mutant strains, the deletions have been done in the *PDR1-3* US50-18C strain. Cells were grown in a mineral medium (MGV) with vitamins (20) supplemented with 2% (w/v) of glucose and incubated on a mechanical shaker at 25°C.

Chemicals

Calcein-AM (acetoxymethyl ester), $DiOC_5$ (3,3′-dipentyloxacarbocyanine iodide), rhodamine 123, and BCECF-AM (2′,7′ -bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester) were purchased from Molecular Probes (Eugene, OR). Stock solutions were made in dimethylsulfoxide (DMSO) for BCECF-AM (100 μM) and in ethanol for $DiOC_5$ (500 μM) and rhodamine 123 (25 μM). Calcein-AM (1 mM) was used directly from the commercial solution. PI (propidium iodide), verapamil, CCCP, 2-deoxyglucose, cycloheximide and sodium fluoride (NaF) were purchased from Sigma Chemical Co. (St. Louis, MO) and sodium azide from Merck (Darmstadt, Germany). The fungicides, benomyl and penconazol were purchased from Riedel-de Haën (Chinosol, Seelzeq, Germany).

Both the inhibitory conditions and the probes, at the concentrations used, were found to be noncytotoxic, by counting colony forming units.

Staining Protocols for Flow Cytometry and Epifluorescence Microscopy Analysis

Cells from an overnight culture (O.D. = 1.0) were harvested, centrifuged, washed twice with ice-cold distilled water and suspended (about 3×10^6 cells/ml) in phosphate-buffered saline (PBS), pH 7.0, for calcein-AM and BCECF-AM staining or in deionized water for rhoda-

mine 123 and $DiOC_5$ staining. Calcein-AM, BCECF-AM, rhodamine 123, and $DiOC_5$ were added to cell suspensions to a final concentration of 10 μM, 5 μM, 500 nM, and 1 μM, respectively. The volumes of cell suspension used were 20 μl for epifluorescence microscopy (EFM) and 300 μl for flow cytometry (FCM). The incubation was carried out at room temperature for calcein-AM, $DiOC_5$, and rhodamine 123 staining and at 30°C for BCECF-AM staining. The incubation time was 30 min for calcein-AM and BCECF-AM and at least 5 and 10 min for $DiOC_5$ and rhodamine 123, respectively. A working solution (20 μg/ml) of PI was also prepared from a concentrated stock solution (500 μg/ml) diluted with PBS. Staining with PI was performed as previously described (21).

FCM analysis was performed on a Epics XL-MCL (Beckman-Coulter Corp., Hialeah, FL) flow cytometer, equipped with an argon-ion laser emitting a 488 nm beam at 15 mW.

For all the probes, the green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass. A minimum of 20,000 cells/sample were analyzed. An acquisition protocol was defined to measure forward scatter (FS), side scatter (SS) and green fluorescence (FL1) on a four decades logarithmic scale. Green fluorescence (FL1, log) was gated in a scattergram of SS log × FS log in order to include in the fluorescence measurements mainly signals originated in intact individual cells and not cell debris or cell clamps. The data were analyzed with the Multigraph software included in the System II acquisition software for the EPICs XL/XL-MCL version 1.0. To evaluate differences in the level of staining between control cells (CC) and cells treated (CT) under inhibitory conditions for PDR proteins, a ratio between the CT and CC mean fluorescence channel number from the FL1 histogram was calculated. These ratio values were used for the detection of AEP by FCM. Ratio values significantly ($P < 0.05$, $n = 5$) higher than 1.00 indicated accumulation of the probe, as a result of the inhibition of the transporter, under the conditions used. This result was interpreted as a positive response and indicative of the presence of a pump for that probe. On the other hand, when the ratio was equal or significantly ($P < 0.05$, $n = 5$) lower than 1.00, this was interpreted as a negative response and indicative of the absence of a pump.

In the study of the involvement of ABC and MFS pumps in the efflux of benomyl, penconazol, and cycloheximide, a ratio between the CT and CC was also calculated. If the treatment with the antimicrobial agent resulted in the induction or overexpression of a pump, for the efflux of the antimicrobial agent and the probe out of the cell, a decrease of the intracellular fluorescence should be observed. Therefore, a ratio significantly ($P < 0.05$, $n = 5$) lower than 1.00, was indicative of efflux. A ratio equal or significantly ($P < 0.05$, $n = 5$) higher than 1.00 was indicative of retention of the probe and of the absence of a pump for the antimicrobial agent.

The epifluorescence microscopy analysis were performed on a Leitz, Laborlux S epifluorescence microscope equipped with a mercury lamp of 50 W and a filter set composed by an excitation filter BP 450-490 a beam splitter FT510 and an emission filter LP520.

Samples of a cell suspension (3×10^6 cells/ml), of each yeast species, nonstained (level of autofluorescence) and stained before and after treatment under inhibitory conditions for PDR proteins, were placed between a slide and a cover slip, after mixing with the antifading reagent (20 μl of cell suspension + 20 μl of antifading reagent). The antifading reagent, Vectashield Mounting Medium for fluorescence H-1000, was purchased from Vector Laboratories.

Cells were observed under simultaneous white light transmitted and blue light epi-illumination with a total magnification of 630×. The digital images were acquired by a 3CCD Color Video Camera (SONY, DXC-9100P), a frame grabber (IMAGRAPH, IMASCAN/Chroma-P) and a software for image archival and management (HOSPITRANS, Fotoscope v1.0).

In the observations by EFM, it was possible to distinguish three increasing levels of cell fluorescence after incubation with the fluorochromes: level 1, identical to the autofluorescence (i.e., nonvisible or almost nonvisible cell fluorescence); level 2, higher than the autofluorescence (visible cell fluorescence); and level 3, much higher than the autofluorescence (very bright cell fluorescence). Cells displaying level 3 fluorescence, never exceeded 4% and could be detected either in nontreated or treated samples under inhibitory conditions for PDR proteins. Once these cells stained with PI they were considered dead and therefore excluded from the counts. For each treated sample, in a total of 200 cells, the number of cells displaying level 2 fluorescence was counted and compared with that in the control sample (nontreated cells). An arbitrary cut-off value of 5% of cells displaying level 2 fluorescence was used to classify the response of the cell population as positive (>5%) and indicative of the presence of a pump for that probe. The results given here are representative of five independent experiments.

As referred to above, the method developed for the detection of AEP either by EFM or by FCM was based on the observation of an increase in cell fluorescence, under inhibitory conditions for PDR proteins. For this purpose, cells were subjected to the following treatments before staining: Cell suspensions, prepared as described above, were incubated separately with verapamil or CCCP. For verapamil, the incubation was carried out at a final concentration of 100 μM and at room temperature for at least 90 min, with agitation. The final concentration used with CCCP was 9 μM and the incubation was carried out at room temperature for about 1 min, with agitation. Verapamil is a known inhibitor of P-glycoprotein in human cells (14,17) while carbonyl cyanide m-chlorophenylhydrazone (CCCP), being a protonophore, collapses the transmembrane ΔpH (22,23) and hence inhibits PDR proteins from the MFS superfamily. In addition, cells were ATP depleted in order to inhibit ABC proteins (12). For this purpose, cells from an overnight culture were centrifuged and resuspended (3×10^6 cells/ml) in MGV medium without glucose and with sodium azide (10 mM) and

2-deoxyglucose (5 mM) for at least 90 min, at room temperature, with agitation (12).

Determination of the Minimum Inhibitory Concentration (MIC) for Cycloheximide, Penconazol, and Benomyl

Cells from an overnight culture (O.D. = 1.0) were centrifuged and resuspended in sterile water (3×10^6 cells/ml) and then plated, in triplicate, as a drop (5 μl) without and with dilution by a 10, 100, and 1000 factor.

Plates were prepared with solid media (21) with 2% glucose (w/v) and different concentrations of cycloheximide, benomyl, or penconazol. The solid media was previously autoclaved and then supplemented with the solutions of the antimicrobial agents, sterilized by filtration with a 0.45 μm filter. After 48 h of incubation at 25°C, the plates were checked for the presence of yeast colonies.

The lowest experimental drug concentration for which no growth was observed in any of the dilutions of cell suspension tested, was considered the MIC value.

Assays With Antimicrobial Agents

Cells of *S. cerevisiae* and *K. marxianus* were suspended in PBS and incubated with cycloheximide, benomyl, or penconazol at room temperature for at least 60 min. The concentrations used for the different drugs were below the minimum inhibitory concentration determined for each yeast species (i.e., for *S. cerevisiae*, cycloheximide 1×10^{-4} mg/l, benomyl 5.8 mg/l and penconazol 10 mg/l and for *K. marxianus,* cycloheximide 1.25 mg/l, benomyl 2.9 mg/l, and penconazol 20 mg/l) and confirmed not to be cytotoxic by counting colony forming units. After these incubations, staining protocols were carried out as described above.

In order to inhibit protein synthesis, 100 mM final concentration of NaF, was used. This concentration was found in preliminary experiments to be inhibitory of protein synthesis in both yeast species.

Statistical Analysis

The data were compared by the Student's *t* test.

RESULTS

Detection of Active Efflux Pumps by Flow Cytometry (FCM)

The presence of ABC or MFS transporters in wild type strains belonging to six different yeast species was investigated by FCM using calcein-AM, BCECF-AM, rhodamine 123, and $DiOC_5$. Typical fluorescence histograms of a control, negative, and positive cell response of *Saccharomyces cerevisiae*, stained with calcein-AM are shown in Figure 1A. As shown in this figure, in cell suspensions treated with verapamil and stained with calcein-AM the levels of green fluorescence were similar to the control cell suspension. However, with this fluorochrome, the cells submitted to ATP depletion conditions displayed an increase in the green fluorescence, suggesting the presence of an ABC protein.

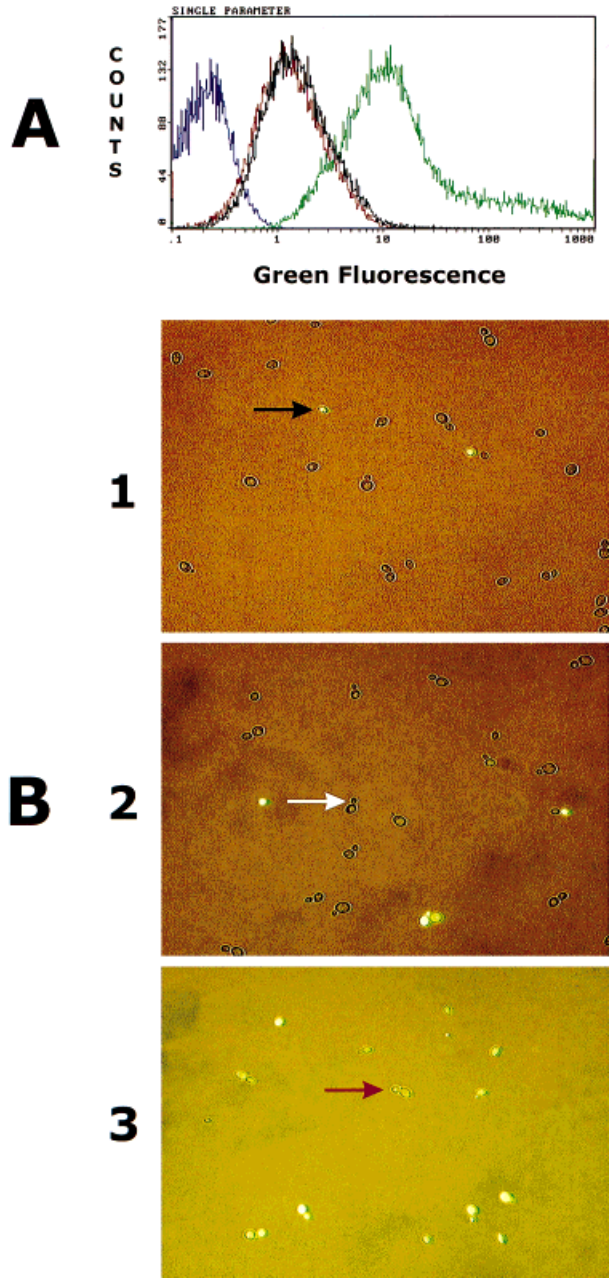

FIG. 1. **(A)** Histograms of *S. cerevisiae* cells green fluorescence before and after staining with calcein-AM. Blue = autofluorescence. Red = control response, cell population stained with the probe without any treatment. Black = negative response of a cell population that under inhibitory conditions (verapamil) stained similar to the control, indicating the absence of an AEP. Green = positive response of a cell population that under inhibitory conditions (ATP depletion) stained more than the control, indicating the presence of an AEP. **(B)** Digital images obtained with epifluorescence and transmitted white light, and 630× magnification. 1 = control, 2 = negative, and 3 = positive response of a cell population. Black, white and red arrows, show examples of levels 3, 1, and 2 of cell fluorescence, respectively, according to Materials and Methods.

In Table 1 the results obtained for the six yeast species selected represent the mean of the fluorescence ratio, as defined in the Materials and Methods section, obtained in

Table 1
Detection of Active Efflux Pumps by Flow Cytometry (FCM) and by Epifluorescence Microscopy (EFM) in Saccharomyces cerevisiae *IGC 4072,* Zygosaccharomyces bailii *ISA 1307,* Kluyveromyces marxianus *IGC 2671,* Debaryomyces hansenii *IGC 2968,* Candida utilis *IGC 2578, and* Issatchenkia orientalis *IGC 3806**

Probes	Inhibitory conditions	*S. cerevisiae*		*K. marxianus*		*D. hansenii*		*Z. bailii*		*C. utilis*		*I. orientalis*	
		FCM[a]	EFM[b]	FCM[a]	EFM[b]	FCM[a]	EFM[b]	FCM[a]	EFM[b]	FCM[a]	EFM[b]	FCM[a]	EFM[b]
Calcein -AM	Verapamil	0.90 ± 0.20	−	1.22 ± 0.21	−	1.27 ± 0.20	−	1.99 ± 0.67†	−	1.80 ± 0.27†	+	1.10 ± 0.11	−
	ATP depletion	6.85 ± 0.10†	+	3.57 ± 1.06†	+	1.16 ± 0.20	−	3.65 ± 1.68†	+	1.25 ± 0.23	−	2.61 ± 0.42†	+
	CCCP	1.48 ± 0.03†	+	0.93 ± 0.17	−	0.71 ± 0.13	−	1.54 ± 0.58	−	1.74 ± 0.24†	+	2.27 ± 0.02†	+
BCECF -AM	Verapamil	0.70 ± 0.10	−	1.31 ± 0.21	−	1.18 ± 0.02†	−	1.86 ± 0.30†	+	0.79 ± 0.32	−	1.90 ± 0.05†	−
	ATP depletion	6.40 ± 1.40†	+	10.33 ± 2.60†	+	1.30 ± 0.23†	−	2.31 ± 0.53†	+	1.20 ± 0.39†	−	2.23 ± 0.20†	+
	CCCP	1.00 ± 0.30	−	7.28 ± 1.34†	+	0.63 ± 0.12	−	1.47 ± 0.24	−	0.83 ± 0.19	−	1.61 ± 0.36†	−
$DiOC_5$	Verapamil	0.73 ± 0.19	−	1.33 ± 0.16†	−	1.31 ± 0.15†	−	0.67 ± 0.13	−	1.56 ± 0.44†	−	0.86 ± 0.15	−
	ATP depletion	0.93 ± 0.11	+	1.68 ± 0.27†	+	1.42 ± 0.21†	+	3.73 ± 1.09†	+	0.99 ± 0.43	−	2.89 ± 0.24†	+
	CCCP	0.26 ± 0.09	−	0.71 ± 0.32	−	0.64 ± 0.23	−	0.49 ± 0.10	−	0.72 ± 0.51	−	0.63 ± 0.02	−
Rhodamine 123	Verapamil	1.41 ± 0.44	+	0.96 ± 0.10	−	0.99 ± 0.03	−	1.92 ± 0.22†	+	1.64 ± 0.13†	−	1.72 ± 0.27†	−
	ATP depletion	2.22 ± 0.38†	+	1.88 ± 0.18†	+	1.42 ± 0.10†	−	1.80 ± 0.12†	+	1.99 ± 0.29†	+	1.29 ± 0.20	−
	CCCP	1.12 ± 0.39	−	0.63 ± 0.09	−	1.00 ± 0.26	−	1.51 ± 0.16†	−	1.68 ± 0.16†	−	1.57 ± 0.20†	−

*The yeasts were stained with calcein-AM, BCECF-AM, rhodamine 123 or $DiOC_5$, under inhibitory conditions for PDR proteins.
[a]FCM, Results are expressed as ratio values estimated by dividing the mean fluorescence intensity for each treated sample by the mean fluorescence intensity of the control cells in the same experiment. The mean values and standard deviation of the ratio of the mean fluorescence intensities of each sample in five independent experiments are presented. †Refers to significantly positive responses ($P < 0.05$).
[b]EFM, Results are expressed as + and − responses according to the criteria described in the Materials and Methods section.

Table 2
Flow Cytometric Assays for the Detection of Active Efflux Proteins in S. cerevisiae *Mutant Strains, Stained With Calcein-AM, BCECF-AM, Rhodamine 123 or $DiOC_5$, Under Inhibitory Conditions for PDR Proteins*

Probes	Inhibitory conditions	Ratio of fluorescence intensity[a]		
		US50-18C	AD12345678	SUPERYOR
Calcein-AM	Verapamil	0.43 ± 0.17	0.42 ± 0.09	1.12 ± 0.11
	ATP depletion	1.27 ± 0.03*	0.81 ± 0.33	1.15 ± 0.06*
	CCCP	0.64 ± 0.19	0.74 ± 0.14	0.71 ± 0.18
BCECF-AM	Verapamil	0.40 ± 0.11	0.92 ± 0.07	1.46 ± 0.10*
	ATP depletion	1.24 ± 0.14*	0.72 ± 0.03	1.21 ± 0.13*
	CCCP	0.54 ± 0.06	1.08 ± 0.02	0.85 ± 0.05
$DiOC_5$	Verapamil	0.69 ± 0.10	1.08 ± 0.54	1.93 ± 0.57*
	ATP depletion	1.46 ± 0.34*	0.78 ± 0.11	2.59 ± 0.56*
	CCCP	0.84 ± 0.17	1.15 ± 0.35	1.22 ± 0.60
Rhodamine 123	Verapamil	1.44 ± 0.07*	0.41 ± 0.09	1.14 ± 0.10*
	ATP depletion	1.27 ± 0.02*	0.83 ± 0.02	1.18 ± 0.15*
	CCCP	1.01 ± 0.18	0.91 ± 0.03	1.04 ± 0.15

[a]Results are expressed as ratio values estimated by dividing the mean fluorescence intensity for each treated sample by the mean fluorescence intensity of the control sample (untreated cells) in the same experiment. The mean values and standard deviation of the ratio of the mean fluorescence intensities of each sample in five independent experiments are presented.
*Refers to significantly positive responses ($P < 0.05$).

five independent experiments. From these results, we could conclude that in a total of 72 responses (for the six yeast species tested with the four probes and three inhibitory conditions) 26% of positive responses were detected with ATP depletion, while treatment with CCCP and verapamil allowed to detect 11% and 15% positive responses, respectively. Ratio values lower or not significantly different than 1 were interpreted as negative responses. Further studies will be needed to understand why in some cases treatment under inhibitory conditions for PDR proteins lead to a decrease in the intracellular fluorescence comparatively to the control.

To further validate the proposed methodology, we used three mutant strains of *S. cerevisiae* namely, US50-18C overexpressing the main ABC transporters known (PDR5, SNQ2 and YOR1), SUPERYOR overexpressing YOR1, a gene coding for an ABC protein that confers resistance to oligomycin, and AD12345678 that is disrupted in the main ABC transporters (19). The latter strain was tested as a negative control, while the other two as positive controls for the presence of ABC proteins.

The results in Table 2 show that, for the strains US50-18C and SUPERYOR, ATP depletion gave rise to a positive response with the four probes tested. These mutant strains when treated with CCCP displayed a negative response. Treatment of SUPERYOR strain with verapamil resulted in positive responses, with exception for cells stained with calcein-AM. With strain US50-18C, the same treatment resulted in a positive response, only with the rhodamine 123 staining.

Table 3
Screening by Epifluorescence Microscopy for the Detection of Active Efflux Pumps in Pichia membranifaciens *IGC 2487,* Dekkera anomala *IGC 5133,* Pichia anomala *IGC 4121,* Rhodotorula mucilaginosa *IGC 4791,* Torulaspora delbrueckii *ISA 326,* Lodderomyces elongisporus *ISA 1421,* Saccharomycodes ludwigii *ISA 1083,* Kloeckera apiculata *ISA 1189,* Candida sheatae *IGC 3504, and* Schizosaccharomyces pombe *G2**

	Calcein-AM			BCECF-AM			Rhodamine 123			$DiOC_5$		
Inhibitory conditions	Verap.	ATP depletion	CCCP	Verap.	ATP depletion	CCCP	Verap.	ATP depletion	CCCP	Verap.	ATP depletion	CCCP
P. membra.	−	+	−	−	+	−	−	+	−	+	−	−
D. anomala	+	+	+	+	−	+	−	−	−	−	−	−
P. anomala	−	+	−	−	+	+	+	+	+	−	−	−
R. mucilaginosa	+	+	+	−	−	+	−	−	−	−	−	−
T. delbrueckii	−	−	+	+	+	+	−	+	−	−	+	+
S. pombe	+	−	−	−	−	−	+	+	+	−	+	+
L. elongisporus	−	−	+	−	−	+	+	−	+	−	+	+
S. ludwigii	+	+	+	−	−	+	−	+	+	−	+	−
K. apiculata	+	+	+	−	+	−	+	+	−	+	−	−
C. shehatae	−	+	−	−	−	−	−	−	−	−	+	+

*The yeasts were stained with calcein-AM, BCECF-AM, rhodamine 123 or $DiOC_5$, under inhibitory conditions for PDR proteins. Results are expressed as + and − responses according to the criteria described in the Materials and Methods section.

With the mutant strain AD12345678, it was not possible to observe any positive response, consistently with the disruption of the main ABC transporters (Table 2).

In general, the different responses obtained with ATP depleted and CCCP treated cells, either in the mutants or in the wild strains tested, allowed us to further discard the possible interference of ATP depletion on the MFS protein detection and vice versa of CCCP treatment on the ABC protein detection. Based on these results, the positive responses detected by ATP depletion and/or verapamil and by CCCP treatments were considered as indicative of the presence of ABC and MFS proteins, respectively.

Epifluorescence Microscopic (EFM) Assays

In a second approach, we attempted to verify if the same results could be obtained by EFM, a less objective and reliable technique but more available than FCM.

In general, for the samples where it was possible to observe a positive response by FCM, the same was also observed by EFM. In Figure 1B, we present digital images of the samples corresponding to the histograms shown in Fig. 1A. Again, and consistently with the histograms, under ATP depletion, but not with verapamil treatment, an increase in the intracellular fluorescence was detectable.

As can be seen in Table 1, the results obtained with EFM were in most of the cases in agreement with those obtained by FCM. However, some false negative (21%) and false positive (3%) responses were observed with EFM. The former can be explained by the lower sensitivity of EFM compared with FCM. On the other hand, the false positive results can be due to unspecific staining or accumulation of the fluorochromes in some organelles, most probably in the mitochondria. This intracellular compartmentation of the dye when visualized by EFM was interpreted as an increase of intracellular fluorescence, compared to the control. However, such an increase in cell fluorescence was not observed when the same sample was analyzed by FCM. Consequently, in this particular situation, EFM give rise to a misleading classification of the result as a positive response. Such fluorescence interferences could be attenuated if the microscopic observation of the cell samples was carried out in the absence of extracellular fluorochrome.

Getting a negative response by EFM cannot be interpreted as conclusive for the absence of AEP. The same conclusion can be drawn when a negative response is obtained by FCM, since the lack of detection can be related to the use of an inappropriate probe or of inhibitory conditions.

Following the results referred to above, the detection method developed by EFM was used to screen for the detection of ABC or MFS transporters in wild type strains of ten other yeast species. The results in Table 3 show, 22, 20, and 13 positive responses, indicating the presence of AEP, in cell populations treated under ATP depletion and with CCCP and verapamil, respectively, in a total of 40 responses for each treatment.

Table 4 summarizes all the results obtained for the 16 yeast species tested, classifying the proteins detected in the ABC or MFS families.

Involvement of ABC and MFS Pumps in the Efflux of Benomyl, Penconazol, and Cycloheximide

The minimum inhibitory concentration (MIC) for the antimicrobial agents, benomyl, penconazol, and cycloheximide, was estimated for the 16 yeast species under study. The results showed that among all the yeast species (data not shown), two of them (*Kluyveromyces marxianus* and *Saccharomyces cerevisiae*) exhibited resistance to one of the drugs, while they were sensitive to the other two: *K. marxianus* was resistant to cycloheximide (MIC $>$ 200 mg/l) and *S. cerevisiae* was resistant to benomyl (MIC = 100 mg/l). In the previous section, experimental evidence was presented supporting the presence in both species of ABC or MFS proteins. The possible involvement of these proteins in the resistance of *K.*

Table 4
*Number of Responses Determined by Flow Cytometry (FCM) and/or by Epifluorescence Microscopy (EFM), Indicative of the Presence of a Pump for the Several Yeast Species**

	#ABC proteins				#MFS proteins	
	ATP depletion		Verapamil		CCCP	
Yeast species	FCM	EFM	FCM	EFM	FCM	EFM
Saccharomyces cerevisiae IGC 4072	3	4	0	1	1	1
Kluyveromyces marxianus IGC 2671	4	4	1	0	1	1
Debaryomyces hansenii IGC 2968	3	1	2	0	0	0
Zygosaccharomyces bailii ISA 1307	4	4	3	2	1	0
Candida utilis IGC 2578	2	1	3	1	2	1
Issatchenkia orientalis IGC 3806	3	3	2	0	3	1
Pichia membranifaciens IGC 2487	n.d.[a]	3	n.d.	1	n.d.	0
Dekkera anomala IGC 5133	n.d.	1	n.d.	2	n.d.	2
Pichia anomala IGC 4121	n.d.	3	n.d.	1	n.d.	2
Rhodotorula mucilaginosa IGC 4791	n.d.	1	n.d.	1	n.d.	2
Torulaspora delbrueckii ISA 326	n.d.	3	n.d.	1	n.d.	3
Schizosaccharomyces pombe G2	n.d.	2	n.d.	2	n.d.	2
Lodderomyces elongisporus ISA 1421	n.d.	1	n.d.	1	n.d.	4
Saccharomycodes ludwigii ISA 1083	n.d.	3	n.d.	1	n.d.	3
Kloeckera apiculata ISA 1189	n.d.	3	n.d.	3	n.d.	1
Candida shehatae IGC 3504	n.d.	2	n.d.	0	n.d.	1

*The responses were detected under inhibitory conditions for ABC or MFS proteins and classified according to the criteria defined in material and methods. The results were taken from Tables 1 and 3.
[a]n.d., results not determined.

marxianus to cycloheximide and of *S. cerevisiae* to benomyl was investigated. For such purposes, calcein-AM and BCECF-AM were selected, the cells being stained either in the absence or in the presence of the drugs at concentrations below the respective MIC value.

Cells of *S. cerevisiae* and *K. marxianus*, when stained after incubation with benomyl and cycloheximide, respectively, displayed a significant decrease ($P < 0.05$, $n = 5$) in cell fluorescence (Fig. 2). On the other hand, none of the treatments with the other drugs seemed to alter significantly the intracellular fluorescence of both probes (Fig. 2).

The observed decrease in cell fluorescence is not consistent with the involvement of the active efflux pump(s) detected in nontreated cells, under inhibitory conditions for PDR proteins, reported in the previous section. Indeed, in that case, we should observe an increase in the intracellular fluorescence. The staining of the cells loaded with the drug should lead to an increase of the retention of the probe due to a competition with the drug for the same carrier.

Hypothetically, the observed fluorescence decrease could be related to cytoplasmatic interferences with the staining mechanism, including inhibition of the esterase activity by the intracellularly accumulated drug. Actually, the fluorescence intensity of calcein and BCECF obtained with cell free extracts of *S. cerevisiae* and *K. marxianus* was identical either in the absence or in the presence of benomyl and cycloheximide, respectively (data not shown). Finally, the observed decrease in the intracellular fluorescence could be associated to the transport of the probes to the vacuoles, which are more acidic than the cytoplasm. If that was the case, a decrease in the fluorescence of BCECF but not of calcein should be observed. Since the response presented by the two probes was similar, this hypothesis was discarded.

Therefore, these results reinforced the interpretation of the involvement of a membrane protein, able to mediate the efflux of the probe, which could be induced after incubation with the drug and responsible for the observed fluorescence decrease. Actually, when the treatment was carried out in the presence of NaF, an inhibitor of protein synthesis in *S. cerevisiae* and *K. marxianus* (data not shown), the decrease in fluorescence was not observed (Fig. 2), indicating that the cell response was dependent on de novo protein synthesis. Moreover, the possibility of interference of the drug with the staining mechanism as a consequence of a decline of the membrane permeability to the probes, was further discarded since, in that case, the same fluorescence decrease should be observed independently of the presence of NaF.

Unexpectedly, for *S. cerevisiae,* when in the same assay (incubation with NaF in the presence of benomyl) BCECF-AM was used instead of calcein-AM, a significant increase in the intracellular fluorescence was observed. This fluorescence change could suggest either an intracellular alkalization associated to the inhibition of protein synthesis and/or a competition of benomyl with BCECF(-AM) for a pump constitutively expressed. However, the first hypothesis was rejected when we obtained a fluorescence level similar to the control when the cells were incubated with NaF in the absence of benomyl (considering the standard deviations). Further studies are needed to confirm the second hypothesis.

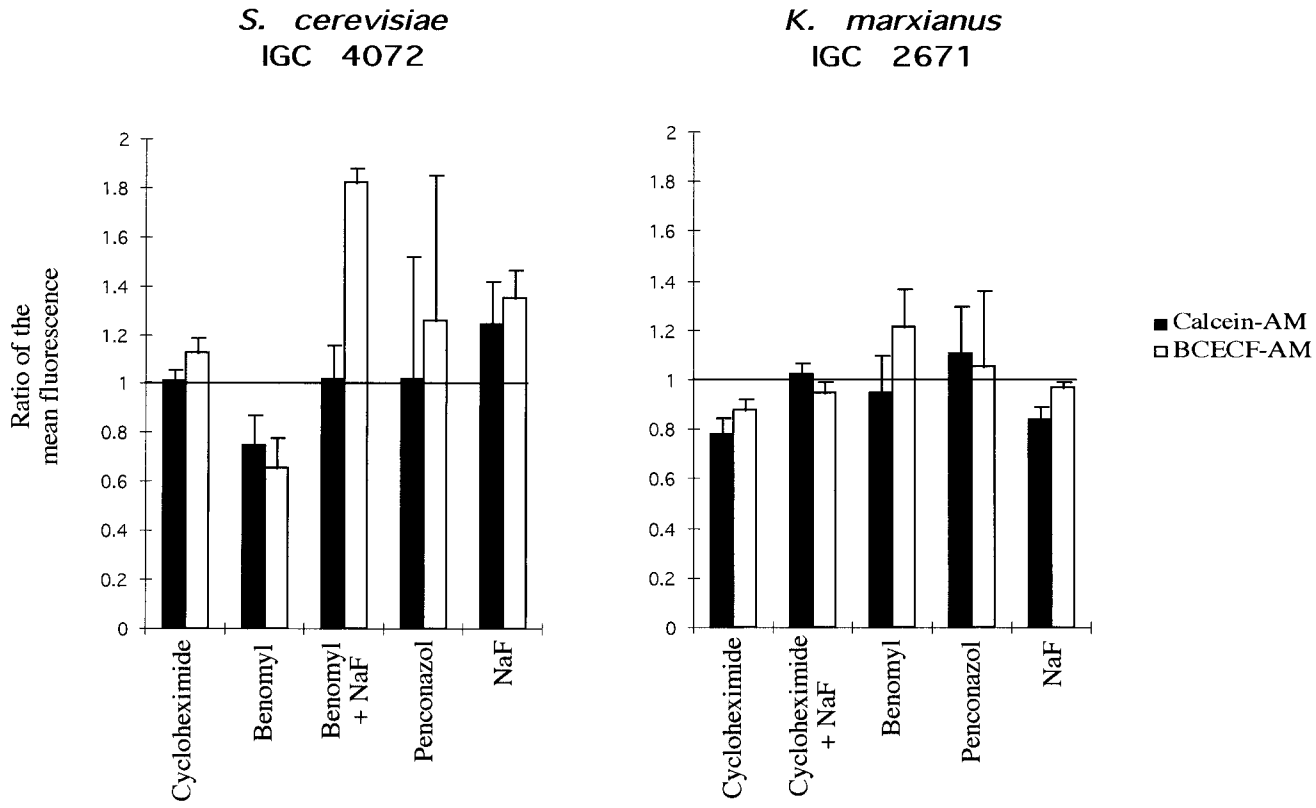

FIG. 2. Ratio of the mean fluorescence intensities of *S. cerevisiae* and *K. marxianus*, stained with calcein-AM or BCECF-AM. Before staining, the cells were incubated with the antimicrobial agents in the absence or presence of NaF. Ratio values were estimated by dividing the mean fluorescence intensity for each sample treated with the antimicrobial agents by the mean fluorescence intensity of the control cells in the same experiment. The horizontal line is the reference ratio. The results obtained refer to mean values and standard deviation of the ratio of the mean fluorescence intensities of each sample in five independent experiments ($P < 0.05$).

DISCUSSION

For all the 16 yeast species (wild type strains) studied, evidence was presented in this work for the occurrence of transport proteins (at least for one of the probes used), that were inhibited by conditions with known effects on PDR proteins. The use of rhodamine 123 and $DiOC_5$ in studies like the present one may be controversial, since the staining mechanism can be affected by different interferences. Calcein-AM and BCECF-AM, in contrast, did not give rise to false positive results (Table 1) and have much more stable fluorescence, permitting a much more reliable end point analysis. Therefore the latter probes appear more adequate for this kind of studies.

Concern with the detection of eventual artifacts due to pH changes was taken. Actually, it is known that intracellular pH changes can alter fluorescence either by shifting the emission maxima or by altering drug transport and retention (16). Although intracellular pH changes appear unlikely, since the extracellular buffer used was PBS (pH = 7.2), BCECF-AM (pH-sensitive probe) and calcein-AM (pH-insensitive probe) were selected to exclude this hypothesis. In most cases, identical responses were obtained with both probes either by EFM or FCM. As is shown in Tables 1 and 3, in a total of 48 responses (obtained with three inhibitory conditions in 16 species), 30 responses coincident for the two probes were obtained by EFM, while by FCM, 10 coincident responses were obtained in a total of 18. These results refuted the possibility that the pH-sensitive probe was detecting pH changes instead of functional AEP. Inconsistent results for the same yeast species with those two probes can be explained by the presence of a pump with specificity only for one of the probes.

If we rely on the results obtained by FCM in *Saccharomyces cerevisiae,* the transport of esterified (acetoxymethyl ester) and/or nonesterified forms of BCECF and calcein, as well as of rhodamine 123, seems to be inhibited by ATP depletion. Treatment with CCCP only resulted in an inhibition of calcein/calcein-AM transport. These results could indicate the presence of PDR proteins both from ABC and/or MFS families that have already been reported in *S. cerevisiae* (3, 6, 7). The responses that were detected in the present work could be given by one or more of the 18 putative ABC proteins detected by in silico analysis (7, 8). Also in *S. cerevisiae*, by analysis in silico, at least 23 putative proteins, belonging to the MFS family have been described (7,8). From this protein family, only the ATR1 (SNQ1) and SGE1 (NOR1), which confer resis-

tance to crystal violet, have been physiologically characterized (3,5).

For the yeast *Schizosaccharomyces pombe*, the results obtained by EFM showed that, in the presence of CCCP and in the absence of ATP, the efflux of rhodamine 123 and $DiOC_5$ was inhibited. In this species, treatment with verapamil only inhibited the transport of rhodamine 123 and calcein-AM/calcein. Again, these results as a whole indicate the presence of one or more proteins belonging to both ABC and MFS families. Although these results were obtained by EFM, a less reliable technique, they are in agreement with previous publications (1,5).

In the present study, an arbitrary cut-off of 5% or more of cells displaying level 2 fluorescence was used to classify the response of the cell population as positive. In all the cell populations classified as displaying a positive response, the number of cells displaying level 2 fluorescence was significantly different from that in the control ($P < 0.05$). However, in some of the cell populations with a negative response it was detected a small number of cells displaying level 2 fluorescence. Although, this might be of biological importance since those rare subpopulations may lead to the predominance of resistant cells in the total population, it was not taken into account in the present work.

Until now, membrane proteins possibly involved in PDR, have not been reported for any of the other fourteen yeast species studied in this work. Nevertheless, our results led us to predict the presence in these yeast species of proteins belonging either to ABC or MFS families, therefore suggesting that the presence of AEP may be generalized in many other yeast species. The physiological role of such cell attribute and its possible involvement on drug cell resistance mechanisms, remain to be elucidated. Besides the ability to transport the fluorescent probes tested, the nature of other possible substrates of AEP (e. g. ions or toxic subproducts of cell metabolism) is unknown.

In the light of the results obtained, it was not possible to point out an universal probe for the detection of AEP. The probe to be selected will depend on the yeast species and on the particular drug used. However, the screening method developed enabled us to ascertain the existence of transporters for the probes used, in all the species studied, belonging either to the ABC (inhibited by verapamil and/or ATP depletion) or MFS (inhibited by CCCP) families. The lack of consistency between the results obtained with ATP depleted cells and verapamil treated cells, can be explained by the different sensitivity of yeast ABC proteins to verapamil.

Regarding the possible involvement of AEP in the transport of the antimicrobial agents tested, the results obtained with calcein-AM and BCECF-AM showed that in *S. cerevisiae* the presence of benomyl, but not cycloheximide and penconazol, induced a significant decrease in the intracellular fluorescence. This result is consistent with the resistance of this species to benomyl evaluated by the MIC. For *Kluyveromyces marxianus*, cells treated with cycloheximide, but not with benomyl or penconazol, also exhibited a decrease in fluorescence comparatively with untreated cells (Fig. 2). This was again consistent with the higher resistance of that species to cycloheximide evaluated by MIC. The hypothesis of fluorescence decrease due to interference of the drugs in the esterase activity or other cytoplasmatic activity, as well as probe uptake by the vacuoles were discarded. Rather, it appears that protein induction after incubation with a drug for which the species exhibits resistance occurs in both species. This interpretation was reinforced by the results obtained in the presence of NaF.

Our results also suggest that, in the yeast species studied and at least for the antimicrobial agents tested, one of the mechanisms underlying drug resistance could be the induction or overexpression of AEP by the drug. These pumps would transport the drug out of the cell, hence reducing their toxicity. On the other hand, the yeast sensitivity to a specific drug might be due to the absence of pump induction or overexpression and hence drug retention.

The occurrence of cross resistance typical of the PDR phenotype in the yeast species studied was not evaluated. For such a purpose, further studies including the use of radiolabeled drugs will be carried out.

In summary, the results presented in this work suggest that the EFM, although less sensitive than FCM, can be used as a preliminary assay to distinguish clear positive cases, in particular using the probes calcein-AM and BCECF-AM. This preliminary assay could be helpful, namely, for laboratories without a flow cytometer, specially in the food industry, since these PDR proteins can also be involved in the resistance to food preservatives (10). Another advantage of the EFM technique is the use of lower sample volumes, which makes it a more economical analysis. With the recent implementation of fluorescence quantitative microscopy systems, the EFM developed might emerge as an efficient alternative to FCM. On the other hand, the FCM developed in the present study is undoubtedly the method of choice, allowing quantitative and objective analysis of large and heterogeneous populations, hence constituting a powerful tool for the rapid screening of ABC and MFS transporter activity in yeast. Assuming that some of the AEP detected in the wild strains are PDR proteins, our results point to an innate resistance of these microorganisms to a great variety of unrelated drugs. This knowledge can have important practical implications either from a clinical, agricultural or biotechnological point of view.

ACKNOWLEDGMENTS

We are grateful to Professor Manuel T. Silva for his critical reading of the manuscript and all the helpful comments and suggestions. We thank Professor André Goffeau who kindly provided us the *S. cerevisiae* mutant strains. We thank Mr. Alexandre Salvador and Ms. Cristina Freitas for their helpful assistance in the acquisition of flow cytometric data.

LITERATURE CITED

1. Balzi E, Goffeau A. Multiple or pleiotropic drug resistance in yeast. Biochim Biophys Acta 1991;1073:241–252.
2. Lewis K, Hooper DC, Ouellette M. Multidrug resistance pumps provide broad defense. ASM News 1998;63:605–610.
3. Ehrenhofer-Murray AE, Würgler FE, Sengstag C. The *Saccharomyces cerevisiae* SGE1 gene product: a novel drug-resistance protein within the major facilitator superfamily. Mol Gen Genet 1994;244:287–294.
4. Fath MJ, Kolter R. ABC transporters: bacterial exporters. Microbiol Rev 1993;57:995–1017.
5. Goffeau A, Park J, Paulsen IT, Jonniaux J, Dinh T, Mordant P, Saier MH Jr. Multidrug-resistant transport proteins in yeast: complete inventory and phylogenic characterization of yeast open reading frames within the major facilitator superfamily. Yeast 1997;13:43–54.
6. van der Rest M, Kamminga AH, Nakano A, Anraku Y, Poolman, Konings N. The plasma membrane of *Saccharomyces cerevisiae*: Structure, function and biogenesis. Microbiol Rev 1995;52:30004–322.
7. André B. An overview of membrane transport proteins in *Saccharomyces cerevisiae*. Yeast 1995;11:1575–1611.
8. Oullete M, Lewis K, Hooper D. Eukaryotic microbial multidrug resistance pumps. ASM News 1998;63:664–667.
9. Peever TL, Milgroom MG. Fungicide resistance — Lessons for herbicide resistance management? Weed Technol 1995;9:840–849.
10. Piper P, Mahé Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, Mühlbauer M, Coote P and Kuchler K. The Pdr12 ABC transporter is required for development of weak organic acid resistance in yeast. EMBO J 1998;17:4257–4265.
11. Ordónez JV, Wehman NM. Amphotericin B susceptibility of *Candida* species assessed by rapid flow cytometric membrane potential assay. Cytometry 1995;22:154–157.
12. Feller N, Broxterman HJ, Wahrer DCR, Pinedo HM. ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): No inhibition by intracellular glutathione depletion. FEBS Letters 1995; 368:385–388.
13. Holló Z, Homolya L, Davis CW, Sarkadi B. Calcein accumulation as a fluorometric functional assay of the multidrug transporter. Biochim Biophys Acta 1994;1191:384–388.
14. Homolya L, Holló Z, Germann UA, Pastan I, Gottesman MM, Sarkadi B. Fluorescent cellular indicators are extruded by multidrug resistance proteins. J Biol Chem 1993;268:21493–21496.
15. Wall DM, Hu XF, Zalcberg JR, Parkin JD. Rapid functional assay for multidrug resistance in human tumor cell lines using the fluorescent indicator fluo-3. J Natl Cancer Inst 1991;83:206–207.
16. Krishan A. Rapid determination of cellular resistance-related drug efflux in tumour cells. In: Darzynkiewicz Z, Robinson P, Crissman HA, editors. Methods in cell biology, vol. 42. San Diego: Academic Press; 1994. p 21–30.
17. Wadkins RM, Houghton PJ. Kinetics of transport of dialkyloxacarbocyanines in multidrug-resistant cell lines overexpressing P-glycoprotein: interrelationship of dye alkyl chain length, cellular flux, and drug resistance. Biochemistry 1995;34:3858–3872.
18. Haugland RP: Handbook of fluorescent probes. Eugene, OR: Molecular Probes Inc.; 1996.
19. Decottignies A, Grant AM, Nichols JW, de Wet H, McIntosh, Goffeau A. ATPase and multidrug transport activities of the overexpressed yeast ABC Yor1p. J Biol Chem 1998;273:12612–12622.
20. van Uden N. Transport-limited fermentation and growth of *Saccharomyces cerevisiae* and its competitive inhibition. Arch Microbiol 1967;58:155–168.
21. Prudêncio C, Sansonetty F, Côrte-Real M. Flow Cytometric Assessment of cell structural and functional changes induced by acetic acid in the yeast, *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*. Cytometry 1998;31:307–313.
22. Beauvoit B, Rigoulet M, Raffard G, Canioni P, Guérin B. Differential sensitivity of the cellular compartments of *Saccharomyces cerevisiae* to protonophoric uncoupler under fermentative and respiratory energy supply. Biochemistry 1991;30:11212–11220.
23. Noshiro A, Purwin C, Laux M, Nicolay K, Scheffers A, Holzer H. Mechanism of stimulation of endogenous fermentation in yeast by carbonyl cyanide m-chloropheyilhydrazone. J Biol Chem 1987;262: 14154–14157.