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The Pleitropic Drug ABC Transporters from *Saccharomyces cerevisiae*

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Abstract

The *Saccharomyces cerevisiae* genome contains 16 genes encoding full-size ABC transporters. Each comprises two nucleotide binding folds (NBF) alternating with transmembrane domains (TM). We have studied in detail three plasma membrane multidrug exporters: Pdr5p (TC3.A.1.205.1) and Snq2p (TC3.A.1.205.2) which share NBF-TM-NBF-TM topology as well as Yor1p (TC3.A.1.208.3) which exhibits the reciprocal TM-NBF-TM-NBF topology. The substrate specificity of Pdr5p, Snq2p and Yor1p are largely, but not totally, overlapping as shown by screening the growth inhibition by 349 toxic compounds of combinatorial deletants of these three ABC genes. Multiple deletion of 7 ABC genes (*YOR1, SNQ2, PDR5, YCF1, PDR10, PDR11* and *PDR15*) and of two transcription activation factors (*PDR1* and *PDR3*) renders the cell from 2 to 200 times more sensitive to numerous toxic coumpounds including antifungals used in agriculture or medicine. The use of the *pdr1-3* activating mutation and when necessary of the *PDR5* promoter in appropriate multideleted hosts allow high levels of expression of Pdr5p, Snq2p or Yor1p. These overexpressed proteins exhibit ATPase activity *in vitro* and confer considerable multiple drug resistance *in vivo*. The latter property can be used for screening specific inhibitors of fungal and other ABC transporters.

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The Discovery of the Yeast ABC Transporters and of their Major Regulators

The first ABC transporter gene discovered in *Saccharomyces cerevisiae* was shown to encode the full-size Ste6p plasma membrane protein exporting the *a*-mating pheromone (Mcgrath and Varshafsky, 1989; Kuchler *et al*., 1989,). The first half-size ABC transporter gene, *ADP1*, was unravelled by the systematic sequencing of chromosome III (Purnelle *et al*., 1991). Adp1p belongs to the historic "*white*" family determining the eye color of *Drosophila*. Its function in yeast is still unknown today. During the next few "pregenomic" years, four additional transporters were reported. The *SNQ2* gene was discovered in a screen for mutants resistant to nitroxiquinoline oxides and other mutagens (Servos *et al*., 1993). The overexpressed *PDR5* gene was found to confer resistance to cycloheximide and many other drugs (Balzi *et al*., 1994; Bissinger *et al*., 1994; Hirata *et al*., 1994). The *YCF1* gene was detected as responsible for a cadmium resistance phenotype due to accumulation of glutathione-heavy metal conjugates in vacuole (Szczypka *et al.,*1994, Li *et al*., 1996). The mutated *YOR1* gene was reported as conferring oligomycin and anionic drugs sensitivity (Katzmann *et al*., 1995, Cui *et al*., 1996).

Table 1. The yeast ABC transporters according to the Transporter Classification (TC) system (Saier, 2000)

3.A.1.203	The Peroxysomal Fatty Acyl CoA Transporter (FAT) Family
3.A.1.203.2	PAT1/PAL1/PXA1 and PAT2/PAL2/PXA2: long chain fatty acyl COA importer
3.A.1.204	The Eye Pigment Precursor Transporter (EPP) Family
undetermined	ADP1: unknown substrate
3.A.1.205	The Pleiotropic Drug Resistance (PDR) Family
3.A.1.205.1	PDR5: cycloheximide/pleiotropic drug exporter
3.A.1.205.2	SNQ2: mutagen/pleiotropic drug exporter
3.A.1.205.3	PDR12: weak acid exporter.
undetermined	PDR10, PDR15 and YOR011: unknown substrates.
3.A.1.206	The Sex Pheromone Exporter (STE) Family
3.A.1.206.1	STE6: a-factor sex pheromone exporter
3.A.1.207	The Conjugate Transporter (CT1) Family
3.A.1.207.1	YCF1: vacuolar metal resistance factor
3.A.1.207.2	BAT1: vacuolar bile acid transporter
3.A.1.207.3	BPT1: vacuolar bilirubin transporter
undetermined	YHL035: unknown substrate
3.A.1.208	The Conjugate Transporter (CT2) Family
3.A.1.208.3	YOR1: oligomycin/pleiotropic drug exporter
3.A.1.209	The Major Histocompatibility Peptide Transporter (TAP) Family
undetermined	TAP1/TAP2: unknown substrate
3.A.1.212	The Mitochondrial Fe/S Protein Exporter (MPE) Family
3.A.1.212.1	ATM1: mitochondrial putative iron transporter
3.A.1.213	The Putative Transporter Family of Unknown Function
3.A.1.213.1	PDR11 and YOR011: unknown function
3.A.1.213.2	YOL075: unknown function
3.A.1.213.3	MDL1 and MDL2: unknown function

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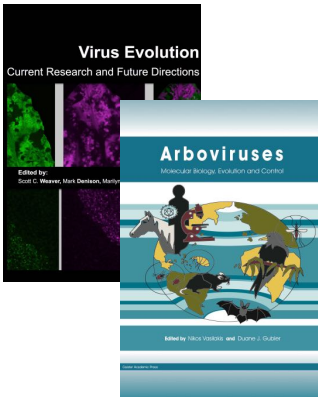

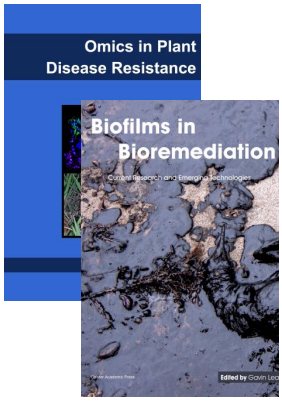

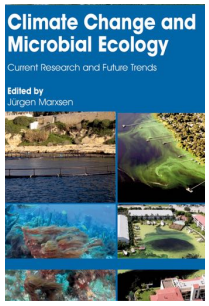

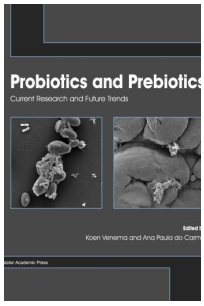

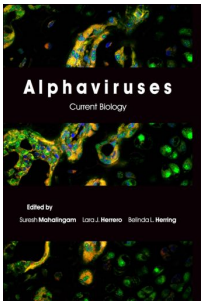

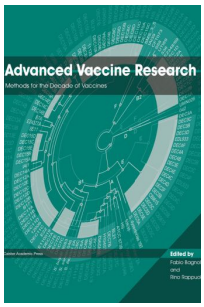

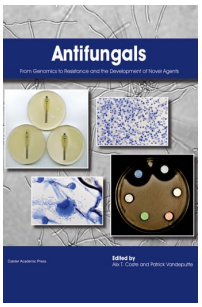

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Table 2. Drug Sensitivity of ABC and PDR Deletants

	ΔPDR5	ΔSNQ2	ΔYOR1	ΔPDR5 ΔSNQ2	ΔPDR5 ΔSNQ2 ΔYOR1	ΔPDR1 ΔPDR3
FUNGICIDES						
Azoles						
bitertanol	+	-	-	+	⊕	+
diclobutrazol	+	-	-	+	⊕	+
flusilazole	⊕	-	-	+	+	+
imazalil nitrate	+	+	+	+	⊕	+
itraconazole	+	+/-	-	+	⊕	+
ketoconazole	+	+/-	-	+	+	⊕
prochloraz	+	-	-	+	⊕	+
tebuconazole	+	-	-	+	+	⊕
triadimenol	+	-	-	⊕	+	+
miconyole	+/-	+/-	+	+	⊕	+/-
expoxiconyole	+	-	+	+	⊕	+
triadimylonb	+	+/-	-	⊕	+	+
Imidothiazole						
2-mercaptobenzothiazole	+/-	+/-	-	+	⊕	+
Dithiocarbamates						
maneb	+/-	-	+	-	⊕	-
nabam	-	-	+	-	⊕	-
zineb	-	-	-	-	⊕	-
metham Na	-	+	+	-	⊕	-
ferbam	-	-	+	-	⊕	-
thiram	-	-	+	-	⊕	-
Polyenes						
amphotericin B	-	-	-	-	-	+
nystatin	+/-	-	+	+	⊕	+
Pyrimidynyl carbinols						
fenarimol	+	-	-	+	+	⊕
nuarimol	+	-	-	+	+	⊕
Anilinopyrimidines						
cyprodinil	-	+	-	+	-	⊕
mepanipyrim	-	-	+	-	+	-
Morpholines						
tridemorph	+	-	-	+	⊕	+
fenpropidin	-	-	-	⊕	+	+
fenpropimorph	-	-	-	⊕	+	+
Strobilurine						
azoxystrobin	+	-	+/-	+	+	⊕
krezoxim	+	-	-	+	+	⊕
Benzimidazoles						
carbendazim	-	⊕	-	+/-	+/-	-
benomyl	-	⊕	-	-	-	-
Others						
chlorothanolil (carbonitrile)	-	-	+	+	⊕	+
dodine (guanidine)	-	-	+	+/-	⊕	+/-
phenapronil	⊕	-	-	+	+	+
chinomethionat	-	-	-	+/-	⊕	+
fenaminosulf	-	-	-	-	⊕	-
diphenylamine (anilide)	-	-	-	+/-	⊕	-
naftifine (allylamine)	-	-	-	⊕	+	+
CGA64251	+	-	-	+	⊕	+
soraphen (macrolide)	+	-	-	+	⊕	-
tecoram	+/-	+/-	-	+/-	+	⊕
HERBICIDES AND PESTICIDES						
Urea derivatives						
chlorbromuron	⊕	-	+	-	+	+
chloroxuron	+	+	-	⊕	+	+
chlorotoluron	-	-	+	-	⊕	+
difenoxuron	+	-	+	+	+	⊕
diuron	-	+	+	+	+	⊕
fluometuron	+	-	+	+	⊕	+
isoproturon	-	-	-	-	⊕	-
linuron	-	+	+	-	+	⊕
metobromuron	-	-	+	-	⊕	-
monuron	-	-	⊕	+	+	+
Anilides						
propanil	-	-	⊕	-	+	+
nirit	+/-	+	-	⊕	+	+
dinitrorhodan benzene	-	+/-	-	⊕	+	+
dicofol	-	-	-	-	-	⊕
ANTIBIOTICS AND ANTISEPTICS						
anisomycin	+	-	+	+	⊕	+
antimycin A1	+/-	-	+	-	⊕	+
chloramphenicol	+	-	-	+	+	⊕
cycloheximide	⊕	-	-	+	+	+
erythromycin	-	-	⊕	-	+	+
oligomycin	+	+	⊕	-	+	+
tetracycline	+	-	+	+	⊕	+
thiolutin	-	-	+	+	⊕	+
tunicamycin	-	-	+/-	+	⊕	+
xenomycinA	+	-	+	+	⊕	-
crystal violet	-	-	-	-	+/-	⊕
ANTICANCER						
daunorubicin	+	-	-	+	⊕	+
doxorubicin	+	-	+	+	⊕	+
trifluoperazine	⊕	-	-	-	-	-
FLAVONOIDS						
baicalein	+	+	⊕	+	+	+
fisetin	-	-	⊕	-	+	+
myricetin	-	-	⊕	-	+	+
quercetin	-	-	⊕	-	+	+
IONOPHORES						
FCCP	-	+/-	+	-	⊕	-
A23187	+	+	-	+	+	+
monensin	⊕	-	-	-	⊕	+
nigericin	+	-	-	+/-	⊕	+
valinomycin	⊕	-	+	-	-	+
ANILINES						
aniline	+/-	-	+	⊕	+	+/-
p-aminopdiphenylamine	-	-	-	+	⊕	-
p-amino-p-methoxy-diphenylamine	-	+/-	-	-	⊕	-
phenylhydrazine	-	-	+	-	⊕	+
1-phenyl-3-pyrazolidone	-	-	-	-	⊕	-
DETERGENTS						
Cationic						
tetradecyl trimethyl-ammonium bromide	-	-	-	+	⊕	+
hexadecyl trimethyl-ammonium bromide	-	-	+	+	⊕	+
n-dodecyl trimethyl-ammonium bromide	-	-	+/-	+	⊕	+
Zwitterionic						
Z 3-10	+	-	+	+	⊕	+
Z 3-12	+	-	+	+	⊕	+
Z 3-14	+	-	+	+	⊕	-
Non ionic						
mega 10	-	-	-	-	⊕	+
polyoxyethylene 8 lauryl ether	-	-	-	-	-	⊕
polyoxyethylene 9 lauryl ether	-	-	-	-	-	⊕
genapol	-	-	-	-	-	⊕
thesit	-	-	-	-	-	⊕
triton X-100	-	-	-	-	-	⊕
triton X-114	-	-	-	-	-	⊕
brij58	+	-	-	+	+/-	⊕
brij35	+	-	-	+	+	⊕
n-dodecylglucoside	+	-	-	+	⊕	+
n-octylglucoside	+	-	-	+	+	⊕
n-dodecylmaltoside	+	-	+	+	⊕	-
Anionic						
n-lauryol sarcosine	-	-	+	-	⊕	+
SDS	-	-	-	-	⊕	-
Bile acids						
taurocholic acid	-	-	-	-	-	⊕
glycochenodeoxycholic acid	-	-	-	-	+	⊕
lithocholic acid	-	-	-	-	⊕	+
taurodeoxycholic acid	-	-	-	-	-	⊕
MISCELLANEOUS						
caprylic acid	-	-	-	+/-	⊕	-
n-decylamine	-	-	-	+	⊕	+
4-nitroquinoline-N-oxide	-	-	-	+/-	⊕	+
8-hydroxyquinoline	-	-	-	+	⊕	+
malachite green	-	-	-	-	⊕	+
menadione	-	-	-	-	⊕	+
resazurin	-	+	-	+	⊕	-
rhodamine B	-	-	-	-	⊕	-
rhodamine 6G	+	-	-	+	+	⊕
tetranitrotetrazolium chloride blue	-	-	-	+	⊕	+
2,3,5-triphenyltetrazolium chloride	⊕	-	-	+	+	+/-
anthron	-	-	-	+	⊕	+
chlorophenol red	-	-	-	+	+	⊕
7-chloro-4-nitrobenzo-2-oxa-1,3-diazole	+/-	-	+	+	⊕	+
helminthosporol	-	⊕	-	-	+	+
4-nitrophenol	+	-	-	⊕	+	+
LYSOSOMOTROPIC AMINOESTERS[A]						
EPT-8	-	-	-	-	⊕	-
EPT-10	-	-	-	-	+/-	⊕
EPT-12	-	-	-	-	-	⊕
EPT-14	-	-	-	-	-	⊕
EPT-16	-	-	-	-	-	-
DM-9	-	-	⊕	-	-	-
DM-11	-	-	-	-	⊕	-
DM-13	-	+	-	-	⊕	-
DM-15	-	-	-	-	-	-
DE-11	-	-	-	-	+	⊕
PY-11	-	-	-	-	⊕	-
PYI-11	-	+/-	-	-	⊕	-
AT-12	-	-	-	+	⊕	-

[a]EPT: n octyl or decyl or dodecyl or tetradecyl or hexadecyl α(1, 2, 4-triazol-1-yl) propionate, DM : (2-dimethylaminoethyl dodecanoate or dodecanoate or tetradecanote or hexadecanoate), DE-11 : (2-diethylaminoethyl dodecanoate), PY-11 : (2-(1-pyrrolidino) ethyl dodecanoate), PYI-11 : (2-(1-methyl-pyrrolidin-2-yl) ethyl dodecanoate), AT-12 : (1-(n-dodecyl)-1, 2, 4-triazole).

Data are compiled from Kolaczkowski *et al.*, 1998. Chemical characterization of the toxic compounds as well as the origin of the products and the strains can be found in the same reference. + means that the halo of death cells around the disc containing the toxic compound is significantly larger (thus the strain is more sensitive) in the tested deletant compared to the isogenic parental strain. +/- means a slight increase of sensitivity at the borderline of significance. - means no detected difference in sensitivity. The most prominent increase of sensitivity among the five tested strains is circled. When two deletants have similar sensitivity, the simplest one is circled. For tested steroids on PDR5 deletants see Kolaczkowski *et al.*, 1997. For flavonoid test, see Conseil *et al.*, 2000.

Upon completion of the yeast genome project, 11 additional full-size ABC genes were identified (Decottignies *et al.*,1994). Transported substrates of only three of them have been identified up to now: vacuolar bile acids *for BAT1* (Ortiz *et al.*, 1997) vacuolar bilirubin for *BTP1* (Petrovic *et al.*, 2000) and weak organic acids for *PDR12* (Piper *et al.*, 1998) The substrates of 8 ABC transporters (Pdr10p, Pdr11p, Pdr15p, YNR070, YOR11, YOL075, YHL035, YKR103/104 –a putative pseudo-gene) are still unknown. However, the amino acid sequence of Pdr10p, Pdr11p and Pdr15p is so similar to that of the powerful Pdr5p pleiotropic drug transporter that they can be suspected by guilt of association to also be multidrug dealers.

On the other hand, numerous allelic mutations responsible for pleiotropic drug resistance had been mapped near the chromosome VII centromere (Rank and Bech-Hansen, 1973). This locus called *PDR1* was identified as a transcription factor from the Zn2Cys6 family (Balzi *et al.*, 1987) regulating the expression of *PDR5* (Balzi *et al.*, 1994), *SNQ2* (Decottignies, *et al.,* 1995, 1998; Mahé, *et al.*, 1996) and *YOR1* (Decottignies *et al.*, 1998, Katzmann et al., 1995). The same ABC genes were found to be also controlled by *PDR3*, a transcription factor homologous to

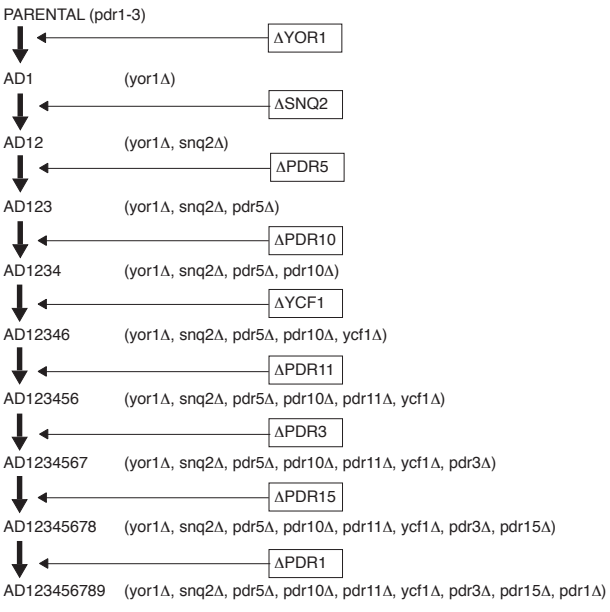


Figure 1. Multiple deletions of yeast ABC transporters and regulators.

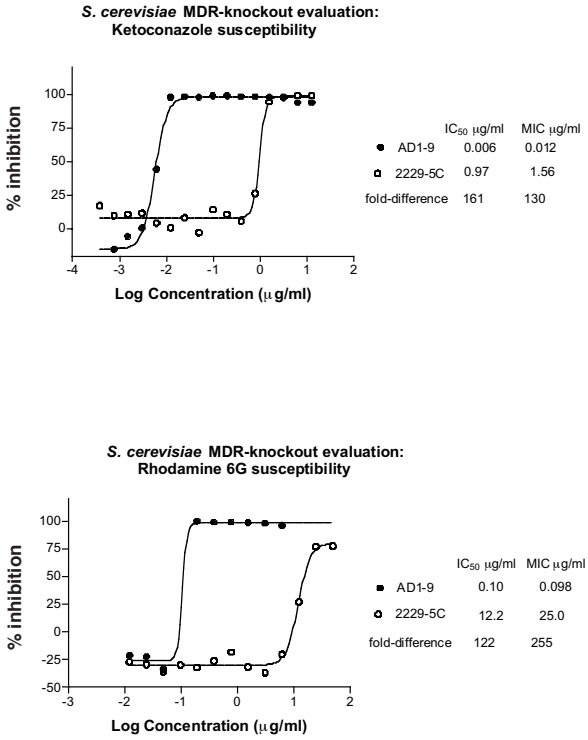


Figure 2. *S. cerevisiae* MDR-knockout evaluation.

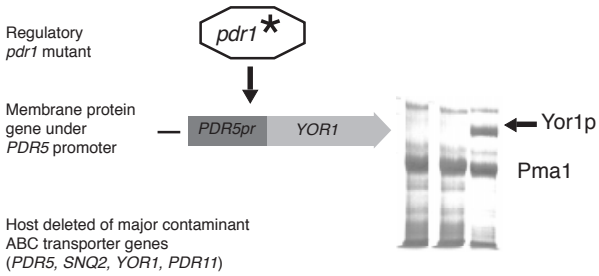
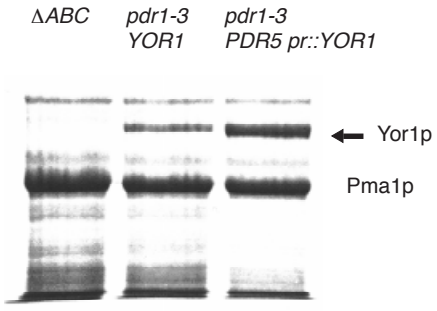


Figure 3. Yeast PDR as a system for overexpression of membrane proteins.



OLIGOMYCIN RESISTANCE	0.1	4	≥ 30	μg/ml

Figure 4. Overproduction of YOR1p

Table 3. Examples of multidrug deleted *S. cerevisiae* strains

Strains	Genotype
IL125-2B	*MATα, PDR1, his1*, parental of the DRI9-T8 *pdr1-3* mutant
2229-5C	*MAT*a, *PDR1, ura3*
D1	*MAT*a, *Δpdr1::URA3*
DRI9-T8	*MATα, pdr1-3, his1*
US50-18C	*MATα, pdr1-3, ura3, his1,* obtained by cross between DRI9-T8 and 2229-5C
D1-3/3	US50-18C disrupted by *pdr1- Δ1::URA3*
DRI9-T7	*MATα, pdr3-2, his1*
FM11	*MAT*a, *PDR1, ade2-2, lys1-1, his4-480, met8-1, SUP4-3*
2-20	*MAT*a, *pdr1-2, ade2-2, lys1-1, his4-480, met8-1, SUP4-3*
D286-2A	*MAT*a, *PDR1, ade1, his*
BOR2-XI	*MAT*a, *pdr1-6, ade1, his1*
US54-17B	*MATα, pdr1-2, ura3, his4*
D1-2	*MATα, pdr1-Δ1::URA3, his4*
JG200	*MAT*a, *PDR1, leu2, his4, can1-100*
JG204	*MAT*a, *pdr1-7, leu2, his4, can1-100*
US50-D5[a]	*pdr5-Δ1::URA3*
US50-D2[a]	*snq2::Tn10-LUK*
US50-D25[a]	*pdr5- Δ1::URA3, snq2::Tn10-LUK*
AD1[a]	*Δyor1::hisG*
AD2[a]	*Δsnq2::hisG*
AD3[a]	*pdr5-Δ2::hisG*
AD4[a]	*Δpdr10::hisG-URA3-hisG*
AD5[a]	*Δpdr11::hisG-URA3-hisG*
AD6[a]	*Δycf1::hisG-URA3-hisG*
AD10[a]	*Δyap1::hisG-URA3-hisG*
AD12[a]	*Δyor1::hisG, Δsnq2::hisG*
AD23[a]	*Δsnq2::hisG, pdr5-Δ2::hisG*
AD13[a]	*Δyor1::hisG, pdr5-Δ2::hisG*
AD26[a]	*Δsnq2::hisG, Δycf1::hisG*
AD34[a]	*pdr5-Δ2::hisG, Δpdr10::hisG-URA3-hisG*
AD36[a]	*pdr5-Δ2::hisG, Δycf1::hisG-URA3-hisG*
AD123[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG*
AD124[a]	*Δyor1::hisG, Δsnq2::hisG, Δpdr10::hisG*
AD126[a]	*Δyor1::hisG, Δsnq2::hisG, Δycf1::hisG-URA3-hisG*
AD136[a]	*Δyor1::hisG, pdr5-Δ2::hisG, Δycf1::hisG-URA3-hisG*
AD1234[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG*
AD1236[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δycf1::hisG-URA3-hisG*
AD1237[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, pdr3-Δ2::hisG*
AD1246[a]	*Δyor1::hisG, Δsnq2::hisG, Δpdr10::hisG, Δycf1::hisG-URA3-hisG*
AD12346[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δycf1::hisG-URA3-hisG*
AD12367[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δycf1::hisG, pdr3-Δ2::hisG*
AD12467[a]	*Δyor1::hisG, Δsnq2::hisG, Δpdr10::hisG, Δycf1::hisG, pdr3-Δ2::hisG*
AD23456[a]	*Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG*
AD123467[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δycf1::hisG, pdr3-Δ2::hisG*
AD123456[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG*
AD124567[a]	*Δyor1::hisG, Δsnq2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG*
AD234567[a]	*Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG*
AD1234567[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG*
AD234568[a]	*Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, Δpdr15::hisG*
AD1234568[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, Δpdr15::hisG*
AD2345678[a]	*Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG, Δpdr15::hisG*
AD12345678[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG, Δpdr15::hisG*
SUPERYOR[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ3::PDR5PROM-YOR1-PDR5STOP, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG*
AD123456789[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG, Δpdr15::hisG, pdr1-Δ3::hisG*
AD124567/CK1[a]	*Δyor1::hisG, Δsnq2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG, Δyck1::hisG*
AD124567/CK1/CK2[tsa]	*Δyor1::hisG, Δsnq2::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG, Δyck1::hisG, yck2[ts]*
PDR5-6HIS[a]	*Δyor1::hisG, Δsnq2::hisG, pdr5-Δ4::PDR5-6HIS, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, pdr3-Δ2::hisG, Δpdr15::hisG*
FY1679-28C	*MAT*a, *PDR1, PDR3, ura3-52, trp1, leu2-Δ1, his3-Δ200, GAL2*
FY1679-28C/EC	FY1679-28C disrupted by *pdr1- Δ2::TRP1*
FY1679-28C/TD	FY1679-28C disrupted by *pdr3-Δ1::HIS3*
FY1679-28C/TDEC	FY1679-28C/TD disrupted by *pdr1- Δ2::TRP1*

[a] In these strains, the deletions have been done in *pdr1-3* US50-18C.

PDR1 (Delaveau *et al*., 1994, Mahe et al.,1996). The regulatory properties of these transcription factors were recently reviewed (Kolaczkowska and Goffeau, 1999). A series of activating mutations (such as *pdr1-3* or *pdr3-7*) were shown to correspond to single amino acid substitutions in either *PDR1* (Carvajal *et al*., 1997) or *PDR3* (Nourani *et al*., 1997).

These mutations confer not only strong drug resistance phenotypes (Balzi *et al*., 1991) but also produce remarkable levels of overexpressed Pdr5p, Snq2p and Yor1p in the plasma membrane (Decottignies *et al*., 1994, 1995,1998).These mutations also increase the mRNA level of several other genes (De Risi et al, 2000)

We will review in this paper some data from our

Table 4. Susceptibility of the multideleted AD1-9 strain for various antifungal agents

	IC_{50}'s			MIC's		
Inhibitors	wild-type 2229-5C	AD1-9	Fold-Difference	wild-type 2229-5C	AD1-9	Fold-Difference
Brefeldin A	195	3.53	55	200.00	6.25	32
Clotrimazole	0.51	0.0061	83	1.56	0.012	128
Crystal Violet	0.09	0.026	3.3	0.07	0.05	1.5
Cycloheximide	0.02	0.006	3.0	0.05	0.01	4.1
Dazomet	11.90	6.19	1.9	12.50	6.25	2.0
Dichlone	4.68	0.90	5.2	12.50	3.13	4.0
Dichlorophen	11.26	0.39	29	12.50	0.78	16
Dinocap	>200	14	14	200.00	25.00	8.0
Econazole Nitrate	0.092	0.002	46	0.20	0.00	63
Fluconazole	5.09	0.184	28	12.5	0.391	32
Fluphenazine	8.50	2.0	4.3	12.50	6.25	2.0
8-Hydroxyquinoline	8.46	3.49	2.4	25.00	6.25	4.0
Imazalil	0.19	0.009	21	0.78	0.03	31
Ketoconazole	0.97	0.006	162	1.56	0.01	130
Maneb	0.96	0.399	2.4	1.56	0.78	2.0
Miconazole	0.0429	0.0015	29	0.391	0.0031	126
Nocodazole	3.37	0.48	7.1	6.25	0.78	8.0
Polyoxin D	>200	~15	13	>200	25.00	8.0
Propiconazole	0.16	0.002	78	1.56	0.00	521
Rhodamine 6G	12.20	0.100	122	25.00	0.10	255
Sanguinarine	2.39	0.440	5.4	3.13	0.78	4.0
Staurosporine	0.67	0.018	37	0.78	0.02	33
Sulfometuron methyl	4.83	0.971	5.0	6.25	1.56	4.0
Terbinafine	3.27	0.253	13	6.25	0.39	16
Thiabendazole	>100	12.5	8.0	>100	14.0	7.2

[a]C50 and MIC are listed as μg/ml. Determinations were the average of duplicate points.

laboratory illustrating the multidrug resistance properties of Pdr5p, Snq2p and Yor1p. We will also show that their control by *PDR1/PDR3* can be manipulated to study the specificity of the yeast multidrug transporters and their biochemical properties

Phylogenic Classification of Yeast Multidrug Transporters

The yeast genome encodes for 22 putative ABC transporters(16 full-size and 6 small-size) one of which (YKR103/104) is a pseudogene (Decottignies and Goffeau, 1997). In this original phylogenetic classification three phylogenetic clusters and seven subclusters were distinguished. Other classifications have been proposed (Michaelis and Berkower, 1995; Bauer *et al*., 1999). An abundance of classifications leads to confusion. The International Union of the Biochemical and Molecular Biology Societies is presently considering to endorse the Transport Classification system developed by Milton Saier (2000). The ABC Efflux Porters (mostly Eukaryotic) have been classified in 12 families. Among those, four do not have a member in *Saccharomyces cerevisiae*: the Multidrug Resistance Exporter (MDR) Family, TC 3.A.1.201; the Cystic Fibrosis Transmembrane Conductance Exporter (CFTR) Family, TC 3.A.1.202; the Cholesterol/Phospholipid/Retinal (CPR) Flippase Family TC 3.A.1.211 and the Heavy Metal Transporter (HMT) Family, TC 3.A.1.210.

The *S. cerevisiae* members of the ABC-Efflux Porter families are listed in Table 1. A total of 12 yeast ABC transporters are still of unknown function. We have classified the genes of five of them (*ADP1, PDR10, PDR15, YOR011* ,*YHL035, TAP1 and TAP2*) in established phylogenetic families. The other five (*MDL1* and *MDL2*, *YOL075, PDR11* and *YOR011*) have been temporarily classified in three different new families of unknown function.

Table 5. Multidrug Sensitivity of Doubly Deleted *PDR1* and *PDR3* Strains

STRAINS	RhB	Rh6G	CHX	OLI	MICO	KETO	ITRA	ARS	ET BR	CRYS VIOL	4-NQO	SMM	TX-100
US50-18C	>1000	100	2	4	2	20	>100	15	1	>20	5	>6.5	>2000
D1-3/3	500	10	0.03	0.25	0.25	1	4	10	1	1	1	500	
FY	500	100	0.09	1	0.1	1	25	4	1	1	>6.5	2000	
FYΔ*PDR1*	500	100	0.045	0.5	0.25	4	1	500					
FYΔ*PDR 3*	500	50	0.09	1	0.1	2	1	>2000					
FYΔPDR1ΔPDR3	100	2.5	0.03	0.5	0.01	<0.1	<1	2	<0.3	1	>6.5	<50	

US50-18C contains the pdr1-3 mutation, D1-3/3 is its closest parental, the FY series is isogenic
(ARS in μM, TX is in μl/ml, all other drugs were tested in liquid culture for MNC (maximum non inhibitory concentrations) expressed in μg/ml

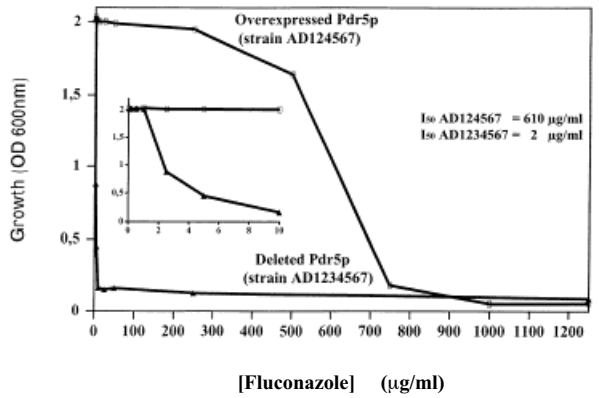


Figure 5. Fluconazole sensitivity.

Overlapping Substrates

A simple procedure for assessing the toxic substrates of ABC transporters has been developed (Kolaczkowski *et al.*,1996, 1998). Sensitivity is measured by the size of the halo of death cell surrounding a paper disc on which a drop of toxic compound is deposited. These discs are put on top of a lawn of either a parental or deleted strain in the tested ABC gene(s). When an ABC transporter exports a given drug, its deletant becomes more sensitive. A total of 349 toxic compounds have been tested by this method in six different strains, either singly deleted in *PDR5, SNQ2* or *YOR1* or doubly deleted in *PDR5+SNQ2* or triply deleted in *PDR5+SNQ2+YOR1*. In addition, an isogenic strain doubly deleted in *PDR1+PDR3* was tested. Table 2 reports these data in a qualitative way.

Several important conclusions can be brought:

1. There is considerable overlapping of substrates between *PDR5*, *SNQ2* and *YOR1* as shown by the fact that full sensitivity is obtained only when double or triple deletants are tested. Striking examples are: itraconazole, miconazole, nystatin, antimycin, nigericine, tetradecylammonium bromide which show considerable promiscuity.
2. Certain substrates show however specificity for a given ABC transporter. Striking examples are cycloheximide, benomyl, fluxilazole, nuarimol, phenapromil, soraphen for Pdr5p or quinoline oxides, resazurin for Snq2p or propanil, ferbam, thiram for Yor1p.
3. The variety of chemical structures handled by all the ABC transporters tested is impressive. It is not possible to identify specific chemical features favoring transport other than general hydrophobicity or amphiphilicity.
4. For some drug, the doubly deletant *PDR1 + PDR3* is more sensitive than the triply deleted *PDR5+SNQ2+YOR1*. This indicates the existence of unidentified multidrug resistance genes regulated by *PDR1/*PDR3 other than the three studied ABC transporters. Clear examples of this is chloromphenicol, rhodamine 6G, taurodeoxycholic acid and polyethylene detergents.

Hypersensitive Multideleted Strains

In an attempt to construct a strain with hypersensitivity to as many drugs as possible, we have deleted sequencially the multidrug ABC transporters *YOR1, SNQ2, PDR5, PDR10, YCF1, PDR11* and *PDR15.* We also deleted *PDR3* and *PDR1*, the two major homologous transcription factors involved in the activation of five ABC transporters and of several major facilitators possibly involved in drug transport (Decottignies *et al.*, 1998). In strains not deleted in *PDR1*, the hyperactivated mutant allele *pdr1-3* was introduced in order to increase expression of its transcription targets which were not deleted (Figure 1). In total, near 100 different isogenic combinatorial deletions (or overexpressions when *pdr1-3* is present) were created. The major ones are listed in Table 3. They are freely available for non commercial purposes (please contact ghislain@fysa.ucl.ac.be).

The growth sensitivity of one of the most exhaustively deleted strain AD1-9, deleted in seven ABC transporters and two transcription factors was tested for three dozens of compounds of different structure and different mode of toxic action. Two measurements for growth inhibition were carried out: the minimal total inhibitory (MIC) and half inhibition (IC50) concentrations. These two indexes provide coherent values (Table 4). They both identify as the most powerful inhibitors of the parental strain : crystal violet, cycloheximide, econazole, miconazole which inhibit growth below µM concentrations. Compared to the parental strain, the IC50 of the AD1-9 multiple deletant to crystal violet was decreased only 3.3 fold, but that to ketoconazole was decreased 160 fold and that to rhodamine 6G was 135 fold lower (Figure 2). The corresponding decrease of MIC ranged from 1.5 fold for crystal violet to 521 fold for propiconazole.

In general, compounds with positive charges such as crystal violet, dazomet, fluphenazine or maneb, showed lower sensitivity of the AD1-9 than the hydrophobic azoles.

It should be pointed out that the combined deletion of *PDR1* and *PDR3* are sufficient to increase considerably the sensitivity to inhibitors whereas each of these two single deletions have relatively little effect (Table 5). However, the AD1-9 strain in which both regulatory *PDR1* and *PDR3* genes as well as the seven ABC *genes YOR1, SNQ2, PDR5, YCF1, PDR10, PDR11* and *PDR15,* are deleted seems to be more slightly sensitive to several inhibitors than the strain doubly deleted in *PDR1* and *PRD3*. This indicates on one hand that some multi-drug ABC genes are not controlled by *PDR1/PDR3* and on the other hand that the major targets of *PDR1/PDR3* involved in resistance to the tested drugs were deleted in our strain AD1-9.

These hypersensitive multideleted knock-outs can be used for novel antifungal screens to detect low abundance or weakly-active lead structures, which are missed by the classic pharmaceutical screens. Our approach is very similar to that previously developed for the construction of hypersensitive bacterial strains (Hsieh et al.1998)This simple innovation may lead to enormous benefits such as the intracellular increase of the antifungal drug levels and the development by chemical engineering of antifungals with decreased susceptibility to ABC efflux pumping.

The Overexpression of Single ABC Transporters

The overlapping structure and function of several members of the yeast ABC transporter family is an handicap for the biochemical and physiological study of single ABC transporters. Combinatorial double deletions of two out of three of the major transporters *YOR1, SNQ2* and *PDR5*, in strains containing the activating mutation *pdr1-3* has allowed (Decottignies *et al*., 1998) to isolate and purify in a solubilized active form each of the three isolated ABC transporter, *Pdr5p, Snq2p* and *Yor1p*. This protocol allowed to demonstrate and characterize their ATPase activity (Decottignies *et al.* 1994, 1995, 1998). In the case of *Yor1p*, the level of expression had to be increased by using the *PDR5* promoter which is much more reactive to the *pdr1-3* activation than the *YOR1* promoter (Figure 3). The SUPERYOR strain so created was shown to be over 400 times more reactive to oligomycin than the deleted strain (Figure 4). The content of the plasma membrane in *YOR1p* was increased considerably up to the level of *Pma1p* (H^+-ATPase) which in the parental strain is the major constituent of the plasma membrane amounting to 10 to 20% of the membrane proteins.

This genetic purification by deletion of major contaminants combined to integrative shuffling of the PDR5 promoter in front of an ABC genes of interest in a strain containing the activating *pdr1-3* mutation is a convenient procedure for the study of single ABC transporters. For instance *Pdr5p* overexpression in a *pdr1-3* background in which six other ABC transporters had been deleted provides a strain with 300-fold increased resistance to fluconazole compared to that of an isogenic Pdr5p deletant (Figure 5). This strain is presently used by Brian Monk (University of Otago, NZ) for screening a 1.8 million member membrane-impermeant D-octapeptide combinatorial library, components which by inhibition of Pdr5p would render the cell more sensitive and be used as adjuvants reducing the minimal inhibitort concentration of antifungals (Monk and Goffeau, 2000). Similar constructions with heterologous ABC transporters (and in particuflar those from fungal pathogens) are underway.

Conclusion

The study of the substrate specificity of the major multidrug ABC pumps from yeast: *Pdr5p*, *Snq2p* and *Yor1p* has lead to the construction of multideleted strains with high sensitivity to hundreds of toxic drugs. These hypersensitive strains are used for novel screenings of antifungals.

In addition, genetic constructs of multideleted strains have been achieved in which single specific ABC proteins can be overexpressed in the plasma membrane of multideleted strains using the remarkable properties of activation of the *PDR5* promoter by the *pdr1-3* point mutation in the transcription factor *PDR1*. This system allows complete purification of the solubilized ABC transporter by histidine tagging and nickel-chelating chromatography. This opens the possibility to reconstitute ABC proteins in phospholipid vesicles and to study transport as well as mechanistic and structural properties. It also allows screening for specific ABC inhibitors which have the potency of increasing considerably the efficiency of antifungals when used as adjuvants.

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