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Multiple-Drug-Resistance Phenomenon in the Yeast *Saccharomyces cerevisiae*: Involvement of Two Hexose Transporters

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In the yeast *Saccharomyces cerevisiae*, multidrug resistance to unrelated chemicals can result from overexpression of ATP-binding cassette (ABC) transporters such as Pdr5p, Snq2p, and Yor1p. Expression of these genes is under the control of two homologous zinc finger-containing transcription regulators, Pdr1p and Pdr3p. Here, we describe the isolation, by an in vivo screen, of two new Pdr1p-Pdr3p target genes: *HXT11* and *HXT9*. *HXT11* and *HXT9*, encoding nearly identical proteins, have a high degree of identity to monosaccharide transporters of the major facilitator superfamily (MFS). In this study, we show that the *HXT11* product, which allows glucose uptake in a glucose permease mutant (*rag1*) strain of *Kluyveromyces lactis*, is also involved in the pleiotropic drug resistance process. Loss of *HXT11* and/or *HXT9* confers cycloheximide, sulfomethuron methyl, and 4-NQO (4-nitroquinoline-*N*-oxide) resistance. Conversely, *HXT11* overexpression increases sensitivity to these drugs in the wild-type strain, an effect which is more pronounced in a strain having both *PDR1* and *PDR3* deleted. These data show that the two putative hexose transporters Hxt11p and Hxt9p are transcriptionally regulated by the transcription factors Pdr1p and Pdr3p, which are known to regulate the production of ABC transporters required for drug resistance in yeast. We thus demonstrate the existence of genetic interactions between genes coding for two classes of transporters (ABC and MFS) to control the multidrug resistance process.

Transmembrane solute transport is ensured in all eukaryotic cells by a set of proteins embedded in the plasma and the internal membranes. Most transport proteins characterized to date catalyze the uptake of solutes across the plasma membrane. Other plasma membrane transporters mediate extrusion of intracellular compounds into the medium, while others, located in intracellular membranes, catalyze efflux from or within the mitochondria, vacuole, peroxisomes, or secretion organelles. These membrane proteins are generally classified in three main categories: channels, facilitators (also named transporters, permeases, or carriers), and pumps (ATPases). Among them, one protein family of particular biological importance is the nonproton ATPase family encoding ATP-binding cassette (ABC) transporters, which appear to be conserved in all living organisms ranging from bacteria to humans (1, 13, 32). Alterations of certain ABC transporters can cause human genetic disorders such as cystic fibrosis (36), Zellweger syndrome (19), X-linked adrenoleukodystrophy (30), and the multidrug-resistance (MDR) phenotype shown by tumor cells which acquire resistance to a variety of chemotherapeutic agents. MDR phenotype is frequently linked to the increased expression, sometimes by gene amplification, of an integral membrane protein, a member of the ABC transporter family. This protein, called P-glycoprotein, functions as an ATP-dependent efflux pump for drugs (18).

In the yeast *Saccharomyces cerevisiae*, a phenotype resembling the mammalian MDR exists and is known as pleiotropic drug resistance (PDR) (3, 4). Recently, three yeast counterparts of the P-glycoprotein gene were identified: *PDR5* (5, 7), *SNQ2* (14, 28, 38), and *YOR1* (24). When produced in large quantities, these ABC transporters confer resistance to several unrelated drugs. Overproduction of Pdr5p confers resistance to a protein synthesis inhibitor (cycloheximide) or the acetolactate synthase inhibitor (sulfomethuron methyl), whereas overproduction of Snq2p allows tolerance of the mutagen 4-NQO (4-nitroquinoline-*N*-oxide) and sulfomethuron methyl; finally, high levels of Yor1p enable yeast to grow on elevated oligomycin concentrations.

Transcription regulation of *PDR5*, *SNQ2*, and *YOR1* requires at least two regulatory genes encoding homologous zinc finger proteins, Pdr1p and Pdr3p (2, 16). Furthermore, several uncharacterized genetic loci, such as *PDR7* and *PDR9*, were found to control *PDR5* expression (17). *PDR5*, *SNQ2*, and *YOR1* were demonstrated to be under tight transcriptional control by Pdr1p and Pdr3p, mediated by *cis*-acting elements in their promoters (15, 22, 24, 28). Disruption of both *PDR1* and *PDR3* results in a significant decrease in *PDR5*, *SNQ2*, and *YOR1* expression. This effect, which is particularly dramatic for *PDR5*, leads to a heightened drug hypersensitivity. These findings indicate that these two regulators functionally overlap. Transcriptional regulation of *PDR3* has also been shown to involve Pdr1p and Pdr3p itself via an autoregulatory loop. It has been proposed that Pdr1p confers a rapid response to cellular toxin exposure, by increasing transcription of membrane transporters, and this response is enhanced by inducing *PDR3* expression. Further, Pdr3p may potentiate drug resistance through additional production of membrane transporters and by its own activation (15).

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In the frame of our systematic search to identify additional Pdr3p targets, we have found that *HXT11* and *HXT9* are under the transcriptional control of both Pdr1p and Pdr3p. These two genes encode two nearly identical proteins belonging to the hexose transporter family (*HXT*). Hxt11p and Hxt9p are the first membrane proteins that do not belong to the ABC transporter family that have been found to be activated by the *PDR* regulators Pdr1p and Pdr3p. In this study, we also show that the *HXT11* gene product is involved in the PDR process.

MATERIALS AND METHODS

Strains and growth conditions. The *S. cerevisiae* strains used in this study were isogenic to FY1679-28C (**a** *ura3-52 trp1Δ63 leu2Δ1 his3Δ200*), FY1679-28C Δ*pdr3* (**a** *ura3-52 leu2Δ1 trp1Δ63 pdr3*::*HIS3*), and FY1679-28C Δ*pdr1* Δ*pdr3* (**a** *ura3-52 leu2Δ1 pdr1*::*TRP1 pdr3*::*HIS3*). The strains FY1679-28C and W303-1A (**a** *ade2-1 can1-100 his3-11,15 leu2-3,118 trp1-1 ura3-1*) had deletions of *HXT11* and *HXT9* or *HXT11* alone, leading to W303-1A Δ*hxt11* Δ*hxt9* (**a** *ade2-1 can1-100,15 leu2-3 ura3-1 hxt11*::*TRP1 hxt9*::*HIS3*), W303-1A Δ*hxt11* (**a** *ade2-1 can1-100,15 leu2-3 ura3-1 hxt11*::*TRP1*), and FY1679-28C Δ*hxt11* Δ*hxt9* (**a** *ura3-52 hxt11*::*TRP1 leu2Δ1 hxt9*::*HIS3*).

Strains were grown at 30°C in minimal medium supplemented with the appropriate nutritional requirements. Sugars were added after autoclaving of the medium at a final concentration of 2%. Galactose inductions were performed as described in the work of Delahodde et al. (15). The composition of the synthetic medium for X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates is as reported in the work of Dang et al. (12).

Kluyveromyces lactis strains were PM6-13A (*MAT***a** *uraA1-1 trpA1-1 ade2-1 rag1-1*) and MW270-7B (*MAT***a** *uraA1-1 leu2 metA1-1 RAG1*). Rag phenotype was tested on GAA (5% glucose complete medium containing 5 mM antimycin A) plates.

Escherichia coli TG1 [K-12 Δ(*lac-pro*) *supE thi hsdD5/F′ traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15] was used for plasmid constructions and production of glutathione-*S*-transferase (GST)–Pdr3p*Eco*47III fusion protein.

Screening of the yeast fusion libraries. The fusion libraries were constructed by insertion of genomic *Sau*3AI DNA fragments into two shuttle vectors, YEp366 and YEp367 (12), leading to two different reading frames with respect to the *E. coli lacZ* gene missing its own ATG. In order to isolate a yeast gene differentially expressed in the absence or the presence of Pdr3p, we transformed (with the libraries) the strain FY1679-28C Δ*pdr3*, rescued by the plasmid pYE-PDR3 (URA3) containing *PDR3* under the control of the inducible *GAL1* promoter (15). About 95,000 transformants were plated on X-Gal medium containing galactose as the carbon source. On this medium, Pdr3p is produced at a high level, and the *lacZ* fusions activated by this transcription factor were functional. Blue colonies appearing after 2 to 4 days of incubation at 30°C were replica plated on glucose–X-Gal medium, and those colonies which turned white were further subjected to 5-fluoroorotic acid treatment to cure the plasmid containing *PDR3* and then retested on galactose–X-Gal medium in parallel with the uncured strain to confirm that the blue-colony response was linked to the presence of Pdr3p. Plasmid DNA was then extracted, transferred to *E. coli*, and used to transform FY1679-28C Δ*pdr3* containing (or not) pYE-PDR3. Only the fusions which again exhibited differential coloration in these two genetic contexts were further analyzed by sequencing.

Plasmids, oligonucleotides, and DNA manipulation. Plasmids pHXT11Z and pHXT9Z were isolated from the YEp367 library. They contained 735 bp of nontranslated sequence of each *HXT* gene and 815 bp of the open reading frame upstream of the *lacZ* gene. Plasmid pFL38-H11Z was constructed by insertion of the *Eco*RI fragment from pHXT11Z containing *HXT11* fused to the β-galactosidase gene into the ARS-CEN plasmid pFL38 described in the work of Bonneaud et al. (9). Deletion of the PDR element (PDRE) (*Sac*II site) was done by digestion of pFL38-H11Z with *Sac*II and T4 polymerase. After ligation, deletion of PDRE (ΔPDRE) was confirmed by linearization of the plasmid by *Bsp*EI.

Plasmid constructions containing *HXT1* or *HXT11* under the control of the *PGK* promoter, pCJ-HXT1 and pCJ-HXT11, respectively, were obtained by insertion of PCR fragments, amplified with two oligonucleotides at the N- and C-terminal regions of *HXT1* or *HXT11* containing *Bam*HI sites, into the *Bgl*II site of pEMBLye(30/2), previously described in the work of Banroques et al. (6). The primers used were *HXT1*-ATG (5′CAGCTGGATCCATGAATTCAACTC CCGA3′) and *HXT1*-Ct (5′CGAGTGGATCCGATGTTGAAGCAGCAGCG 3′) for *HXT1* construction or *HXT11*-ATG (5′GACACGGATCCTCAATA TCATGTCAGG3′) and *HXT11*-Ct (5′TCACCCTGTCAACTCGTGTAGC3′) for *HXT11* construction.

Plasmids pYE-PDR3, pFL44-PP3, pFL38-PP3, and Yep24-PDR1 are described in the work of Delahodde et al. (15). The mutated form of PDR3 (pdr3-9) was encoded in the ARS-CEN plasmid pFL38-PP3 (32a). GST-Pdr3p*Eco*47III is a truncated version of GST-Pdr3p, leading to the fusion of the 415 N-terminal amino acids of Pdr3p to GST. This fusion was produced as described in the work of Delahodde et al. (15).

Plasmid constructions containing *PDR3* or *PDR1* under the control of the *PGK* promoter, BFG1-PDR3 and BFG1-PDR1, respectively, were obtained by insertion of PCR fragments, amplified with two oligonucleotides at the N- and C-terminal regions of *PDR3* or *PDR1* containing *Bam*HI sites, into a *Bam*HI site of BFG1 previously described in the work of Chardin et al. (10). The primers used for *PDR1* were *PDR1*-ATG (5′GCGTGGATCCCCGCAAGCATTCTCAG TGGCC3′), and the universal primer and that used for *PDR3* were described in the work of Delahodde et al. (15).

HXT11 (*LGT3*) was cloned by in vivo complementation of the *rag1* mutation of *K. lactis* (impaired in the low-affinity glucose permease [43]) by using an *S. cerevisiae* genomic library made in the *K. lactis-S. cerevisiae* shuttle vector pSK1, as previously reported (34). *HXT11* was also cloned in *K. lactis* centromeric plasmid vector KCp491, generating KCp491-HXT11 (34). Its nucleotide sequence was determined. *HXT1* was part of the pR1S-1 plasmid (34).

Northern blot analyses. Total RNAs were extracted by a hot-phenol method (37), separated on a 1% agarose gel containing 6% formaldehyde, blotted to a nylon membrane (Hybond N$^+$; Amersham), and hybridized to DNA probes by standard methods. The membranes were probed with a 1-kb *Eco*RI-*Hin*dIII fragment of *ACT1*, with a 0.5-kb fragment (extending from bp −254 to +245) of *HXT11* and a 3.5-kb *Hin*dIII fragment of *PDR5*. Probes were radiolabelled by random priming (Nonaprime kit; Appligene).

Gene disruptions. The 5.2-kbp *Bam*HI-*Bgl*II fragment containing the entire *HXT11* gene was cloned into the *Bam*HI site of pBR322; this step was followed by *Kpn*I digestion and by removal of protruding 3′ termini. The internal 800-bp fragment thus eliminated was replaced by an 800-bp *Pst*I-*Eco*RI blunt fragment containing *TRP1*. The resulting 2.15-kbp *Nar*I-*Xho*I fragment was then used to disrupt the *HXT11* chromosomal locus by transformation of strain W303-1A to tryptophan prototrophy. Southern blot analysis of independent transformants, using the appropriate probe and restriction digest, allowed us to determine whether *HXT11* was correctly disrupted by integration of the modified gene.

The fragment used to delete *HXT9* was constructed as follows: a pKS(+) Bluescript vector containing a *Hin*dIII fragment encoding HXT11 was digested with *Hpa*I, treated with alkaline phosphatase, and then ligated to a *Pvu*II fragment containing the *HIS3* yeast selectable marker. The modified plasmid, called pCJHXT11::HIS3, was digested with *Hin*dIII, and the fragment containing the disrupted *HXT11* gene was used to transform the strain W303-1A Δ*hxt11*. Stable [HIS3$^+$] transformants were selected, and the site of chromosomal integration was determined by PCR analysis with discriminating oligonucleotides. Both constructions were also used to delete *HXT11* and *HXT9* in FY1679-28C.

Measurement of glucose uptake. Glucose uptake was determined according to the method of Walsh et al. (41). Cells were grown to an optical density at 600 nm of 2.0 in 2% glucose complete medium or in Ura$^-$ selective medium for the transformant (2% glucose minimal medium). The rate of D-[U-^{14}C]glucose uptake was determined by a 5-s incubation over the glucose concentration range of 0.2 to 160 mM.

β-Galactosidase activity determinations. The β-galactosidase assays of Tables 1 and 2 were performed with crude extracts as described in the work of Miller (29). The β-galactosidase activity reported in Table 3 was determined with crude extracts by using the chemiluminescent LumiGAL β-galactosidase detection kit (Clontech). Each value reported is the average of determinations of at least four independent transformants.

DNase I footprinting analyses. The DNase I protection assay was performed as reported in the work of Delahodde et al. (15). The oligonucleotides used to generate the *HXT11* promoter fragment are *HXT11*-AM (5′CCATTATTG CATTGCCTCCGC3′) and *HXT11*-AV (5′GCATTGTTCGTGTGGTCAGC 3′). The GST-Pdr3p*Eco*47III fusion was produced and purified as indicated in the work of Delahodde et al. (15).

Nucleotide sequence accession number. The nucleotide sequence of *HXT11* appears in the EMBL-GenBank nucleotide sequence database as *ScLGT3*, under accession no. X82621.

RESULTS

Expression library screening. We have adapted the screening method developed by Dang et al. (12) to isolate yeast genes regulated by the Pdr3p transcription activator. The screen is based on the use of an expression library in which random *Sau*3AI genomic fragments were cloned upstream of an ATG-less form of the *lacZ* reporter gene. Fusion libraries, representing two of the three reading frames, were used to transform a yeast strain in which production of the transcription activator, Pdr3p, is under the control of UAS_{Gal}. The Pdr3p-regulated fusions were selected on the basis of their differential coloration on X-Gal medium when *PDR3* is expressed or not (see Materials and Methods).

A total of 95,000 transformants were plated on galactose medium supplemented with X-Gal. Two percent of the colonies turned blue on this medium between day 2 and day 4. All of these colonies were retested to confirm the association be-

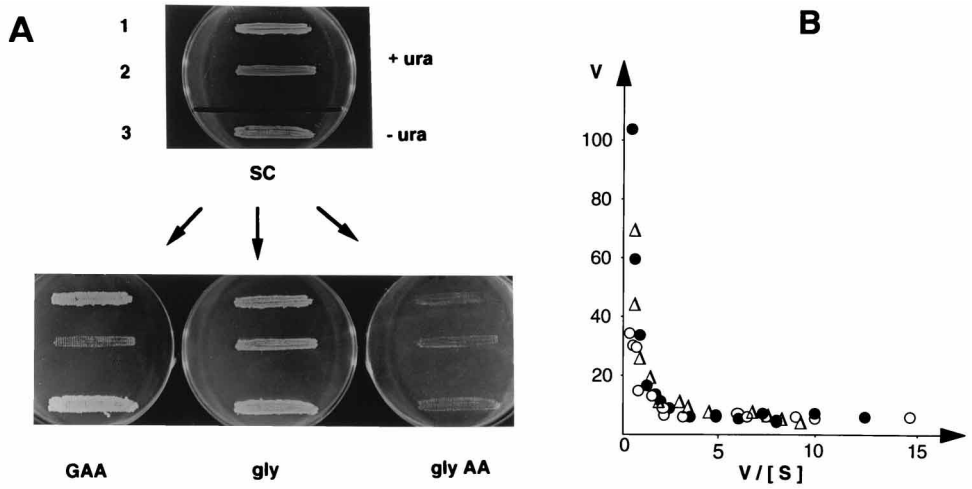


FIG. 1. Hxt11p restores glucose uptake in a *K. lactis rag1* mutant. (A) Complementation of the *rag1* mutation of *K. lactis* by *S. cerevisiae HXT11*. Strains were grown on synthetic complete (SC) 2% glucose medium. Transformants were grown on uracil-deficient medium and then replica plated to GAA (5% complete glucose medium, 5 mM antimycin A), Gly (2% glycerol), and GlyAA (2% glycerol, 5 mM antimycin A) plates. 1, MW270-7B (*RAG1*); 2, PM6-13A (*rag1*); 3, PM6-13A transformed with KCp491-HXT11. (B) Eadie-Hofstee plots of glucose uptake in the *rag1* mutant and its derivative containing the *S. cerevisiae HXT11* gene. Glucose uptake was measured as described in Materials and Methods. Uptake rate (V) is expressed as nanomoles of glucose per milligram (dry cell weight) per minute; glucose concentration (S) is millimolars. Δ, *K. lactis* wild-type strain MW270-7B; ○, *rag1* mutant strain PM6-13A; ●, *rag1* mutant strain transformed with KCp491-HXT11.

tween β-galactosidase activity and the presence of Pdr3p. Finally, genomic sequences from 28 plasmids were found to be regulated by Pdr3p and were selected for further characterization.

Sequence analyses were performed, and we found that seven fusions had occurred at the same position (+815 bp) in two genes, *HXT11* and *HXT9*. They present 97.3% base pair identity in their coding sequences and encode nearly identical open reading frames of 567 and 569 amino acids, respectively. These proteins belong to a family of monosaccharide facilitators (Hxtp/Gal2p) which includes the low-affinity glucose permease of *K. lactis* (Rag1p [43]) and the mammalian glucose transporters (Glutp [8, 21]). The HXT transporter subfamily of *S. cerevisiae* is composed of 20 very similar proteins which have 60 to 99% identity but differ in substrate specificity and regulation of expression (8, 26). A comparison of the predicted Hxt11p protein with other known HXT transporters revealed an average of 65% identity with Hxt1p, Hxt2p, Hxt3p, and Rag1p; 70% identity with Hxt4p and Hxt6p/7p; and 64% identity with Gal2p, a transporter required for galactose utilization (31, 39).

So far, all the known yeast genes regulated by the transcription factors Pdr1p and Pdr3p (except for *PDR3* itself) belong to the ABC transporter family. Since Hxt11p and Hxt9p belong to the major facilitator superfamily (MFS) class of membrane proteins, we intended to define the relationship between these proteins and the PDR phenotype.

Hxt11p restores glucose uptake in a *rag1* mutant of *K. lactis*. In *K. lactis*, the expression of the *RAG1* gene, coding for the apparent single low-affinity inducible glucose transporter, is necessary for growth on high-glucose medium in the presence of the respiratory inhibitor antimycin A (Rag^+ phenotype [43]). Few *S. cerevisiae* genes restoring growth on high-glucose medium in the absence of respiration in the *K. lactis rag1* mutant have been isolated. Two of them were characterized, *HXT4*/*LGT1* (34) and *HXT11*/*LGT3* (this work).

Figure 1A shows that *HXT11* cloned in the *K. lactis* centromeric vector KCp491 could complement the *rag1* mutation of *K. lactis*. The *rag1* mutant strain transformed with the KCp491-*HXT11* plasmid could not grow on glycerol in the presence of antimycin A, indicating that Hxt11p did not prevent drug entrance into the cells (Fig. 1A). To test the possibility that Hxt11p, like Rag1p, restores glucose transport, we examined glucose uptake kinetics as a function of glucose concentration in the *rag1* mutant strain transformed with KCp491-*HXT11*. The results, shown in Fig. 1B, indicate that *HXT11* of *S. cerevisiae* was able to restore, to a wild-type level, low-affinity glucose transport, which is greatly reduced in the *rag1* strain. These data, although obtained in a heterologous system, suggest that Hxt11p could act as a glucose permease or could regulate hexose transporter function.

Expression of *HXT11* and *HXT9* is not induced by glucose. It has been reported that expression of some *HXT* genes depends on the glucose induction pathway (33). Expression of *HXT1*, *HXT2*, *HXT3*, and *HXT4* exhibits different types of glucose induction in response to variable glucose amounts. Therefore, we first determined whether *HXT11* expression was inducible by glucose. For this purpose, we used the *HXT11-lacZ* fusion gene and monitored its expression in wild-type cells grown on different carbon sources. The results, shown in Table 1, re-

TABLE 1. *HXT11* expression in wild type on different carbon sources[a]

Carbon source	β-Galactosidase activity (U)
2% Gal	2.3 ± 0.7
2% Raf	3.4 ± 1.2
0.1% Glu	2.8 ± 0.8
0.2% Glu	2.0 ± 0.8
0.5% Glu	1.6 ± 0.5
1% Glu	1.9 ± 0.15
2% Glu	1.5 ± 0.4
4% Glu	1.6 ± 0.5
8% Glu	1.5 ± 0.6

[a] The wild-type strain FY1679-28C was transformed with the pHXT11Z plasmid encoding the HXT11-LacZ fusion protein. Cells were grown to mid-log phase in minimal medium containing different carbon sources at the concentrations indicated. β-Galactosidase activity was determined as reported in the work of Miller (29). Each value is the average (± the standard deviation) of four independent transformants tested. Gal, galactose; Raf, raffinose; Glu, glucose.

TABLE 2. In vivo expression of *HXT11* and *HXT9* promoters as a function of Pdr3p or Pdr1p production[a]

Medium	Plasmid	β-Galactosidase activity (U) (± SD) with plasmid encoding:	
		HXT9-LacZ	HXT11-LacZ
Glucose	pFL38	2.1 ± 0.3	1.3 ± 0.2
	pFL38-PP3	2.0 ± 0.3	1.4 ± 0.3
	pFL38-PP3A13	11.3 ± 3	13.1 ± 3.8
	pFL44-PP3	11.2 ± 1.2	12.9 ± 2.1
	YEp24-PDR1	12.8 ± 2.7	10.4 ± 1.2
Galactose	pYE-PDR3	123.0 ± 51.5	80.2 ± 15.5
	pYE-DP1/8-2	1.1 ± 0.3	0.9 ± 0.2

[a] The strain FY1679-28C Δ*pdr1* Δ*pdr3* containing the reporter plasmid pHXT11Z or pHXT9Z encoding HXT11-LacZ or HXT9-LacZ, respectively, was transformed with different plasmids encoding PDR3 or PDR1 to measure, in vivo, the effects of variation of Pdr3p or Pdr1p amounts. Pdr3p was produced from its own promoter on a multicopy plasmid (pFL44-PP3) or on an ARS-CEN plasmid (pFL38-PP3). Pdr3p was also produced from the strong *GAL1* promoter (pYE-PDR3); pYE-DP1/8-2 was the empty corresponding plasmid. pFL38-PP3A13 encoded a mutated form of PDR3 (pdr3-9) which conferred a high degree of drug resistance on the cell (32a). YEp24-PDR1 is a multicopy plasmid encoding Pdr1p from its promoter. β-Galactosidase activity was determined as described in the work of Miller (29). Each value is the average of four independent transformants tested.

TABLE 3. In vivo importance of PDRE in the *HXT11* promoter[a]

Strain	Plasmid	β-Galactosidase activity	
		PDRE	ΔPDRE
FY1679-28C	BFG1	61 ± 2	42 ± 2
	BFG1-PDR3	3,850 ± 54	284 ± 6
	BFG1-PDR1	2,665 ± 102	233 ± 5
FY1679-28C Δ*pdr1* Δ*pdr3*	BFG1	37 ± 3	33 ± 1

[a] The wild-type strain FY1679-28C or the isogenic strain FY1679-28C Δ*pdr1* Δ*pdr3* carrying the plasmid BFG1 encoding Pdr3p (BFG1-PDR3) or Pdr1p (BFG1-PDR1) was transformed with the wild-type (PDRE) or the mutated (ΔPDRE) ARS-CEN plasmid encoding HXT11-LacZ (pFL38-H11Z). β-Galactosidase activity was measured in crude extracts for four independent transformants. Values are shown in 100 relative light units, normalized with the optical density, ± the standard deviations.

vealed a very low level of expression of *HXT11* in all the carbon sources tested. Notably, *HXT11* expression was not induced by glucose but rather was slightly reduced when cells were grown on glucose rather than on galactose. In addition, expression of *HXT11* was increased in cells grown on raffinose (sugar hydrolyzed outside the cell, providing a very low extracellular concentration of glucose). These results indicated that *HXT11* expression was not controlled by the glucose induction pathway, in contrast to some other *HXT* genes. Similar results were obtained with the *HXT9-lacZ* fusion (data not shown), which did not seem surprising since similarity between *HXT11* and *HXT9* also extends to the 5′ noncoding region (94% base pair identity over 800 bp), suggesting that these two genes have similar regulatory signals.

The Pdr3p and Pdr1p transcriptional factors control *HXT11* and *HXT9* expression. To study the regulation of *HXT11* and *HXT9* by Pdr3p and Pdr1p, we measured the expression of *HXT11-lacZ* and *HXT9-lacZ* fusions by in vitro β-galactosidase assays in strains producing different levels of *PDR* regulators.

Plasmids carrying the two fusions were used to transform the strain with deletions of both *PDR1* and *PDR3* (Δ*pdr1* Δ*pdr3*) and expressing different levels of Pdr3p. Variations in levels of Pdr3p production were obtained by using centromeric or 2μm plasmids. Results presented in Table 2 clearly show that, in the absence of Pdr3p (pFL38 or pYE-DP1/8-2), the two fusions presented similar basic levels of expression on both glucose and galactose. A 5- to 50-fold increase in β-galactosidase activity was measured when Pdr3p was expressed at high levels (pFL44-PP3, pYE-PDR3). A fivefold increase in β-galactosidase activity was also obtained with the mutated PDR3 encoded in the centromeric plasmid pFL38-PP3A13 (pdr3-9). These results indicated that *HXT11* and *HXT9* were regulated by the transcriptional factor Pdr3p under the conditions tested.

The product of the *PDR1* gene (Pdr1p) coregulates with Pdr3p all the known *PDR* target genes: *PDR5* (22), *PDR3* (15), *SNQ2* (14, 28), and *YOR1* (24). To determine whether Pdr1p is also able to control *HXT11* and *HXT9* expression, the double null mutant strain (Δ*pdr1* Δ*pdr3*) carrying either the *HXT11-lacZ* or *HXT9-lacZ* construct was transformed with a multicopy plasmid encoding Pdr1p (YEp24-PDR1) and the β-galactosidase activity was measured. The results (Table 2) show that overproduction of Pdr1p caused a fivefold increase in β-galactosidase activity, similar to that found with Pdr3p. Similar results were obtained with the reporter gene encoded on an ARS-CEN plasmid (Table 3). Such findings confirmed that *HXT11* and *HXT9* were positively regulated by the Pdr1p and Pdr3p transcriptional factors.

Analyses of *HXT11-HXT9* transcript levels. To confirm the activator role of Pdr1p and Pdr3p in *HXT11* and *HXT9* transcription, we performed Northern blotting experiments. Cross-hybridization of the *HXT11* probe with other structurally related *HXT* transcripts has been ruled out by designing a specific probe. For this purpose, we chose a probe of *HXT11*, taking advantage of the weak similarity of the N-terminal regions of all the *HXT* genes (−254 to +245 bp from the initiation codon). Total RNAs were isolated from the wild-type strain and the double null strain Δ*pdr1* Δ*pdr3* or Δ*hxt9* Δ*hxt11* grown on galactose and expressing different amounts of Pdr3p and Pdr1p. Northern blot analyses are shown in Fig. 2. We observed that, as previously noticed for *PDR5*, *HXT11-HXT9* mRNA levels are elevated in response to increased gene dosage of *PDR3* or *PDR1*. (i) Upon loss of *PDR1* and *PDR3*, a reduction in *HXT11-HXT9* mRNA quantity was observed (Fig. 2, lanes 1 and 2), and (ii) a CEN plasmid encoding Pdr3p was able to restore *HXT11-HXT9* expression in the null strain (Fig. 2, lanes 7 and 8). An increase in *HXT11-HXT9* transcripts was also seen when Pdr3p or Pdr1p was overproduced compared to

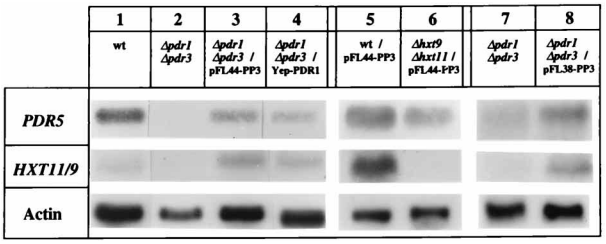


FIG. 2. Northern blot analyses of *HXT11-HXT9* expression. Total RNAs were prepared from the wild-type (wt) strain FY1679-28C (lane 1), the isogenic strain with deletions of *PDR1* and *PDR3* (Δ*pdr1* Δ*pdr3*, lanes 2 and 7), the Δ*pdr1* Δ*pdr3* strain transformed with plasmids encoding *PDR3* (pFL44-PP3, lane 3, and pFL38-PP3, lane 8) or *PDR1* (YEp24-PDR1, lane 4), and FY1679-28C (lane 5) or the isogenic Δ*hxt11* Δ*hxt9* strain (lane 6), both transformed with pFL44-PP3. Cells were grown on galactose minimal medium, and total RNAs were extracted; used for RNA blotting assays; and probed with *PDR5*, *HXT11*, and *ACT1* sequences.

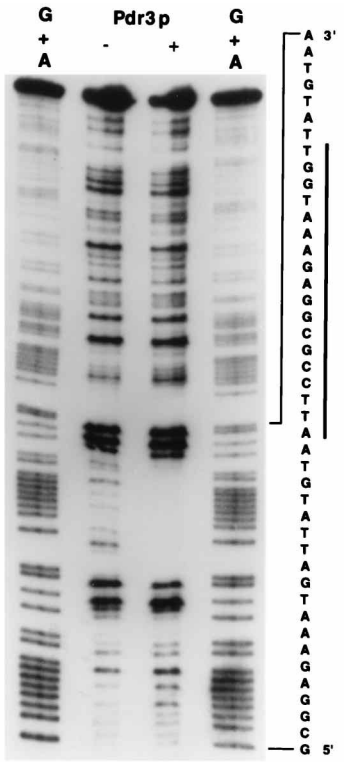


FIG. 3. DNase I footprinting analysis of the *HXT11* promoter. The *HXT11* promoter fragment extending from −640 to −252 bp from the initiation codon was generated by PCR and end labelled on the noncoding strand. It was then subjected to DNase I digestion (2 and 4 U) with (+) or without (−) prior incubation with 1.5 μg of the purified *E. coli* GST-Pdr3p*Eco*47III fusion protein and analyzed through a denaturing polyacrylamide gel. DNase I-produced fragments were loaded on the gel along with a Maxam-Gilbert reaction (G+A) of the same fragment. The heavy vertical line indicates the 18-bp sequence protected by the fusion protein.

the wild-type strain (Fig. 2, lanes 1, 3, and 4), an effect which totally disappeared in the strain with deletions of both *HXT9* and *HXT11* (Fig. 2, lanes 5 and 6). Similar results were obtained with cells grown on glucose (data not shown). All these data were consistent with the previous findings for the *HXT11*- and *HXT9-lacZ* constructs and confirmed the positive regulation of *HXT11-HXT9* expression by Pdr3p and Pdr1p.

Pdr3p recognizes at least one binding site in the *HXT11* promoter in vitro and in vivo. To investigate if Pdr3p is directly involved in the transcriptional activation of *HXT11*, we have performed a DNase I protection assay. Previous data have characterized a *cis* element (PDRE; 5′TCCGCGGA3′) common to all promoters of the *PDR3* targets: *PDR5* (22), *PDR3* (15), and *SNQ2* (28). Analysis of *HXT11* and *HXT9* promoter sequences revealed the existence of an analogous motif. Therefore, we carried out DNase I protection assays with a PCR-generated fragment of the *HXT11* promoter extending from −640 to −252 bp and the *E. coli*-expressed GST-Pdr3p*Eco*47III fusion protein.

Figure 3 shows that bacterially produced GST-Pdr3p protected an 18-bp-long DNA segment, extending from −534 to −517 bp and encompassing a canonical PDRE sequence, 5′TCCGCGGA3′. Since *PDR5* and *SNQ2* promoter sequences contain at least three related PDRE sequences (15, 23, 28), we further investigated the *HXT11* promoter. Two other putative Pdr3p binding sites, differing at one position (5′TCCGCGaA3′ and 5′TCCGtGGA) from the consensus PDRE and located at 24 and 206 bp, respectively, downstream of the protected site, were not recognized by the GST-Pdr3p fusion protein in vitro, even with increasing amounts of GST-Pdr3p (data not shown).

The in vivo function of the identified PDRE was assessed by deleting 2 bp in the Pdr3p binding site (5′TCCGCGGA3′, resulting in 5′TCCGGA3′; ΔPDRE) of the *HXT11-lacZ* reporter plasmid and measuring the corresponding β-galactosidase activity in the presence of high levels of Pdr1p or Pdr3p. Table 3 clearly shows that overexpression of Pdr3p (BFG1-PDR3) or Pdr1p (BFG1-PDR1) leads to a very high level of β-galactosidase activity with the wild-type *HXT11-lacZ* reporter plasmid, which is reduced to 10% of this value when PDRE is converted to ΔPDRE. In the absence of *PDR1* and *PDR3* (Δ*pdr1* Δ*pdr3*), either reporter plasmid leads to the same basic level of β-galactosidase activity. The large decrease in β-galactosidase activity with the mutated reporter plasmid did not reach the wild-type level, suggesting either remnant weak binding properties of the mutated PDRE or the existence of a weak secondary PDRE site. As mentioned above, such a secondary site could not be detected in vitro with the GST-Pdr3p*Eco*47III fusion protein.

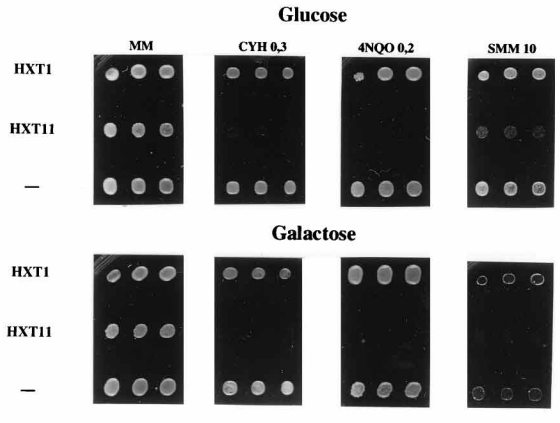
***HXT11* and *HXT9* are involved in the drug resistance process.** *HXT11* and *HXT9* are the first identified Pdr1p-Pdr3p targets of the MFS family which encode putative hexose transporters. To test whether this property was relevant to the drug resistance phenomenon, we have firstly overexpressed *HXT11*, secondly constructed a set of isogenic yeast strains lacking either *HXT11* or both *HXT11* and *HXT9*, and thirdly examined PDR on two carbon sources (glucose and galactose).

(i) Overproduction of Hxt11p enhances drug sensitivity. To explore further the role of Hxt11p in the PDR phenomenon, we overproduced Hxt11p and a closely related hexose transporter, Hxt1p (65.5% amino acid identity). *HXT11* and *HXT1* were placed under the control of the *PGK* promoter on a 2μm plasmid to allow a high level of expression. Cells in which Hxt11p was overproduced were more sensitive to the external presence of drugs such as cycloheximide, sulfomethuron methyl, and 4-NQO compared to cells with a wild-type level of Hxt11p (FY1679-28C) or when Hxt1p was overproduced either on glucose or on galactose (Fig. 4A). For instance, a concentration of 0.5 μg of cycloheximide per ml was sufficient to inhibit growth of the wild-type strain on minimal medium. This value, defined as the MIC, was decreased to 0.2 μg/ml in cells overproducing Hxt11p but not in cells overproducing Hxt1p (Fig. 4A). Cycloheximide hypersensitivity was not associated with sugar conditions since similar results were obtained with glucose or galactose as the carbon source. Figure 4B shows the MIC scored after 3 days on minimal medium supplemented with cycloheximide, sulfomethuron methyl, or 4-NQO. For these three drugs, a twofold decrease in drug concentration tolerance was measured in cells overproducing the Hxt11p transporter. By contrast, in the Δ*pdr1* Δ*pdr3* isogenic strain, Hxt11p overproduction did not significantly decrease the sensitivity to cycloheximide; nevertheless, a threefold increase in 4-NQO sensitivity was observed. These results suggested that high levels of Hxt11p, but not Hxt1p, enhanced the drug sensitivity of strains expressing different levels of Pdr5p and Snq2p ABC transporters.

(ii) Deletion of *HXT11* and *HXT9* enhances drug resistance. To confirm the involvement of these *HXT*s in the PDR process, we have tested the drug tolerance of a set of isogenic yeast strains lacking either *HXT11* or both *HXT11* and *HXT9* and grown on glucose or on galactose.

We observed (Fig. 5A) that the wild-type strain (W303-1A) did not grow in the presence of 2 μg of sulfomethuron methyl per ml whereas the isogenic Δ*hxt11* Δ*hxt9* mutant strain was

A



B

Strain	Overexpressed gene	MIC(μg/ml)		
		CYH	4-NQO	SMM
Wild Type	*HXT1*	0.5	0.4	2
	HXT11	0.2	0.2	<2
	—	0.5	0.4	2
Δpdr1Δpdr3	*HXT11*	0.03	0.06	<2
	—	0.04	0.2	<2

FIG. 4. Overproduction of Hxt11p enhances drug sensitivity. The wild-type strain (FY1679-28C) was transformed with the plasmid containing *HXT1* (pCJHXT1) or *HXT11* (pCJHXT11) under the control of the *PGK* promoter or with the vector alone (pEMBLye30/2) (—). (A) Drug resistance test of the different strains spotted on minimal medium (MM) (glucose or galactose) ± sulfomethuron methyl (SMM), cycloheximide (CYH), or 4-NQO and scored after 3 days of incubation at 30°C. (B) MIC for cell growth determined after 3 days of incubation. Abbreviations are as defined for panel A.

much more resistant, tolerating up to 6 μg of this drug per ml. An increase in drug tolerance was also observed with cycloheximide and 4-NQO (Fig. 5B). The strain carrying the single deletion Δ*hxt11* presented an intermediate phenotype for all these drugs. No difference in drug tolerance of these strains was seen with cells grown on glucose or on galactose (Fig. 5B). These results strongly indicate that the hexose transporters Hxt11p and Hxt9p were involved in the PDR process.

DISCUSSION

We have found that Pdr1p and Pdr3p, two similar transcription factors which control the production of several ABC membrane pumps involved in the PDR phenomenon in yeast, also control *HXT11* and *HXT9* expression. These very similar genes, located on chromosome XV and chromosome X, respectively, encode putative hexose transporters of the MFS. In this study, we have shown that these transporters were not transcriptionally induced by glucose but were activated by Pdr1p and Pdr3p. Hxt11p, even translated from a low-copy-number plasmid, restored low-affinity glucose uptake in the *K. lactis rag1* mutant, suggesting that it can function as a glucose transporter or can regulate other hexose transporter functions. In addition, we have shown that overproduction of this protein in *S. cerevisiae* enhanced drug sensitivity of the cells while disruption of both *HXT11* and *HXT9* rendered the cells more resistant to different drugs. Identification of *HXT11* and *HXT9* as target genes of *PDR1-PDR3*-mediated drug resistance is an important piece of information for the understanding of the PDR process in *S. cerevisiae.*

A new kind of target gene regulated by Pdr1p and Pdr3p. With the exception of *PDR3* itself, all the Pdr1p-Pdr3p target genes identified so far encode ABC transporters. *PDR5* (22), *SNQ2* (14, 28), and *YOR1* (24) each encode an ABC pump which, upon overexpression, leads to resistance against a variety of structurally unrelated cytotoxic compounds. These ABC transporters are involved in a wide range of energy-dependent transport events across the plasma membrane and are thought to function as drug efflux pumps (27). The present work demonstrates that Pdr1p and Pdr3p can also activate expression of *HXT11* and *HXT9*, two genes belonging to the MFS. Typically, members of this superfamily contain 12 putative membrane-spanning domains with a relatively large hydrophilic region between membrane-spanning domains 6 and 7. Many members of this superfamily exhibit structural conservations within membrane-spanning domains 3, 5, 7, 8, and 11, which have been postulated to form amphipathic α-helices and assemble into a pentagonal pore forming the channel through which glucose is transported (for a review, see reference 8). As revealed by yeast genome sequencing, 20 *HXT*-related genes exist in *S. cerevisiae*. The roles of many members of the *HXT* family remain obscure. This large family (1, 26, 32) includes proteins for which the hexose specificity and transport properties are well known (for a review, see reference 8). Recent data have raised the question of the actual role of all of these transporters in sugar catabolism (1, 26, 32). The very low expression level of some of these genes appears to be inconsistent with a direct role as catabolic transporters (8, 25). Our data showing that Hxt11p can restore glucose uptake in a *rag1* mutant of *K. lactis* suggest that this protein can act as an active glucose transporter or can regulate the functions of other *HXT* transporters. However, the facts that (i) *HXT11* and *HXT9* are very poorly expressed (this study) and (ii) deletion of the seven genes *HXT1* to *HXT7* of *S. cerevisiae* prevents growth on glucose (35) clearly indicate that *S. cerevisiae* cannot rely on *HXT11* and *HXT9* to transport glucose in nonappropriate inducible conditions and raise the question of their function in the cell. Interestingly, the existence of such inducible transporters in *S. cerevisiae* has recently been hypothesized (35).

Pdr1p and Pdr3p activate *HXT11* and *HXT9* transcription by interacting with a canonical binding site (PDRE, 5′TCCGC GGA3′) located −532 bp upstream from the ATG codon. The recent availability of the complete sequence of the yeast genome allowed us to look for other *HXT* genes carrying a PDRE. Two candidates, *HXT12* and *HXT3*, are worth mentioning. They possess the canonical sequence, located at −532 and −388 bp, respectively, from their ATG codon. *HXT12* is, however, interrupted by a frameshift before the first potential *Sau*3AI cloning site; therefore, no *lacZ* fusion gene could be functional. *HXT3* is known to be highly expressed on glucose and repressed on galactose (33). This feature can reasonably explain why *HXT3*, which contains several *Sau*3AI sites, was not revealed by our initial screening procedure which was carried out on galactose medium; alternatively, *HXT3* could not be regulated by Pdr1p and Pdr3p, a point which clearly deserves further investigation.

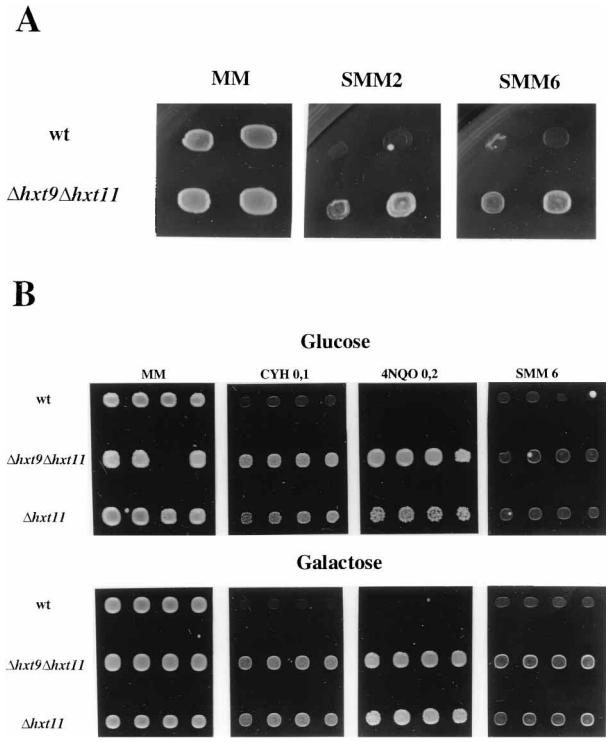


FIG. 5. *HXT11* and *HXT9* are involved in the drug resistance process. (A) Resistance to sulfomethuron methyl (SMM) on glucose plates was tested for the wild-type (wt) strain W303-1A and the isogenic strain with both *HXT11* and *HXT9* deleted (Δ*hxt11* Δ*hxt9*). MM, minimal medium. (B) Resistance to sulfomethuron methyl, 4-NQO, and cycloheximide was tested for the wild-type (wt) strain W303-1A and isogenic strains with both *HXT11* and *HXT9* deleted (Δ*hxt11* Δ*hxt9*) or *HXT11* alone deleted (Δ*hxt11*). Four colonies from each strain were spotted on minimal medium containing glucose or galactose and the appropriate amino acids (MM) and/or supplemented with different drugs: cycloheximide (CYH), 4-NQO, and sulfomethuron methyl (SMM).

***HXT11* and *HXT9* are involved in the PDR process.** It is remarkable that *HXT11-HXT9* or *HXT11* deletions on one hand, and Hxt11p overproduction on the other hand, indicate that the presence of Hxt11p makes cells more sensitive to drugs. This multidrug sensitivity is in direct opposition to the MDR process, since both phenomena, *HXT11* sensitivity and *PDR5* resistance, are regulated by the same transcription factors, Pdr1p and Pdr3p. Interestingly, Hxt11p cannot be replaced by Hxt1p for this function, suggesting some sort of specificity in relation to the specific Pdr1p-Pdr3p regulation. Different functions of these transporters can be postulated. First, Hxt11p and Hxt9p could negatively influence ABC pump function, providing a kind of feedback regulation. It is now well known that eukaryotic ABC transporters are regulated post-translationally during trafficking or membrane targeting. Therefore, it is possible that the absence of Hxt11p or overproduction of Hxt11p, but not Hxt1p, might interfere with the final distribution of Pdr5p and Snq2p in the membrane. Second, as the true physiological role of the MDR ABC transporters is unknown, it could be that the physiological substrate of the ABC transporters is something that is under glucose control, thus coupling *HXT* function to *PDR* excretion. This hypothesis does not seem likely, since the presence of Hxt11p confers the same level of drug sensitivity in the presence or in the absence of glucose in the medium. Furthermore, the absence of Hxt11p and Hxt9p rendered the cell much more resistant to drugs and tolerant to the same concentration of drugs when cells were grown on glucose or on galactose. These results lend more credence to a third hypothesis in which these *HXT* transporters would allow the translocation of other classes of compounds across the membrane. It would not be so surprising that drastic changes in the membrane properties due to the overproduction of ABC transporters would have to be compensated for, notably the permeability of the membrane, by overproduction of other membrane proteins in resistant cells. Notably, preliminary results of our systematic search of Pdr1p-Pdr3p target genes revealed mainly genes coding for membrane proteins (32b).

The roles of many members of the *HXT* family remain obscure. We have shown that two of them are directly related to the PDR phenomenon. Previous observations have implied relationships between drug resistance and hexose transport processes (11, 40). Such a multigene family of glucose transporters closely resembles the mammalian system, six glucose carriers (*GLUT*) of which have already been identified (20). It has been shown that expression of some *GLUT* genes is increased in human cancers (44) and in response to stress (42). Although differential expression in cells and tissues can explain an array of physiological roles of the multiple *GLUT* genes in mammals, there is not yet a clear explanation for the multitude of hexose transporters in *S. cerevisiae*. In this study, we provide new evidence that some *HXT* genes are regulated by some specific nonsugar transcription factors. The coregulation of these *HXT* and multidrug transporter genes, together with their individual activities, provides new insights for the understanding of the *PDR* network and its mechanism of action.

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