YEAST
Yeast **15**, 1595–1608 (1999)

FLR1 Gene (ORF YBR008c) is required for Benomyl and Methotrexate Resistance in *Saccharomyces cerevisiae* and its Benomyl-induced Expression is Dependent on Pdr3 Transcriptional Regulator

NUNO BRÔCO[1], SANDRA TENREIRO[1], CRISTINA A. VIEGAS[1] AND ISABEL SÁ-CORREIA[1]*

[1]*Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal*

In this work we report the disruption of a *Saccharomyces cerevisiae* ORF YBR008c (*FLR1* gene) within the context of EUROFAN (EUROpean Functional Analysis Network) six-pack programme, using a PCR-mediated gene replacement protocol as well as the results of the basic phenotypic analysis of a deletant strain and the construction of a disruption cassette for inactivation of this gene in any yeast strain. We also show results extending the knowledge of the range of compounds to which *FLR1* gene confers resistance to the antimitotic systemic benzimidazole fungicide benomyl and the antitumor agent methotrexate, reinforcing the concept that the *FLR1* gene is a multidrug resistance (MDR) determinant. Our conclusions were based on the higher susceptibility to these compounds of *flr1Δ* compared with wild-type and on the increased resistance of both *flr1Δ* and wild-type strains upon increased expression of *FLR1* gene from a centromeric plasmid clone. The present study also provides, for the first time, evidence that the adaptation of yeast cells to growth in the presence of benomyl involves the dramatic activation of *FLR1* gene expression during benomyl-induced latency (up to 400-fold). Results obtained using a *FLR1–lacZ* fusion in a plasmid indicate that the activation of *FLR1* expression in benomyl-stressed cells is under the control of the transcriptional regulator Pdr3p. Indeed, *PDR3* deletion severely reduces benomyl-induced activation of *FLR1* gene expression (by 85%), while the homologous Pdr1p transcription factor is apparently not involved in this activation. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — *FLR1* gene; ORF YBR008c; *PDR1*/*PDR3*; benomyl; methotrexate; *Saccharomyces cerevisiae*

INTRODUCTION

As a contribution to the functional analysis of novel yeast genes discovered by systematic sequencing of *Saccharomyces cerevisiae* genome we have disrupted ORF YBR008c within the context of EUROFAN (EUROpean Functional Analysis Network) six-pack programme (Oliver, 1996; Oliver *et al*., 1998). In this work, we report the disruption of ORF YBR008c in two different backgrounds (FY1679, the reference strain of the yeast genome project, and W303), using a polymerase chain reaction (PCR)-mediated gene replacement protocol (Wach *et al*., 1994; Wach, 1996), and the construction of a disruption cassette for inactivation of this gene in any yeast strain. We also describe the results of the basic phenotypic analysis of a deletant strain and the construction of a plasmid carrying the *FLR1* gene. While the EUROFAN six-pack programme was in progress, ORF YBR008c was reported to be involved in *YAP1*-mediated resistance to fluconazole (*FLR1* gene), cycloheximide and 4-nitroquinoline-N-oxide (4-NQO) (Alarco *et al*., 1997). In the present work, we show results indicating that the *FLR1* gene is also required for resistance to benomyl and

*Correspondence to: I. Sá-Correia, Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal. Tel: +351-1-8417682; fax: +351-1-8480072; e-mail: pcisc@alfa.ist.utl.pt
Contract/grant sponsor: BIOTECH EUROFAN program of the EU; Contract/grant number: BIO4-CT95-0080; Contract/grant number: BIO4-CT97-2294.
Contract/grant sponsor: Fundação para a Ciência e a Tecnologia (FCT), FEDER and PRAXIS XXI program; Contract/grant number: PRAXIS/PCN/C/BIO/79/96; Contract/grant number: BD/9633/96; Contract/grant number: BIC/14709/97.

CCC 0749–503X/99/151595–14$17.50

Received 15 March 1999
Accepted 11 July 1999

methotrexate, based on the increased susceptibility of the deletion mutant to these inhibitory compounds and on the increased resistance of both the deletion mutant and the wild-type strain upon *FLR1* overexpression. Using this same approach, we confirmed the involvement of the *FLR1* gene in yeast resistance to cycloheximide, 4-NQO and fluconazole, as previously found by Alarco *et al.* (1997) by *YAP1* overexpression. Interestingly, ORF YBR008c was predicted to code for an integral membrane protein with 12 potential transmembrane segments, belonging to family 1 of homologues of transporters belonging to the major facilitator superfamily (MFS), which are required for multiple drug resistance (MDR) (André, 1995; Nelissen *et al.*, 1995, 1997; Paulsen *et al.*, 1998). On the basis of the complete yeast genome sequence and using different criteria, the multidrug: H^+ antiporters comprise about 24 proteins (Nelissen *et al.*, 1997; Goffeau *et al.*, 1997; Paulsen *et al.*, 1998), although the involvement of the vast majority as MDR determinants remains unknown and their physiological functions and apparent redundancy remain unclear.

Benomyl and other systemic benzimidazole fungicides, which act against phytopathogenic fungi, are extensively used in agriculture and horticulture to provide crop protection against a wide range of diseases (Davidse, 1986; Adams, 1997). Following widespread benomyl use, benomyl-resistant strains of many fungal pathogens have emerged, reducing the usefulness of this fungicide in agriculture (Nachmias and Barash, 1976; Adams, 1997). A number of resistance mechanisms have been proposed, including decreased rate of uptake/increased efflux of the fungicide (Nachmias and Barash, 1976; Nare *et al.*, 1994). Many transport systems play an important role in conferring MDR, presumably due to the catalysis of energy-dependent extrusion of a large number of structurally and functionally unrelated compounds out of the cells (Balzi and Goffeau, 1994; Roepe *et al.*, 1996; Bolhuis *et al.*, 1997; Kolaczkowski and Goffeau, 1997). In yeast, the proton-motive-force-dependent multidrug efflux systems identified to date belong to the major facilitator superfamily (MFS) and are involved in the symport, antiport or uniport of various substrates (Paulsen *et al.*, 1996; Pao *et al.*, 1998). Other known determinants associated with multidrug resistance are other membrane proteins belonging to the ATP-binding-cassette (ABC) superfamily, that utilize ATP hydrolysis to drive drug extrusion, and factors for transcriptional regulation of all these multidrug transporters (Balzi and Goffeau, 1994; Kolaczkowski and Goffeau, 1997). In yeast, multiple or pleiotropic drug resistance (PDR) can be controlled by the function of Pdr1p and Pdr3p, two homologous proteins which belong to the Zn_2Cys_6 family of transcriptional regulators (Balzi and Goffeau, 1991, 1995; Delaveau *et al.*, 1994; Katzmann *et al.*, 1996; Carvajal *et al.*, 1997). Most of the target genes for Pdr1p/Pdr3p identified so far comprise membrane transporters of the ABC protein superfamily (Balzi and Goffeau, 1994, 1995; Wolfger *et al.*, 1997), although it was recently shown that two hexose transporters are also controlled by Pdr1p/Pdr3p (Nourani *et al.*, 1997a). Mutations at *PDR1* and *PDR3* transcriptional regulator loci, such as *pdr1-3* and *pdr3-7* gain of function mutations, respectively, lead to pleiotropic drug resistance due to the increased activation of target genes encoding ABC drug efflux pumps (Carvajal *et al.*, 1997; Nourani *et al.*, 1997b). Transcription regulation within the PDR network requires a PDR-responsive element (PDRE) consensus motif, which is present in the promoters of PDR-responsive genes (Katzmann *et al.*, 1995, 1996; Mahé *et al.*, 1996; Wolfger *et al.*, 1997; Nourani *et al.*, 1997b). The *FLR1* gene promoter has a PDRE motif, the degenerate element 5′-TCCGC GCA-3′, at position −440 from the putative translational start site. In this report, we show results indicating that PDRE-mediated regulation also includes *FLR1*. By using a *FLR1–lacZ* fusion in a plasmid, we found that *FLR1* expression is very low during growth in the absence of benomyl stress but it is strongly activated (about 400-fold) during benomyl-induced latency, this activation being specifically dependent on Pdr3p, while the homologous Pdr1p transcription factor is apparently not involved.

MATERIALS AND METHODS

Strains, media and plasmids

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. YBR008c (*FLR1* gene) deletion was carried out in two EUROFAN reference yeast strains FY1679 and W303. The haploid strains FY23 (MAT**a**) and FY73 (MATα) (the progenitors of FY1679) were also used to determine the mating type of deletant or wild-type haploid cells. The strain FY1679-28C/EC is a

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Name	Genotype	Source or reference
FY1679	*MAT***a**/α, *ura*3-52/*ura*3-52, *trp1Δ63*/+, *leu2Δ1*/+, *his3Δ200*/+, *GAL2*$^+$/*GAL2*$^+$	EUROFAN
FY23	*MAT***a**, *ura*3-52, *trp1Δ63*, *leu2Δ1*, *GAL2*$^+$	EUROFAN
FY73	*MAT*α, *ura*3-52, *his3Δ200*,*GAL2*$^+$	EUROFAN
W303	*MAT***a**/α, *ura*3-1/*ura*3-1, *leu*2-3,112/*leu*2-3,112, *his*3-11, 15, 15/ *his*3-11, 15, 15, *trp*1-1/*trp*1-1, *ade1-2*/*ade1-2*, *can1-100*/*can1-100*	EUROFAN
W303-ISC02a	*MAT*α, *flr1*Δ::*KANMX4*, *ura*3-1, *leu*2-3,112, *his*3-11, 15, 15, *trp*1-1, *ade1-2*, *can1-100*	This work
W303-ISC02b	*MAT***a**, *ura*3-1, *leu*2-3,112, *his*3-11, 15, 15, *trp*1-1, *ade1-2*, *can1-100*	This work
W303-ISC02c	*MAT***a**, *ura*3-1, *leu*2-3,112, *his*3-11, 15, 15, *trp*1-1, *ade1-2*, *can1-100*	This work
W303-ISC02d	*MAT*α, *flr1*Δ::*KANMX4*, *ura*3-1, *leu*2-3,112, *his*3-11, 15, 15, *trp*1-1, *ade1-2*, *can1-100*	This work
FY1679-28C	*MAT***a**, *PDR1*, *PDR3*, *ura3-52*, *leu2-Δ1*, *trp1-Δ63*, *his3Δ200*, *GAL2*$^+$	Thierry *et al*., 1990
FY1679-28C/TDEC	*MAT***a**, *pdr1-Δ2::TRP1*,*pdr3Δ::HIS3*, *ura3-52*, *leu2-Δ1*, *trp1-Δ63*, *his3Δ200*, *GAL2*$^+$	Delaveau *et al*., 1994
FY1679-28C/EC	*MAT***a**, *pdr1-Δ2::TRP1*,*PDR3*, *ura3-52*, *leu2-Δ1*, *trp1-Δ63*, *his3Δ200*, *GAL2*$^+$	Delaveau *et al*., 1994
EC60	*MAT***a**, *PDR1 (from IL125-2B)*, *pdr3Δ::HIS3*, *ura3-52*, *leu2-Δ1*, *trp1-Δ63*, *his3Δ 200*, *GAL2*$^+$	Carvajal *et al*., 1997
EC61	*MAT***a** *pdr1-3*, *pdr3Δ::HIS3*, *ura3-52*, *leu2-Δ1*, *trp1-Δ63*, *his3Δ200*, *GAL2*$^+$	Carvajal *et al*., 1997

derivative of wild-type FY1679-28C and carries the *pdr1Δ* deletion (Carvajal *et al*., 1997), the strain FY1679-28C/TDEC carries *pdr1Δpdr3Δ* double deletion (Carvajal *et al*., 1997) and strains EC60 and EC61 resulted from the integration of the wild-type *PDR1* gene (of IL125-2B) or of this gene with the gain of function mutant *pdr1-3*, respectively, into the double mutant *pdr1Δpdr3Δ* (Carvajal *et al*., 1997). For routine culture, *S. cerevisiae* strains were cultivated in YPD medium (2% yeast extract, 1% peptone and 2% glucose). This rich medium, containing 2% glycerol instead of glucose (YPG), was used to test growth on a non-fermentable carbon source. Glucose minimal medium (SD) contained (per litre): 6·7 g Yeast Nitrogen Base without amino acids (Difco), 5 g glucose and the auxotrophic requirements. When required, 200 mg/l of geneticin (G418, Sigma) was added to YPD medium. The susceptibility of yeast strains to antifungal, drugs and other metabolic inhibitors was compared using minimal medium MM2 or in MM2, lacking either uracil (MM2-U) or leucine (MM2-L), depending on the plasmid carried by the strain. MM2 medium contained (per litre): 1·7 g yeast nitrogen base without amino acids or NH_4^+ (Difco), 20 g glucose, 2·65 g $(NH_4)_2SO_4$, 80 mg adenine, 10 mg histidine, 10 mg leucine, 20 mg tryptophan and 20 mg uracil.

Escherichia coli strains XL1 blue and JM109 were used as plasmid hosts. The bacteria were grown either in LB (Sigma) medium or in an alternative rich medium (5 g/l yeast extract; 10 g/l bactotryptone; 5 g/l NaCl; 1 g/l glucose; 0·7 g/l K_2HPO_4 and 0·3 g/l KH_2PO_4) supplemented with 50 µg/ml ampicillin or 30 µg/ml kanamycin, when necessary.

Centromeric plasmids carrying the wild-type *PDR1* or the *pdr1-3* alleles ligated to pRS315 (Sikorski and Hieter, 1989) were derived as described by Carvajal *et al.* (1997). Centromeric plasmids carrying the wild-type *PDR3* or the *pdr3-7* alleles ligated to pFL38 (Bonneaud *et al*., 1991) were derived as described by Delaveau *et al.* (1994) and Nourani *et al.* (1997b), respectively.

Cloning procedures were carried out by standard methods (Sambrook *et al*., 1989). Transformation of yeast cells was performed by the method of Gietz *et al*. (1992), slightly modified.

Table 2. Oligonucleotides used in this study.

Name	Sequence*
A1	5′-CCGGCATGCAGAAGGTAGAAGAGTTACGG-3′
A2	5′-GACGGCCATAGCGTGCAGTT-3′
A3	5′-TTGGCTTGGCCTATATGGGG-3′
A4	5′-GCGGCATGCGGCTTTGACAGTGGAACAGC-3′
L1/S1	5′-CAGCTCTTTACGAGGCTAAAATATCTACATCCTTATGCCGCGGCCGCATAGGCCACTAGT GGATCTG-3′
L2/S2	5′-ACGTAAACTTATGTGGAACATCTTTGTCGACAGATGGACGCGGCCGCCAGCTGAAGCTTC GTACGC-3′
K2	5′-CGACTGAATCCGGTGAGA-3′
K3	5′-CCTCGACATCATCTGCCC-3′
GF1	5′-CCCAAGCTTCTGCTACTTACCGAACTTGCA-3′
GF2	5′-CCCGGATCCACGATAGTGTGTCTGTACGT-3′

*The sequence complementary to the MCS of pFA6a–*kanMX4* is underlined.

*ORF YBR008c (*FLR1 *gene) disruption and cloning of YBR008c replacement cassette*

Disruption of ORF YBR008c in FY1679 was performed by the short flanking homology (SFH) strategy (Wach *et al.*, 1994); details are given in Huang *et al.* (1997) and Pearson *et al.* (1998). Briefly, the disruption cassette, consisting of a dominant resistance marker, *kanMX4*, which confers resistance to geneticin in yeast, flanked by short homology regions to the target ORF, was prepared by PCR using the pFA6–*kanMX4* plasmid (Wach *et al.*, 1994) as template and two primers (L1/S1 and L2/S2, Table 2) containing, at the 5′ end, 40 nucleotides homologous to the flanking region of the ORF followed by the *Not*I site and, at the 3′ end, 28 nucleotides homologous to pFA6a–*kanMX4*. A 1579 bp PCR fragment was generated using the following amplification conditions: 10 min denaturation at 94°C followed by the addition of *Taq* DNA polymerase at 0°C; 3 min at 94°C, 2 min at 57°C, 2 min at 74°C for three cycles; 2 min at 94°C, 2 min at 62°C, 2 min at 74°C for 30 cycles; 10 min final elongation at 74°C. This SFH–PCR product was directly used to transform the FY1679 strain and transformants were obtained on YPD plates with 200 mg/l geneticin. To verify the correct replacement of the gene by the deletion cassette, two independent PCRs were carried out with primers A1+A2+K2 and A3+A4+K3 (Table 2 and Figure 1), using genomic DNA of the deletion mutant candidate as template. Genomic DNA from the wild-type strain was used as the control.

In order to obtain a replacement cassette that can be used for the systematic inactivation of ORF YBR008c in any *S. cerevisiae* strain, it was necessary to create longer homologous sequences on both sides of the *kanMX4* module. This YBR008c replacement cassette (LFH) was obtained by PCR amplification with *Pwo* DNA polymerase using genomic DNA isolated from the heterologous deletant strain and two primers, A1 and A4 (Table 2), that were designed to be located approximately 700 and 400 bp upstream and downstream of the start and the stop codons, respectively. Amplification parameters were as follows: 2 min at 94°C; 1 min at 94°C, 2 min at 57°C, 2 min at 72°C for 10 cycles; 1 min at 94°C, 2 min at 57°C and 2 min+20 s at 72°C for each cycle for 15 cycles; 7 min at 72°C. The PCR product was cloned in the *Sph*I site of the pFL38 vector (Bonneaud *et al.*, 1991). The primers used in the disruption cassette construction were designed to have a replacement of the Kan^r module in the opposite sense of the ORF. Nevertheless, the existence of an ATG at the end of the *kan* gene, in the antisense strand, can be problematic. Thus, we have chosen to have the *kan* gene in the same orientation as the ORF. Consequently, the *kan*MX4 module was cleaved using the *Not*I site that was introduced by PCR, blunted with Klenow and ligated. The clone having the *kan* gene with the same orientation of the ORF YBR008c was chosen by restriction analysis. The deletion cassette was excised from pFL38 and recloned into the pUG7 plasmid in the *Eco*RV restriction site. The resulting construction was

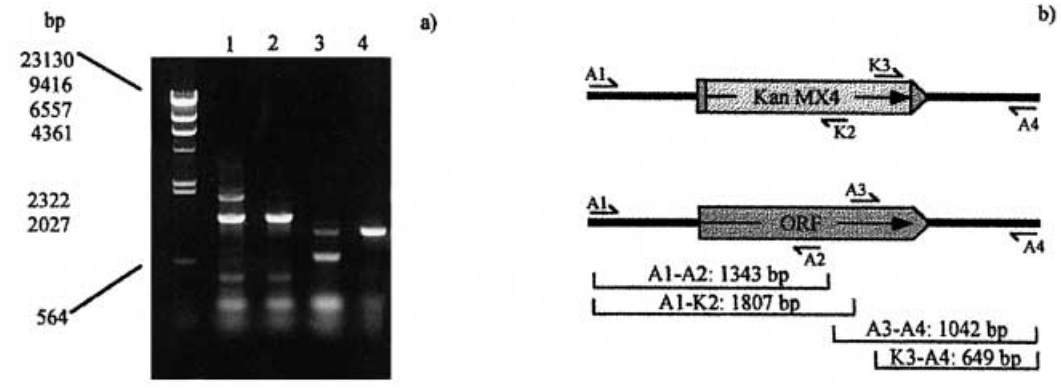

Figure 1. Confirmation of ORF YBR008c (*FLR1* gene) disruption by analytical PCR. (a) Agarose gel electrophoresis of PCR products obtained with six different primers: lanes *1* and *2* (A1, A2 and K2) or lanes *3* and *4* (A3, A4 and K3) using as template genomic DNA of lanes *1* and *3* (*FLR1* deletion mutant in W303) and lanes *2* and *4* (wild-type W303). (b) Schematic representation of analysis strategy, showing location of the primers used (sequences in Table 2) and the size of PCR products.

named pYORC_YBR008c plasmid and deposited in the EUROFAN collection (EUROSCARF, Frankfurt).

New FY1679 and W303 YBR008c deletants were obtained with the *Not*I digestion product of the pYORC_YBR008c plasmid. Correct replacement of the *FLR1* gene on the genomic locus was verified by PCR, as described above (Figure 1). These strains were deposited in the EUROFAN collection (EUROSCARF).

*Construction of a plasmid carrying the ORF YBR008c (*FLR1 *gene)*

The wild-type *FLR1* gene (ORF YBR008c) was cloned by the gap-repair technique (Rothstein, 1991) into pFL38. After confirming, by sequencing, that no mutations were incorporated during PCR amplification in the LFH cassette cloned into pFL38, the DNA fragment obtained after excision of the *kan*MX4 module with *Not*I was used to transform FY1679. Selection of Ura^+ and $G418^s$ transformants was performed, followed by recovery of the rescued plasmids in *Escherichia coli*. The presence of the *FLR1* gene in the resulting plasmid was confirmed by restriction analysis and PCR and this plasmid, named pYCG_YBR008c, was also deposited in EUROSCARF.

Tetrad dissection and homozygous YBR008c deletant construction

The diploid strains FY1679 and W303 and the respective deleted mutants were sporulated using standard procedures. At least 10 tetrads were dissected in the FY1679 background and two tetrads in the W303 background. Spores were then checked for germination and their genotype determined. FY1679 homozygous YBR008c deletant was obtained by crossing **a** and α haploid deletants and the zygote was isolated using a micromanipulator. Sporulation of the homozygous deletant was checked by dissecting six tetrads. Strains were also deposited in EUROSCARF.

Basic phenotypic tests

Growth tests were performed on haploid YBR008c deletion mutants of both mating types, resulting from the sporulation of the FY1679 heterozygous deletion mutant, and on the corresponding wild-type FY23 and FY73. Cells first grown on solid YPD medium for 1 day were used to inoculate agarized YPD, YPG and SD plates to obtain isolated colonies. The plates were incubated at 15°C, 30°C and 37°C and the size of the colonies of the haploid deletion mutant and the haploid wild-type was compared after 2–12 days of incubation, depending on the test conditions.

Multidrug susceptibility assays

The susceptibility tests were carried out, using a complete tetrad (two wild-type spores and two deleted spores) resulting from the deleted W303 heterozygous sporulation. To compare the susceptibility to antifungals, drugs and other metabolic inhibitors of the two wild-type and the two

deletion mutant (*flr1Δ*) strains, cells were grown on minimal medium MM2-agar plates supplemented with suitable concentrations of the different compounds. When a susceptibility phenotype was detected in the two *flr1Δ* strains, one wild-type strain and one deleted strain belonging to the same tetrad were transformed with either pYCG_YBR008c or the cloning vector, and the effect of the specific metabolic inhibitor on the growth of the transformants was also compared on MM2-U agar plates. All the metabolic inhibitors used for the susceptibility testing were purchased from Sigma Aldrich (Química S.A., Spain) and the stock solutions were solubilized in DMSO, with the exception of fluconazole (DIFLUCAN; in saline solution). DMSO concentration in the growth media was kept below 0·1% (w/v); this concentration had no detectable effect on growth and was also added to the control medium without inhibitors. The cells used to inoculate MM2-U or MM2 agar plates, supplemented with the various metabolic inhibitors, were exponential cells grown in the same liquid medium without drugs until culture $OD_{600}=0{\cdot}2\pm0{\cdot}02$ was reached, followed by dilution with H_2O to obtain cell suspensions with a standardized $OD_{600}=0{\cdot}05\pm0{\cdot}005$. This cell suspension or the diluted (1:5) suspension were applied as spots (4 µl) onto the surface of the agarized media and incubated at 30°C for 3–5 days, depending on the severity of growth inhibition. The specific effect of benomyl on the growth of the different transformants examined was assessed in agarized and in liquid MM2 medium lacking either uracil (MM2-U) (range 12·5–32·5 mg/l) or leucine (MM2-L) (range 15–27·5 mg/l) for maintenance of plasmids. The strains examined were W303-ISC02b (wild-type) and W303-ISC02a (*flr1*Δ) transformed with recombinant plasmids carrying *PDR1, PDR3*, these genes with the gain of function mutations *pdr1-3* or *pdr3-7,* or the *FLR1* gene, or with the respective cloning vectors pRS315 or pFL38. The growth curves were also compared at 30°C, 250 rev min^{-1} in liquid media supplemented with benomyl, by measuring culture OD_{600}. Specific growth rates were calculated by least-square fitting to the linear parts of the semilog growth plots; at least five experimental values were used and the correlation coefficients were above 0·97.

FLR1 *expression assays*

The levels of *FLR1* expression were compared during the growth under benomyl stress or in the absence of benomyl, of strain W303 or strain FY1679 and its isogenic derivatives carrying either *pdr1Δ, pdr3Δ* or *pdr1Δpdr3Δ or pdr3Δpdr1-3* mutations. They were assessed based on β-galactosidase (β-gal) activity of a *FLR1–lacZ* fusion plasmid present in the cells. This construction was obtained using a PCR fragment overlapping the promotor region, the translation initiation codon and a short portion of the coding region of the *FLR1* gene, from positions −979 to +33 bp. This PCR fragment was generated using oligonucleotides GF1 and GF2 (Table 2), which introduced, respectively, a 5′*Hin*dIII and a 3′*Bam*HI site for directional cloning into the centromeric plasmid YCpAJ152, cleaved with *Hin*dIII and *Bam*HI (André *et al.*, 1993). It was confirmed by DNA sequencing that the *FLR1* coding region was in frame with the *lacZ* gene and that no mutations had occurred during PCR amplification.

Cells transformed with the *FLR1-lacZ* plasmid construction were first cultivated in MM2-U medium at 30°C, 250 rev min^{-1} until mid-exponential growth (culture $OD_{600}=0{\cdot}4\pm0{\cdot}05$) and then resuspended in MM2-U (250 ml in 500 ml Erlenmeyer flasks; initial $OD_{600}=0{\cdot}05\pm0{\cdot}01$) supplemented (or not) with the desired concentrations of benomyl followed by incubation under identical conditions. Growth was followed by measuring culture OD_{600}. Culture samples were harvested at adequate time intervals and cells were filtered and kept at −20°C until used to assay β-gal activity. For β-gal assays, the cell pellets were resuspended in 5000 µl of Z buffer (Viegas *et al.*, 1994) to obtain a standardized OD_{600} of $0{\cdot}5\pm0{\cdot}2$ and the OD_{600} of this cell suspension was registered. Assays were based on the method of Miller, as previously described (Viegas *et al.*, 1994) and β-gal units (U) were defined as the increase in $A_{420}\times(\text{min}.OD_{600})^{-1}\times1000$.

RESULTS

*Generation of YBR008c (*FLR1 *gene) deletant strains and basic phenotypic analysis*

One of the chromosomal copies of ORF YBR008c was disrupted by a gene replacement method in strains FY1679 and W303, as described in Materials and Methods. Correct replacement of the target gene at the genomic locus was verified by PCR in the two backgrounds (Figure 1 and results not shown) and, after strain deposition this was re-confirmed at the EUROSCARF. Both strains

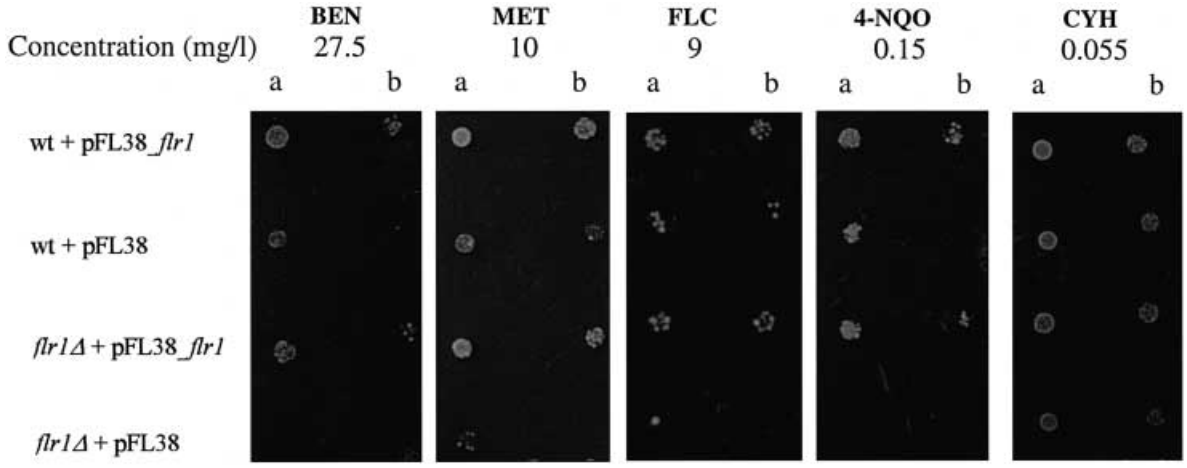

Figure 2. Comparison of the susceptibility to the various metabolic inhibitors, at the indicated concentrations, of strains W303-ISC02a (*flr1Δ*) and W303-ISC02b (wild-type), harbouring either the recombinant plasmid with *FLR1* into pFL38 (plasmid pYCG_YBR008c) or the cloning vector, using the experimental procedures described in Material and Methods. The *FLR1* gene is required for resistance to benomyl (BEN) and methotrexate (MET), in addition to fluconazole (FLC), 4-nitroquinoline-N-oxide (4-NQO) and cycloheximide (CYH). The cell suspension used to prepare the spots in (b) was a 1/5 dilution of the cell suspension used in (a).

were induced to sporulate and subjected to tetrad analysis. For the FY1679 background, at least 10 tetrads were dissected and analysed for viability, mating type segregation of auxotrophic markers and the geneticin-resistance phenotype. The results revealed that *FLR1* gene is non-essential; all the four spores from each tetrad of both mating type analysed were viable and two were geneticin-resistant. FY1679 haploid strains, deleted for YBR008c, did not display any evident growth phenotype on YPD, YPG or SD at 15°C, 28°C or 37°C (results not shown).

FLR1 *gene is a MDR determinant*

The susceptibility to several antifungals, drugs and other metabolic inhibitors of strains corresponding to the four spores of a complete tetrad resulting from the deleted W303 sporulation was compared by a spot test growth inhibition assay. A slight but consistently increased susceptibility to benomyl, methotrexate, fluconazole, 4-NQO and cycloheximide of the two *flr1Δ* mutants compared with the two wild-type strains was observed (results not shown). The role of *FLR1* gene in yeast resistance to all these metabolic inhibitiors was confirmed, based in the increase of resistance of both *flr1Δ* (W303-ISC02a) and wild-type (W303-ISC02b) strains whenever the expression of *FLR1* was restored or increased by the introduction of the centromeric plasmid pYCG_YBR008c carrying the *FLR1* gene (Figure 2).

Activation of FLR1 *expression by benomyl requires* PDR3 *gene*

The expression of the *FLR1* gene was found to be very low during the growth of *S. cerevisiae* strains W303 and FY1679-28C in MM2-U medium lacking benomyl, as monitored based on β-galactosidase activity of a *FLR1–lacZ* fusion plasmid construction present in the cells (Figures 3 and 4). However, *FLR1* gene expression was strongly activated in the two genetic backgrounds (up to 400-fold in both backgrounds) during benomyl-induced latency, reaching maximal values when benomyl-inhibited exponential growth started (Figure 4). During exponential growth with benomyl, β-galactosidase values steeply decreased, accompanying the increase of cell concentration, from the referred maximal values, although maintaining levels above those estimated for exponential unstressed cells (Figure 4). For highly inhibitory concentrations of benomyl (7·5–12·5 mg/l) that did not allow W303 growth after 27 h of incubation, the level of *FLR1* activation did not reach values as high as those observed with 5 mg/l of benomyl, a concentration that allowed exponential growth to start after a period of

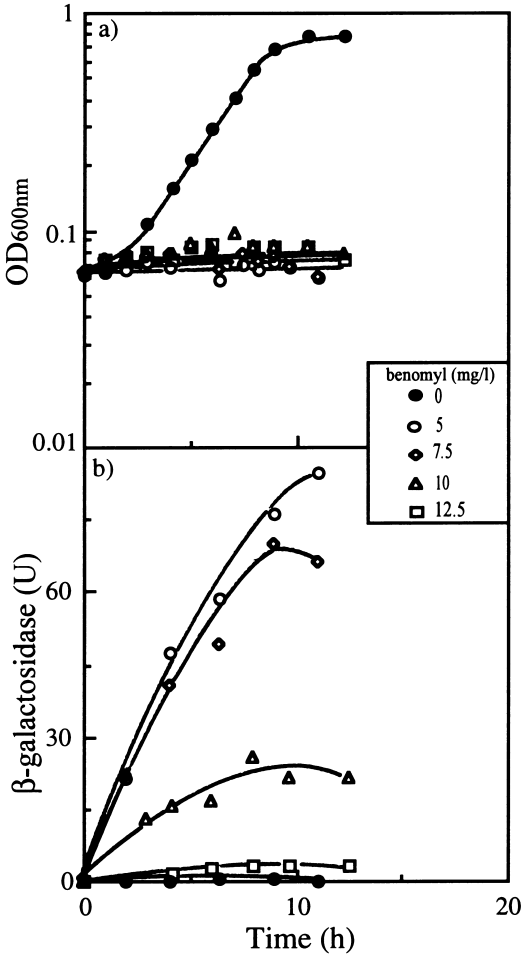

Figure 3. (a) Growth curves and (b) β-galactosidase activity of cells of *S. cerevisiae* W303 harbouring a *FLR1–lacZ* fusion plasmid, in MM2-U medium supplemented with increasing concentrations of benomyl (mg/l): 0 (●), 5 (○), 7·5 (◇), 10 (△) and 12·5 (□). Cells used as inoculum were grown in the absence of benomyl.

latency of approximately 15 h (Figure 3 and results not shown).

Benomyl-induced activation of *FLR1* was found to be highly dependent on the product of the *PDR3* gene. In fact, *PDR3* gene disruption in FY1679-28C severely reduced (up to 85%) benomyl-induced activation of *FLR1* expression, while the disruption of *PDR1* had no detectable effect on the activation of *FLR1* expression by benomyl (Figures 4b and 5). Consistently, no significant effect on *FLR1* expression under benomyl stress was found due to the additional deletion of *PDR1* or to the introduction of a gain of function mutation *pdr1-3* in cells with the *PDR3* gene deleted (Figures 4b and 5). However, the very low basal levels of *FLR1* expression were apparently reduced by the elimination of either *PDR1* or *PDR3* (by 70%) and nearly abolished by the elimination of both genes (Figures 4a *and* 5), although the introduction of a *pdr1-3* gain of function mutation in the strain carrying a *pdr3*Δ deletion did not lead to the upregulation of the basal expression of *FLR1* gene (Figure 5).

Effect of increased expression of PDR1/PDR3 *on benomyl resistance*

Since the activation of *FLR1* expression in yeast cells responding to benomyl stress was found to be PDRE-mediated, we have also examined the effect of the two homologous transcription factors, Pdr1p and Pdr3p, in yeast resistance to benomyl. This comparative analysis was based on a spot test growth inhibition assay using MM2-L or MM2-U agar plates in the case of plasmids carring the *PDR1* or *PDR3* genes, respectively. The increase of the number of *PDR1* gene copies or the expression of the gain of function mutation *pdr1-3* in both the wild-type strain and in the *flr1Δ* mutant led to an identical and significant increase of the resistance to benomyl, whenever the *FLR1* gene was functional or not, being the increase of benomyl resistance more pronounced with *pdr1-3* compared with *PDR1* (Figure 6). These results indicate that *PDR1*-mediated resistance to benomyl is not exerted via the *FLR1* gene. Compared with the effect of *PDR1*, *pdr1-3,* the increased expression of *PDR3* or of the gain of function mutation *pdr3-7* led to a slighter increase of benomyl resistance that was only detectable at highly inhibitory benomyl concentrations and more clearly with *pdr3-7* (Figure 7). Due to the different sensitivity to benomyl of the *flr1Δ* mutant compared with the wild-type strain, the effect of the increased expression of *PDR3* or *pdr3-7* became evident at different concentrations of benomyl (Figure 7). Contrary to expectations, the slight increase of benomyl tolerance by the increased expression of *PDR3* and *pdr3-7* did not appear to differ significantly in the wild-type and in the *flr1Δ* deletion mutant, at least within the range of benomyl concentrations examined using the referred experimental approach (Figure 7).

DISCUSSION

As predicted based on structural considerations (André, 1995; Nelissen *et al.*, 1995, 1997; Goffeau

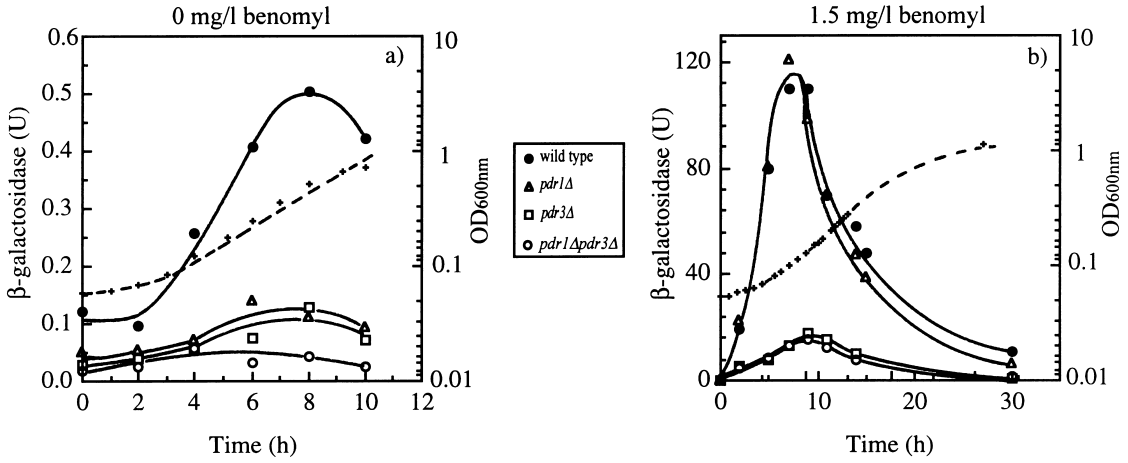

Figure 4. β-galactosidase activity from the *FLR1–lacZ* fusion plasmid present in cells of strains FY1679-28C (wild-type) (●), FY1679-28C/TDEC (*pdr1*Δ*pdr3*Δ) (○), EC60 (*pdr3*Δ) (□) and FY1679-28C/EC (*pdr1*Δ) (△) during growth in MM2-U medium supplemented with (a) 0 or (b) 1·5 mg/l of benomyl. Cells used as inoculum were grown in the absence of benomyl. The growth curves shown (+) as an indication of growth-dependent *FLR1* expression in benomyl-stressed (1·5 mg/l) and unstressed cells are those obtained with wild-type strain.

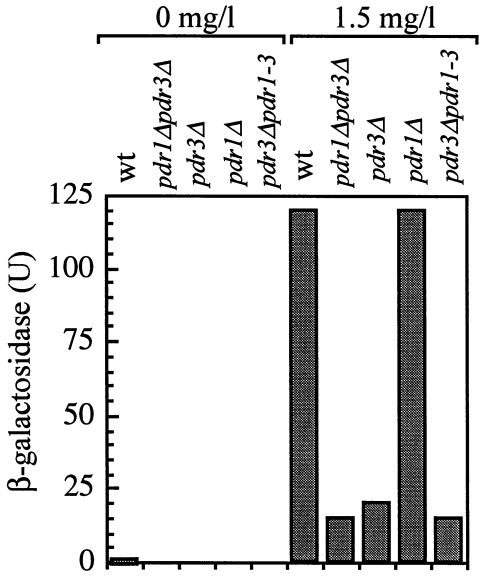

Figure 5. Comparison of the maximum levels of basal (0 mg/l benomyl) and benomyl (1·5 mg/l)-induced expression of the *FLR1* gene in wild-type FY1679-28C and in mutants with *PDR1*, *PDR3* or both *PDR1* and *PDR3* genes deleted, or in a strain with the *PDR3* gene deleted and a gain-of-function mutation *pdr1-3*. Expression values were monitored by measurement of β-galactosidase activity from the *FLR1–lacZ* fusion present in cells of the different strains harvested after approximately 8 h and 7 h (Figure 4) of incubation in benomyl-supplemented and unsupplemented medium, respectively.

et al., 1997; Paulsen *et al.*, 1998), we show results reinforcing the idea that ORF YBR008c (the *FLR1* gene) is a MDR determinant, extending the range of compounds to which the *FLR1* gene confers resistance to the antimitotic systemic benzimidazole fungicide, benomyl, and the dihydrofolate reductase inhibitor methotrexate, widely used as an antitumour agent. We also show results confirming that the *FLR1* gene is required for resistance to fluconazole, 4-NQO and cycloheximide, as previously found by Alarco *et al.* (1997), based on the distinct pattern of increased resistance to these inhibitors due to *YAP1* overexpression in wild-type and *flr1Δ* strains. However, our conclusions were based on the higher susceptibility to these metabolic inhibitors of *flr1Δ* compared with wild-type and on the increased resistance of both *flr1Δ* and wild-type strains upon increased expression of *FLR1* gene from a centromeric plasmid clone. Interestingly, the protein encoded by the *FLR1* gene is closely related to MDR proteins from *C. albicans* BEN^r, also involved in resistance to benomyl and methotrexate (Fling *et al.*, 1991), *C. maltosa* CYHR, which also confers resistance to cycloheximide and methotrexate (Ben-Yaacov *et al.*, 1994), *S. pombe* CAR1, which confers resistance to amiloride (Jia *et al.*, 1993) and *C. dubliniensis* CdMDR1, which confers resistance to fluconazole, being the fluconazole-resistant isolates also less susceptible to 4-NQO and methotrexate (Moran *et al.*, 1998).

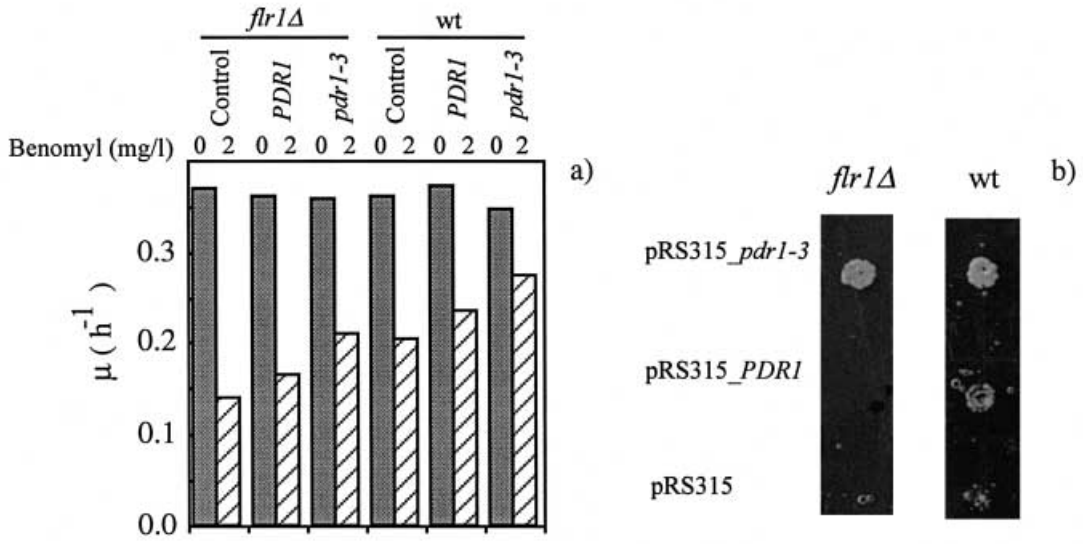

Figure 6. (a) Specific growth rates (h^{-1}), compared in MM2-L medium either supplemented (▨) (2 mg/l) or not (▩) with benomyl, of strains W303-ISC02b (wild-type) or W303-ISC02a (*flr1Δ*) transformed with recombinant plasmids with: *PDR1* or *PDR1* with the gain-of-function mutation, *pdr1-3*, into pRS315, or transformed with the cloning vector alone (control); (b) comparison of the susceptibility to benomyl of these same yeast transformants, by a spot test growth inhibition assay, on MM2-L-agarized medium supplemented with 25 mg/l of benomyl, as described in Materials and Methods.

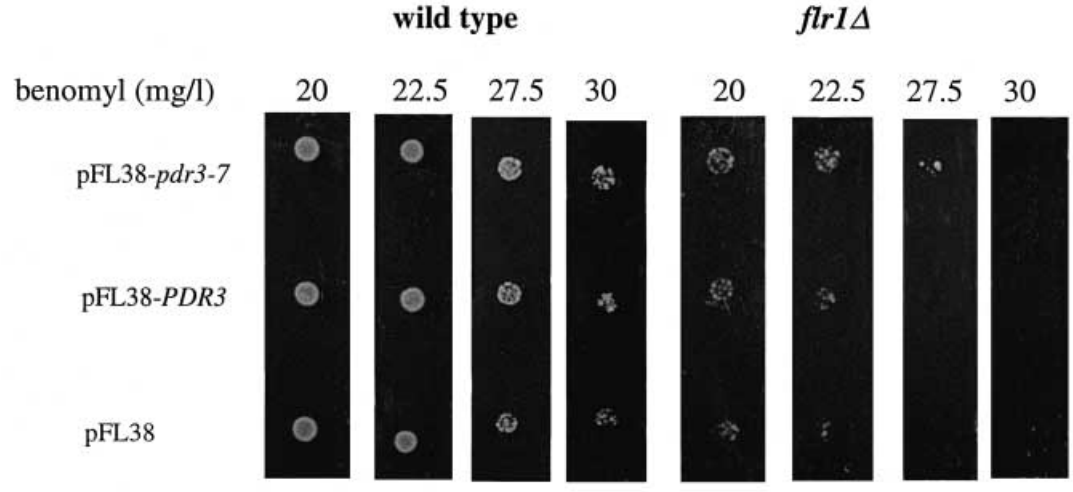

Figure 7. Effect of the increased expression of *PDR3* gene or the gain-of-function mutation *pdr3-7* on the susceptibility to benomyl of W303-ISC02b (wild-type) and W303-ISC02a (*flr1Δ*) by the introduction of recombinant plasmids with these genes into pFL38, by a spot test inhibition assay on MM2-U agar medium supplemented with 20–30 mg/l benomyl, as described in Materials and Methods.

The prevalence and apparent redundancy of proton-motive-force-dependent multidrug systems in a diversity of organisms, protecting a cell from the effects of toxic compounds, raises the question of what their normal physiological role in the cell is. A few evidences appear to suggest that they may play other roles than detoxification, such as the transport of a particular substrate, owing to their

ability to confer cross-resistance to several unrelated drugs that do not have a common structure or mechanism of action, only fortuitous. Significantly, it was recently proved that the membrane protein encoded by ORF YLL028w, which is also a member of cluster II of family 1 of the MFS–MDR transporters closely related to the FLR1 protein (Nelissen *et al*., 1997), is involved in polyamine transport across the vacuolar membrane (Tomitori *et al*., 1999). Indeed, the role of MDR protein overexpression in decreasing the intracellular retention of a variety of metabolic inhibitors is not necessarily the result of the direct active translocation of the toxic compounds, but can be due to the alteration of the electrical membrane potential ($\Delta\psi$) and/or the intracellular pH (pHi). In fact, the modification of the electrochemical proton gradient across the plasma membrane or internal membranes indirectly alters translocation and intracellular retention of hydrophobic drugs that are cationic, weakly basic and/or react with intracellular targets in a pHi- or $\Delta\psi$-dependent manner (Roepe *et al*., 1996).

The primary mode of action of benzimidazoles in fungi and in cells of many other eukaryotic organisms appears to be the inhibition of microtubule-mediated cellular functions, leading to the disruption of the mitotic spindle and consequent marked inhibition of nuclear division (Davidse, 1986; Adams, 1997). Resistance to benzimidazoles developed rapidly in the 1970s, following their widespread use as sprays. Therefore, the study of the mechanisms underlying the acquisition of resistance to benzimidazoles in pathogenic fungal species of agricultural importance or in model species like *S. cerevisiae* is of high interest. Studies with several fungi indicate that resistance is frequently a consequence of an alteration in the fine structure of the β-monomer of tubulin, which results in a decreased affinity for benzimidazoles (Adams, 1997). However, the decreased rate of uptake/increased efflux of the fungicide have also been proposed as a resistance mechanism (Nachmias and Baresh, 1976; Nare *et al*., 1994). The expression of drug extrusion systems is often induced by the drugs themselves (Paulsen *et al*., 1996; Bolhuis *et al*., 1997). This suggests that the specificity of a transporter for a particular group of unrelated compounds may also derive from differential gene expression induced by these drugs, and not simply be the result of differences in drug recognition by the transporters. The present study provides, for the first time, evidence that during benomyl-induced latency, when yeast cells are adapting to growth in the presence of the fungicide, *FLR1* gene expression is dramatically induced. There is a lack of information concerning the regulation of gene expression or enzyme activity during the extended period of latency preceeding exponential growth in the presence of a number of metabolic inhibitors. However, the physiological adaptation of cells that have been grown in the absence of inhibitors during this lag phase, such as the physiological phenomenon observed in this study during benomyl-induced latency, is critical to their eventual recovery and entrance in exponential growth. Among the few examples reported in the literature is the increase of the activity of plasma membrane H^+-ATPase during the period of latency induced by octanoic acid in yeast (Viegas *et al*., 1998).

Full activation of the *FLR1* gene during benomyl-induced latency was found to require a functional *PDR3* gene, while the homologous Pdr1p transcription factor is apparently not involved. However, although severely reducing benomyl-induced activation of the *FLR1* gene (by 85%) during benomyl-induced latency, *PDR3* deletion did not lead to the complete elimination of *FLR1* activation by benomyl. This suggests that there are *PDR3*-independent mechanisms involved in the full activation of *FLR1* by benomyl, namely the possible direct action of Yap1p, in concert with Pdr3p, on *FLR1* gene (Alarco *et al*., 1997). Contrary to expectations, no significant difference was observed in resistance to benomyl upon the increased expression of either *PDR3* or *pdr3-7* in wild-type or *flr1Δ* mutant strains. Indeed, the increased expression of this transcription factor only led to a slight increase of yeast benomyl resistance, while the effect of *PDR1*, and especially *pdr1-3*, was significant. However, and consistently with all the results reported in this work, the absence of a functional *FLR1* gene had no effect on Pdr1- or Pdr1-3-mediated benomyl resistance, which is probably due to *PDR1* target genes such as those encoding ABC drug efflux pumps.

In the absence of benomyl stress, yeast cells express extremely low *FLR1* levels, *FLR1* expression being reduced in both *pdr3Δ* and *pdr1Δ* mutants and almost abolished in the *pdr1Δpdr3Δ* mutant. Since transcriptional regulation of *PDR3* gene is known to involve control by Pdr1p (Delahodde *et al*., 1995), it is possible that the apparent contribution of the *PDR1* gene to the basal *FLR1* expression may be at least partially

indirect, via the *PDR3* gene. Indeed, basal expression was not upregulated in the *pdr3Δ* deletion mutant carrying the gain-of-function mutation *pdr1-3*. In addition, the strong benomyl-induced *FLR1* expression was apparently independent of the *PDR1* gene. The exact molecular basis for these observations was not clarified in the present work. It is possible that the comparison of the transcription levels of both *PDR1* and *PDR3* genes in benomyl-stressed and unstressed cells may bring some light to this complex problem.

A number of observations reported in this work indicate that *FLR1* expression in benomyl-stressed cells is under the control of Pdr3p, while Pdr1p is apparently not involved. These results reinforce the concept that, *in vivo*, Pdr1p and Pdr3p do not represent a strictly redundant gene family of transcription activators (Delahodde *et al*., 1995). Significantly, the only PDRE motif present in *FLR1* promotor, the sequence element 5′-TCCGCGCA-3′, was identified as a potential Pdr3p target site in certain PDR genes (Mahé *et al*., 1996). *FLR1* expression was also proved to be under the control of YAP1p (Alarco *et al*., 1997). Importantly, a functional link between the Yap1-dependent stress response pathway and Pdr1p/Pdr3p-dependent development of pleiotropic drug resistance in yeast was recently described (Wendler *et al*., 1997). Although it remains to be proved, benomyl-induced expression of *FLR1* may be exerted by YAP1p via the *PDR3* gene, as thought to be the case for the activation of target genes mediating diazaborine and 4-NQO resistance in yeast (Wendler *et al*., 1997).

ACKNOWLEDGEMENTS

We are grateful to B. André, Université Libre de Bruxelles, for his friendly support throughout ORF YBR008c disruption, the cognate gene cloning and the *lacZ* gene fusion construction. We also express our gratitude to A. Goffeau and E. Balzi, Université Catholique de Louvain, and C. Jacq, ENS-CNRS, Paris, for their encouragement and advice in this study and for the donation of Pdr1 and Pdr3 plasmid clones and deletion strains. This research was supported by the European Union BIOTECH EUROFAN I and II projects (contracts BIO4-CT95-0080 and BIO4-CT97-2294) and by 'Fundação para a Ciência e a Tecnologia' (FCT), FEDER and PRAXIS XXI Programme (Project PRAXIS/PCN/C/BIO/79/96 and Grants BD/9633/96 and BIC/14709/97 to S. Tenreiro and N. Brôco, respectively).

REFERENCES

Adams, D. J. (1997). Drug and pesticide resistance in fungi. In Hayes, J. D. and Wolf, C. R. (Eds), *Molecular Genetics of Drug Resistance*. Harwood Academic, Amsterdam, pp. 31–80.

Alarco, A.-M., Balan, I., Talibi, D., Mainville, N. and Raymond, M. (1997). AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires *FLR1* encoding a transporter of the major facilitator superfamily. *J. Biol. Chem.* **272,** 19 304–19 313.

André, B. (1995). An overview of membrane transport proteins in *Saccharomyces cerevisiae*. *Yeast* **11,** 1575–1611.

André, B., Hein, C., Grenson, M. and Jauniaux, J. C. (1993). Cloning and expression of the *UGA4* gene coding for the inducible GABA-specific transport protein of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **273,** 17–25.

Balzi, E. and Goffeau, A. (1991). Multiple or pleiotropic drug resistance in yeast. *Biochim. Biophys. Acta* **1073,** 241–252.

Balzi, E. and Goffeau, A. (1994). Genetics and biochemistry of yeast multidrug resistance. *Biochim. Biophys. Acta* **1187,** 152–162.

Balzi, E. and Goffeau, A. (1995). Yeast multidrug resistance: the PDR network. *J. Bioenerg. Biomembr.* **27,** 71–76.

Ben-Yaacov, R., Knoller, S., Caldwell, G. A., Becker, J. M. and Koltin, Y. (1994). *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. *Antimicrob. Agents Chemother.* **38,** 648–652.

Bolhuis, H., van Veen, H. W., Poolman, B., Driessen, A. J. M. and Konings, W. N. (1997). Mechanisms of multidrug transporters. *FEMS Microbiol. Rev.* **21,** 55–84.

Bonneaud, N., Ozier-Kalogeropoulos, O., Li, G., Labouesse, M., Minvielle-Sebastia, L. and Lacroute, F. (1991). A family of low and high copy replicative and single-stranded *S. cerevisiae*/*E. coli* shuttle vectors. *Yeast* **7,** 609–615.

Carvajal, E., van den Hazel, H. B., Cybularz-Kolaczkowska, A., Balzi, E. and Goffeau, A. (1997). Molecular and phenotypic characterization of yeast *PDR1* mutants that show hyperactive transcription of various ABC multidrug transporter genes. *Mol. Gen. Genet.* **256,** 406–415.

Davidse, L. C. (1986). Benzimidazole fungicides: mechanism of action and biological impact. *Ann. Rev. Phytopathol.* **24,** 43–65.

Delahodde, A., Delaveau, T. and Jacq, C. (1995). Positive autoregulation of yeast transcription factor Pdr3p which is involved in control of drug resistance. *Mol. Cell. Biol.* **15,** 4043–4051.

Delaveau, T., Delahodde, A., Carvajal, E., Subik, J. and Jacq, C. (1994). *PDR3*, a new yeast regulatory gene, is homologous to *PDR1* and controls the multidrug resistance phenomenon. *Mol. Gen. Genet.* **244,** 501–511.

Fling, M. E., Kopf, J., Tamarkin, A., Gorman, J. A., Smith, H. A. and Koltin, Y. (1991). Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. *Mol. Gen. Genet.* **227,** 318–329.

Gietz, D., Jean, A. S., Woods, R. A. and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* **20,** 1425.

Goffeau, A., Park, J., Poulsen, I. T., Jonniaux, J.-L., Dinh, T., Mordant, P. and Saier, M. H. Jr (1997). Multidrug-resistant transport proteins in yeast: complete inventory and phylogenetic characterization of yeast open reading frames within the major facilitator superfamily. *Yeast* **13,** 43–54.

Huang, M.-E., Cadieu, E., Souciet, J.-L. and Galibert, F. (1997). Disruption of six novel yeast genes reveals three genes essential for vegetative growth and one required for growth at low temperature. *Yeast* **13,** 1181–1194.

Jia, Z.-P., McCullough, N., Wong, L. and Young, P. G. (1993). The amiloride resistence gene, *car1*, of *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **241,** 298–304.

Katzmann, D. J., Hallstrom, T. C., Voet, M., Wysoch, W., Golin, J., Volckaert, G. and Moye-Rowley, W. S. (1995). Expression of an ATP-binding cassette transporter-encoding gene (*YOR1*) is required for oligomycin resistance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15,** 6875–6883.

Katzmann, D. J., Hallstrom, T. C., Mahé, Y. and Moye-Rowley, W. S. (1996). Multiple Pdr1p/Pdr3p binding sites are essential for normal expression of the ATP binding cassette transporter protein-encoding gene *PDR5*. *J. Biol. Chem.* **271,** 23 049–23 054.

Kolaczkowski, M. and Goffeau, A. (1997). Active efflux by multidrug transporters as one of the strategies to evade chemotherapy and novel practical implications of yeast pleiotropic drug resistance. *Pharmacol. Ther.* **76,** 219–242.

Mahé, Y., Parle-McDermott, A., Nourani, A., Delahodde, A., Lamprecht, A. and Kuchler, K. (1996). The ATP-binding cassette multidrug transporter Snq2 of *Saccharomyces cerevisiae*: a novel target for the transcription factors Pdr1 and Pdr3. *Mol. Microbiol.* **20,** 109–117.

Moran, G. P., Sanglard, D., Donnelly, S. M., Shanley, D. B., Sullivan, D. J. and Coleman, D. C. (1998). Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. *Antimicrob. Agents Chemother.* **42,** 1819–1830.

Nachmias, A. and Barash, I. (1976). Decreased permeability as a mechanism of resistance to methyl benzimidazol-2-yl carbamate (MBC) in *Sporobolomyces roseus*. *J. Gen. Microbiol.* **94,** 167–172.

Nare, B., Liu, Z., Prichard, R. K. and Georges, E. (1994). Benzimidazoles, potent anti-mitotic drugs-substrates for P-glycoprotein transporter in multidrug-resistant cells. *Biochem. Pharmacol.* **48,** 2215–2222.

Nelissen, B., Mordant, P., Jonniaux, J.-L., De Wachter, R. and Goffeau, A. (1995). Phylogenetic classification of the major superfamily of membrane transport facilitators, as deduced from yeast genome sequencing. *FEBS Lett.* **377,** 232–236.

Nelissen, B., De Wachter, R. and Goffeau, A. (1997). Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **21,** 113–134.

Nourani, A., Wesolowski-Louvel, M., Delaveau, T., Jacq, C. and Delahodde, A. (1997a). Multiple-drug-resistance phenomenon in the yeast *Saccharomyces cerevisiae*: involvement of two hexose transporters. *Mol. Cell. Biol.* **17,** 5453–5460.

Nourani, A., Papajova, M., Delahodde, A., Jacq, C. and Subik, J. (1997b). Clustered amino acid substitutions in the yeast transcription regulator Pdr3p increase pleiotropic drug resistance and identify a new central regulatory domain. *Mol. Gen. Genet.* **256,** 397–405.

Oliver, S. G. (1996). A network approach to the systematic analysis of yeast gene function. *Trends Genet.* **12,** 241–242.

Oliver, S. G., Winson, M. K., Kell, D. B. and Baganz, F. (1998). Systematic functional analysis of the yeast genome. *TIBTECH* **16,** 373–378.

Pao, S. S., Paulsen, I. T. and Saier, M. H. Jr (1998). Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* **62,** 1–34.

Paulsen, I. T., Brown, M. H. and Skurray, R. A. (1996). Proton-dependent multidrug efflux systems. *Microbiol. Rev.* **60,** 575–608.

Paulsen, I. T., Sliwinski, M. K., Nelissen, B., Goffeau, A. and Saier, M. H. Jr (1998). Unified inventory of established and putative transporters encoded within the complete genome of *Saccharomyces cerevisiae*. *FEBS Lett.* **430,** 116–125.

Pearson, B. M., Hernando, Y. and Schweizer, M. (1998). Construction of PCR-ligated long flanking homology cassettes for use in the functional analysis of six unknown open reading frames from the left and right arms of *Saccharomyces cerevisiae*. *Yeast* **14,** 391–399.

Roepe, P. D., Wei, L., Hoffman, M. M. and Fritz, F. (1996). Altered drug translocation mediated by MDR protein: direct, indirect, or both? *J. Bioenerg. Biomembr.* **28,** 541–554.

Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* **194,** 281–301.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Sikorski, R. S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122,** 19–27.

Thierry, A., Fairhead, C. and Dujon, B. (1990). The complete sequence of the 8·2 kb segment left of *MAT* on chromosome III reveals five ORFs, including a gene for a yeast ribokinase. *Yeast* **6,** 521–534.

Tomitori, H., Kashiwagi, K., Sakata, K., Kakinuma, Y. and Igarashi, K. (1999). Identification of a gene for a polyamine transport in yeast. *J. Biol. Chem.* **274,** 3265–3267.

Viegas, C. A., Supply, P., Capieaux, E., van Duck, L., Goffeau, A. and Sá-Correia, I. (1994). Regulation of the expression of the H^+-ATPase genes *PMA1* and *PMA2* during growth and effects of octanoic and decanoic acid in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1217,** 74–80.

Viegas, C. A., Almeida, P. F., Cavaco, M. and Sá-Correia, I. (1998). The H^+-ATPase in the plasma membrane of *Saccharomyces cerevisiae* is activated during growth latency in octanoic acid-supplemented medium accompanying the decrease in intracellular pH and cell viability. *Appl. Environ. Microbiol.* **64,** 779–783.

Wach, A. (1996). PCR-synthesis of marker cassettes with long flanking homology for gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **12,** 259–265.

Wach, A., Brachat, A., Pohlmann, R. and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10,** 1793–1808.

Wendler, F., Bergler, H., Prutej, K., Jungwirth, H., Zisser, G., Kuchler, K. and Högenauer, G. (1997). Diazaborine resistance in the yeast *Saccharomyces cerevisiae* reveals a link between *YAP1* and the pleiotropic drug resistance genes *PDR1* and *PDR3*. *J. Biol. Chem.* **272,** 27 091–27 098.

Wolfger, H., Mahé, Y., Parle-McDermott, A., Delahodde, A. and Kuchler, K. (1997). The yeast ATP binding cassette (ABC) protein genes *PDR10* and *PDR15* are novel targets for the Pdr1 and Pdr3 transcriptional regulators. *FEBS Lett.* **418,** 269–274.

