THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 20, Issue of May 15, pp. 12612–12622, 1998

Printed in U.S.A.

ATPase and Multidrug Transport Activities of the Overexpressed Yeast ABC Protein Yor1p*

(Received for publication, October 24, 1997, and in revised form, March 12, 1998)

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The *Saccharomyces cerevisiae* genome encodes 15 full-size ATP binding cassette transporters (ABC), of which *PDR5*, *SNQ2*, and *YOR1* are known to be regulated by the transcription factors Pdr1p and Pdr3p (pleiotropic drug resistance). We have identified two new ABC transporter-encoding genes, *PDR10* and *PDR15*, which were up-regulated by the *PDR1–3* mutation. These genes, as well as four other ABC transporter-encoding genes, were deleted in order to study the properties of Yor1p. The *PDR1–3* gain-of-function mutant was then used to overproduce Yor1p up to 10% of the total plasma membrane proteins. Overexpressed Yor1p was photolabeled by [γ-^{32}P]2′,3′-*O*-(2,4,6-trinitrophenyl)-8-azido-ATP ($K_{0.5}$ = 45 μM) and inhibited by ATP (K_D = 0.3 mM) in plasma membranes. Solubilization and partial purification on sucrose gradient allowed to detect significant Yor1p ATP hydrolysis activity (~100 nmol of $P_i \cdot min^{-1} \cdot mg^{-1}$). This activity was phospholipid-dependent and sensitive to low concentrations of vanadate (I_{50} = 0.3 μM) and oligomycin (I_{50} = 8.5 μg/ml).

***In vivo*, we observed a correlation between the amount of Yor1p in the plasma membrane and the level of resistance to oligomycin. We also demonstrated that Yor1p drives an energy-dependent, proton uncoupler-insensitive, cellular extrusion of rhodamine B. Furthermore, cells lacking both Yor1p and Pdr5p (but not Snq2p) showed increased accumulation of the fluorescent derivative of 1-myristoyl-2-[6-*(NBD)aminocaproyl*]phosphatidylethanolamine.**

Despite their different topologies, both Yor1p and Pdr5p mediated the ATP-dependent translocation of similar drugs and phospholipids across the yeast cell membrane. Both ABC transporters exhibit ATP hydrolysis *in vitro*, but Pdr5p ATPase activity is about 15 times higher than that of Yor1p, which may indicate mechanistic or regulatory differences between the two enzymes.

The yeast *YOR1*[1] gene confers oligomycin resistance on overexpression in a 2-μm plasmid (1). Its nucleotide sequence reveals an ORF of 1477 amino acids encoding an ABC protein highly homologous to mammalian transporters such as the multidrug resistance-conferring enzyme MRP (BLAST (see Ref. 2) sequence homology score: $p = e^{-228}$), the organic anion transporter cMOAT ($p = e^{-216}$), the sulfonylurea receptor ($p = e^{-164}$), and the cystic fibrosis transmembrane conductance regulator CFTR ($p = e^{-132}$). Yor1p is a "full-size" ABC transporter with the topology $(TM\text{-}NBF)_2$ (3, 4). It consists of two homologous halves, with each containing a putative ATP-binding domain (NBF) and a transmembrane domain of six membrane spans (TM). Cui *et al.* (5) showed that Yor1p confers resistance to a series of drugs including reveromycin A and suggested that Yor1p may be involved in the cellular efflux of organic anions including the fluorescent dye rhodamine B. They also showed that incubation with reveromycin A increases the *YOR1* mRNA level. The transcription of *YOR1* is controlled by the homologous pair of transcription factors Pdr1p/Pdr3p. The level of *YOR1* transcription is decreased by the deletion of either *PDR1* or *PDR3* and increased in the presence of the gain-of-function *PDR1* alleles (1).

In this paper, we have investigated the transport activity of Yor1p. Building on previous studies, which indicated that the $(TM\text{-}NBF)_2$-type Yor1p, together with the $(NBF\text{-}TM)_2$-type Pdr5p and Snq2p ABC transporters, are overexpressed in the *PDR1–3* mutant plasma membrane (6–8), the *PDR1–3* mutant has been used as a tool that enhances the Yor1p protein level. As another investigative tool, we constructed a set of isogenic strains, in the *PDR1–3* mutant, with multiple deletions of homologous ABC genes since, in situations where two or more proteins located in the same subcellular compartment share a common substrate, a clear phenotype is only seen when all the corresponding genes are deleted, as illustrated by the work of Mahé *et al.* (9), who showed that Pdr5p and Snq2p have an overlapping transport capacity for steroids. We deleted the yeast ABC transporter-encoding genes known or suspected to

* This work was supported by grants from the Service des Affaires Scientifiques, Techniques et Culturelles, Pôles d'Attraction Interuniversitaires, the Fonds National de la Recherche Scientifique, and the Foundation for Research Development of South Africa, and by NATO Collaborative Exchange Research Grant CRG940493 and National Institutes of Health Grant GM52410 (to J. W. N.) and a National Institutes of Health minority predoctoral fellowship (to A. M. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: YOR1, yeast oligomycin resistance; ORF, open reading frame; ABC, ATP-binding cassette; PDR, pleiotropic drug resistance; Pma1p, H^+-plasma membrane ATPase; PDRE, Pdr1p/Pdr3p response element; M-C_6-NBD-PE, 1-myristoyl-2-[6-*(NBD)aminocaproyl*]phosphatidylethanolamine; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; SDC, synthetic complete glucose medium; YD, rich glucose medium; YG, rich glycerol medium; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; TNP, trinitrophenyl; TNP-8-azido-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-8-azido-adenosine triphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); TM, transmembrane; NBF, nucleotide-binding fold; CFTR, cystic fibrosis transmembrane regulator.

TABLE I
Yeast strains used in this study

Strains	Genotype	Reference
IL125–2B	*MATα, PDR1, his1*, parental of the DRI9-T8 *PDR1–3* mutant	Balzi *et al.* (24)
2229–5C	*MAT***a**, *PDR1, ura3*	Balzi *et al.* (24)
US50–18C	*MATα, PDR1–3, ura3, his1*, obtained by cross between DRI9-T8 and 2229–5C	Balzi *et al.* (24)
D1–3/3	US50–18C disrupted by Δ*pdr1::URA3*	Balzi *et al.* (24)
AD1[a]	Δ*yor1::hisG*	This study
AD2[a]	Δ*snq2::hisG*	This study
AD3[a]	Δ*pdr5::hisG*	This study
AD4[a]	Δ*pdr10::hisG*	This study
AD5[a]	Δ*pdr11::hisG*	This study
AD6[a]	Δ*ycf1::hisG*	This study
AD12[a]	Δ*yor1::hisG,* Δ*snq2::hisG*	This study
AD23[a]	Δ*snq2::hisG,* Δ*pdr5::hisG*	This study
AD13[a]	Δ*yor1::hisG,* Δ*pdr5::hisG*	This study
AD123[a]	Δ*yor1::hisG,* Δ*snq2::hisG,* Δ*pdr5::hisG*	This study
AD1234[a]	Δ*yor1::hisG,* Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG*	This study
AD23456[a]	Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG*	This study
AD123456[a]	Δ*yor1::hisG,* Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG*	This study
AD124567[a]	Δ*yor1::hisG,* Δ*snq2::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG,* Δ*pdr3::hisG*	This study
AD234567[a]	Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG*, Δ*ycf1::hisG*, Δ*pdr3::hisG*	This study
AD1234567[a]	Δ*yor1::hisG,* Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG,* Δ*pdr3::hisG*	This study
AD234568[a]	Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG,* Δ*pdr15::hisG*	This study
AD1234568[a]	Δ*yor1::hisG,* Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG,* Δ*pdr15::hisG*	This study
AD2345678[a]	Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG,* Δ*pdr3::hisG,* Δ*pdr15::hisG*	This study
AD12345678[a]	Δ*yor1::hisG,* Δ*snq2::hisG,* Δ*pdr5::hisG,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG,* Δ*pdr3::hisG,* Δ*pdr15::hisG*	This study
SUPERYOR[a]	Δ*yor1::hisG,* Δ*snq2::hisG,* Δ*pdr5::PDR5PROM-YOR1-PDR5STOP,* Δ*pdr10::hisG,* Δ*pdr11::hisG,* Δ*ycf1::hisG,* Δ*pdr3::hisG*	This study
FY1679–28C	*MAT***a**, *PDR1, PDR3, ura3–52, trp1, leu2-Δ1, his3-Δ200, GAL2*	C. Fairhead, B. Dujon
FY1679–28C/EC	FY1679–28C disrupted by Δ*pdr1::TRP1*	Delaveau *et al.* (44)
FY1679–28C/TD	FY1679–28C disrupted by Δ*pdr3::HIS3*	Delaveau *et al.* (44)
FY1679–28C/TDEC	FY1679–28C/TD disrupted by Δ*pdr1::TRP1*	Delaveau *et al.* (44)

[a] In this study, the deletions have been done in the *PDR1–3* US50–18C strain.

be controlled by the transcription factors Pdr1p and Pdr3p. The *YCF1* gene, which encodes a glutathione *S*-conjugate pump (10), was also deleted. The multiply deleted mutants have allowed the demonstration that Yor1p and Pdr5p share several substrates, which include fluorescent phosphatidylethanolamine, rhodamine B, and oligomycin, even though previous studies had concluded that Pdr5p was not involved in oligomycin resistance (11, 12). The pumping of phospholipids is in line with reports of "flippase" activity with several human and mouse ABC transporters (13–16). It is also in agreement with the defective phospholipid accumulation of two new mutant yeast alleles, *PDR1–11* and *pdr3–11* (17). Construction of a stronger Yor1p-overexpressing strain allowed us to detect vanadate- and oligomycin-sensitive ATPase activity associated with Yor1p while no UTPase activity was detectable. Despite similar nucleotide binding specificities and transport capacities for Yor1p and Pdr5p, the Yor1p enzyme showed 15 times lower levels of ATP hydrolysis rate than Pdr5p.

EXPERIMENTAL PROCEDURES

Chemicals—*n*-Dodecyl β-D-maltoside was purchased from Boehringer Mannheim; bovine serum albumin, 2-deoxy-D-glucose, and oligomycin were from Sigma; *o*-nitrophenyl-β-D-galactopyranoside and rhodamine B were from Merck; molecular weight markers (range 53,000–212,000 Da) and *Taq* polymerase were from Amersham Pharmacia Biotech; yeast extract was purchased from both KAT and Difco. M-C_6-NBD-PE, dioleoylphosphatidylcholine, and *N*-rhodamine-dioleoylphosphatidylethanolamine were from Avanti Polar Lipids Inc. (Alabaster, AL). TNP-8-azido-ATP and the γ-^{32}P species were synthesized as described previously (18, 19). All other reagents were of analytical grade.

Yeast Strains—The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. Multiple deletions were performed sequentially in the US50–18C *PDR1–3* strain by repeated use of the *hisG-URA3-hisG* cassette followed by selection of the *ura3* auxotrophic marker with 5-fluoroorotic acid (20). The plasmids for the deletion of *PDR5* (12), *SNQ2*, and *YOR1* (1) genes were kindly provided by W. S. Moye-Rowley (Department of Physiology and Biophysics, University of Iowa, Iowa City, IA). For the deletion of *PDR10*, *PDR11*, *PDR15*, *PDR3*, and *YCF1* genes, we amplified fragments of the promoter and the ORF 3′-end of each gene (Table II). The gene promoters were cloned into the *Eco*RI/*Bam*HI sites of pSK, the ORF ends were cloned into the *Bam*HI/*Xba*I sites (except for the *PDR11* gene; see Table II), and the *Bam*HI/*Bgl*II *hisG-URA3-hisG* cassette was cloned into the *Bam*HI site.

Linearized fragments of the plasmids were used to transform the yeast for deletion of *PDR5* (*Bam*HI, *Sal*I), *SNQ2* (*Sst*I, *Sal*I), *YOR1* (*Sac*II/*Bam*HI), *PDR10* (*Eco*RI/*Not*I), *PDR11* (*Kpn*I/*Not*I), *PDR15* (*Eco*RI/*Not*I), YCF1 (*Eco*RI/*Not*I), and *PDR3* (*Eco*RI/*Not*I). The deletion of *PDR5*, *SNQ2*, and *YOR1* genes was checked by Southern blotting analysis. Deletion of the other genes was checked by PCR screening: yeast cells from a 1.5-ml overnight rich glucose medium (YD: 2% yeast extract, 5.8% glucose) culture were washed, resuspended in 200 μl of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA and broken by vortexing for 2 min with glass beads and 300 μl of phenol-chloroform (50:50). After a second phenol-chloroform extraction and a washing with 1 volume of ether, 2 μl of cell extract were used for the PCR analysis. Recovery of the *ura3* marker was monitored by plating onto 5-fluoroorotic acid 10^7 cells of an overnight YD culture. The resistant cells were screened by PCR as described above. The deletions of *PDR5*, *SNQ2*, and *YOR1* were monitored by Southern analysis.

The SUPERYOR strain was constructed as follows: 1129 bp of the *PDR5* promoter (from position −1141 to −12) and 505 bp of the *PDR5* ORF end (from +4026 to +4531) were amplified by PCR using the following primer sequences: 5′-CCATCGATGGTCCGTCATATACG and 5′-CCCCCGGGTCTTTCGAACGAGCG (promoter) and ′-CCCCCGGG-TCTGCTTGTCATTTC and 5′-GCTCTAGACTTGGAGAGTTTACC (ORF end) to which restriction sites had been added (underlined sequences). The PCR fragments were cut by either *Cla*I and *Sma*I (*PDR5* promoter) or *Xba*I and *Sma*I (*PDR5* end) and cloned into *Cla*I-*Xba*I-cleaved pSK (pSK::*PDR5PROM-PDR5STOP*). The *YOR1* DNA was prepared as follows. the pEGH452 cosmid (kind gift of Hervé Tettelin) containing 35.4 kb of *S. cerevisiae* chromosome VII was cut by *Mlu*I. The 5.7-kb fragment, containing the *YOR1* ORF, was blunted with Klenow

TABLE II
PCR amplifications for disruption constructs

Gene	First primer[a,b]	Second primer	Restriction sites[b]	Size[c]
				bp
PDR10	P: 5′-GGAATTCCTGCCTGACTTACAGATAC	5′-CGGGATCCCGAGTTTGAACTTGAGGGC	*Eco*RI/*Bam*HI	709
	S: 5′-CGGGATCCCGGTCAAATGAGGAGCAGAAG	5′-GCTCTAGAGCATCCATCTACGTGAATACG	*Bam*HI/*Xba*I	912
PDR15	P: 5′-GGAATTCCGCCCAGCCTTTTATACCT	5′-CGGGATCCCTTGAGCTCGAGCTCCG	*Eco*RI/*Bam*HI	952
	S: 5′-CGGGATCCCGGAGGTTGTAGGTGCCG	5′-GCTCTAGAGCCACACCAAGGGCTAAT	*Bam*HI/*Xba*I	845
PDR11	P: 5′-GCTCTAGAGCCAGCCAAAATAGCGTCAAAT	5′-CGGGATCCCGAACGAAAACGGACGGAGAAG	*Xba*I/*Bam*HI	970
	S: 5′-GGAATTCCGCAGACGGTGTATGTGTT	5′-CGGAATTCCGAAAAGAGGTGGTGAGTGTGT	*Eco*RI/*Eco*RI	1137
YCF1	P: 5′-CGGGATCCCGCCCTTCAGGAGATCTACA	5′-GGAATTCCATTCATGTATGATCTCCAG	*Eco*RI/*Bam*HI	827
	S: 5′-CGGGATCCCGGTCGTTTAGACTCTATTAC	5′-GCTCTAGAGCCGATGGTCAAGATAGTTC	*Bam*HI/*Xba*I	1041
PDR3	P: 5′-CGGGATCCCGCACTTT**CAT**TGCGGTC	5′-GGAATTCCGGGCTTGGATACCTTCGC	*Eco*RI/*Bam*HI	523
	S: 5′-CGGGATCCCGCATCTTGGGTGCTGTT	5′-GCTCTAGAGCCACTGCTGACAAACTC	*Bam*HI/*Xba*I	907

[a] The primer sequences for the PCR amplifications were chosen in the promoter (P) and in the ORF 3′-end (S) sequence of each gene.
[b] Restriction sites were added for the cloning (underlined sequences).
[c] Size of the PCR-amplified DNA fragments.

and cloned into *Eco*RV-cleaved pSK. The pSK::*YOR1* plasmid was cut by *Nhe*I and *Sna*BI to generate a 5-kb fragment, which was purified, blunted with Klenow, and cloned into *Sma*I-cleaved-pSK::*PDR5PROM-PDR5STOP*. The pSK::*PDR5PROM-YOR1-PDR5STOP* resulting plasmid was cut by *Cla*I and *Not*I and used to transform the AD124567 strain. The transformed yeasts were plated on 0.75 μg/ml oligomycin. Replacement of the *PDR5* chromosomic allele by the modified *YOR1* gene was checked by PCR using primer sequences corresponding to both the *YOR1* and the *PDR5* ORFs.

Transcriptional Activity of the Yeast ABC Genes—Transcriptional activity of the yeast ABC genes was measured after transformation of the FY1679–28C/EC (*pdr1*Δ) strain with 2 centromeric plasmids. The first plasmid carries either no allele (pRS315), or the wild type (pRS315::*PDR1*), or the mutant allele (pRS315::*PDR1–3*) of the *PDR1* gene as described previously (21). The second plasmid (pSEYC102) bears a translational fusion of the ABC gene promoters and the *Escherichia coli lacZ* gene. The *PDR5-lacZ* fusion (12), the *SNQ2-lacZ* fusion (8), and the *YOR1-lacZ* construct (1) have been described previously. For the construction of the *PDR11-lacZ* fusion, 687 bp of the *PDR11* gene, including 633 bp of the promoter, were amplified by PCR using the primers 5′-CGGGATCCCGATCAAAGGTGACTGAAGC and 5′-GGAATTCCACTTTGACGCCCCTTTATGC (restriction sites are underlined). The PCR-amplified promoter was cloned into a *Bam*HI-*Eco*RI-cleaved pSEYC102 vector. The same strategy was used to clone 680 bp of the *PDR10* promoter using the primer sequences: 5′-GGAATTCCTGCCTGACTTACAGATAC and 5′-CGGGATCCCA**CAT**CCTAACAACTATG and 952 bp of the *PDR15* gene, including 897 bp of the promoter, with the primer sequences: 5′-GGAATTCCGCCCAGCCTTTTATACCT and 5′-CGGGATCCCTTGAGCTCGAGCTCCG. β-Galactosidase activity was measured as described by Sambrook *et al.* (22) on cell extracts from yeast grown in synthetic complete glucose medium (SDC: 0.7% yeast nitrogen base, 2% glucose, complete amino acid supplements) lacking uracil and leucine.

Complementation of Deleted yor1 Strains by the YOR1 Gene on a Centromeric Plasmid—The pEGH452 cosmid was cut by *Mlu*I. The 5.7-kb restriction fragment containing *YOR1*, 0.4 kb of the gene promoter including the Pdr1p/Pdr3p binding site, and 0.9 kb downstream of the STOP codon was blunted with Klenow, cloned into the *Sma*I site of pRS316 (pRS316::*YOR1*) and used to transform the AD13 and AD1234568 strains for testing complementation of oligomycin and rhodamine B resistance on plates.

Isolation of Plasma Membranes—Plasma membranes were isolated from the particulate fraction pelleted at 15,000 × *g* for 40 min after selective precipitation of mitochondria at pH 5.2 (23).

Solubilization of Plasma Membrane Proteins and Centrifugation on a Continuous Sucrose Gradient—AD1234567 and SUPERYOR plasma membrane proteins (5 mg/ml) were solubilized with 0.2% (w/v) *n*-dodecyl-β-D-maltoside in the presence of 8 mM Tris-HCl, pH 7.5 (7) and the solubilized proteins (7 mg) were separated on a 33.3-ml linear sucrose gradient as described previously (8).

Nucleoside Triphosphatase Assays—Nucleotide hydrolysis of plasma membrane-enriched fractions was measured by incubation at 35 °C in a final volume of 100 μl containing 6 mM NTP, 7 mM $MgSO_4$, 10 mM NaN_3, 50 mM MES, 50 mM MOPS, and 50 mM Tris, adjusted to the right pH with either HCl or NaOH. In the sucrose gradient fractions, the NTP hydrolysis was measured in the presence of 6 mM NTP, 7 mM $MgCl_2$, 50 mM MES, 50 mM MOPS, 50 mM Tris (pH adjusted with either HCl or NaOH) and 150 μg/ml asolectin. Assays were carried out as described previously (7).

Photolabeling—Plasma membrane preparations (0.075 or 0.15 mg/ml) in 25 mM MES/tetramethyl ammonium hydroxide, pH 6.0, 1 mM $MgCl_2$, 5 mM EGTA, and 20% (v/v) glycerol, containing the indicated amount of [γ-^{32}P]TNP-8-azido-ATP and ATP were photolabeled using a xenon lamp and toluene filters as described previously (18). The samples were processed, subjected to SDS-PAGE and autoradiography, and the radioactivity quantitated by imaging (19).

Drug Resistance Assays—The strains were tested for drug resistance on solid medium containing 1% yeast extract (Difco) and either 2% glucose and rhodamine B, or 4% glycerol and oligomycin dissolved in ethanol. Drug resistance assays after yeast transformation with the *YOR1* gene-containing plasmid were performed on solid synthetic media containing either 2% glucose or 4% glycerol plus 0.7% yeast nitrogen base supplemented with amino acids lacking uracil. A 32-well replicator was used for plating and drug resistance was scored after 3–4 days at 30 °C as described previously (24, 25).

Rhodamine B Fluorescence Measurements in Intact Cells—Four-ml YD cultures were inoculated with ~50 × 10^6 cells from an overnight preculture and incubated for 3 h at 30 °C. Culture aliquots of 750 μl (~40 × 10^6 cells/ml) were washed three times with buffer A (50 mM Hepes-NaOH, pH 7.0), resuspended in 2 ml of buffer A containing 100 μg/ml rhodamine B and either 5 mM D-glucose or 5 mM 2-deoxy-D-glucose and incubated for 2 h at 28–30 °C. A 1.5-ml sample was pelleted and washed three times with buffer A. The cell pellet resuspended in 800 μl of water was maintained on ice until cell fluorescence was measured using an SLM Aminco 48000 S spectrofluorimeter. The excitation wavelength was 555 nm (slit of 4 nm), and the emission wavelength was 575 nm (slit of 4 nm).

In rhodamine B extrusion experiments, cells from 3.5 ml of YD culture (~40 × 10^6 cells/ml) were washed three times with buffer A, incubated in 2 ml of buffer A containing 100 μg/ml rhodamine B and 5 mM 2-deoxy-D-glucose for 2 h at 28–30 °C and then washed three times with buffer A and resuspended in 1.5 ml of buffer A. Rhodamine B extrusion was measured in response to either 10 mM D-glucose, 4% ethanol, or no carbon source (control). At the indicated times, the fluorescence of 300 μl of both the cell-free supernatant and the cell pellet were measured.

Yeast Cell Labeling with NBD-phosphatidylethanolamine—Lipid vesicles including 50 μM total lipids comprising M-C_6-NBD-PE (40 mol%), dioleoylphosphatidylcholine (58 mol%), and *N*-rhodamine-dioleoylphosphatidylethanolamine (2 mol%) were prepared as described previously (17). Phospholipid concentrations were determined by the lipid phosphorus assay (26). For internalization of M-C_6-NBD-PE, yeast cells were grown overnight in SDC at 30 °C, diluted, and allowed to grow to an A_{600} of 0.2–0.3. Donor vesicles containing the fluorescent lipids were added to the yeast cells and incubated for 30 min at 37 °C. Cells were washed three times with ice-cold $SCNaN_3$ (SDC lacking glucose but containing 2% sorbitol and 20 mM sodium azide) prior to analysis by fluorescence microscopy and flow cytometry.

Fluorescence Microscopy—Fluorescence microscopy was performed on a Zeiss Axiovert microscope equipped with barrier filters that allowed no detectable crossover of NBD and rhodamine fluorescence. The fluorescence image was enhanced with a VE1000-SIT image-intensify-

TABLE III
Measurement of PDR5, YOR1, SNQ2, PDR10, PDR15, and PDR11 transcription activity mediated by pdr1Δ, PDR1, and PDR1–3 alleles

	Pdr1/3p-binding consensus[a]	pRS315[b]	pRS315::*PDR1*	pRS315::*PDR1–3*
PDR5-lacZ	$^{-492}$**TTCCG**C**GGA**A^{-483} $^{-376}$**TTCCG**T**GGA**A^{-367} $^{-314}$C**TCCGCGGA**A^{-305}	29.2 ± 3	147 ± 48	2473 ± 550
YOR1-lacZ	$^{-402}$**TTCC**AC**GGA**A^{-393}	15.6 ± 7	21.5 ± 11	530 ± 119
SNQ2-lacZ	$^{-602}$A**TCCGCGGA**G^{-593} $^{-581}$T**TCCGCGGA**T^{-572} $^{-543}$T**TCCGCGCA**C^{-534}	45.5 ± 7.5	61.5 ± 3	301 ± 55
PDR10-lacZ	$^{-408}$C**TCCGTGGA**A^{-399} $^{-339}$T**TCC**AC**GGA**C^{-330}	0.16 ± 0.07	0.36 ± 0.05	4.1 ± 1.9
PDR15-lacZ	$^{-443}$T**TCCGCGG**GA^{-434} $^{-380}$T**TCCGCGGA**G^{-371}	2.4 ± 0.5	4.1 ± 1.3	6.0 ± 2.8
PDR11-lacZ	$^{-205}$T**TCCG**CA**GA**T^{-196}	0.21 ± 0.04	0.28 ± 0.06	0.18 ± 0.03

[a] The conserved residues of Pdr1/3p binding motifs found in the promoter regions of the genes are shown in boldface type, and the palindromic sequences are underlined.
[b] The measurements were done in the FY1679/EC (*pdr1Δ*) strain as described under "Experimental Procedures." The β-galactosidase activities, measured in four independent yeast transformants, are expressed with the standard errors.

ing camera (DAGE-MTI Inc., Michigan City, IN), digitized, and stored. Image manipulation and editing were performed with Metamorph software (Universal Imaging Corp., West Chester, PA).

Flow Cytometry—Flow cytometric analysis of the M-C_6-NBD-PE labeled cells was performed with a FACScan cytometer (Becton-Dickinson Immunocytochemistry, San Jose, CA) equipped with an argon laser operating at 488 nm. Ten μl of a 50 μg/ml stock solution of propidium iodide was added to approximately 4×10^5 cells in 200 μl of $SCNaN_3$ immediately prior to dilution (~3 times) and flow cytometric analysis. Ten thousand cells were analyzed without gating during acquisition. Analysis was performed with Lysis II (Becton-Dickinson Immunocytochemistry Systems) software. A dot plot of forward scatter *versus* the red fluorescence channel (propidium iodide) was used to set a gate that excluded dead cells from the analysis. The remaining live cells were plotted on a histogram with the green fluorescence (M-C_6-NBD-PE) plotted on a log scale, and the mean (F_{cell}) and standard deviation of the fluorescence intensity of the live cells calculated.

Other Methods—The protein content was measured as described by Lowry *et al.* (27) with bovine serum albumin as the standard. The protein samples were electrophoresed on SDS-polyacrylamide gel according to Laemmli (28) and stained with either Coomassie Blue or silver. The yeast cells were transformed as described by Kuo and Campbell (29), and bacteria (DH5α strain) transformation was performed by electroporation using a Bio-Rad Gene Pulser apparatus, following the manufacturer's instructions.

RESULTS

Overexpression of Yor1p and Genetic Purification—The low level of expression of *YOR1* in wild-type yeast (1) precludes characterization of its properties. Traditional overexpression of plasma membrane proteins using strong promoters and multicopy vectors often causes mistargeting and stimulates accumulation of intracellular membranes (30). These problems have been overcome in a new approach, which has allowed dramatically enhanced overexpression of Pdr5p and Snq2p ABC transporters in the yeast plasma membrane (6–8). The method takes advantage of point mutations in the transcription factor-encoding genes *PDR1*/*PDR3*, which activate the transcription of their target genes. The target gene promoters contain typical binding sequences called PDREs (for PDR response elements) which correspond to the 5′-TCCG(C/T)GGA-3′ consensus sequence (12, 31, 32) (Table III). An inherent problem to this approach is the simultaneous overexpression of several Pdr1p-regulated proteins, including other ABC transporters. It was therefore necessary to identify all potentially interfering proteins and eliminate them by gene deletion. Systematic sequencing of the yeast genome has revealed new ORFs, which encode a total of 15 full-size ABC transporters (3) including the *PDR10* (33), *PDR15* (34), and *PDR11* (35) genes whose promoters display at least one putative PDRE. Table III shows the transcription activity mediated by the *PDR5*, *YOR1*, *SNQ2*, *PDR10*, *PDR15*, and *PDR11* promoters in the presence of *PDR1* wild type-, null, or mutated *PDR1–3* allele as measured by the β-galactosidase activity of fusion constructs. The *PDR11* promoter-mediated transcription activity was weak and not significantly affected by Pdr1p, possibly because the 5′-TCCG-C**A**GA-3′ sequence in the promoter was insufficient for Pdr1p/Pdr3p recognition. The *PDR1–3* mutation slightly increased the efficiency of the *PDR15* gene promoter despite the presence of a perfect Pdr1p/Pdr3p-binding site. In contrast, the *PDR1–3* allele increased the efficiency of the *PDR10* promoter 11-fold. Note, however, that the putative PDREs of the *PDR10*, *PDR15* and *PDR11* gene promoters have yet to be verified experimentally. In the presence of the wild-type allele of *PDR3*, the *PDR1–3* mutation increased expression of β-galactosidase 25 times for the *YOR1* promoter, 17 times for the *PDR5* promoter, and 5 times for the *SNQ2* promoter.

Table III also shows that the number of putative PDREs per promoter did not correlate with the extent of the induction of β-galactosidase activity mediated by Pdr1p. Although the *PDR5* and *SNQ2* promoters each have three PDREs (12, 31, 32), *PDR5-lacZ* expression was enhanced 5 times by the wild-type Pdr1p while the expression of the *SNQ2-lacZ* fusion was increased only 1.4 times upon the addition of the wild-type *PDR1* allele. Under the same conditions, the induction of the other translational *lacZ* fusions showing 1 or 2 putative PDREs varied between 1.4 (*YOR1-lacZ*) and 2.2 (*PDR10-lacZ*). A further important point is that the β-galactosidase activities of *PDR5-*, *SNQ2-*, and *YOR1-lacZ* fusions reported here in the presence of wild type Pdr3p are quite different to when Pdr3p is absent (21).

In agreement with the results of Table III, Fig. 1 shows that the *PDR1–3* mutant plasma membrane (compared with that from either wild type or *pdr1Δ* strains) dramatically overexpressed a 160-kDa Coomassie Blue-stained band, which mainly comprised Pdr5p, Snq2p, and Yor1p. In order to free Yor1p from membrane contaminants of similar size and possibly overlapping function, we deleted *PDR5*, *SNQ2*, *PDR10*, and *PDR15* yeast ABC transporter-encoding genes whose expression was also induced by the *PDR1–3* mutation. The *PDR11* gene whose product is a constituent of the 160-kDa overexpressed band (8) was also deleted. *YCF1* was deleted because this gene is involved in drug resistance (36, 37).

Yor1p Binds TNP-8-azido-ATP and Shows ATPase Activity—Using the AD234567 multiply deleted strain overexpressing Yor1p, we started to investigate the Yor1p potential NTP hydrolysis activity. Pdr5p has an NTPase activity, which was distinguished from that of the H^+-pump Pma1p by its

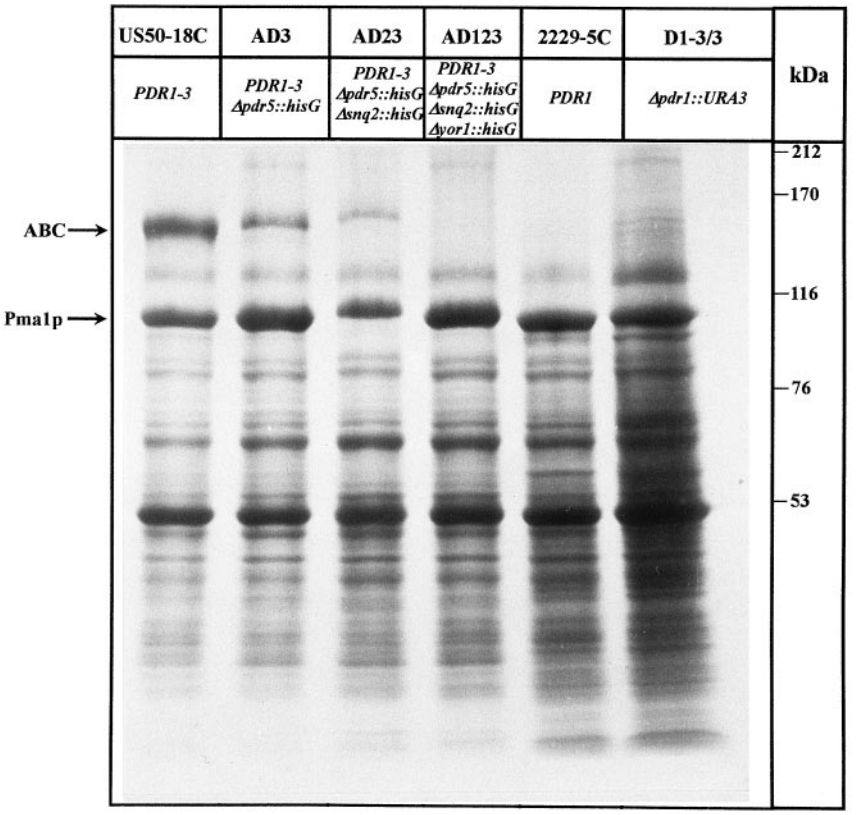

FIG. 1. **Pdr5p, Snq2p, and Yor1p are overexpressed in the *PDR1–3* mutant plasma membrane.** Plasma membrane-enriched fractions were separated onto 7–14% SDS-PAGE and stained with Coomassie Blue. From *left* to *right*: US50–18C (*PDR1–3*), AD3 (*PDR1–3, pdr5*Δ), AD23 (*PDR1–3, pdr5*Δ, *snq2*Δ), AD123 (*PDR1–3, pdr5*Δ, *snq2*Δ, *yor1*Δ), 2229–5C (*PDR1*), and D1–3/3 (*pdr1*Δ). Each lane contains 150 μg of protein.

broader nucleotide specificity and pH dependence (7, 8). With similar protein levels in the plasma membrane, the UTPase activity of Pdr5p was up to 10-fold higher than that of Pma1p (Fig. 2), which is the major ATPase in the yeast plasma membrane. The situation with Yor1p-enriched plasma membrane (AD234567) was very different since no significant difference in the NTPase activity was detected compared with the Yor1p-depleted strain (AD1234567). The same results were obtained with the AD2345678/AD12345678 strains. However, in the SUPERYOR strain obtained by fusion of the *PDR5* promoter to the *YOR1* ORF, a survey of pH from 5.0 to 8.5 revealed that some very low ATPase activity could be associated with SuperYor1p above pH 7.0 (data not shown).

Partial purification of SuperYor1p by centrifugation on sucrose gradient, separated it from H^+-ATPase (Fig. 3*A*). This allowed us to detect ATPase activity associated with the SuperYor1p-enriched fractions (fractions 6–12) after subtraction of the contaminating ATPase activity (~75 nmol of $P_i \cdot min^{-1} \cdot mg^{-1}$) measured in the corresponding fractions of the AD1234567 Yor1p-depleted strain (Fig. 3*B*). Detection of the ATPase activity required the presence of 150 μg/ml asolectin, which was found to be optimal concentration (data not shown). By scanning the SDS-polyacrylamide gel, we found good correlation between the SuperYor1p band intensity and its ATPase activity (Fig. 3*B*). The enzyme was able to hydrolyze ATP from pH 7.0 to 8.5 with similar efficiency (Fig. 3*C*). ATPase activity was strongly sensitive to vanadate (I_{50} ~ 0.3 μM) and inhibited by oligomycin concentrations above 3 μg/ml (Fig. 3, *D* and *E*).

Recently, the fluorescent ATP analogue, TNP-ATP, was shown to bind tightly to several proteins including the purified recombinant NBD1 and NBD2 of mouse MDR P-glycoprotein (38, 39), a synthetic NBD1 of human CFTR (40), and the Chinese hamster P-glycoprotein (41). Photolabeling of plasma membranes enriched in Yor1p (AD234567) and Pdr5p (AD124567) with [γ-^{32}P]TNP-8-azido-ATP is shown in Fig. 4*A*. The labeling of Pma1p is given for comparison. The two ABC

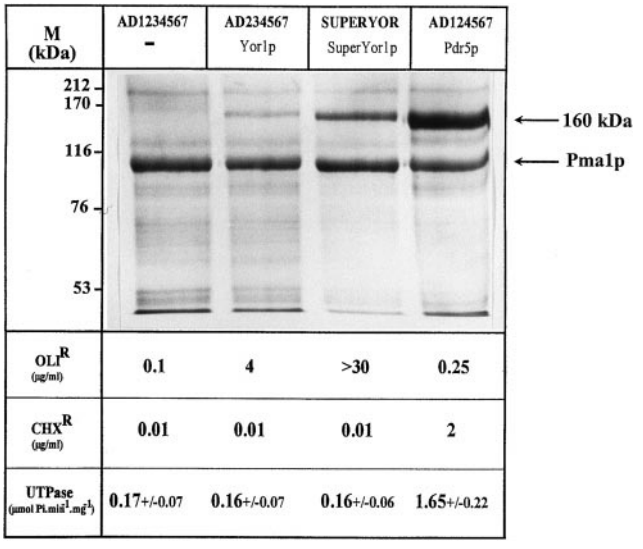

FIG. 2. **The level of Yor1p in the plasma membrane is enhanced by fusing the *PDR5* promoter to the *YOR1* ORF.** One hundred μg of plasma membrane-enriched fractions were separated onto 7%-SDS-PAGE and stained with Coomassie Blue. From *left* to *right*: AD1234567 (*yor1*Δ, *snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ), AD234567 (*snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ), SUPERYOR (*yor1*Δ, *snq2*Δ, Δ*pdr5::PDR5PROM-YOR1-PDR5STOP, pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ), AD124567 (*yor1*Δ, *snq2*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ). The nature of the 160-kDa overexpressed protein is given in each case. The maximum non-inhibitory concentrations of drug for growth on either oligomycin or cycloheximide are shown. We could not determine the corresponding oligomycin concentration for the SUPERYOR strain because of its poor solubility in rich glycerol medium (YG: 2% yeast extract, 4% glycerol) plates. The UTPase activities of the plasma membrane-enriched fractions at pH 6.3 are given in the *last row*.

transporters exhibited a very similar dependence on nucleotide concentration for photolabeling ($K_{0.5}$ = 45 and 46 μM for Yor1p and Pdr5p, respectively), whereas Pma1p required a slightly

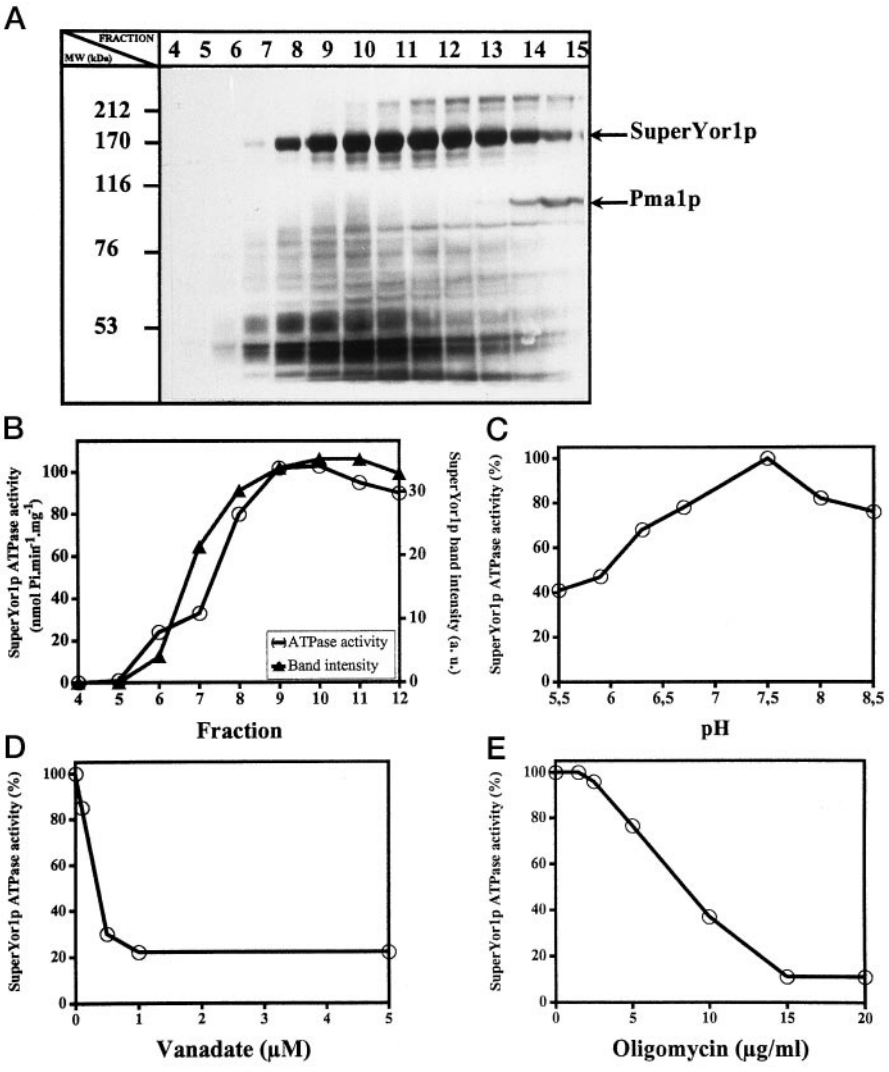

FIG. 3. **Solubilized and partially purified SuperYor1p hydrolyses ATP.** Solubilized plasma membrane proteins (7 mg) from strains SUPERYOR (*yor1*Δ, *snq2*Δ, Δ*pdr5::PDR5PROM-YOR1-PDR5 STOP*, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ) and AD1234567 (*yor1*Δ, *snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ) (data not shown) were separated by centrifugation through a continuous sucrose gradient. Fifty µl of fractions 4–15 were analyzed by SDS-PAGE and stained with silver (*A*). The polyacrylamide gel was scanned and the intensity of the SuperYor1p band was estimated using the Image Master 1D program (Amersham Pharmacia Biotech) (*B*, *closed triangles*). Ten µl of fractions 4–12 (SUPERYOR gradient) were assayed for ATPase activity at pH 7.5 as described under "Experimental Procedures" and, each time, the contaminating ATPase activity measured in the corresponding fractions of the deleted strain (AD1234567) was subtracted (*B*, *open circles*). The activities were calculated for three independent gradients of each strain. Similarly, the effects of pH (*C*), vanadate at pH 7.5 (*D*), and oligomycin at pH 7.5 (*E*) on SuperYor1p ATPase activity were estimated.

higher concentration range ($K_{0.5}$ = 93 µM). The effect of competitor ATP on the photolabeling is shown in Fig. 4*B*. The concentration of TNP-nucleotide chosen was close to the $K_{0.5}$ value for the ABC transporters. It has been shown previously that the labeling reaction in the presence of ATP approximates an equilibrium situation and the " true " dissociation constants for ATP (K_D(ATP)) can be derived from the observed inhibition curves ($K_{0.5}$(ATP)) using the equation: $K_D(\text{ATP}) = K_{0.5}(\text{ATP})/\{1 + ([\text{TNP-8-azido-ATP}]/K_{0.5}(\text{TNP-8-azido-ATP}))\}$ (19). The labeling of all three pumps is inhibited by ATP in the millimolar concentration range, yielding K_D values of 0.3 mM for both the ABC transporters and the proton pump. This value is in good agreement with the K_m for activation of proton transport (42). Note, however, that the TNP-8-azido-ATP binding on Pdr5p and Yor1p proteins has yet to be verified experimentally.

Overexpressed Yor1p Confers Increased Resistance to Oligomycin in Vivo—Overexpressed Yor1p has been reported to confer resistance to oligomycin (1). The analysis of the multiple deletions shows that oligomycin resistance of the *PDR1–3* mutant strain is also dependent on the presence of Pdr5p. Unlike AD1 (*yor1*Δ) and AD12 (*yor1*Δ*snq2*Δ) strains, the AD13 (*yor1*Δ*pdr5*Δ) strain does not grow on plates containing 0.25 µg/ml oligomycin (Fig. 5). Previous studies showed unmodified oligomycin sensitivity in single *PDR5* deletants (11, 12) but Kolaczkowski *et al.* (43) showed that oligomycin is a competitive inhibitor of Pdr5p-mediated transport of rhodamine 6G. As also shown by Fig. 5, Pdr1p more strongly influences oligomycin resistance than its homolog Pdr3p (compare the FY1679/EC (*pdr1*Δ) and FY1679/TD (*pdr3*Δ) strains). This is in agreement with previous observations (12) even though no difference in oligomycin resistance between *pdr1*Δ and *pdr3*Δ strains was observed by Delaveau *et al.* (44). As also shown by Fig. 2, oligomycin resistance was increased 40 times in the strain AD234567 which overexpresses Yor1p and was further increased by a factor of more than 8 in the SUPERYOR strain. These data indicate that, at the cell level, the drug transport properties of Yor1p are conserved during overexpression.

Overexpressed Yor1p Confers Resistance to Rhodamine B—Cui *et al.* (5) recently found that deletion of *YOR1* increases the cellular content of rhodamine B (which is negatively charged, unlike the Pdr5p substrate rhodamine 6G; see Ref. 43). Analysis of the multiply deleted mutants revealed that rhodamine B resistance is mediated by both Yor1p and Pdr5p. The deletion of *PDR5* did not allow cell growth in the presence of 500 µg/ml rhodamine B and deletion of both *PDR5* and *YOR1* (AD13) increased drug sensitivity so that the cells fail to grow in 250 µg/ml rhodamine B (Fig. 5). Finally, the combined deletion of the *YOR1*, *SNQ2*, *PDR5*, *PDR10*, *PDR11*, *PDR15*, and *YCF1* genes (strains AD1234568 and AD12345678) further reduced the resistance to rhodamine B since growth was diminished at 50 µg/ml rhodamine B and abolished at 100 µg/ml rhodamine B. Pdr1p affects rhodamine B and oligomycin resistance more drastically than Pdr3p (FY1679/EC (*pdr1*Δ) compared with FY1679/TD (*pdr3*Δ)). Thus, Pdr1p and Pdr3p me-

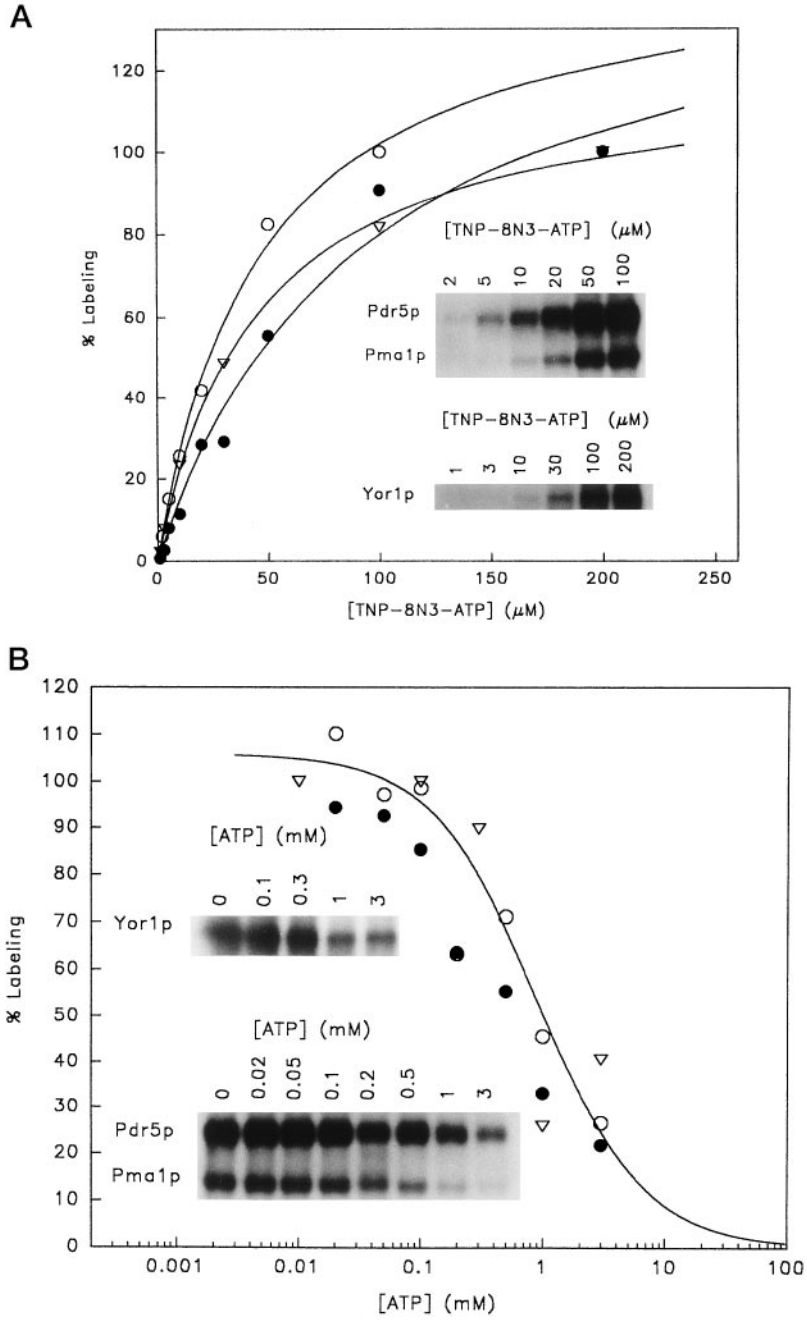

FIG. 4. **TNP-8N3-ATP photolabels Yor1p and Pdr5p and the labeling is inhibited by ATP.** Photolabeling was performed with the concentrations of [γ-^{32}P]TNP-8-azido-ATP shown (*A*) or with 45 or 50 μM [γ-^{32}P]TNP-8-azido-ATP, for Yor1p (strain AD234567) or Pdr5p (AD124567) and Pma1p (AD124567), respectively, and the concentrations of ATP shown (*B*). *Open triangles*, Yor1p (AD234567); *open circles*, Pdr5p (AD124567); *closed circles*, Pma1p (AD124567). In *A*, the lines show the best fit to the data. The $K_{0.5}$(TNP-8-azido-ATP) values are given in the text. In *B*, the line shows the best fit to the data obtained for Pdr5p and yielded a $K_{0.5}$(ATP) of 0.9 mM. That for Yor1p was similar. The value for Pma1p was 0.6 mM. The "true" K_D values are given in the text. The *insets* show autoradiographs of the relevant gel bands.

diate rhodamine B and oligomycin resistance through both Pdr5p and Yor1p. Notice that Yor1p and Pdr5p share other common substrates, including the fungicide miconazole (data not shown).

The involvement of Yor1p in rhodamine B and oligomycin resistance of *pdr5*Δ strains was confirmed by complementation of the AD13 (*yor1*Δ*pdr5*Δ) and the AD1234568 (*yor1*Δ, *snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr15*Δ) strains with the *YOR1* gene. Transformation of both strains with the *YOR1* gene on centromeric plasmid allowed recovery of growth on both 0.5 μg/ml oligomycin and 250 μg/ml rhodamine B as shown for the AD3 and AD234568 strains (data not shown).

The Efflux of Rhodamine B in Yeast Cells Overexpressing Yor1p Is Energy-dependent—We therefore used the Yor1p-overexpressing strain AD2345678 (*snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ, *pdr15*Δ) and its isogenic control, deleted in *YOR1,* AD12345678 (*yor1*Δ, *snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ, *pdr15*Δ) to demonstrate the involvement of Yor1p in the rhodamine B cell content. In the *yor1*Δ strain, rhodamine B accumulation was slightly lowered (82%) by deoxyglucose compared with glucose (100%). However, in the presence of overexpressed Yor1p, rhodamine B accumulation was much higher (58%) in energy-starved cells (deoxyglucose), while glucose caused a drastic reduction (to 8%) in the cellular rhodamine B content (data not shown). Fig. 6*A* shows the Yor1p-mediated energy-dependent extrusion of rhodamine B from pre-loaded cells incubated with deoxyglucose, which depletes the intracellular ATP (45). The addition of glucose caused rapid extrusion of rhodamine B from the Yor1p-expressing cells, while no glucose-dependent effect was observed in the *yor1*Δ cells. Fig. 6*B* shows the difference in the supernatant fluorescence ($F_{glucose} - F_{control}$) in the same experiment and establishes the Yor1p energy-dependent extrusion of rhodamine B out of the cell. Addition of 30 μM protonophore FCCP in the presence of the respiratory substrate ethanol as the sole energy source completely abolished rhodamine B transport. while, in

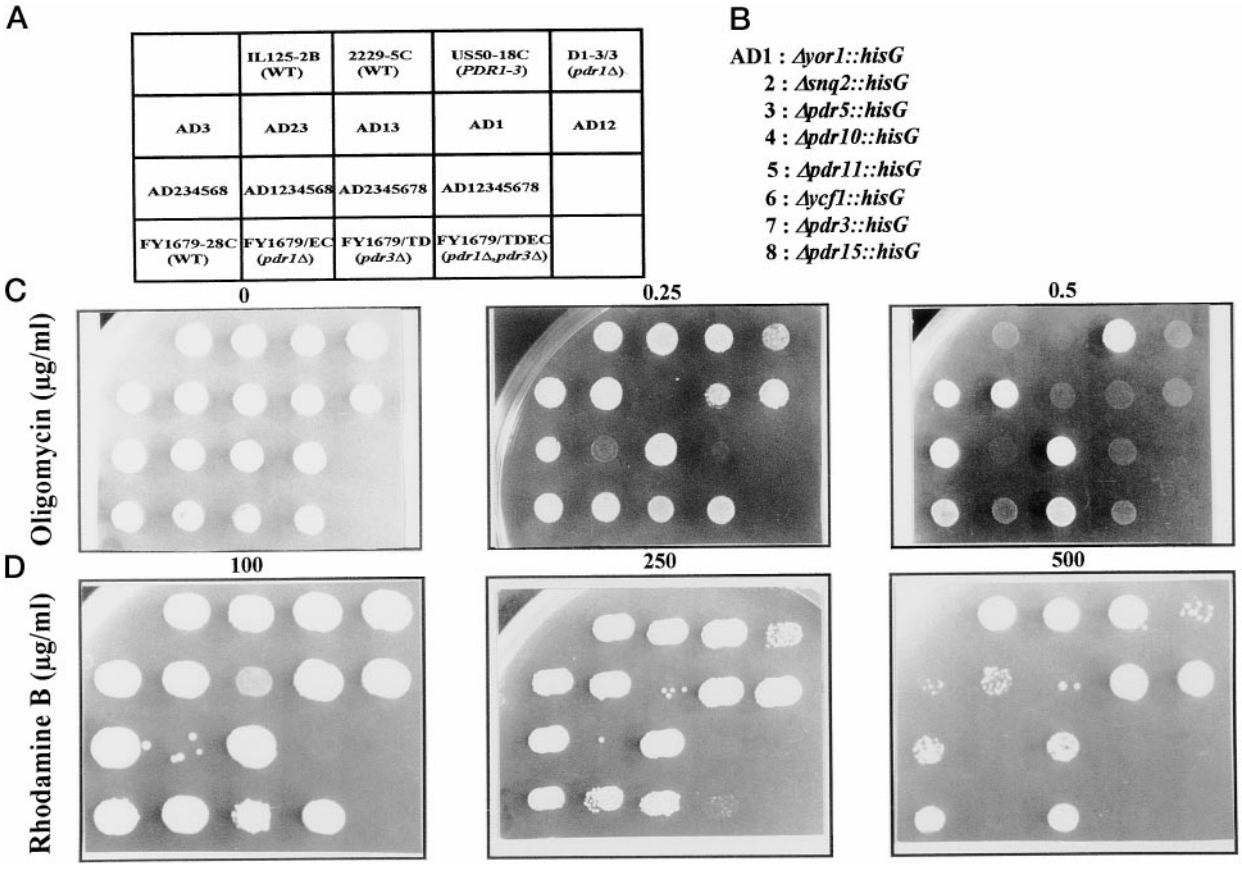

FIG. 5. **Yor1p and Pdr5p confer *in vivo* resistance to oligomycin and rhodamine B.** For oligomycin resistance tests, cells were spotted onto YG plates containing either 0, 0.25 or 0.5 μg/ml (*C*). Resistance to rhodamine B was tested on YD plates containing 100, 250, or 500 μg/ml (*D*). Plates were incubated for 3 days at 30 °C. The names of the tested strains are shown in *A*. *B* describes the nomenclature used for the *PDR1–3* multiply deleted strains.

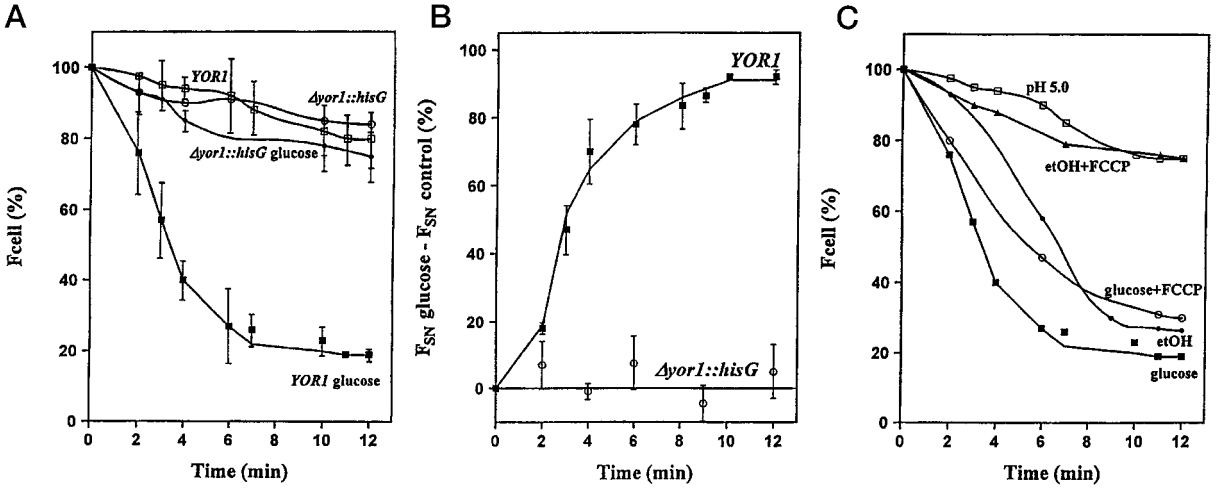

FIG. 6. **Yor1p mediates ATP-dependent efflux of rhodamine B *in vivo*.** Pre-loaded deenergized cells were incubated at 30 °C and the efflux of rhodamine B was measured after the addition of either 10 mM glucose or 4% ethanol, while no energy source was added in the control fraction. At the indicated times, cells were pelleted and the fluorescence was measured in both the supernatant and the cell pellet. *A*, fluorescence of the cell pellet; *closed squares*, strain AD2345678 (*snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ, *pdr15*Δ) + glucose; *open squares*, AD2345678 control; *closed circles*, strain AD12345678 (*yor1*Δ, *snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ, *pdr15*Δ) + glucose; *open circles*, AD12345678 control. *B*, difference between the supernatant fluorescence after addition of glucose (F_{SN} *glucose*) and in the control fraction (F_{SN} *control*); *closed squares*, strain AD2345678; *open circles*, strain AD12345678. *C*, fluorescence of the cell pellet in the AD2345678 strain; *closed squares*, glucose; *closed circles*, ethanol; *open circles*, glucose + 30 μM FCCP; *closed triangles*, ethanol + 30 μM FCCP; *open squares*, pH gradient (pH 7.0 inside the cell and pH 5.0 outside). The *bars* represent the standard deviations.

the presence of glucose, FCCP only slightly affected Yor1p-mediated rhodamine B transport (Fig. 6*C*). This may be explained either by the partial involvement of oxidative phosphorylation in the ATP formation of glucose-grown cells and/or by a possibly higher use of cellular ATP by the Pma1p H^+-ATPase under these conditions. In the absence of an energy source, the application of a pH gradient of 2 units (pH 7.0 inside the cell and pH 5.0 outside the cell) did not cause rhodamine B extrusion (Fig. 6*C*).

These data indicate that the energy required for drug efflux

by Yor1p is not provided by the proton motive force across the plasma membrane. This is in agreement with previous studies showing the ATP requirement for drug transport by several other ABC transporters, including the yeast Pdr5p (43) and Ycf1p (10), the lactococcal MDR transporter (46), and the mammalian MRP (47) and MDR1 (48).

Yor1p and Pdr5p Are Involved in M-C_6-NBD-phosphatidylethanolamine Accumulation in Vivo—Several mammalian ABC transporters translocate phospholipids (13–16). A "flip-flop" of hydrophobic drugs from the inner leaflet to the outer one has been proposed as part of the mechanism of drug transport (49). We have tested whether overexpressed Yor1p was involved in the transport of a fluorescent phospholipid analog, M-C_6-NBD-PE. Cell fluorescence was measured by flow cytometry after the incubation of yeast cells with M-C_6-NBD-PE (Fig. 7*A*) and analyzed by fluorescence microscopy (Fig. 7*B*). The average accumulation of M-C_6-NBD-PE in the *PDR1–3* mutant strain (US50–18C) was about 13% of the *PDR1* parent strains (IL125–2B and 2229–5C). In similar experiments, another mutant, *PDR1–11,* accumulated 1–2% of its isogenic parent.[2] A strain in which the *PDR1* gene was deleted (D1–3/3) accumulated about 70% more M-C_6-NBD-PE than the *PDR1* parent strains (Fig. 7*A*). It therefore seems likely that Pdr1p activates the expression of genes encoding proteins that decrease the steady-state accumulation of M-C_6-NBD-PE by either increasing its efflux or decreasing its influx.

Thus, to determine if any of the seven ABC transporters included in this study were responsible for the efflux of M-C_6-NBD-PE, we analyzed the multiply deleted strains. Based on the average M-C_6-NBD-PE fluorescence intensity for 10,000 cells, the *PDR1–3* strain accumulated only 8% of the *pdr1*Δ strain. Deletion of *YOR1* (AD1) or *PDR5* (AD3) in the *PDR1–3* strain resulted in an increase to 13% M-C_6-NBD-PE accumulation. The fluorescence intensity of the doubly deleted strain *yor1*Δ *pdr5*Δ (AD13), was increased to 36% of the *pdr1*Δ strain (60% of the *PDR1* wild-type strain), a value slightly higher than if the two effects were additive. Single deletions of *SNQ2* (AD2), *PDR10* (AD4), *PDR11* (AD5), *YCF1* (AD6), or *PDR15* (AD8) had no effect on M-C_6-NBD-PE accumulation. Thus, of the ABC transporters tested, only Pdr5p and Yor1p appeared to transport M-C_6-NBD-PE.

DISCUSSION

This study provides some new information on the control of *YOR1* and other yeast ABC genes by the transcription factors Pdr1p and Pdr3p. The expression of *lacZ* gene fusions with *PDR10*, *PDR15*, and *PDR11* gene promoters containing putative PDREs reveals that *PDR10* may be a target of Pdr1p/Pdr3p transcription factors. However, its level of expression was very low compared with that of *PDR5*, *SNQ2*, and *YOR1*, whose transcription was greatly enhanced by the *PDR1–3* mutation. Transcription of the *PDR15* gene, despite the presence of one perfect Pdr1p/Pdr3p-binding site (PDRE) in its promoter, was only increased 2 fold by the *PDR1–3* mutation. The transcription level of the *PDR11* gene was not modified upon addition of either the wild type *PDR1* or the mutated *PDR1–3* allele, possibly because the PDRE sequence in its promoter is not sufficient for Pdr1p/Pdr3p binding (TCCGC**A**GA instead of TCCG(T/C)GGA).

In the *PDR1–3* gain-of-function mutant, the *PDR5* gene promoter gave the highest absolute β-galactosidase activity among the fusion products with five different Pdr1p-regulated gene promoters. This is consistent with the SDS-PAGE analysis of plasma membrane-enriched fractions of multiply deleted strains. The latter analysis also confirmed that Pdr5p, Snq2p, and Yor1p ABC transporters are the major components of the 160-kDa overexpressed protein band in the *PDR1–3* mutant.

Characterization of Yor1p, a minor protein of the plasma membrane, required both the overexpression of the Yor1p protein and also the deletion of related ABC transporters, in particular those suspected to be activated by the *PDR1–3* mutation. We were able to delete up to 7 full-size yeast ABC

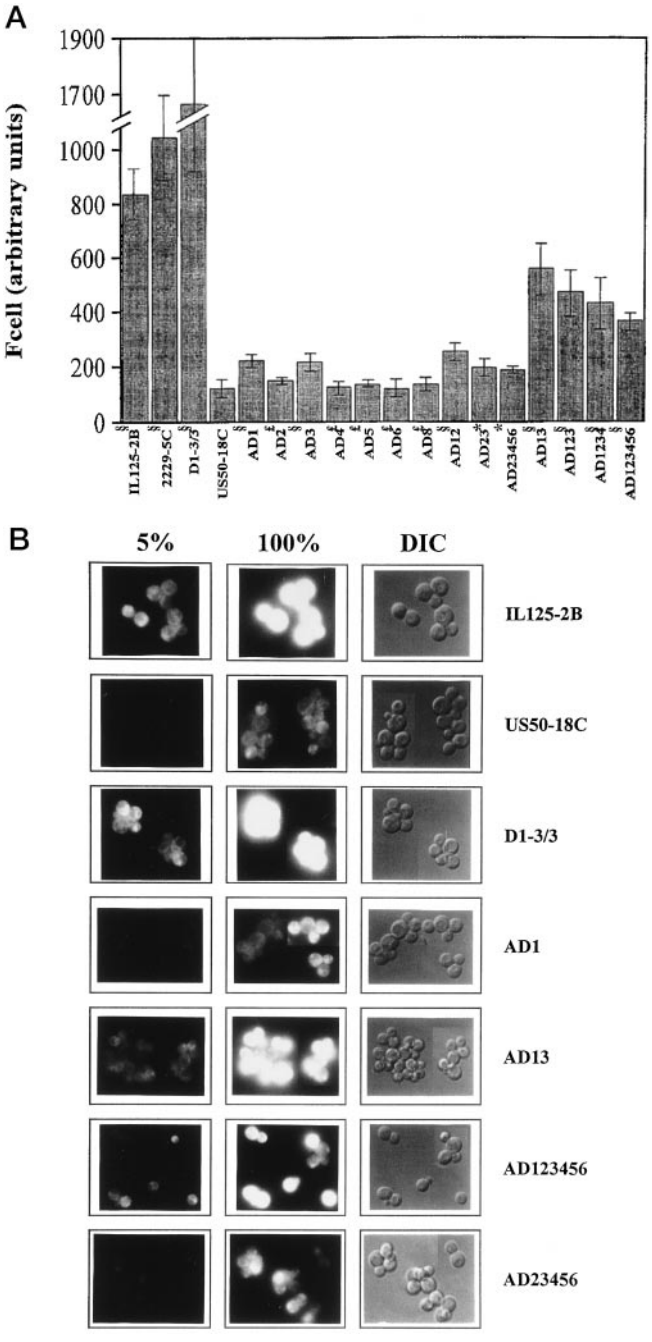

FIG. 7. **Yor1p and Pdr5p are involved in M-C_6-NBD-PE accumulation *in vivo*.** After M-C_6-NBD-PE internalization, cells were submitted to both flow cytometric analysis and fluorescence microscopy. *A*, flow cytometric analysis of the M-C_6-NBD-PE-labeled cells. Fcell is the mean of the fluorescence intensity of a population of ~10,000 live cells. The *bars* represent the standard deviations. According to unpaired Student's *t* test, values are statistically different from value for US50–18C strain with $p < 0.01$ (§); with $p < 0.05$ (*); values are not significantly different from value for US50–18C strain since $p > 0.1$ (£). *B*, fluorescence microscopy: 5% means that a neutral density filter was used to reduce the excitatory light by 95%. In the second column (100%), the filter was removed. The *last column* shows the differential interference contrast optics.

[2] A. M. Grant and J. W. Nichols, unpublished observations.

transporters without impairing growth.

Membrane-bound Yor1p was labeled with TNP-8-azido-ATP *in vitro* in a saturable manner and the labeling was inhibited by ATP. Interestingly, the $K_{0.5}$ for TNP-8-azido-ATP that we found for Yor1p (about 45 μM) is very close to the value found by Liu and Sharom for TNP-ATP binding to human P-glycoprotein (43 μM) (50). The nucleotide binding properties of Pdr5p and Yor1p appeared very comparable.

Investigation of the drug transport properties of intact cells overexpressing Yor1p showed that the Yor1p enzyme was active *in vivo* and required energy. The oligomycin resistance was increased 40 times in the strain overexpressing Yor1p and further increased by about 8-fold in the SUPERYOR strain, indicating that overexpression of Yor1p does not change its oligomycin transport capability *in vivo*. Absence of Yor1p or Pdr5p caused increased sensitivity to both oligomycin and rhodamine B. These effects were additive when both proteins were missing. A requirement for energy was demonstrated by the strong efflux of rhodamine B from Yor1p-enriched energy-starved cells on glucose addition. The energy required for this process appears to be provided by ATP rather than ΔpH. Finally, absence of either Yor1p or Pdr5p resulted in increased accumulation of a fluorescent phosphatidylethanolamine. Again, the effect was more pronounced when both ABC transporters were deleted, indicating that the transporters may act independently. Conversely, none of the 5 other ABC transporters, including the overexpressed Snq2p transporter, exhibited this activity.

In plasma membranes from the AD234567 Yor1p-expressing strain, it was difficult to measure ATPase activity distinct from that of Pma1p. Enhancing the level of Yor1p by fusing the *YOR1* ORF to the *PDR5* promoter in the *PDR1–3* mutant (SUPERYOR strain) allowed us, however, to detect ATPase activity in solubilized and partially purified SuperYor1p fractions. The SuperYor1p activity of ~100 nmol of $P_i \cdot min^{-1} \cdot mg^{-1}$ was sensitive to both vanadate and oligomycin, establishing that we are not dealing with either P-type or F-type ATPase contaminants. The use of the *PDR1–3* gain-of-function allele of *PDR1* has already allowed characterization of the Pdr5p and Snq2p NTPase activities (7, 8). Surprisingly, Pdr5p, Snq2p, and (Super)Yor1p NTPase activities show distinct characteristics; Pdr5p hydrolyzes all Mg-NTPs over a broad pH range, whereas Snq2p and Yor1p hydrolyze ATP preferentially. The pH profile of Yor1p ATPase activity is also very broad (with an optimum at pH 7.5), while it is much sharper for Snq2p (pH 6.3). Only Pdr5p and Yor1p ATPase activities are oligomycin-sensitive, while vanadate was shown to inhibit all three enzyme ATPase activities. Finally, taking into account the relative amount of each transporter in the plasma membrane, one can estimate that the Yor1p ATPase activity is more or less 15 times lower than that of Pdr5p or Snq2p.

As the pumping capacity (and specificity) of the Yor1p and Pdr5p transporters appears similar *in vivo*, the low ATPase activity of Yor1p (~0.1 μmol of $P_i \cdot min^{-1} \cdot mg^{-1}$) compared with that of Pdr5p (~1.5 μmol of $P_i \cdot min^{-1} \cdot mg^{-1}$) in similar conditions may indicate that the purified Yor1p transporter is a more highly coupled pump than Pdr5p. The high ATPase activity and apparently low pumping capacity of ABC drug transporters (51–54) has been a puzzling feature. For instance, Pdr5p ATPase activity is very high in the apparent absence of drugs or other substrates, slightly stimulated by substrates, and shows a broad nucleotide specificity despite an *in vivo* requirement for ATP for transport (43). The poor NTPase activity of Yor1p might be due to the fact that an activation factor required for ATPase activity is lost during the plasma membrane preparation or that some phospholipids block the Yor1p ATPase activity when tested in membranes. Another possibility is that Yor1p, like its ortholog CFTR, has low ATPase activity (~0.05 μmol of $P_i \cdot min^{-1} \cdot mg^{-1}$) that is sufficient for the control of channel gating (55). Very recently, the close human ortholog of Yor1p, MRP, was purified and shown to hydrolyze ATP at a rate of ~0.3 μmol of $P_i \cdot min^{-1} \cdot mg^{-1}$ in the presence of 6 mM Mg-ATP (56). If we consider that, in our preparations, the "purified" SuperYor1p amounts to 20% of the total proteins, one can estimate that SuperYor1p may exhibit an ATP hydrolysis rate of ~0.5 μmol of $P_i \cdot min^{-1} \cdot mg$ $SuperYor1p^{-1}$.

The mediation of phospholipid efflux by yeast Pdr5p and Yor1p is consistent with recent reports that identified other ABC transporters as phospholipid transporters or flippases. These include mouse mdr1 (16) and mdr2 (13, 14) and human MDR1 (16) and MDR3 (15, 16). Phospholipid transport by MDR1 and mdr1 is not head-group or glycerol backbone-specific, whereas the MDR3 P-glycoprotein transports phosphatidylcholine exclusively. The specificity of Yor1p and Pdr5p for phospholipids other than phosphatidylethanolamine remains to be seen, but it could be different for the two pumps. It seems possible that the high steady state of ATP hydrolysis by Pdr5p and the low ATPase activity of Yor1p may be related to activation of the former with certain yeast phospholipids, which may not be substrates or even inhibitors of the latter. Anyway, it is intriguing that Pdr5p and Yor1p share similar phospholipid translocation properties which were not observed for Snq2p.

Finally, we wish to point out that, taking advantage of the strong *PDR5* promoter associated with the gain-of-function *PDR1–3* mutation, we have developed an important new tool for overexpression of ABC transporters in the yeast plasma membrane. This system allows the overexpression of functional yeast Yor1p at a level that represents more than 10% of total plasma membrane proteins. For comparison, overexpression of the human MDR1 from an high copy number expression vector in yeast by Mao and Scarborough (57) yielded protein amounts to only 0.4% of the total yeast membrane proteins. We anticipate that the PDR system, which dramatically overexpresses the functional yeast Yor1p in the plasma membrane without associated intracellular trafficking problems, and the use of the *PDR5* promoter in strains deleted in the majority of the full-size ABC transporter-encoding genes provides a prototype for high level expression of orthologous ABC transporters of medical interest.

Acknowledgments—We thank W. S. Moye-Rowley for the plasmid gift and J. Nader for useful technical assistance. We acknowledge E. Balzi, B. van den Hazel, M. Kolaczkowski, M. A. do Valle-Matta, and A. Cybularz-Kolaczkowska for helpful comments. We are grateful to B. van den Hazel for sharing unpublished observations and W. S. Moye-Rowley for constant interest in our work. We acknowledge B. C. Monk for critical reading of the manuscript.

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ATPase and Multidrug Transport Activities of the Overexpressed Yeast ABC Protein Yor1p

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J. Biol. Chem. 1998, 273:12612-12622.
doi: 10.1074/jbc.273.20.12612
